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VSEBINA / CONTENTS

Izvirni znanstveni članki / Original research articles

Pregledni znanstveni članki / Review articles

Kratke vesti/Short Communications

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Effect of plant growth regulators on the growth and direct shoot formation from leaf explants of the hybrid *Phalaenopsis* 'Pink'

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ABSTRACT

Phalaenopsis orchids are one of the most beautiful flowering plants. The objective of this study was to identify the best plant growth regulator combination and medium for the growth and direct shoot formation from leaf explants of the hybrid *Phalaenopsis* 'Pink'. Leaf tips segments from *in vitro* young plants were cultured on half-strength Murashige and Skoog (MS) and Vacin and Went (VW) media supplemented with different concentrations of auxin [α-napthaleneacetic acid (NAA)] and cytokinins [6-benzylaminopurine (BAP) or thidiazuron (TDZ)]. The explants that were cultured on 0 mg l^{-1} NAA and 3 mg l^{-1} BAP supplemented to half-strength MS medium formed shoots successfully within 10 weeks of culture with 5 % regenerants and 50 % survival frequency. The explants cultured on 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} TDZ supplemented to half-strength MS medium developed calluses and shoots within 11 weeks of culture with 25 % regenerants and 90 % survival frequency. Future research needs to be directed to find out the shortest time of shoot regeneration to produce viable plants with a high survival frequency.

Key words: *Phalaenopsis*; shoot formation; α-napthaleneacetic acid; 6-benzylaminopurine; thidiazuron

IZVLEČEK

UČINEK RASTLINSKIH RASTNIH REGULATORJEV NA RAST IN NEPOSREDNO TVORBO POGANJKOV IZ LISTNIH IZSEČKOV PRI KRIŽANCU *Phalaenopsis* 'Pink'

Orhideje iz rodu *Phalaenopsis* so med najlepšimi cvetnicami**.** Namen te raziskave je bil ugotoviti najprimernejšo kombinacijo rastlinskih rastnih regulatorjev in gojišč za rast in neposredno tvorbo poganjkov iz listnih izsečkov križanca *Phalaenopsis* 'Pink'. Izsečki vrhnjih delov lista mladih, *in vitro* vzgojenih rastlin so bili gojeni na polovičnem Murashige in Skoog (MS) ter Vacin in Went (VW) gojišču, in dodane so bile različne koncentracije auksina [α-naftalenocetna kislina (NAA)] in citokinina [6-benzilaminopurin (BAP) ali tidiazurona (TDZ)]. Izsečki, ki so bili gojeni na polovičnem MS gojišču z dodatkom 0 mg l^{-1} NAA in 3 mg l^{-1} BAP so v desetih tednih uspešno tvorili poganjke s 5 % nastalih regenerantov in 50 % preživetjem. Izsečki, ki so bili gojeni na polovičnem MS gojišču z dodatkom 0.5 mg l^{-1} NAA in 1.5 mg l^{-1} TDZ so tvorili kalus in poganjke v enajstih tednih s 25 % nastalih regeneratov in 90 % preživetjem. Potrebne so še nadaljnje raziskave za določitev najkrajšega časa regeneracije poganjkov, ki bi dale vitalne rastline z dobrim preživetjem.

Ključne besede: *Phalaenopsis*; tvorba poganjkov; α-naftalenocetna kislina; 6-benzilaminopurin; tidiazuron

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1 INTRODUCTION

Species from the genus *Phalaenopsis*, Orchidaceae, also known as "moth orchids", are one of the most popular, beautiful and unique flowering plants in the world due to their large, colourful, durable flowers and their adaptability to room condition. They have a very high economic value in Europe, Asia and other continents. They are commercially grown for the production of the cut flowers and potted plants (Košir et al., 2004; Niknejad et al., 2011). *Phalaenopsis* orchids are distributed throughout Southeast Asia with a few species extending from Taiwan, Indian State of Sikkim to Australia and the Pacific (Teob, 1989). *Phalaenopsis* hybrids are the result of intensive breeding of plants for increasing market value as cut flowers and even more as potted plants. The duration and intensity of flowering depend mostly on the genotype and the breeding technology, which greatly influences the vitality of the plants. Species from the genus *Phalaenopsis* are difficult to propagate vegetatively as they are monopodial epiphytic orchids (Košir et al., 2004). The characteristics of seedlings propagated by ordinary vegetative means are not uniform and propagation through tissue culture has been the most desired way of propagation (Košir et al., 2004).

The main objective of tissue culture is the development of protocols to regenerate whole plants from single cells, plant cells without cell walls (protoplasts) and calluses (Chen et al., 2000). This technique is based on the principle of totipotency and has contributed a large scale to the production of plants of economic importance such as orchid and other ornamental plants. However, conventional breeding is slow and difficult as it requires almost two to three years for completing a life cycle (Niknejad et al., 2011; Purohit, 2005).

Many researchers have developed *in vitro* protocols for *Phalaenopsis* (Chen et al., 2000; Lu, 1993). Some authors also have reported frequent callus formation as an intermediate phase just prior to somatic embryogenesis or regeneration to protocorms (Ishii et al., 1998). Researchers observed the formation of embryonic callus when they used 73 % of *Phalaenopsis* shoot-tip explants excised from flower stalk buds and by culturing for seven months on New Dogashima Medium (NDM) containing plant growth regulators (PGRs) such as auxin (α-napthaleneacetic acid (NAA)) and cytokinin (6-benzylaminopurine (BAP)) (Tokuhara and Mii, 2001). Leaf sections from *in vitro* young plants of *Phalaenopsis gigantea* J.J.Sm. on NDM supplemented with cytokinins such as BAP, thidiazuron (TDZ) and kinetin (KIN) alone and in combination with auxin (NAA) were cultured and the development of callus and protocorm-like-bodies (PLBs) from explants within six weeks of culture was observed (Niknejad et al., 2011). Direct regeneration without undesirable callus formation shortens the time period needed for regeneration and reduces the possibility of the occurrence of somaclonal variation (Košir et al., 2004). However, more research needs to be done to find out the best medium and PGR with the right concentration that could provide an efficient shoot formation from *Phalaenopsis* in short period of time (Arditi and Ernst, 1993; Park et al., 2001). The objective of this research was to identify the best PGR combination and medium for the growth and direct shoot formation of the hybrid *Phalaenopsis* 'Pink' from leaf explants.

2 MATERIALS AND METHODS

2.1 Plants material and culture conditions

Laboratory experiments were carried out during March to August 2014 at plant tissue culture laboratory, Rapee Sagarik Orchid Garden, Department of Horticulture, Faculty of Agriculture, Kasetsart University, Thailand. Three months old *in vitro* young plantlets of hybrid orchid (*Phalenopsis* 'Pink') purchased from the Salaya Orchid Company in Nonthaburi, Thailand were used as donor plants. Flower stalk explants were used to produce donor plants that were cultured on Vacin and Went (VW) medium supplemented with 10 g l^{-1} sucrose, 15 g l^{-1} banana extract and 15 g l^{-1} potato extract for regeneration and multiplication (Zahara et al., 2016).

In this study, half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and VW medium (Vacin and Went, 1949) were used as the basal medium for the growth and direct shoot formation. Leaf tips segments from donor plants were cut about 1 cm in diameter, and placed on half-strength MS or VW medium containing two NAA concentrations (0 and 0.5 mg l^{-1}) supplemented with either five concentrations of BAP (0, 1, 2, 3 and 4 mg l^{-1}) or five concentrations of TDZ (0, 0.5, 1, 1.5 and 2 mg l^{-1}). The pH of the medium was adjusted to 5.6 for MS medium and 5.2 for VW medium with 1 mol KOH or 1 mol HCl prior to autoclaving for 15 minutes at 121 °C. The media were placed in sterile vials (25 ml) prior to autoclaving. Subsequently, all explants were placed onto the surface of either half-strength MS or VW medium for five

months and kept in a culture room at 25 °C during the light and dark phase in a 15-h photoperiod under the light intensity of 40 µmol m^{-2} s^{-1} provided by cool white florescent tubes. Subculture was done in every 21 days onto the same PGR combination and medium.

2.2 Induction of direct shoot regeneration

Leaf explants (1 cm × 1 cm) were used to test the effects of NAA (0 and 0.5 mg l^{-1}) with different concentrations of BAP (0, 1, 2, 3 and 4 mg l^{-1}) or TDZ (0, 0.5, 1, 1.5 and 2 mg l^{-1}) supplemented to half-strength MS or VW medium on the growth and direct shoot formation. A total of 10 vials each with two leaf explants were used in each treatment combination. Data were recorded after five months of culture.

2.3 Experimental design and data analysis

The vials were arranged in a completely randomized design with 20 replications per treatment. Each experimental treatment combination consists of two factors: NAA concentrations and different concentrations of BAP or TDZ in either half-strength MS or VW medium. *In vitro* growth was evaluated 150 days after cultures were initiated. The parameters recorded were leaf length, leaf width, percentage of regenerants and survival frequency of explants. The data were subjected to analysis of variance (ANOVA) and significant differences among the treatments were tested using two-way ANOVA and means were separated by Duncan multiple range test (DMRT) at $p \leq 0.05$ using SAS 9.1.3 (SAS Institute, Cary, NC, USA).

3 RESULTS

3.1 Effects of NAA and BAP supplemented to half-strength MS medium on the growth and direct shoot formation of leaf explants

The combination of NAA and BAP supplemented to half-strength MS medium significantly affected the growth and direct shoot formation of leaf explants (Table 1). After five months of culture, most of the leaf explants showed an increase in leaf length and survival frequency with increasing BAP concentration, regardless of NAA concentration. However, leaf width did not significantly change in most of the explants. Leaf length was the highest (2.43 ± 0.01 cm) at 0 mg l^{-1} NAA in combination with 4 mg l^{-1} BAP with 50 % survival rate. Leaf width of the hybrid *Phalaenopsis* 'Pink' did not increase when cultured on NAA and BAP supplemented to half-strength MS medium as it was similar in all concentrations of NAA and BAP, except for the concentrations of 0.5 mg l^{-1} NAA with 2, 3 and 4 mg l^{-1} BAP where leaf width was comparatively smaller. Somatic embryos were formed directly from the cut edge/wounding area (5 %) at 0 mg l^{-1} NAA and 3 mg l^{-1} BAP combination culture with 50 % survival rate (Figs. 1a & 2). Shoots also directly formed at 0.5 mg l^{-1} NAA and 2 mg l^{-1} BAP within 10–11 weeks of culture with 40 % survival frequency. Overall, the combination of 0 mg l^{-1} NAA and 3 mg l^{-1} BAP supplemented to half-strength MS medium showed optimal growth and direct shoot formation from leaf explants of the hybrid *Phalaenopsis* 'Pink' (Fig. 2).

Table 1: Effects of NAA and BAP supplemented to half-strength MS medium on leaf formation, percentage of regenerants and survival frequency of the hybrid *Phalaenopsis* 'Pink' leaf explants after five months of culture

NAA (mg l^{-1})	BAP (mg l^{-1})	Leaf length (cm)	Leaf width (cm)	Regenerants (%)	Survival frequency (%)
0	0	2.11 ± 0.009 def	1.20 ± 0.02 a	0.00 ± 0.00 b	25.04 ± 0.14 d
0	1	2.21 ± 0.01 bcde	1.21 ± 0.01 a	0.00 ± 0.00 b	30.20 ± 0.29 c
0	2	2.23 ± 0.01 bcd	1.23 ± 0.003 a	0.00 ± 0.00 b	30.70 ± 0.19 c
0	3	2.26 ± 0.01 bc	1.23 ± 0.006 a	5.00 ± 0.00 a	50.00 ± 0.31 a
0	4	2.43 ± 0.01 a	1.26 ± 0.01 a	0.00 ± 0.00 b	50.60 ± 0.26 a
0.5	0	1.00 ± 0.00 g	1.21 ± 0.01 a	0.00 ± 0.00 b	10.00 ± 0.14 e
0.5	1	2.06 ± 0.01 f	1.20 ± 0.00 a	0.00 ± 0.00 b	30.00 ± 0.28 c
0.5	2	2.10 ± 0.07 ef	1.00 ± 0.00 b	5.00 ± 0.00 a	40.00 ± 0.14 b
0.5	3	2.15 ± 0.03 cdef	1.00 ± 0.00 b	0.00 ± 0.00 b	40.00 ± 0.14 b
0.5	4	2.28 ± 0.01 b	1.00 ± 0.00 b	0.00 ± 0.00 b	40.00 ± 0.00 b
F		***	***	*	***

Data are means ± standard error (SE) of 20 replicates. Means followed by the same letter in each column do not differ by Duncan's multiple range test at $p \leq 0.05$. * and *** indicate significance at $p \leq 0.05$ and $p \leq 0.001$, respectively.

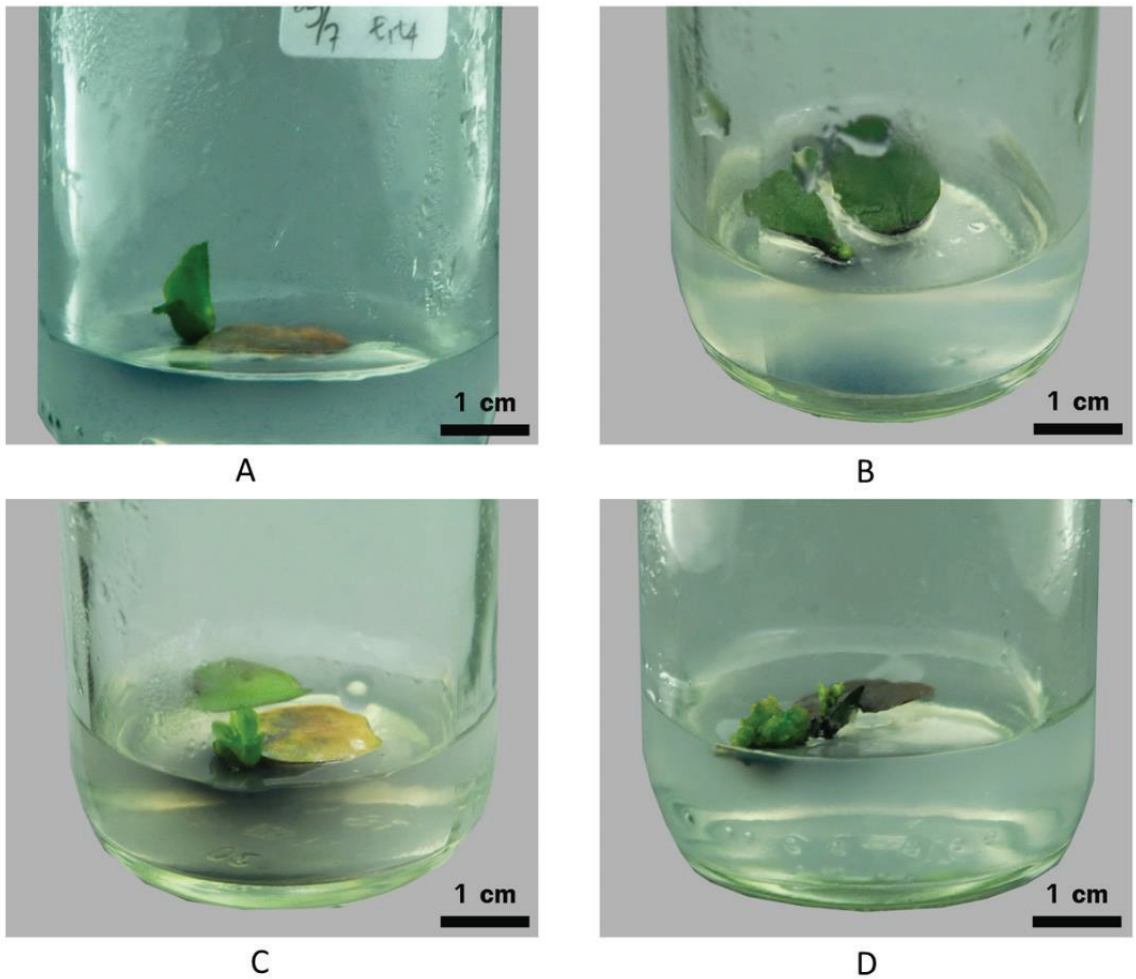

Figure 1: Effect of plant growth regulators on the growth and direct shoot formation from leaf explants of the hybrid *Phalaenopsis* 'Pink': A – Shoot formation at 0 mg l^{-1} NAA + 3 mg l^{-1} BAP supplemented to half-strength MS medium, B – Callus formation at 0 mg l^{-1} NAA + 1 mg l^{-1} BAP supplemented to VW medium, C – Shoot formation at 0 mg l^{-1} NAA + 2 mg l^{-1} TDZ supplemented to half-strength MS medium, D – Callus formation at 0.5 mg l^{-1} NAA + 1.5 mg l^{-1} TDZ supplemented to half-strength VW medium

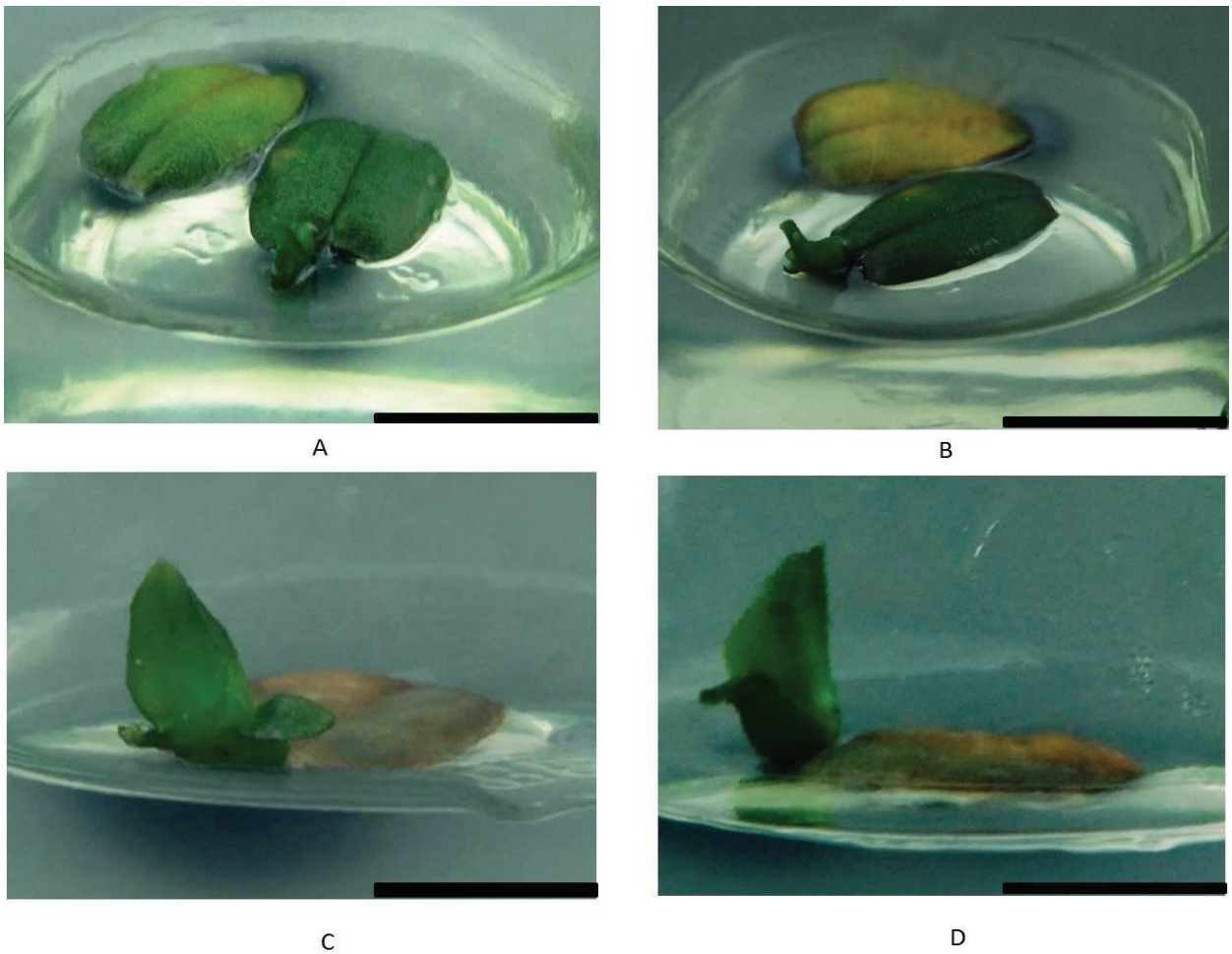

Figure 2: Direct shoot formation from leaf explants of the hybrid *Phalaenopsis* 'Pink' without callus-mediated at 0 mg l^{-1} NAA + 3 mg l^{-1} BAP supplemented to half-strength MS medium: A – Shoot formation after 10 weeks of culture, B – Shoot formation after 15 weeks of culture, C and D – Shoot formation after 20 weeks of culture (*bar* = 1.0 cm)

3.2 Effects of NAA and BAP supplemented to VW medium on the growth and direct shoot formation of leaf explants

Explants growth in terms of leaf length and leaf width was significantly affected by the combination of NAA and BAP supplemented to VW medium (Table 2). After five months of culture, leaf length and survival frequency showed increasing trend with increasing NAA and BAP concentration. In contrast, leaf width decreased with increasing BAP concentration regardless of NAA concentration. The combination of 0.5 mg l^{-1} NAA and 3 mg l^{-1} BAP resulted in the maximum leaf length (2.66 ± 0.02 cm). Leaf width was showed to be optimal in the combination of 0 mg l^{-1} NAA with 0 and 1 mg l^{-1} BAP as well as in the combination of 0.5 mg l^{-1} NAA with 0 mg l^{-1} BAP. Callus was directly formed in the combination of 0 mg l^{-1} NAA and 1 mg l^{-1} BAP from the cut edge after 15 weeks of culture and it maintained the same size until 20 weeks of growth (5 months) (Fig. 1b). Overall, the combination of 0 mg l^{-1} NAA and 1 mg l^{-1} BAP supplemented to VW medium showed optimal growth and direct shoot formation from the hybrid *Phalaenopsis* 'Pink' leaf explants.

Table 2: Effects of NAA and BAP supplemented to VW medium on leaf formation, percentage of regenerants and survival frequency of the hybrid *Phalaenopsis* 'Pink' leaf explants after five months of culture

NAA (mg l^{-1})	BAP (mg l^{-1})	Leaf length (cm)	Leaf width (cm)	Regenerants (%)	Survival frequency (%)
0	0	2.00 ± 0.01 d	1.30 ± 0.008 ab	0.00 ± 0.00 b	30.00 ± 0.20 c
0	1	2.01 ± 0.01 d	1.27 ± 0.006 ab	5.00 ± 0.00 a	40.00 ± 0.31 b
0	2	2.17 ± 0.02 bcd	1.25 ± 0.006 bc	0.00 ± 0.00 b	40.00 ± 0.14 b
0	3	2.18 ± 0.01 bcd	1.25 ± 0.006 bc	0.00 ± 0.00 b	40.00 ± 0.20 b
0	4	2.22 ± 0.01 bc	1.19 ± 0.004 c	0.00 ± 0.00 b	50.00 ± 0.14 a
0.5	0	1.54 ± 0.02 e	1.32 ± 0.01 a	0.00 ± 0.00 b	20.00 ± 0.14 d
0.5	1	1.68 ± 0.07 e	1.00 ± 0.00 d	0.00 ± 0.00 b	30.00 ± 0.14 c
0.5	2	2.13 ± 0.01 cd	1.00 ± 0.00 d	0.00 ± 0.00 b	40.00 ± 0.31 b
0.5	3	2.66 ± 0.02 a	1.00 ± 0.00 d	0.00 ± 0.00 b	40.00 ± 0.14 b
0.5	4	2.30 ± 0.01 b	1.00 ± 0.00 d	0.00 ± 0.00 b	50.00 ± 0.20 a
F		***	***	*	***

Data are means ± standard error (SE) of 20 replicates. Means followed by the same letter in each column do not differ by Duncan's multiple range test at $p \leq 0.05$. * and *** indicate significance at $p \leq 0.05$ and $p \leq 0.001$, respectively.

3.3 Effects of NAA and TDZ supplemented to half-strength MS medium on the growth and direct shoot formation of leaf explants

In this experiment, the combination of NAA and TDZ supplemented to half-strength MS medium showed significant effect on the growth and direct shoot formation of leaf explants (Table 3). After five months of culture, higher concentration of NAA and TDZ resulted in greater leaf length, leaf width and percentage of survival frequency. Callus and shoots were directly formed from the leaf surface (adaxial leaf surface) and the cut edge region after 8–11 weeks of culture, respectively (Figs. 1c, 3 & 4). Leaf length (2.63 ± 0.008 cm) and leaf width (1.31 ± 0.004 cm) was found to be optimal in the combination of 0 mg l^{-1} NAA and 2 mg l^{-1} TDZ with 10 % regenerants and 60 % survival frequency. Shoots were directly formed after 8 weeks of culture in the combination of 0 mg l^{-1} NAA and 2 mg l^{-1} TDZ (Fig. 3). The combinations of 0 mg l^{-1} NAA and 1 mg l^{-1} TDZ as well as 0.5 mg l^{-1} NAA and 2 mg l^{-1} TDZ also resulted in 5 % regenerants within 11 weeks of culture. The highest frequency of regenerants (25 %) was obtained in the presence of 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} TDZ within 11 weeks of culture with 90 % survival frequency (Table 3), and most of the explants formed callus (Fig. 4). Overall, the combination of 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} TDZ supplemented to half-strength MS medium showed good results across the greatest number of recorded parameters for the growth and direct shoot formation of leaf explants.

Table 3: Effects of NAA and TDZ supplemented to half-strength MS medium on leaf formation, percentage of regenerants and survival frequency of the hybrid *Phalaenopsis* 'Pink' leaf explants after five months of culture

NAA (mg l^{-1})	TDZ (mg l^{-1})	Leaf length (cm)	Leaf width (cm)	Regenerants (%)	Survival frequency (%)
0	0	2.21 ± 0.01 d	1.00 ± 0.00 d	0.00 ± 0.00 d	40.00 ± 0.31 f
0	0.5	2.38 ± 0.007 c	1.00 ± 0.00 d	0.00 ± 0.00 d	40.00 ± 0.31 f
0	1	2.40 ± 0.008 c	1.00 ± 0.00 d	5.00 ± 0.14 c	50.00 ± 0.31 e
0	1.5	2.60 ± 0.01 ab	1.22 ± 0.008 b	0.00 ± 0.00 d	50.00 ± 0.14 e
0	2	2.63 ± 0.008 a	1.31 ± 0.004 a	10.00 ± 0.14 b	60.00 ± 0.24 d
0.5	0	2.40 ± 0.008 c	1.15 ± 0.01 c	0.00 ± 0.00 d	50.00 ± 0.14 e
0.5	0.5	2.41 ± 0.005 c	1.21 ± 0.005 b	0.00 ± 0.00 d	70.00 ± 0.31 c
0.5	1	2.42 ± 0.007 c	1.22 ± 0.004 b	0.00 ± 0.00 d	80.00 ± 0.20 b
0.5	1.5	2.58 ± 0.005 ab	1.25 ± 0.003 b	25.10 ± 0.13 a	90.00 ± 0.14 a
0.5	2	2.52 ± 0.007 b	1.21 ± 0.003 b	5.00 ± 0.20 c	90.00 ± 0.14 a
F		***	***	***	***

Data are means ± standard error (SE) of 20 replicates. Means followed by the same letter in each column do not differ by Duncan's multiple range test at $p \leq 0.05$. *** indicates significance at $p \leq 0.001$.

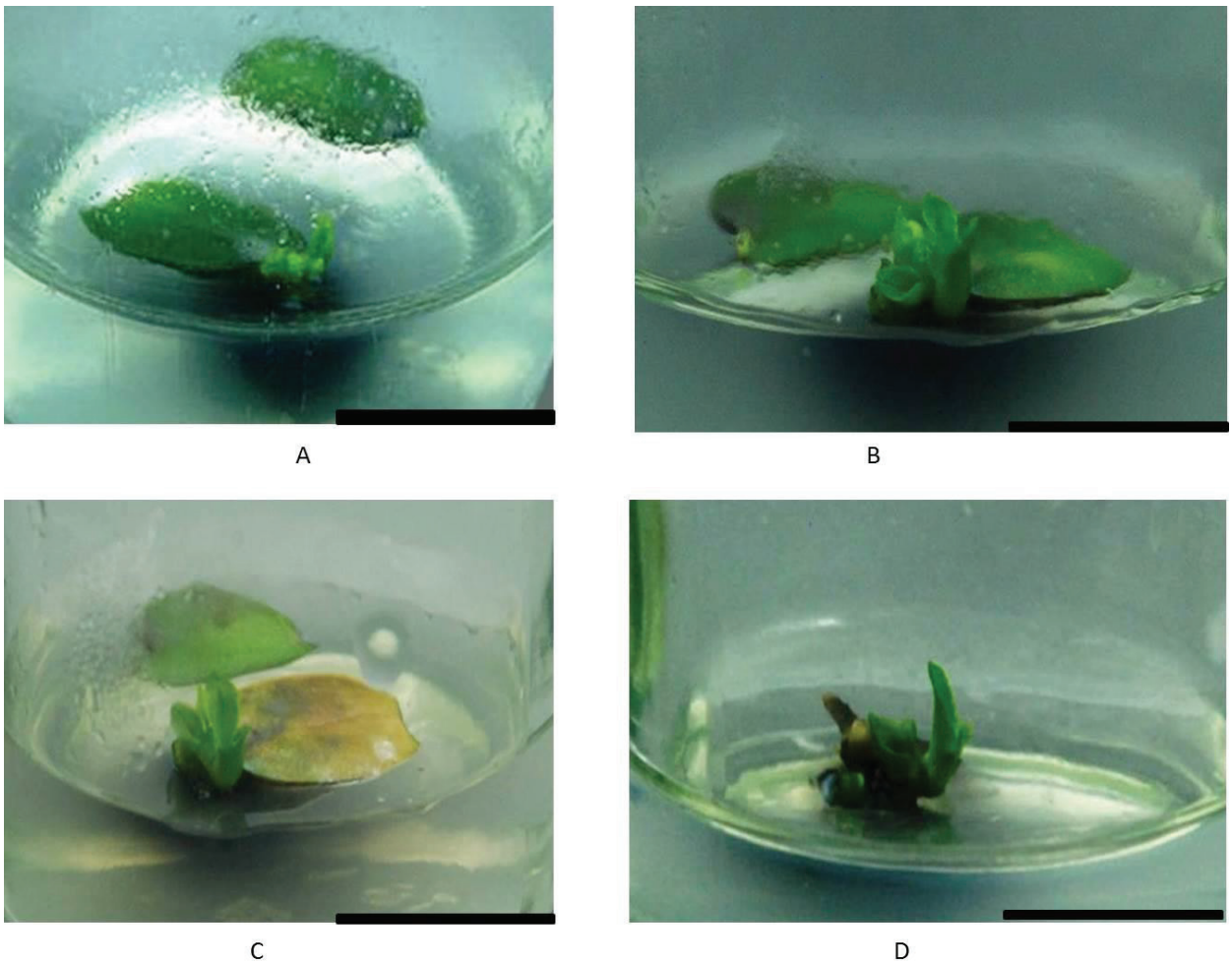

Figure 3: Direct shoot formation from leaf explants of the hybrid *Phalaenopsis* 'Pink' without callus-mediated at 0 mg l^{-1} NAA + 2 mg l^{-1} TDZ supplemented to half-strength MS medium: A – Shoot formation after 8 weeks of culture, B – Shoot formation after 12 weeks of culture, C – Shoot formation after 16 weeks of culture, D – Shoot formation after 20 weeks of culture (*bar* = 1.0 cm)

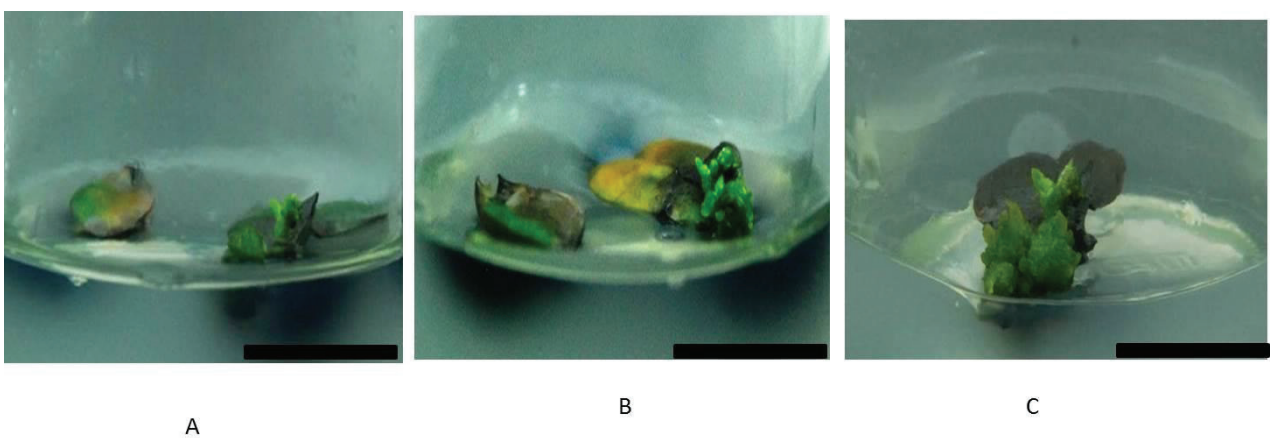

Figure 4: Callus regeneration from leaf explants of the hybrid *Phalaenopsis* 'Pink' at 0.5 mg l^{-1} NAA + 1.5 mg l^{-1} TDZ supplemented to half-strength MS medium: A – Callus formation after 12 weeks of culture, B – Callus formation after 16 weeks of culture, C – Callus formation after 20 weeks of culture (*bar* = 1.0 cm)

3.4 Effects of NAA and TDZ supplemented to VW medium on the growth and direct shoot formation of leaf explants

After five months of culture, the combination of NAA and TDZ supplemented to VW medium showed significant effect on leaf length whereas leaf width was unaffected (Table 4). Higher leaf length and percentage survival rate was obtained with higher concentrations of NAA and TDZ. The length of leaf was found to be optimal in the combination of 0 mg l^{-1} NAA and 1.5 mg l^{-1} TDZ (2.40 ± 0.01 cm) with 30 % survival frequency, as well as in the combination of 0.5 mg l^{-1} NAA and 2 mg l^{-1} TDZ (2.32 ± 0.01 cm) with 50 % survival frequency (Fig. 1d). Overall, the combination of 0.5 mg l^{-1} NAA and 2 mg l^{-1} TDZ supplemented to VW medium showed better performance for explant growth.

Table 4: Effects of NAA and TDZ supplemented to VW medium on leaf formation, percentage of regenerants and survival frequency of the hybrid *Phalaenopsis* 'Pink' leaf explants after five months of culture

NAA (mg l^{-1})	TDZ (mg l^{-1})	Leaf length (cm)	Leaf width (cm)	Regenerants (%)	Survival frequency (%)
0	0	2.00 ± 0.00 d	1.00 ± 0.00	0.00±0.00	20.00 ± 0.14 c
0	0.5	2.00 ± 0.00 d	1.00 ± 0.00	0.00 ± 0.00	20.00 ± 0.12 c
0	1	2.10 ± 0.01 cd	1.00 ± 0.00	0.00 ± 0.00	30.00 ± 0.31 b
0	1.5	2.40 ± 0.01 a	1.00 ± 0.00	0.00 ± 0.00	30.00 ± 0.20 b
0	2	2.20 ± 0.03 bc	1.00 ± 0.00	0.00 ± 0.00	30.00 ± 0.40 b
0.5	0	2.00 ± 0.00 d	1.00 ± 0.00	0.00 ± 0.00	10.00 ± 0.14 d
0.5	0.5	2.22 ± 0.02 bc	1.00 ± 0.00	0.00 ± 0.00	30.00 ± 0.17 b
0.5	1	2.22 ± 0.02 bc	1.00 ± 0.00	0.00 ± 0.00	30.00 ± 0.17 b
0.5	1.5	2.26 ± 0.008 b	1.00 ± 0.00	0.00 ± 0.00	50.00 ± 0.17 a
0.5	2	2.32 ± 0.01 ab	1.00 ± 0.00	0.00 ± 0.00	50.00 ± 0.10 a
F		***	ns	ns	***

Data are means ± standard error (SE) of 20 replicates. Means followed by the same letter in each column do not differ by Duncan's multiple range test at $p \leq 0.05$. ns and *** indicate non-significant and significance at $p \leq 0.001$.

4 DISCUSSION

There are various effects of PGR on embryogenesis (Koh and Loh, 2000). It has been observed that depending on the PGR composition in the culture medium, either somatic embryogenesis or organogenesis can be induced (Jimenez, 2005). The combination with appropriate concentration of NAA and BAP and the composition of macro- and microelements in the culture medium are of key importance for micropropagation of *Phalaenopsis* on a commercial scale, especially to stimulate shoot regeneration (Tokuhara and Mii, 1993).

In this study, the combination of 0 mg l^{-1} NAA and 3 mg l^{-1} BAP as well as 0.5 mg l^{-1} NAA and 2 mg l^{-1} BAP supplemented to half-strength MS medium resulted in 5 % regenerants within 10–11 weeks of culture with 50 % and 40 % survival frequency, respectively. Shoots were formed directly from wounding area and continued to proliferate until 20

weeks of culture (Fig. 2). Subculture was carried out periodically in every three weeks to prevent senescence or necrosis that might be caused by the phenolic compound released from the wounding area of the explants (Kuo et al., 2005). In this study, the growth and proliferation of plantlets were superior in the presence of higher BAP concentration compared with NAA. It has been reported that higher auxin (NAA) concentration compared with cytokinin (BAP) generally leads to root formation on the cut edge, embryogenesis and adventitious root formation whereas higher cytokinin (BAP) concentration generally leads to adventitious shoot formation, auxiliary shoot proliferation (by stimulating cell enlargement or division and growth of lateral bud) and cell cycle control (George and Sherington, 1984; Gaspar et al., 2003; Moharami et al., 2014). The combination of 0 mg l^{-1} NAA and 1 mg l^{-1} BAP supplemented to VW medium resulted in 5 % regenerants where callus directly appeared from the basal region near the wounding area. It has been reported that the combination of 0.5 mg l^{-1} NAA and 2 mg l^{-1} BAP supplemented to modified MS medium was the most suitable combination for direct shoot formation without callus-mediated regeneration (Košir et al., 2004).

The presence of cytokinin (TDZ) was reported to play an important role in callus induction, subculture and subsequent plant regeneration of *Phalaenopsis* (Mok et al., 1982; Chen et al., 2000). In this study, the combination of 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} TDZ supplemented to half-strength MS medium resulted the highest percentage (25 %) of regenerants and calluses which were formed within 11 weeks of culture with 90 % survival frequency. The combination of 0 mg l^{-1} NAA and 2 mg l^{-1} TDZ supplemented to half-strength MS medium produced the highest leaf number and leaf width with 10 % of regenerants (formed after 8 weeks of culture) with 60 % survival rate (Fig. 3). In *Phalaenopsis*, it has been reported that TDZ promoted repetitive embryogenesis from zygotic protocorms (Chen and Chang, 2004) and induced a higher frequency of regeneration from leaf explants compared with BAP and KIN, since leaf tips had a higher capacity to form embryos than other leaf regions (Chen et al., 2000; Chen and Chang, 2001; Kuo et al., 2005).

5 CONCLUSION

This study showed that the combination of 0 mg l^{-1} NAA and 3 mg l^{-1} BAP supplemented to half-strength MS medium was optimal for direct shoot regeneration with 5 % regenerants and 50 % of survival frequency. The combination of 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} TDZ supplemented to half-strength MS medium proved to be optimal concentration in enhancing the callus formation with 25 % regenerants and 90 % of survival frequency. However, further research needs to be undertaken to find out the shortest time of direct shoot regeneration without callus mediated regeneration and to produce viable acclimatized plants with a high survival frequency.

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7 REFERENCES

Arditti J.R., Ernst. (1993). *Micropropagation of Orchids*. Wiley-Interscience, New York, USA.

Chen Y.C., Chang C., Chang W.C. (2000). A reliable protocol for plant regeneration from callus culture of *Phalaenopsis*. *In Vitro Cellular & Developmental Biology – Plant*, *36*, 420–423. doi:10.1007/s11627-000-0076-5

Chen J.T., Chang W.C. (2001). Effects of auxins and cytokinin on direct somatic embryogenesis from leaf explants of *Oncidium* 'Grower Ramsey'. *Plant Growth Regulation, 34*, 229–232. doi:10.1023/A:1013304101647

Chen J.T., Chang W.C. (2004). Induction of repetitive embryogenesis from seed derived protocorms of *Phalaenopsis amabilis* 'Formosa Shimadzu'. *In Vitro Cellular & Developmental Biology – Plant, 40*, 209–293. doi:10.1079/IVP2003527

Gaspar T.H., Kevers C., Faivre-rampant O., Crevecoeur M., Penel C., Greppin H., Dommes J. (2003). Changing concepts in plant hormones action. *In*

Vitro Cellular & Developmental Biology – Plant, 39, 85–106. doi:10.1079/IVP2002393

George F.E., Sherrington P.D. (1984). Plant Propagation by Tissue Culture: *Handbook and directory of commercial laboratories*. Exegetics Ltd., Edington, England, UK, 1984. pp. 709.

Ishii Y., Takamura T., Goi M., Tanaka M. (1998). Callus induction and somatic embryogenesis of *Phalaenopsis*. *Plant Cell Reports, 17*, 446–450. doi:10.1007/s002990050423

Jimenez V.M. (2005). Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. *Plant Growth Regulation, 47*, 91–110. doi:10.1007/s10725-005-3478-x

Košir P., Škof S., Luthar Z. (2004). Direct shoot regeneration from nodes of *Phalaenopsis* orchids. *Acta Agriculturae Slovenica, 83*, 233–242.

Koh W.L., Loh C.S. (2000). Direct somatic embryogenesis, plant regeneration and *in vitro* flowering in rapid cycling *Brassica napus*. *Plant Cell Reports, 19*, 1177–1183. doi:10.1007/s002990000268

Kuo H.L., Chen J.T., Chang W.C. (2005). Efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* 'Little Steve'. *In Vitro Cellular & Developmental Biology – Plant, 41*, 453–456. doi:10.1079/IVP2005644

Lu C.Y. (1993). The use of thidiazuron in tissue culture. *In Vitro Cellular & Developmental Biology – Plant, 29*, 92–96. doi:10.1007/BF02632259

Moharami L., Hosseini B., Ravandi E.G., Jafari M. (2014). Effects of plant growth regulators and explant types on *in vitro* direct plant regeneration of *Agastache foeniculum*, an important medicinal plant. *In Vitro Cellular & Developmental Biology – Plant, 50*, 707–711. doi:10.1007/s11627-014-9640-2

Mok M.C., Mok D.W.S., Armstrong D.J., Shudo K., Isogai Y., Okamoto T. (1982). Cytokinin activity of N-Phenyl-N'- (1,2,3-thidiazuron-5-yl) urea (thidiazuron). *Phytochemistry, 21*, 1509–1511. doi:10.1016/S0031-9422(82)85007-3

Murashige T., Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum, 15*, 473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Niknejad A., Kadir M.A., Kadzimin B.S. (2011). *In vitro* plant regeneration from protocorms-like bodies (PLBs) and callus of *Phalaenopsis gigantea* (Epidendroidaceae: Orchidaceae). *African Journal of Biotechnology, 10*, 11808–11816.

Park S.Y., Murthy H.N., Paek K.Y. (2001). Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. *In Vitro Cellular & Developmental Biology – Plant, 38*, 162–172.

Purohit S.S. (2005). *Plant tissue culture*. Student Edition, Singapore. pp. 100–125.

Teob E.S. (1989). *Orchid of Asia.* Times Books International, Singapore.

Tokuhara K., Mii M. (1993). Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Reports, 13*, 7–11. doi:10.1007/BF00232306

Tokuhara K., Mii M. (2001). Induction of embryonic callus and cell suspension culture from shoot tips excised from flower stalk Buds of *Phalaenopsis* (Orchidaceae). *In Vitro Cellular & Developmental Biology – Plant, 37*, 457–461. doi:10.1007/s11627-001-0080-4

Vacin E., Went F.W. (1949). Some pH changes in nutrient solutions. *Botanical Gazette, 110*, 605–613. doi:10.1086/335561

Zahara M., Datta A., Boonkorkaew P. (2016). Effects of sucrose, carrot juice and culture media on growth and net CO_2 exchange rate in *Phalaenopsis* hybrid 'Pink'. *Scientia Horticulturae, 205*, 17–24. doi:10.1016/j.scienta.2016.04.011

doi:10.14720/aas.2018.111.1.02 Original research article / izvirni znanstveni članek

A comparative study of using a wooden storage box and storage platform for white yam tuber storage

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ABSTRACT

The study was undertaken to evaluate the performances of an experimental box and platform, as storage structures for white yam tubers (*Dioscorea rotundata* Poir.). The criteria used for evaluation were the degree of mass loss during storage, tuber sprouting and rotting during the 20 weeks storageperiod: March - June 2015. Measurements of temperatures and relative humidity of the storage environment were taken three times daily during the period. Mass loss in each tuber was measured weekly while sprouts were removed from tubers weekly. Results show that the average temperature and relative humidity in the experimental box were 29.7 °C and 78.6 % respectively, while for the platform, they were 30.7 °C and 76.5 %., respectively. Rotting was completely absent on tubers in both storage approaches. White yam tubers stored in the box exhibited a cumulative mass loss of 9 %, while on the platform, it was15.0 %. The experimental box performed better in respect to mass loss and nutritional composition.

Key words: white yam; *Dioscorea rotundata;* storage techniques; , tuber mass loss; rotting; sprouting

IZVLEČEK

PRIMERJAVA UPORABE LESENIH ZABOJEV IN PLATOJEV PRI HRAMBI GOMOLJEV BELEGA JAMA

V raziskavi je je bila ovrednotena obstojnost gomoljev belega jama (*Dioscorea rotundata* Poir.) med shranjevanjem v lesenih zabojih in na platojih. Kriteriji za ovrednotenje ohranjenosti gomoljev so bili izguba mase, kalitev in gnitje gomoljev v obdobju 20 tednov, od marca do junija 2015. Meritve temperature in relativne zračne vlage v prostorih hrambe so potekale trikrat dnevno v obdobju trajanja poskusa. Meritve izgube mase vsakega gomolja so potekale tedensko, pravtako odstranjevanje kalečih poganjkov. Povprečna temperatura zraka in relativna vlažnost v poskusnih zabojih sta bili 29.7 °C in 78.6 %, v platojij 30.7 °C in 76.5 %. Gnitja gomoljev ni bilo pri nobenem načinu hrambe. Gomolji belega jama, shranjeni v zabojih, so kumulativno izgubili 9 % mase, tisti v platojih pa 15.0 %. Shramba v zabojih se je izkazala boljša glede izgube mase in hranilne vrednosti gomoljev.

Ključne besede: beli jam; *Dioscorea rotundata;* načini hrambe; izguba mase gomoljev; gnitje; kalitev

1 INTRODUCTION

The term yam is used for several economically important species of the genus of *Dioscorea* which belong to the monocotyledonous family Dioscoreaceae. Yams originated in the Far East and today are grown in most of the trpical regions for their edible tubers (enlarged, fleshy, usually underground storage stems). West and Central Africa account for about 94 % of the world production, Nigeria being the major producer (Osunde, 2008).

Most edible yams species reach maturity in 8 to 11 months after planting. As a seasonal crop, harvested yam tubers are stored to meet the demand during the off season period. Adequate aeration, reduction of temperature, protection from direct sunlight and flood, and regular inspection are the basic requirements for successful and long term storage of yam tubers (Orhevba and Osunde, 2006; Sunmonu et al., 2017). Ventilation prevents moisture condensation on the tuber surface and is helpful in removing heat caused by respiration while low temperature is necessary to reduce losses from respiration, sprouting and rotting.

The storage structures may include trench or clamp silos, underground pits, barns of various designs, shelves in specially constructed or improvised sheds,

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raised huts and assorted platforms. Storage techniques include cold storage, improved underground storage and improved yam barns. The popularity of these structures varies from one region to another, and the choice depends mainly on the volume to be stored and the cost (Adeniran et al., 2014).

Techniques and methods developed to reduce the physiological activities and also to protect tubers from post-harvest diseases can include treatments with plant extracts, palm wine and gamma irradiation, chemicals like gibberellic acid (GA), chloroisopropyl phenylcarbamate (CIPC), maleic hydrazide and other chemicals.

2 MATERIALS AND METHODS

2.1 Plant material

Mature white yam (*Dioscorea rotundata* Poir.) tubers from the second harvest period of 2014 growing season were obtained from the yam market in Ilorin. A detailed inspection was carried out in order exclude yam tubers that were mechanically damaged, and also to avoid yams gathered from early harvest period which are quite difficult to store due to high water content and are susceptible to rot. (Opara, 1999).

2.2 Experimental site

A well ventilated open area under a tree shade within the premises of the Department of Agricultural and Biosystems Engineering, University of Ilorin, was selected to simulate farm practice.

2.3 Experimental setup

The experiment was conducted from March to June 2015. The material used in the experiment included: wooden box, 20 fresh tubers of yam, a digital weighing balance, dry and wet bulb thermometer and a platform.

The tested storage techniques were an experimental box and a platform. The demensions of the experimental box (Figure 1) were 1.75 × 1.50 × 0.6 m. All 20 yam tubers were weighed to determine the initial mass. Tubers were arranged without touching each other. Perforations were provided on the sides of the box for adequate ventilation purpose. For the construction of the storage platform, a total of four Y-shaped wooden columns measuring 1.2 m in length and 8 cm in diameter were erected at 0.9 m between the columns. The columns were erected to adepth of 30 cm into the ground. Four *Bambusa vulgaris* Schrad, ex J.C.Wendl. was used to construct the platform on which the yam tubers were stored. Rodent guards installed at the columns bases was made from aluminium sheets. The yam tubers were then heaped on the storage platform and covered with palm leaves.

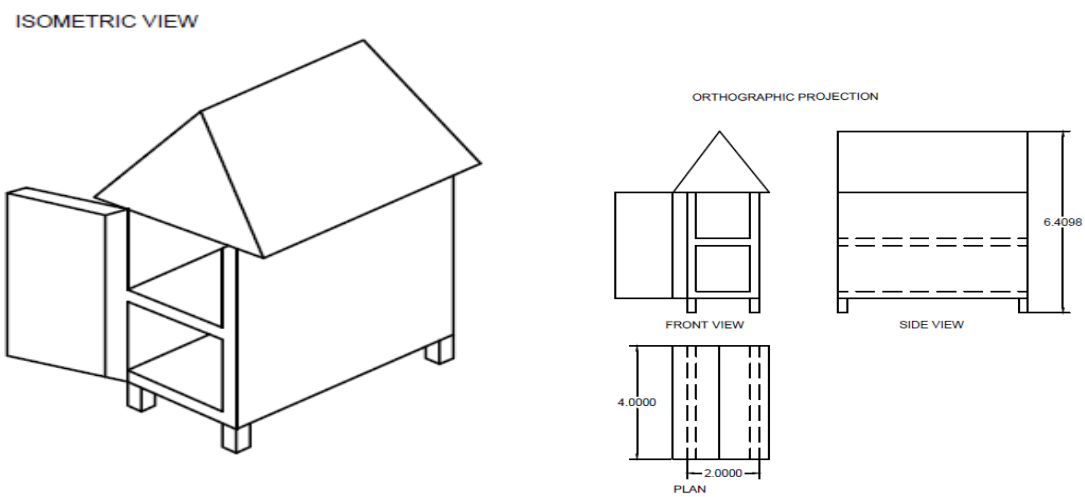

Figure 1: Isometric and orthographic projection of the experimental box for yam storage used in the study

2.4 Studied parameters

2.4.1 Temperature and relative humidity

Temperature readings of the storage structure and environment were taken at 8.00 a.m, 12.00 p.m. and 5.00 p.m. on a daily basis during ten weeks using wet and dry bulb thermometers.
The amount of moisture contained in the atmosphere of the wooden box storage structure was determined with a hygrometer tables and psychometric chart and EL Wifi TH Lascar data logger to ensure the accuracy of the data obtained.

2.4.2 Rotting and sprouting

Evaluation of yam tubers in each storage structure included visual inspection and numerical recording. . The rotting index was determined according to Opara's (1999) formula shown in equation 1:

$$\text{Rotting Index} = \frac{\text{Number of deteriorated tubers}}{\text{Total number of tubers}} \times 100$$

2.4.3 Percentage mass loss determination

The tubers were labeled to enable identification and weighed before the commencement of the experiment. The mass of the tubers was recorded every week and the difference between subsequent masses represented the weekly loss. Mass was measured with the aid of a digital weighing balance having 0.5 g accuracy.

2.4.4 Nutritional value

The nutritional parameters such as moisture, ash and crude fiber contents were determined using Standard Chemical Methods described by Association of Official Analytical Chemistry (AOAC 1996). 2 g of each sample was oven dried at 105 °C for 24 hours in order to determine the moisture content, while the ash content was determined by incinerating 2 g of each sample in a muffle furnace at 500 °C for 2 hours. In the determination of the fat content of the samples, Soxhlet extraction technique was used. (Pearson et al., 1981). Kjedahl method was, however, used to determine the crude protein content of the samples as described by (AOAC, 1996). The percentage content of carbohydrate of the samples was estimated using equation 2.

Carbohydrate content, % = (100-(moisture + ash + fat + crude fibre + crude protein contents)

3 RESULTS AND DISCUSSION

3.1 Temperature and relative humidity variation

The experimental box and platform temperatures are presented in Figure 2. While the temperatures in the experimental box varied from 29.5 to 30.2 °C with an average value of 29.7 °C, those within the platform varied from 30.2 to 31.04 °C with an average value of 30.7 °C. The temperatures within the experimental box were generally lower than those recorded in the platform for all periods throughout the experimentation period..

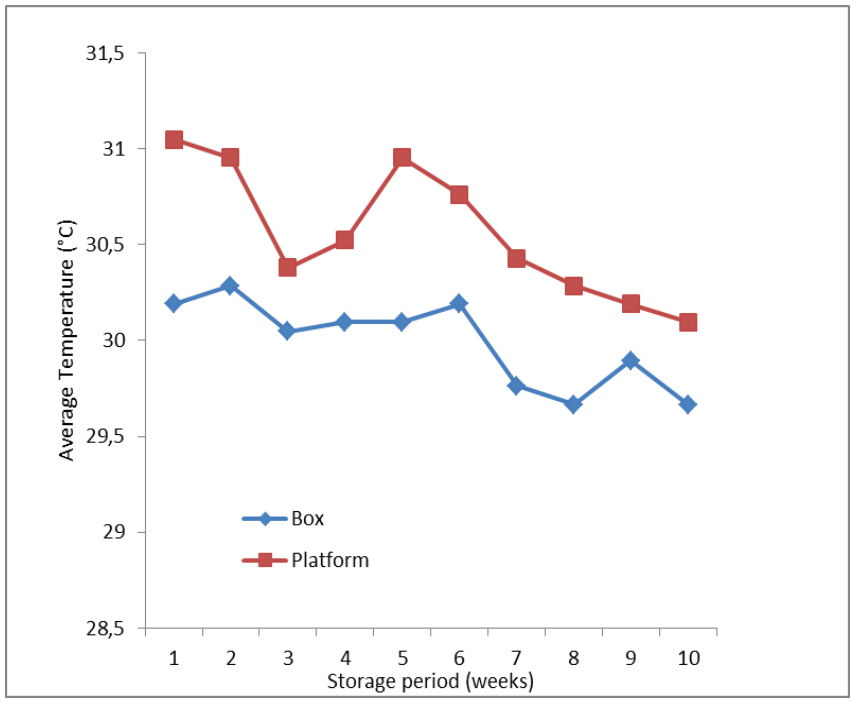

Figure 2: Temperature fluctuations with storage period

Temperature distribution trend is significant in the storage of yam tubers as it has a great influence on massloss High environmental temperature increases respiration rates, enhances significant sprouting activity during the late storage period and consequently causes a significant massloss of the tubers. The reason is that at high temperature, there is the tendency for increased metabolic activity and transpiration process, which are associated with the total energy content of the tuber, all resulting to massloss (Kay, 1973).

The relative humidity ranged from 77 to 80 % with an average value of 78.6 % for the experimental box while for the platform, the range was from 74 to 77 % with an average of 76.5 %. (Figure 3)
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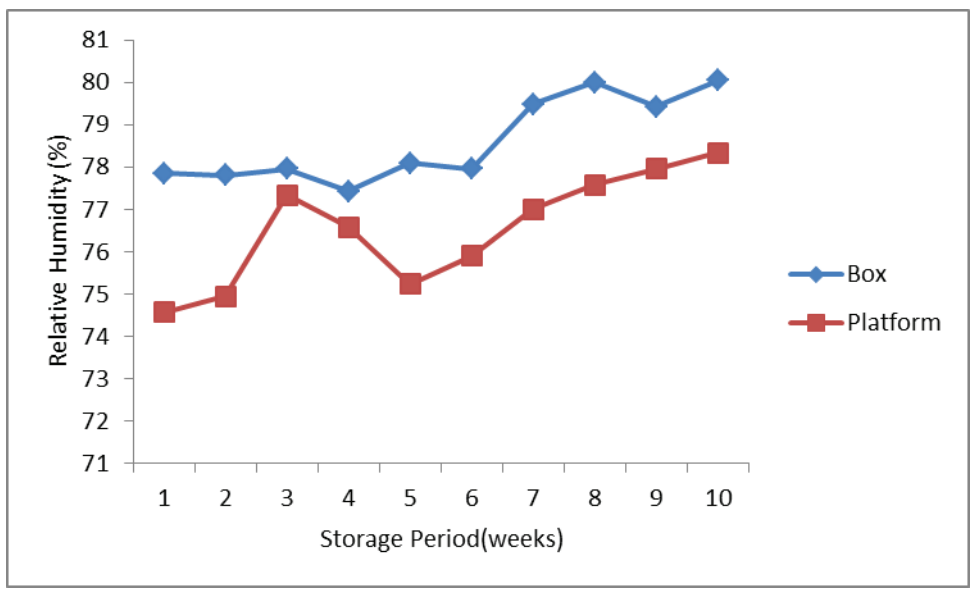

Figure 3: Relative humidity versus storage period

The high humidity values recorded in the evaporative cooling box was attributed to the rainy season at that particular period of the year. The air in contact with it was often laden with moisture and high humidity condenses moisture from air.

3.2 Mass loss

Comparing the cumulative massloss of the tubers stored in the experimental box and those stored on the platform, it was observed that yam tubers in the experimental box sustained a massloss of 1.1 to 9.2 % after 10 weeks of storage, while those in the platform exhibited a massloss ranging from 1.8 to 15.2 % in the same storage period.

The weekly rate and cumulative masslosses observed in the stored yam tubers are presented in Figures 4 and 5 while the statistical analysis is presented in Tables 1 and 2. The values of the weekly masslosses were higher on the platform than in the experimental box throughout the experimentation period. There was a significant difference ($p > 0.05$) between the rates of masslosses in the experimental box and the platform.

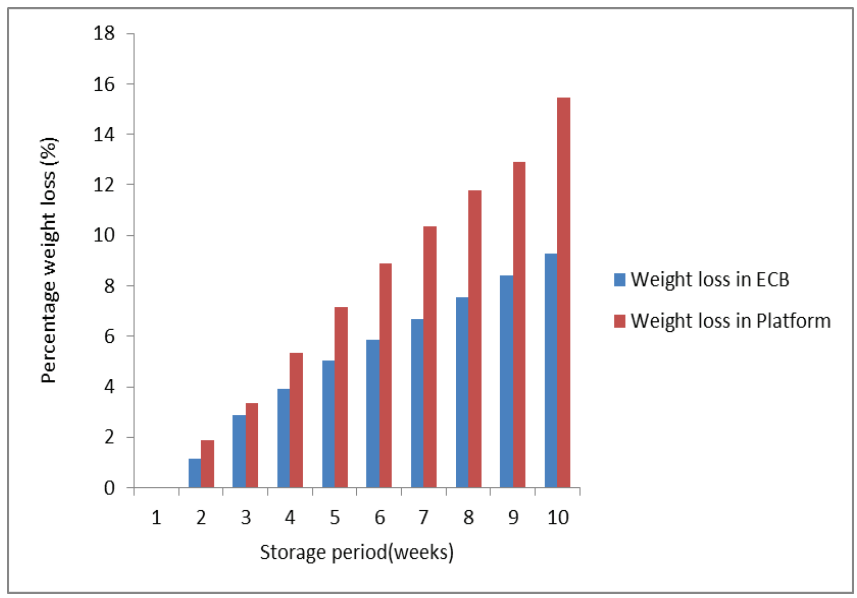

Figure 4: Weekly mass loss

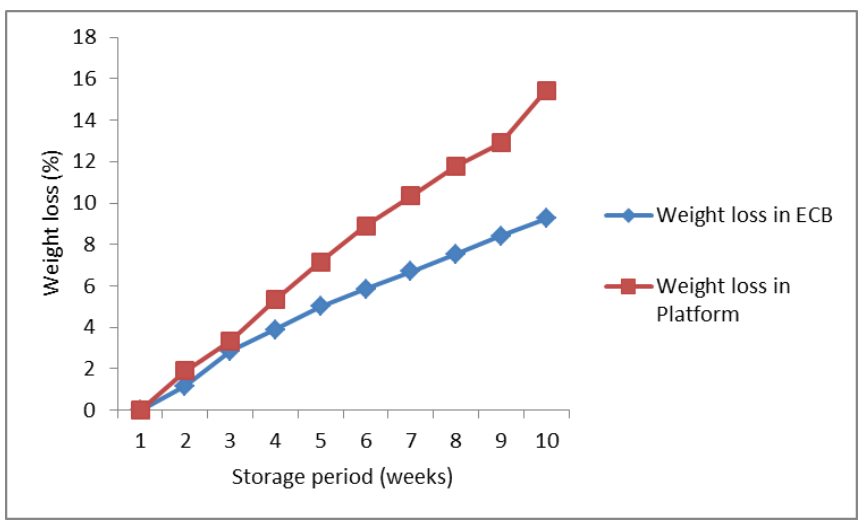

Figure 5: Cumulative mass loss

Table 1: Analysis of variance of the mass loss recorded in the evaporative cooling box

	Sum of Squares	df	Mean Square	F	Sig.
Treatment	.001	8	.000	1.08	0.39
Error	.005	72	.000		
Total	.006	80			

There is no significant different in the weight loss of the at $\alpha = 0.05$

Table 2: Analysis of variance of the massloss recorded on the platform

	Sum of Squares	df	Mean Square	F	Sig.
Treatments	.001	8	.000	2.46	.02
Errors	.002	72	.000		
Total	.003	80			

There is significant different in the weight loss of the second structure $\alpha = 0.05$

3.3 Proximate Analysis

An account on the effect of the storage conditions (evaporative cooling box and platform) on the nutritional composition of white yam selected for the study is given in Table 3. The analysis was done just before and after the storage experiment. The nutritional composition analyzed comprised the ash, carbohydrates, fats and oil, fiber, moisture and protein contents.

Table 3: Proximate analysis of yam tubers before and after storage

Parameters	Before storage	Experimental box	Platform
Moisture Content %	43.26	42.62	34.38
Ash Content %	2.57	2.08	1.64
Fat and Oil %	1.75	1.53	1.41
Crude Fiber %	0.95	2.79	2.23
Carbohydrate Content %	51.47	47.01	32.72

3.4 Physical observation

Rotting of white yam tubers was one of the physiological parameters considered in this study. There was however no incidence of rotting among the white yam tubers stored on the platform and experimental box throughout the period of the experiment. Sprouting is promoted by humid environment and high temperatures. Higher relative humidity and temperatures within the white yam tubers stored in the experimental box were the major factors responsible for the higher level of sprouting in the experimental box than the platform.

4 CONCLUSIONS

Yam tubers were stored in an experimental storage box and storage platform over a period of 20 weeks. While the experimental box maintained an environment of 29.5 °C and 78.6 % relative humidity, the platform environment was 30.7 °C and 76.5 % relative humidity. The tubers stored in the experimental box were observed to have sprouted more than those on the platform but the overall massloss of the yam tubers was more on the platform (15.2 %) than in the experimental box (9.2 %). There was no rotting observed among the tubers stored. The experimental box is able to reduce the massloss which could be an advantage for farmers as yams are priced on massbasis and it may therefore be preferred.

Further work which should involve longer storage periods and determination of the sensory qualities of the stored white yam tuber is recommended.

5 REFERENCES

Adejumo O. I. (1998). Yam storage structures in western Nigeria. *Proceedings of the Nigeria Society of Agricultural Engineers (NSAE) conference*, Bauchi, Nigeria. pp. 143- 151.

Adeniran, K. A., Mijinyawa, Y., Akpenpuun, T. D. and Oseni T. D. (2014). Engineering Properties of Termite Mound Bricks as a Construction Material for Agricultural Buildings. *Journal of Agricultural Engineering and Technology (JAET)*, *22* (4), pp.143 – 150.

Ajadi, B.S., Adeniyi A., Afolabi M. T. (2011). Impact of climate on urban agriculture: Case Study of Ilorin City, Nigeria. *Global Journal of Human Social Science*, 11 (1), pp. 12. 25.

(AOAC), 1996. Official methods of analysis of the association of the official analytical chemists. 19th Edn.,Washington DC.

Food and Agriculture Organization of the United Nations (FAO) (1998). *Storage and processing of Roots and Tubers in the Tropics*. International Institute of Tropical Agriculture (IITA) 2007. Yam research for development. IITA Publication 1, pp. 1-10.

Hahn S.K., D.S.O. Osiru, M.O. Akoroda and J.A. Otoo (1987). Yam production and its future prospect. *Outlook on Agriculture, 16*(3), 105-109. https://doi.org/10.1177/003072708701600302

Kay, D.E. (1973). *Crop and product digest*. Root crops. 2nd Edition, London.

Knoth J. (1993). *Traditional Storage of Yams and Cassava and Its Improvement*. http://goo.gl/1ItQpD

Opara L. U. (1999). Yam Storage In: Bakker–Arekema et al. (eds). *CIGR Handbook of Agricultural Engineering*. Vol. IV. Agro Processing 182 – 214. The American Society of Agricultural Engineers.

Orhevba B. A, Osunde Z. D. (2006)**.** Effects of storage condition and cipc treatments on sprouting and weight loss of stored yam tubers. *Proceedings of the 28th Annual Conference of the Nigerian Institution of Agricultural Engineers*. 28, pp. 352-360.

Orkwor, G.C, R. Asiedu and I.J Ekanayake eds. (1998). Food Yams: Advances in: *Research, IITA*, Ibadan and NRCRI, Umudike, Nigeria. 249p.

Osagie A.U. (1992). *The yam tuber in storage*. Post-Harvest Research Unit, University of Benin, Nigeria pp. 107-173.

Osunde, Z.D. and Orhevha, B. A. (2009). Effect of storage condition and storage period on nutritional qualities of stored yam (Diacorea spp) Tubers. *AJF, 9* (2) 678 – 690.

Osunde Z. D. and M. G. Yisa (2000). Effect of storage structures and storage period on weight loss and sprout growth on stored yams. *Proceedings of the First International Conference and Millenium General Meeting of the Nigerian Institution of Agricultural Engineers held at University of Ibadan*, Ibadan.

Pearson, D., H. Egan, R.S. Kirk and R. Sawyer (1981). *Pearson's chemical analysis of food*. Edinburgh: Churchill Livingston.

Sunmonu, M. O., Odewoleb, M. M., Obajemihic, O. I., Akpenpuund, T. D. and M. Jimoh (2017). Development of Battery Operated Evaporative Cooling System for Storing Perishables. *The Journal of the Association of Professional Engineers of Trinidad and Tobago, 45* (2), 21-25.

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Field performance of maize (*Zea mays* L.) cultivars under drought stress

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ABSTRACT

This research was carried out in 2014 at the Research Farm of the University of Tabriz, Iran. The experiment was arranged as split plot on the basis of randomized complete block with three replicates to assess the effects of four irrigation intervals (irrigations after 60, 80, 100 and 120 mm evaporation) on physiological and agronomical traits of three cultivars of maize (*Zea mays* L.; 'SC704', 'NS640', 'DC303': late, mid and early maturing, respectively). Irrigation intervals and maize cultivars were assigned to the main and sub-plots, respectively. Leaf temperature of all maize cultivars significantly increased, but chlorophyll content index, maximum efficiency of photosystem II, number of grains per plant, 1000 grain mass, plant biomass, grain yield and harvest index significantly decreased with increasing irrigation intervals. Late maturing cultivar ('SC704') was superior in all studied traits, followed by mid ('NS640') and early ('DC303') maturing cultivars. It was concluded that water limitation can potentially reduce performance of maize cultivars in the field, but the extent of this reduction depends on genotype and severity of stress.

Key words: chlorophyll content; leaf temperature; maize; photosystem II; drought stress

IZVLEČEK

USPEVANJE SORT KORUZE (*Zea mays* L.) V RAZMERAH SUŠNEGA STRESA

Raziskava je bila opravljena v sezoni 2014 na Research Farm of the University of Tabriz, Iran. Poskus je bil zasnovan kot poskus z deljenkami na osnovi popolnega naključnega bločnega poskusa s tremi ponovitvami za ovrednotenje učinkov štirih načinov namakanja (namakanje po 60, 80, 100 in 120 mm evaporacije) na osnovi fizioloških in agronomskih lastnosti treh sort koruze (*Zea mays* L.; 'SC704', 'NS640', 'DC303': zgodnje, srednje in pozno dozorevajoča sorta). Načini namakanja so bili vrednoteni na glavnih ploskvah, sorte koruze na podploskvah. Temperatura lista je pri vseh sortah značilno naraščala z večanjem intervala namakanja, lastnosti kot so indeks vsebnosti klorofila, maksimalna učinkovitost fotosistema II, število zrn na rastlino, masa 1000 zrn, biomasa rastlin, pridelek zrnja in žetveni indeks pa so se z večanjem intervala namakanja značilno zmanjšale. Pozno dozorevajoča sorta ('SC704') je bila v vseh preučevanih lastnostih najboljša, sledili sta ji srednje ('NS640') in zgodaj ('DC303') dozorevajoči sorti. Ugotovljeno je bilo, da pomanjkanje vode lahko potencialno zmanjša uspevanje koruze, a je obseg zmanjšanja odvisen od genotipa in jakosti stresa.

Ključne besede: vsebnost klorofila; temperature lista; koruza; fotosistem II, sušni stres

1 INTRODUCTION

Maize (*Zea mays* L.), also known as corn, is an important crop worldwide, not only because it is the third cereal after wheat and rice, but also because of its various uses and increasing demand (Huang et al., 2006). Maize had its origin in a semi-arid area, but it is not a reliable crop for growing under dry-land conditions, with limited or erratic rainfall (Campos et al., 2004). Monneveux et al. (2006) reported that seasonal drought was the most important limiting factor for producing maize in the world.

Iran is placed in arid and semi-arid region and water shortage is one of the basic problems of agriculture in that area. The crop experiences drought stress from late vegetative stages until maturity (Soltani et al., 2001). When full crop requirements are not met, water deficit

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in the plant can develop to a point, where physiological activities, crop growth and yield are affected. The manner in which water deficit affects crop growth and yield varies with crop species and growth period (Badoni et al., 2009). It has been shown that growth of maize is sensitive to water limitation (Aslam et al., 2013) and water deficit can limit the performance of this crop in the field (Ghassemi-Golezani et al*.,* 2011).

Drought stress can reduce the photosynthetic rate indirectly by closure of the stomata or directly by a reduction of the photosynthetic capacity of the leaves (Sabir et al., 2009). Closure of stomata results in higher leaf temperature due to the loss of the ability for transpiration cooling under water limitation (Lu et al., 1997). Increasing leaf temperature could lead to the combination of drought and heat stresses, leading to leaf scorch (Mohammadian et al*.,* 2005). Dalil and Ghassemi-Golezani (2012) reported that leaf temperature and differences in leaf and air temperatures increase with increasing irrigation intervals in Ksc301 cultivar of maize.

Chlorophyll fluorescence is directly related to plant photosynthesis and the physiological state of vegetation. Therefore, chlorophyll fluorescence measurements have become a widely used method to study the functioning of the photosynthetic apparatus and are a powerful, non-destructive and reliable tool in plant physiology for study the effects of stress on PSII photochemistry (Brestic & Zivcak, 2013). Many studies have shown that photochemical efficiency of photosystem II (Fv / Fm) in plants decreases due to deactivation of antennae to prevent damage by harmful radicals that are formed under different stress conditions (Ghassemi-Golezani et al., 2008; Zarco-Tejada et al., 2009). Nevertheless, little information is available on the effect of drought stress on Fv / Fm in maize cultivars.

Studies showed that the ratio of variable/maximum fluorescence (Fv/Fm) is a quantitative measure for the photochemical efficiency of photosystem II (maximum quantum yield of PSII). The accumulation of excessive excitation energy can cause photo-inhibition or photo-oxidation in the photosynthetic apparatus and the reduced values of Fv/Fm indicate that a proportion of PSII reaction centers were damaged (Ghassemi-Golezani and Lotfi, 2015). Fv/Fm is the most common parameter which responds to drought stress (Gregoriou et al., 2007) and can be used as a physiological index for selecting osmotic stress tolerant cultivars (Paul Parkhill et al. 2001). According to Roohi et al. (2013) reduction in Fv/Fm by drought stress was different. It has been reported that most of this variation was due to differences among crop species. Similar results revealed that the components of the photosynthetic apparatus could be damaged significantly in drought sensitive barley genotypes, while drought tolerant genotypes were relatively less affected. On the other hand, the value of Fv/Fm in drought tolerant genotypes was significantly higher than that in drought sensitive genotypes under drought stress (Rong-Hua et al., 2006).

A better understanding of the physiological and agronomical traits of maize under drought stress would help in selecting the promising maize cultivars for drought resistance. We hypothesized that there were differences in physiological and yield responses among maize cultivars under drought stress. Therefore, the comparative study presented here was carried out under different irrigation intervals in order to (i) compare changes in leaf temperature, chlorophyll content and fluorescence, as parameters influencing photosynthesis in maize cultivars and (ii) determine the consequences for yield and yield component of cultivars.

2 MATERIALS AND METHODS

This research was carried out at the Research Farm of the University of Tabriz, Iran (latitude 38.050 N, longitude 46.170 E, Altitude 1360 m above sea level) in 2014. The climate is characterized by mean annual precipitation of 245.75 mm, mean annual temperature of 10 ˚C, mean annual maximum temperature of 16.6 ˚C and mean annual minimum temperature of 4.2 ˚C.

The experimental design was split plot on the bases of randomized complete block in three replicates. Irrigation treatments (I_1, I_2, I_3, I_4: irrigation after 60, 80, 100 and 120 mm evaporation from class A pan, respectively) were located in main plots and cultivars (‘SC704’, ‘NS640’ and ‘DC303’: late, mid and early maturing, respectively) were assigned to sub plots. Seeds were first treated with 2 g kg^{-1} Mancozeb fungicide and then were hand sown in 5 cm depth of a sandy-loam soil with a density of 10 seeds per m^2. At the same time, plots were fertilized with urea (46 % N) at a rate of 200 kg ha^{-1}. Each plot consisted of nine rows of 2.5 m length, spaced 50 cm apart. All plots were irrigated immediately after sowing. Irrigation treatments were applied after seedling establishment. Hand weeding of the experimental area was carried out as required.

All of the physiological measurements were carried out just before irrigation at silking stage (R1). A plant from

each plot was marked and temperature of leaves (top, middle and bottom leaves) was measured using an infrared thermometer (TES 1327, Taiwan). Chlorophyll content index (CCI) was measured by a portable chlorophyll-meter (CCM-200, Opti-Sciences, USA).

Fluorescence emission was monitored from the upper surface of the leaves. Dark-adapted leaves (30 min) were initially exposed to the weak modulate measuring beam, followed by exposure to saturated white light to estimate the initial (F_0) and maximum (Fm) fluorescence values, respectively (Krause & Weis, 1991). Variable fluorescence (Fv) was calculated by subtracting F_0 from Fm. The Fv/Fm ratio measures the efficiency of excitation energy capture by open PSII reaction centers, representing the maximum capacity of light-dependent charge separation in PSII (Rizza et al., 2001).

At maturity, plants in 1 m^2 of each plot were harvested and above ground biomass (plant biomass), number of grains per plant, 1000 grain mass, grain yield per unit area and harvest index. Analysis of variance appropriate to the experimental design was conducted, using MSTATC and SPSS. Means of each trait were compared according to Duncan multiple range test at $P \leq 0.05$. Excel software was used to draw figures.

3 RESULTS

Analysis of variance showed significant effects of irrigation and cultivar on leaf temperature, maximum efficiency of photosystem II (Fv/Fm), grains per plant, plant biomass, grain yield and harvest index ($P \leq 0.01$). The interaction of these factors were also significant for grains per plant, plant biomass, grain yield and harvest index ($P \leq 0.01$), but not for leaf temperature and Fv/Fm ($P > 0.05$). Mean 1000 grain mass was significantly affected by cultivar ($P \leq 0.01$) and interaction of irrigation × cultivar ($P > 0.05$).

Leaf temperature of plants significantly increased under I_3 and I_4 (Fig. 1A), but there was no significant difference between I_1 and I_2 and also between I_3 and I_4 (Fig. 1A). Early maturing cultivar ('DC303') had the highest leaf temperature, which was significantly reduced in mid ('NS640') and late ('SC704') maturing cultivars (Fig. 1B).

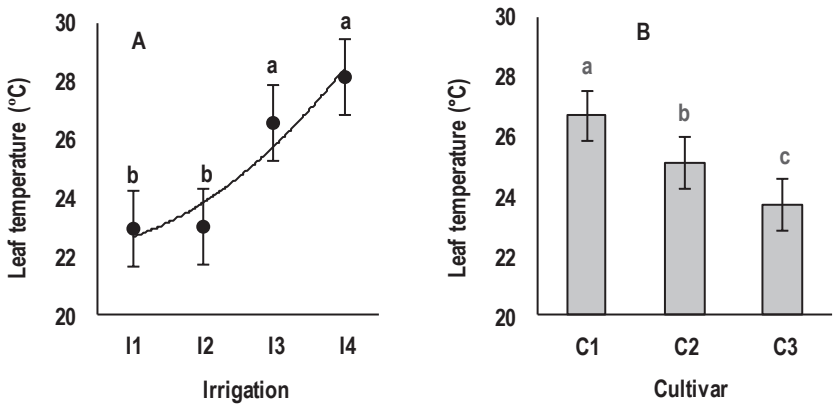

Figure 1: Mean leaf temperature of maize for irrigation treatments (A) and cultivars (B)
I1, I2, I3, I4: Irrigation after 60, 80, 100 and 120 mm evaporation, respectively
C1, C2, C3: 'DC303', 'NS640' and 'SC704' cultivars, respectively

Although, chlorophyll content index (CCI) of maize plants was not significantly affected by irrigation treatments and cultivars, the highest and the lowest CCI were obtained under I_1 and I_4, respectively (Fig. 2A). Chlorophyll content index of late ('SC704') and mid ('NS640') maturing cultivars was greater than that of early maturing cultivar ('DC303') (Fig. 2B).

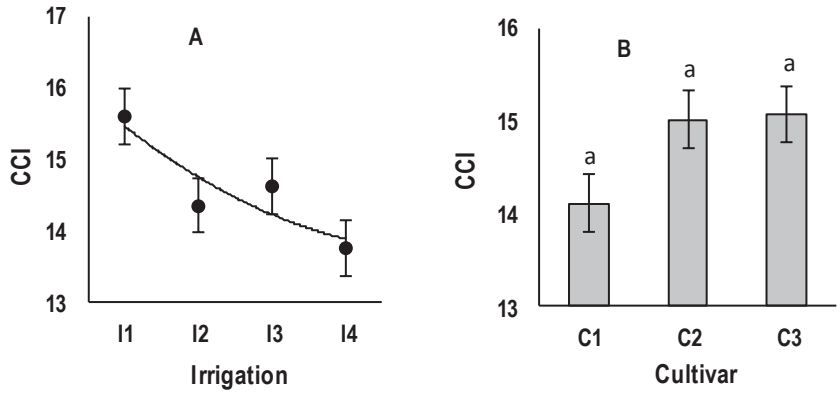

Figure 2: Chlorophyll content index of maize for irrigation treatments (A) and cultivars (B)
I1, I2, I3, I4: irrigation after 60, 80, 100 and 120 mm evaporation, respectively
C1, C2, C3: 'DC303', 'NS640' and 'SC704' cultivars, respectively

Maximum efficiency of PSII (*F*v/*F*m) significantly declined under moderate (I_3) and severe water deficit (I_4), compared with well-watering (I_1) (Fig. 3A). The lowest and the highest *F*v/*F*m were recorded for 'DC303' (early maturing cultivar) and 'SC704' (late maturing cultivar), respectively. However, differences in *Fv/Fm* between 'SC704' and 'NS640' (mid maturing cultivar) and also between 'NS640' and 'DC303' were not statistically significant (Fig. 3B).

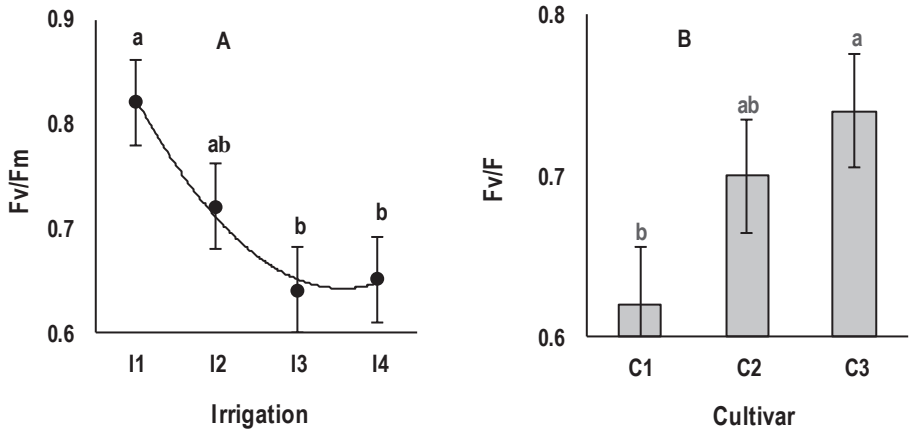

Figure 3: Maximum efficiency of photosystem II in dark- adapted leaves of maize for irrigation treatments (A) and cultivars (B)
I1, I2, I3, I4: irrigation after 60, 80, 100 and 120 mm evaporation, respectively
C1, C2, C3: 'DC303', 'NS640' and 'SC704' cultivars, respectively

Mean number of grains per plant, plant biomass and grain yield per unit area and harvest index of all cultivars decreased with decreasing water availability. The greatest reduction in these traits due to water deficit was observed in 'DC303' (early maturing cultivar), compared with other cultivars (Table 1). The highest grains per plant, 1000 grain mass, biological and grain yields per unit area and harvest index under different irrigation treatments were produced by 'SC704' (late maturing cultivar), followed by 'NS640' (mid maturing cultivar) (Table 1).

Table 1: Means of yield components and grain yield of maize for interaction of irrigation intervals × cultivars

Irrigation treatments	Cultivar	Grains per plant	1000 grain mass (g)	Plant biomass (g/m^2)	Grain yield (g/m^2)	Harvest index (%)
I_1	C_1	351c	132.45e	1770.1c	445.77f	25.18e
	C_2	386b	145.52c	1856.47b	533.98c	29.18c
	C_3	401a	169.45a	1951.1a	628.23a	36.77a
	Mean	379.33	149.14	1859.22	535.99	30.38
I_2	C_1	305f	134.82e	1532.07e	382.09h	24.93e
	C_2	343.67d	143.52cd	1624.4d	474.11e	28.75c
	C_3	389.67b	166.2ab	1762.73c	613.05b	36.1a
	Mean	346.11	148.18	1639.73	489.75	29.93
I_3	C_1	206i	129.81f	1228.9j	258.07j	20.99f
	C_2	290g	138.72d	1374.1h	398.23g	28.97c
	C_3	344d	161.41b	1516.77f	541.21c	34.77b
	Mean	280	143.31	1373.26	399.17	28.24
I_4	C_1	111.33j	128.81f	1102.8k	141.36k	12.81g
	C_2	262.67h	144.44c	1320.9i	360.69i	27.29d
	C_3	317.67e	161.01b	1456.5g	495.51d	34.21b
	Mean	230.57	144.75	1293.4	332.52	24.77
Means for cultivars	C_1	243.33	131.47	1408.47	306.82	20.98
	C_2	320.58	143.05	1543.97	441.75	28.55
	C_3	363.08	164.52	1671.78	569.5	35.46

I_1, I_2, I_3, I_4: Irrigation after 60, 80, 100 and 120 mm evaporation, respectively
C_1, C_2, C_3: Maize cultivars of 'DC303', 'NS640' and 'SC704', respectively

4 DISCUSSION

Increasing leaf temperature with decreasing water availability (Fig. 1A) was the result of stomata closure under drought stress. Under drought stress, rate of water uptake cannot match the transpiration rate and stomata close to maintain the plant water balance (Shahenshah & Isoda, 2010). As a result, leaf temperature rises and may even exceed air temperature (Larcher, 2000). The significant differences among genotypes for leaf temperature (Fig. 1B) indicate appreciable amount of variability among the genotypes. Increasing in leaf temperature due to water loss could reduce net photosynthesis which correlates with a decrease in the activation state of Rubisco in both C_3 and C_4 plants (Salvucci and Crafts-Brandner, 2004).

Decreasing chlorophyll content under drought stress (Fig. 2A) may be partly resulted from low nitrogen uptake (Rimski-Korsakov et al., 2009), oxidative damage of reactive oxygen species (Lotfi et al., 2015) and disorganization of thylakoid membranes (Ladjal et al., 2000). Decreased or unchanged chlorophyll levels during drought stress have been reported in many species, depending on the duration, severity of drought (Anjum et al., 2011) and sensitivity of cultivars (Valifard et al., 2012). The highest chlorophyll content index in late maturing cultivar ('SC704') (Fig. 2B), could be the result of lower leaf temperature in this cultivars (Fig. 1B).

The low *F*v/*F*m values in maize cultivars under drought stress (Fig. 3A) could have resulted from the inactivity

of the reaction centers, which may favor greater energy dissipation in the form of heat and fluorescence, as deduced from the high *F*v/*F*m values (Ghassemi-Golezani and Lotfi, 2015). This may be associated with increased heat sinks (heat sink centers or silent centers), which may absorb light in a similar manner as that of active reaction centers, but are unable to store the excitation energy as redox energy and dissipate their total energy as heat (Hermans et al., 2003). The *F*v/*F*m ratio is frequently used as an indicator of the photo-inhibitor or other injury caused to the PSII complexes (Rohacek, 2002). The higher *F*v/*F*m in late maturing cultivar ('SC704') (Fig. 3B) may be related with lower leaf temperature of this cultivar under different irrigation treatments, compared with other cultivars (Fig. 1B).

Increasing leaf temperature (Fig. 1A) and decreasing chlorophyll content (Fig. 2A) and Fv / Fm (Fig. 3A) are the possible reasons for reduction of biomass accumulation in maize plants under drought stress, which is strongly related with harvest index (Table 1). In other words, the net changes in biomass and harvest index are reflected in grain yield (Gholipoor, 2009). The high plant biomass could lead to the production of more grains per plant and consequently grain yield per unit area (Table 1). This is in agreement with the results of another research about rice (Soni et al*.,* 2013), cowpea (Hosseinian & Majnoun-Hoseini, 2015) and soybean (Ball et al., 2000). Decreasing grain yield per unit area under drought stress can be largely attributed to considerable reduction in number of grains per plant rather than grain mass (Table 1). This is also supported by the other researchers (Gonzalez et al*.,* 2003; Borra´s et al*.,* 2004; Dalil & Ghassemi-Golezani, 2012).

Production of comparatively more grains per plant, heavier grains and higher plant biomass in late maturing cultivar ('SC704') (Table 1) could be attributed to longer period of radiation use and energy store of this cultivar, compared with other cultivars (Sangoi, 2000). These differences resulted in higher grain yield of this cultivar, compared with 'NS640' (mid maturing cultivar) and 'DC303' (early maturing cultivar). Banzinger et al. (2000) reported that number of grains per plant and grain mass could help to determine the grain yield of maize. Variation of these yield components among maize cultivars directly influences grain yield per unit area. The greatest harvest index of 'SC704' under different irrigation intervals (Table 1) suggests a high ratio of grain yield to plant biomass in this cultivar, compared with 'NS640' and 'DC303'. So, harvest index positively related with grain yield and negatively related with plant biomass.

5 CONCLUSION

Drought stress enhances leaf temperature, but reduces chlorophyll content, maximum efficiency of photosystem II, number of grains per plant, grain mass, plant biomass and consequently grain yield and harvest index in maize cultivars, depending on duration of stress. These reductions increase with increasing water limitation. The late maturing maize cultivar ('SC704') showed a superior performance under different irrigation intervals, compared with early and mid-maturing cultivars. Therefore, delayed maturation of maize may be an advantage in areas with sufficient or slightly limited water availability during crop growth and development.

6 REFERENCES

Anjum, S.A., Xie, X., Wang, L., Saleem, M., Man, C., Lei, W. (2011). Morphological, physiological and biochemical responses of plants to drought stress. *African Journal of Agricultural Research, 6*, 2026-2032.

Aslam, M., Zamir, M.S.I., Afzal, I., Yassen, M., Mubeen, M., Shoaib, A. (2013). Drought stress, its effect on maize production and development of drought tolerance through potassium application. *Cercetari Agronomice in Moldova*, *2*, 99-114.

Badoni, A., Nautiyal, M., Gusain, K., Kaur, M., Dhiman, R., Bisht, C., Chauhan, J.S. (2009). Effect of water uptake on germinability in seeds of some medicinal plants. *Journal of American Science, 5*, 123-28.

Ball, R.A., Purcell, L.C., Vories, E.D. (2000). Short-season soybean yield compensation in response to population and water regime. *Crop Science, 40*, 1071-1078. doi:10.2135/cropsci2000.4041070x

Banzinger, M., Edmeades, G.O., Beck, D., Bellon, M. (2000). *Breeding for drought and nitrogen stress tolerance in maize: From theory to practice*. Mexico, D.F.: CIMMYT.

Borra´s, L., Slafer, G.A., Otegui, M.E. (2004). Seed dry weight response to source-sink manipulations in wheat, maize and soybean: a quantitative

reappraisal. *Field Crops Research, 86*, 131-46. doi:10.1016/j.fcr.2003.08.002

Brestic, M., Zivcak, M. (2013). PSII fluorescence techniques for measurement of drought and high temperature stress signal in crop plants: protocols and applications. In G.R. Rout & A.B. Das (Eds), *Molecular stress physiology of plants* (pp. 427). India, Springer. doi:10.1007/978-81-322-0807-5_4

Campos, H., Cooper, M., Habben, J.E., Edmeades, G.O., Schussle, J.R. (2004). Improving drought tolerance in maize view from industry. *Field Crop Research*, *90*, 19-34. doi:10.1016/j.fcr.2004.07.003

Dalil, B., Ghassemi-Golezani, K. (2012). Changes in leaf temperature and grain yield of maize under different levels of irrigation. *Research on Crops*, *13*, 481- 485.

Ghassemi-Golezani, K., Dalil, B., Dabbagh-Mohammadi Nasab, A., Zehtab-Salmasi, S. (2008a). The Response of chickpea cultivars to field water deficit. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 36, 25-28.

Ghassemi-Golezani, K., Dalil, B., Moghaddam, M., Raey, Y. (2011). Field performance of differentially deteriorated seed lots of maize (*Zea mays*) under different irrigation treatments. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *39*, 160-163.

Ghassemi-Golezani, K., Khomari, S., Valizadeh, M., Alyari, H. (2008b). Changes in chlorophyll content and fluorescence of leaves of winter rapeseed affected by seedling vigor and cold acclimation duration. *Journal of Food, Agriculture & Environment*, *6*, 196-199.

Ghassemi-Golezani, K., Lotfi, R. (2015). The impact of salicylic acid and silicon on chlorophyll a fluorescence in mung bean under salt stress. *Russian Journal of Plant Physiology*, *62*, 611–616. doi:10.1134/S1021443715040081

Gholipoor, M. (2009). Evaluating the response of rainfed-chickpea population density in Iran, using simulation. *World Academy of Sciences, Engineering & Technology,* 49, 97-104.

Gonzalez, F.G., Slafer, G.A., Miralles, D.J. (2003). Grain and floret number in response to photoperiod during stem elongation in fully and slightly vernalized wheat. *Field Crops Research, 81*, 17-27. doi:10.1016/S0378-4290(02)00195-8

Gregoriou, K., Pontikis, K., Vemmos S. (2007). Effects of reduced irradiance on leaf morphology, photosynthetic capacity, and fruit yield in olive (*Olea europaeaL.*). *Photosynthetica*, *45*, 172–181. doi:10.1007/s11099-007-0029-x

Hermans, C., Smeyers, M., Rodriguez, R.M., Eyletters, M., Strasser, R.J., Delhaye, J.P. (2003). Quality assessment of urban trees: A comparative study of physiological characterization, airborne imaging and on site fluorescence monitoring by the O-J-I-P-test. *Journal of Plant Physiolog*y, *160*, 81-90. doi:10.1078/0176-1617-00917

Hosseinian, H., Majnoun-Hoseini, N. (2015). Analysis of correlation coefficients between grain yield and yield components in cowpea genotypes under normal and drought conditions stress. *Iranian Journal of Field Crop Science*, *45*, 575-583.

Huang, R., Birch, C.J., George, D.L. (2006). Water use efficiency in maize production. The challenge and improvement strategies. 6th Triennial Conference, Australia.

Krause, G.H., Weis, E. (1991). Chlorophyll fluorescence and photosynthesis: The basics. *Annual* Review of *Plant Physiology* and *Plant Molecular Biology*, *42*, 313-349. doi:10.1146/annurev.pp.42.060191.001525

Ladjal, M., Epron, D., Ducrey, M. (2000). Effects of drought preconditioning on thermo tolerance of photosystem II and susceptibility of photosynthesis to heat stress in cedar seedlings. *Tree Physiology, 20*, 1235–1241. doi:10.1093/treephys/20.18.1235

Larcher, W. (2000). Temperature stress and survival ability of Mediterranean sclerophyllous plants. *Plant Biosystems, 134*, 279-95. doi:10.1080/11263500012331350455

Lotfi, R., Pessarakli, M., Gharavi-Kouchebagh, P., Khoshvaghti, H. (2015). Physiological responses of *Brassica napus* to fulvic acid under water stress: Chlorophyll a fluorescence and antioxidant enzyme activity. *The Crop Journal*, *3*, 434-439. doi:10.1016/j.cj.2015.05.006

Lu, Z., Chen, J., Percy, R.G., Zeiger, E. (1997). Photosynthetic rate, stomatal conductance and leaf area in two cotton species (*Gossypium barbadense* and *Gossypium hirsutum*) and their relation with heat resistant and yield. *Austuralian Journal of Plant Physiology, 24*, 693-700. doi:10.1071/PP97056

Mohammadian, R., Moghaddam, M., Rahimian, H., Sadeghian, S.Y. (2005). Effect of early season drought stress on growth characteristics of sugar beet genotypes. *Turkish Journal of Agriculture and Forestry, 29*, 357-68.

Monneveux, P., Sanchez, C., Beck, D., Edmeds, G.E. (2006). Drought tolerance improvement in topical maize source populations: evidence of progress. *Journal of Crop Science, 46*, 180-191. doi:10.2135/cropsci2005.04-0034

Paul Parkhill, J., Maillet, G., Gullen, J.J. (2001). Fluorescence-based maximal quantum yield for PS II as a diagnostic of nutrient stress. *The Journal of Physiology*, 37, 517-529.

Rizza, F., Pagani, D., Stanca, A.M., Cattivelli, L. (2001). Use of chlorophyll florescence to evaluate the cold acclimation and freezing tolerance of winter and spring oats. *Plant Breeding*, *120*, 389-396. doi:10.1046/j.1439-0523.2001.00635.x

Rong-Hua, L., Pei-Guo, G., Baum, M., Grando, S., Ceccarelli, S. (2006). Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. *Agricultural Science in China*, *5,* 751-757. doi:10.1016/S1671-2927(06)60120-X

Roohi, E., Tahmasebi-Sarvestani, Z., Modarres-Sanavy, S.A.M. Siosemardeh, A. (2013). Comparative study on the effect of soil water stress on photosynthetic function of triticale, bread wheat, and barley. *Journal of Agricultural Science & Technology*, *15*, 215-228.

Rimski-Korsakov, H., Rubio, G., Lavado, R.S. (2009). Effect of water stress in maize crop production and nitrogen fertilizer fate. *Journal of Plant Nutrition, 32*, 565-578. doi:10.1080/01904160802714961

Rohacek, K. (2002). Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationship. *Photosynthetica*, *40*, 13-29. doi:10.1023/A:1020125719386

Sabir, P., Ashraf, M., Hussain, M., Jamil, A. (2009). Relationship of photosynthetic pigments and water relations with salt tolerance of roso millet (*Panicum miliaceum* L.) accessions. *Pakistan Journal of Bot*any, *41*, 2957-2964.

Salvucci, M.E., Crafts-Brandner, J.S. (2004). Inhibition of photosynthesis by heat stress: the activation state of rubisco as a limiting factor in photosynthesis. *Physiologia Plantarum, 120*, 179-86. doi:10.1111/j.0031-9317.2004.0173.x

Sangoi, L. (2000). Understanding plant density effects on maize growth and development: an important issue to maximize grain yield. *Ciencia Rural*, *31*, 159-168. doi:10.1590/S0103-84782001000100027

Shahenshah, Isoda, A. (2016). Effects of water stress on leaf temperature and chlorophyll fluorescence parameters in cotton and peanut. *Plant Production Science*, *13*, 269-278. doi:10.1626/pps.13.269

Soltani, A., Khooie, F.R., Ghassemi-Golezani, K., Moghaddam, M. (2001). A simulation study of chickpea crop response to limited irrigation in a semi-arid environment. *Agricultural Water Management, 49*, 225-37. doi:10.1016/S0378-3774(00)00143-8

Soni, S., Yadav, V.K., Pratap, N., Bhadana, V.P., Ram, T. (2013). Selection criteria, yield relationship with component traits and grouping of tropical japonica, indica lines and derived hybrids of rice (O*ryza sativa* L.). *SAARC Journal of Agriculture*, *11*, 17-32.

Valifard, M., Moradshahi, A., Kholdebarin, B. (2012). Biochemical and physiological responses of two wheat (*Triticum aestivum* L.) cultivars to drought stress applied at seedling stage. *Journal of Agricultural Science & Technology, 14,* 1567-1578.

Zarco-Tejada, P.J., Berni, J.A.J., Suarez, L., Sepulcre-Canto, G., Morales, F., Miller, J.R. (2009). Imaging chlorophyll fluorescence with an airborne narrow-band multispectral camera for vegetation stress detection. *Remote Sensing & Environment*, *113*, 1262–1275. doi:10.1016/j.rse.2009.02.016

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Changes in dry matter, protein percentage and organic matter of soybean-oat and groundnut-oat intercropping in different growth stages in Jilin province, China

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ABSTRACT

One of the most important and sustainable cropping practice is intercropping. The study was conducted under field conditions in the arid Horqine sandy land in Baicheng District, Jilin Province, Northern China in 2011. A randomized complete block design with four replications was used. Treatments comprised different mono cropping and intercropping patterns, TO: sole cropping of oat, TOS-O: oat in the intercropping of oat and soybean, TOG-O: oat in the intercropping of oat and groundnut, TS: sole cropping of soybean, TOS-S: soybean in intercropping of oat and soybean, TG: sole cropping of groundnut, TOG-G: groundnut in the intercropping of oat and groundnut. In mono-cropping systems, oat mono-cropping obtained the highest dry matter and nitrogen accumulation in all growth stages. The maximum protein percentage in all stages except for ripening stage, were for groundnut mono-cropping. Although, the maximum organic matter in ripening stage was achieved in mono-cropping of soybean, the highest one in other stages was related to groundnut mono-cropping. In intercropping patterns, oat in oat-groundnut obtained the highest dry matter in all stages. The highest value of protein percentage and organic matter in heading stage, grain filling stage, and grain dough stage was achieved in groundnut in oat-groundnut intercropping. Furthermore, the maximum value of protein percentage and organic matter in booting stage and ripening stage was related to soybean in oat-soybean intercropping. The results of this study clearly indicate that intercropping oat and groundnut affects the growth rate of the individual species in mixtures as well as the dry matter yield and nitrogen accumulation. This information can help in the adaptation of oat- intercrops for increased forage production in new cropping systems.

Key words: protein percentage; organic matter; soybean; groundnut; oat; intercropping

IZVLEČEK

SPREMEMBA SUŠINE, ORGANSKE MASE IN VSEBNOSTI BELJAKOVIN V VMESNIH POSEVKIH SOJE, OVSA IN ARAŠIDOV V RAZLIČNIH FAZAH RAZVOJA IN RASTI V PROVINCI JILAN, KITAJSKA

Eden najpomembnejših ukrepov za trajnostni način pridelave poljščin je vmesna setev. V raziskavi je bil izveden popolni naključni poljski poskus s štirimi ponovitvami na suhih peščenih tleh v Horkinu, območje Baicheng, v provinci Jilin v severni Kitajski, leta 2011. Obravnavanja so obsegala različne načine setve v čistem posevku in v vmesnem posevku, in sicer: čisti posevek ovsa (TO); setev ovsa v vmesnem posevku s sojo (TOS-O); setev ovsa v vmesnem posevku z arašidi (TOG-O); čisti posevek soje (TS); setev soje v vmesnem posevku z ovsom (TOS-S); čisti posevek arašidov (TG); arašidi v vmesnem posevku z ovsom (TOG-G). Pri setvi čistih posevkov je imel čisti posevek ovsa največjo vsebnost suhe mase in dušika v vseh razvojnih fazah. Največji odstotek beljakovin je bil v vseh fazah razvoja, z izjemo faze zorjenja, v čistih posevkih arašidov. Največja vsebnost organske mase je bila v čistih posevkih soje dosežena v fazi zorenja, v drugih razvojnih fazah pa v čistih posevkih arašidov. V vmesnih posevkih je imel posevek ovsa z arašidi največjo vsebnost suhe mase v vseh fazah rasti in razvoja. Največji odstotek beljakovin in vsebnost organske snovi v fazah latenja, polnjenja zrnja in fazi voščene zrelosti zrnja sta bila dosežena v sistemu setve arašidov z medsetvijo arašidov z ovsom. Največji vsebnosti beljakovin in organske mase v fazah kolenčenja in zorenja sta bili doseženi v vmesnih posevkih soje z ovsom. Rezultati raziskave jasno nakazujejo, da setev ovsa v vmesnem posevku z arašidi vpliva na rast posameznih poljščin v mešanicah kot tudi na pridelek suhe mase in odvzem dušika. Pridobljene izkušnje lahko pomagajo prilagoditvam vmesnih posevkov z ovsom za povečanje pridelave krme v novih načinih pridelave.

Ključne besede: vsebnost beljakovin; sušina; organska masa; soja; arašidi; oves; vmesna setev

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1 INTRODUCTION

Intercropping, the mixed growth of two or more crops, is practiced in more than 28 million hectares of areas sown annually in China. Multiple-cropping systems in China, including intercropping and related practices, have contributed to increased crop productivity due to a more effective utilization of resources compared to monoculture crops (Karimuna et al., 2012).Cereal-legume intercropping system was experimented by many researchers in all over the world (Carr et al., 2004; Lithourgidis et al., 2006; Lauk and Lauk, 2008; Li et al., 2009; Soleymani et al., 2011; Soleymani et al., 2012). The benefits of oat intercropping with other crops are also reported by many researchers (Malezieus et al., 2009; Naumann et al., 2010; Gong et al., 2011; Han et al., 2012). Researchers also reported the improvement of peanut production in intercropping system (Justino and Sodek, 2013). The inclusion of legumes in crop rotations and intercrops can provide increased protein-rich yields and a more sustainable source of nitrogen, while on the other side it saves cost by reducing the requirement for mineral nitrogen application (Crew and Peoples, 2004). On the one hand, monocultures of legumes and cereals do not provide satisfactory results for forage production (Soleymani et al., 2011; Soleymaniand Shahrajabian., 2012). On the other hand, small grain cereals provide high yield in terms of dry mass but they produce forage with low protein content (Lauk and Lauk, 2008). Other benefits of mixtures include greater uptake of water and nutrients, enhanced weed suppression, and increased soil conservation (Li et al., 2009). These systems also protect soil against erosion, improve the use of limited resources, improve forage quality, increase stability of yield and provide higher returns (Javanmard et al., 2009; Lee and Yoon, 2013). Intercropping of legumes with non-legumes results in production of more dry matter and an increase in protein content of the resulting crop, with minimum N fertilizer input (Ijoyah and Fanen, 2012).Caballero and Goicoechea (1986) reported that the most suitable cereal for mixtures with legume is oat (*Avena sativa* L.). Soybean (*Glycine max* (L.) Merrill), which is one of the major legume crops produced worldwide (Garrett et al., 2013; Jing and Chin, 2013; Mazza et al., 2013), is commercially used for its edible oil, proteins, health functional ingredients, and fermented food (Jensen, 1996; Sharma et al., 2013). Materials left after evaporation is the dry matter, while loss in weight upon ignition at certain defined temperature is the organic matter content. This research had three aims. The first was to study the organic matter production in mono-cropping and intercropping patterns. The second aim was to evaluate nitrogen and protein percentage for each treatment. The third aim was to study changes of dry matter in different stages of oat intercropped by soybean and groundnut.

2 MATERIALS AND METHODS

The study was conducted under field conditions in the arid Horqine sandy land in Baicheng District (44°14′-46°18′N, 121°38′-124°22′E), Jilin Province, Northern China in 2011. A randomized complete block design with four replications was used. Treatments comprised different mono cropping and intercropping patterns, TO: sole cropping of oat (*Avena sativa* 'Baiyan2'), TOS-O: oat in the intercropping of oat and soybean (*Glycine max* 'Zao Shu96136'), TOG-O: oat in the intercropping of oat and groundnut (*Arachis hypogaea* 'Baiyuanhual'*)*, TS: sole cropping of soybean, TOS-S: soybean in intercropping of oat and soybean, TG: sole cropping of groundnut, TOG-G: groundnut in the intercropping of oat and groundnut. No nitrogen fertilizer was used in this research. 55 kg ha^{-1} P_2O_5, 45 kg ha^{-1} K_2O, 4.5 kg ha^{-1} $FeSO_4$, 1 kg ha^{-1} H_3BO_3, 1.5 kg ha^{-1} $Na_2MOO_4.2\ H_2O$ were applied as basal fertilizers. An automatic weather station was installed in the experimental field to record daily air temperature and rainfall during growing period. Available nitrogen, phosphorus and potassium at the mentioned depth were 66.6 mg kg^{-1}, 14.2 mg kg^{-1} and 68.2 mg kg^{-1}, respectively. Soil pH was 7.2. No additional fertilizers were used during growth stages. Soybean and groundnut seeds were mixed with rhizobia before plantation. The soybean density in monoculture was 10 × 60 cm with 1 seedling in each hole, which is equivalent to 167 thousand plants per ha. The groundnut density in monoculture was 20 × 60 cm with two seedlings in each hole, equivalent to 167 thousand plants per ha. The seed quantity of oat in monoculture was 200 kg ha^{-1}. In soybean and groundnut monoculture, the distance between two rows was 60 cm, and the distance between seedlings on the row was 10 cm and 20 cm, respectively. Oat seed rate per row for both monoculture and intercropping patterns were the same. In intercropping patterns, the distance between both groundnut and soybean row with oat rows were 20 cm. The ration of both soybean and groundnut intercropping with oat was 2: 2. All seeds were sown by skillful workers on May 17th; furthermore, oat and legumes were harvested on 12th August and 7th September. Intercultural operations such as weeding and plant protection were done when required to ensure and

maintain the normal growth of crop. The amount of nitrogen was determined by Kjeldahl analysis from dry and ground samples, and nitrogen was multiplied by 6.25 to determine protein content. (Pregl, 1945). Dry matter was determined by drying samples for 15 h at 105 ^{o}C; dry matter was expressed as a percentage of the sample at the time of the analysis. Organic matter was determined by ashing for at least 4 h at 500 ^{o}C. All data were statistically treated using Analysis of variance (ANOVA) for randomized complete block design and the means were compared by Duncan's multiple range method using SAS software program ($P \leq 0.05$).

3 RESULTS AND DISCUSSION

3.1 Booting stage

There was no significant difference in nitrogen concentration among cropping patterns. Oat dry matter in booting stage in oat-soybean intercropping was higher than oat yield in oat-groundnut intercropping and other treatments, which had significant differences with other treatments. The maximum nitrogen accumulation in booting stage was also obtained for oat in intercropping of oat and soybean (Table 1). Protein percentage of soybean in oat-soybean intercropping obtained the maximum value (20.95 %). The highest value of organic matter was obtained for soybean in oat-soybean intercropping, followed by ground nut in oat-groundnut intercropping and oat in oat-soybean intercropping. Moreover, there was not any significant difference in organic matter of oat in both oat-groundnut and oat-soybean intercropping. In mono-cropping, the maximum organic matter in booting stage was achieved for groundnut mono-cropping (26.82 %) (Table 3). Using cereals intercropped with legumes improves the value of farming systems, moreover, the selection of appropriate intercropping system remains the best approach (Soleymani and Shahrajabian, 2012).

3.2 Heading stage

In solo-cropping patterns, the highest dry matter in heading stage was obtained for oat mono-cropping, followed by soybean and groundnut mono-cropping. On the one hand, there was no significant difference in dry matter and nitrogen accumulation between dry matter of oat in oat-groundnut and oat-soybean intercropping. In the other hand, oat in oat-groundnut obtained the highest value of dry matter and nitrogen accumulation (Table 1). Mono-cropping of groundnut obtained the maximum value of protein percentage (15.79 %) and organic matter (21.39 %). Groundnut in oat-groundnut intercropping had obtained the maximum value of protein percentage in heading stage, which had significant differences with oat in both oat-soybean and oat-groundnut intercropping; however, its difference with soybean in oat-soybean intercropping was not significant. Crude protein concentration of forage is one the most important criteria for forage quality evaluation (Dordas and Lithourgidis, 2011). Organic matter value of groundnut in oat-groundnut intercropping (21.39 %) in heading stage was significantly higher than in other intercropping treatments. Furthermore, the difference in organic matter of oat in both oat-soybean and oat-groundnut intercropping was not meaningful (Table 3).

3.3 Grain filling stage

In mono-cropping patterns, the highest value of dry matter was obtained for oat, followed by soybean and groundnut. In intercropping patterns, the highest and the lowest dry matter production was related to oat yield in oat-groundnut intercropping, and groundnut in oat-groundnut intercropping. Some other researchers also stated that in intercropping system of cereal with a legume, forage yield is much higher than that of the legume sole crop and forage quality is higher than that of the sole cereal crop (Mariotti et al., 2009; Dordas et al., 2012).The maximum nitrogen accumulation in grain filling stage was achieved in oat mono-cropping, which had significant differences with groundnut and soybean mono-cropping. Oat nitrogen accumulation in oat-groundnut intercropping, which had no meaningful difference with nitrogen accumulation of oat in oat-soybean, obtained the highest value of it (Table 1). The maximum value of protein percentage (16.55 %) and organic matter in grain filling stage (22.57 %) was related to groundnut mono-cropping. Protein percentage for groundnut in oat-groundnut intercropping was higher than those of other treatments. There were significant differences between groundnut in oat-groundnut intercropping and other intercropping patterns in the term of protein percentage. Indeed, there was no significant difference in organic matter for oat in oat-groundnut intercropping and oat in oat-soybean system (Table 3).

3.4 Grain dough stage

The highest production of dry matter and nitrogen accumulation in grain dough stage was obtained for oat mono-cropping. The highest amount of dry matter in grain dough stage was achieved in oat in oat-groundnut intercropping in comparison with those of other intercropping systems; moreover, its differences with other treatments were significant. Oat nitrogen accumulation of oat in oat-groundnut intercropping obtained the maximum value, which had meaningful differences with other treatments. In contrast, nitrogen accumulation for groundnut in oat-groundnut

intercropping, which obtained the minimum value, had no significant difference with the one for soybean in oat-soybean intercropping (Table 2). Groundnut mono-cropping obtained both the maximum protein percentage (12.69 %), and organic matter (17.26 %), followed by soybean mono-cropping and oat mono-cropping, respectively. Among intercropping patterns, the maximum protein percentage and organic matter production was achieved in groundnut in oat-groundnut intercropping, which had no significant difference with the value of soybean in oat-soybean intercropping. Indeed, differences between oat in oat-groundnut and oat-soybean intercropping were not significant (Table 3). Ghanbari-Bonjar and Lee (2003) and Arshad and Ranamukhaarachchi (2012) concluded that intercropping had greater total output for protein content compared to sole cropped of crops.

3.5 Ripening stage

In solo-cropping, the highest dry matter in grain filling stage was related to oat mono-cropping, followed by mono-cropping of soybean and groundnut mono-cropping. On the one side, higher values of nitrogen accumulation were related to oat in oat-groundnut intercropping than those of other intercropping treatments. On the other side, the difference in oat yield in oat-groundnut and oat-soybean was not meaningful (Table 2). The maximum protein percentage in ripening stage was achieved in soybean mono-cropping followed by mono-cropping of groundnut and solo-cropping of oat, respectively. In intercropping treatments, the maximum and the minimum protein percentage was related to soybean in oat-soybean intercropping (13.35 %), and in oat in oat-groundnut intercropping (8.95 %), respectively. But, Li et al. (2009) reported that there were no significant differences in protein content between intercropping and sole cropping. Legume-cereal intercrops have produced higher seed and protein yields than pure cereal crops (Jensen, 1996; Hauggaard-Nilsen et al., 2001; Lauk and Lauk, 2005). The highest and the lowest amount of organic matter were related to soybean mono-cropping (17.36 %), and oat mono-cropping (11.02 %), respectively. Soybean in oat-soybean intercropping obtained the maximum organic matter in ripening stage (18.18 %), which had significant differences with oat in oat-groundnut and oat-soybean intercropping. However, it had no meaningful difference with groundnut in oat-groundnut intercropping (Table 3).

Table 1: Mean comparison for nitrogen concentration (g g^{-1} dry matter), dry matter (g m^{-2}) and nitrogen accumulation (g m^{-2}) in booting stage, heading stage and grain filling stage under different cropping patterns

	Booting stage	Booting stage	Booting stage	Heading stage	Heading stage	Heading stage	Grain filling stage	Grain filling stage	Grain filling stage
Treatment	Nitrogen concentration in booting stage	Dry matter in booting stage	Nitrogen accumulation in booting stage	Nitrogen concentration in heading stage	Dry matter in heading stage	Nitrogen accumulation in heading stage	Nitrogen concentration in grain filling stage	Dry matter in grain filling stage	Nitrogen accumulation in grain filling stage
TO	0.017a	73.53c	1.283b	0.011a	149.7b	1.710b	0.012a	205.7b	2.703b
TOG-O	0.022a	99.83b	1.273a	0.013a	232.2a	3.327a	0.013a	312.9a	4.251a
TOS-O	0.022a	107.8a	2.443a	0.014a	209.5a	3.103a	0.013a	284.6a	3.913a
TG	0.031a	2.400d	0.076c	0.024a	6.233c	0.156c	0.026a	11.27c	0.296c
TOG-G	0.031a	1.833d	0.053c	0.024a	6.400c	0.163c	0.024a	10.53c	0.263c
TS	0.028a	7.100d	0.200c	0.022a	10.07c	0.233c	0.023a	22.60c	0.543c
TOS-s	0.033a	4.300d	0.143c	0.022a	8.133c	0.183c	0.020a	19.53c	0.406c

Mean with the same letter in each column are not significantly different at 5 percent probability level.

Table 2: Mean comparison for nitrogen concentration (g g^{-1} dry matter), dry matter (g m^{-2}) and nitrogen accumulation (g m^{-2}) in grain dough stage and ripening stage under different cropping patterns

	Grain dough stage	Grain dough stage	Grain dough stage	Ripening stage	Ripening stage	Ripening stage
Treatment	Nitrogen concentration	Dry matter	Nitrogen accumulation	Nitrogen concentration	Dry matter	Nitrogen accumulation
TO	0.012a	292.5b	3.610b	0.012a	246.3c	3.187c
TOG-O	0.014a	333.8a	5.003a	0.014a	345.3a	4.953a
TOS-O	0.013a	292.9b	3.880b	0.014a	303.7b	4.370b
TG	0.019a	26.17c	0.530c	0.013a	30.10d	0.430e
TOG-G	0.020a	16.73c	0.350c	0.017a	19.37d	0.346e
TS	0.017a	37.30c	0.660c	0.019a	43.13d	0.880d
TOS-S	0.018a	27.63c	0.520c	0.021a	44.50d	0.950d

Mean with the same letter in each column are not significantly different at 5 percent probability level.

Table 3: Mean comparison for protein percentage (%) and organic matter (%) under different cropping patterns

Treatment	Protein percentage in booting stage	Organic matter in booting stage	Protein percentage in heading stage	Organic matter in heading stage	Protein percentage in grain filling stage	Organic matter in grain filling stage	Protein percentage in grain dough stage	Organic matter in grain dough stage	Protein percentage in ripening stage	Organic matter in ripening stage
TO	10.94b	14.87b	7.121c	9.690c	8.247c	11.22c	7.75c	10.52b	8.103c	11.02c
TOG-O	14.09b	19.16b	8.943bc	12.160bc	8.500c	11.57c	9.37bc	12.03b	8.950bc	12.17bc
TOS-O	16.15b	19.24b	9.280b	12.620bc	8.597c	11.69c	8.34c	11.35b	8.993bc	12.24bc
TG	19.72a	26.82a	15.790a	21.470c	16.55a	22.57a	12.69a	17.26a	8.907bc	12.11bc
TOG-G	19.65a	26.72a	15.720a	21.390c	15.73a	21.40a	13.13a	17.86a	11.24ab	15.28ab
TS	17.89a	24.33a	14.490a	19.170a	14.90ab	20.26ab	11.21ab	15.25a	12.76a	17.36a
TOS-S	20.95a	28.49a	14.410a	19.60a	13.12b	17.85b	11.82a	16.08a	13.36a	18.18a

Mean with the same letter in each column are not significantly different at 5 percent probability level.

4 CONCLUSION

Using cereals intercropped with legumes improves the value of farming systems, moreover, the selection of appropriate intercropping system remains the best approach. Moreover, mixing species in cropping systems may lead to a range of benefits that are expressed on various space and time scales, from a short-term increase in crop yield and quality, to long-term increase in crop yield and quality, to long-term agro-ecosystem sustainability, up to societal and ecological benefits. The results of this study clearly indicate that intercropping oat and groundnut affects the growth rate of the individual species in mixtures as well as the dry matter yield and nitrogen accumulation. This information can help in the adaptation of oat-intercrops for increased forage production in new cropping systems.

5 REFERENCES

Arshad, M., Ranamukhaarachchi, S. L. (2012).Effects of legume type, planting pattern and time of establishment on growth and yield of sweet sorghum-legume intercropping. *Australian Journal of Crop Science*, *6*(8), 1265-1274.

Caballero, R., Goicoechea, E.L. (1986). Utilization of winter cereals as companion crops for common vetch and hairy vetch. In: *Proceedings of the 11th General Meeting of the European Grass*. Fed. Pp. 379-384.

Carr, P.M., Horsley, R.D., Poland, W.W. (2004).Barley, oat and cereal-pea mixtures as dryland forages in the Northern Great Plains.*Agronomy Journal, 96*, 677-684. doi:10.2134/agronj2004.0677

Crews, T. E., and Peoples, M. B. (2004). Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. *Agriculture, Ecosystems & Environment*, *102*,279-297. doi:10.1016/j.agee.2003.09.018

Dordas, C.A., Lithourgidis, A.S. (2011). Growth, yield and nitrogen performance of faba bean intercrops

with oat and triticale at varying seeding ratios. *Grass and Forage Science*, *66*, 569-577. doi:10.1111/j.1365-2494.2011.00814.x

Dordas, C.A., Vlachostergios, D.N., Lithourgidis, A.S. (2012).Growth dynamics and agronomic-economic benefits of pea-oat and pea-barley intercrops. *Crop and Pasture Science*, *63*(1), 45-52. doi:10.1071/CP11181

Garrett, R.D., Lambin, E.F., Naylor, R.L. (2013). Land institutions and supply chain configurations as determinants of soybean planted area and yields in Brazil. *Land Use Policy, 31*, 385-396. doi:10.1016/j.landusepol.2012.08.002

Ghanbari-Bonjar, A., Lee, H.C. (2003).Intercropped wheat (*Triticum aestivum* L.) and bean (*Vicia faba* L.) as whole-crop forage: effect of harvest time on forage yield and quality. *Grass and Forage Science*, *58*, 28-36. doi:10.1046/j.1365-2494.2003.00348.x

Gong, C., ChangZhong, R., LaiChun, G., GuoJun, Z., YueGao, H., and ZhaoHai, Z. (2011). Effects of two spaces and intercropping on forage and crude protein yields of oat (*Avena sativa* L.) and common vetch (*Vicia sativa* L.). *Acta Agronomica Sinica*, *37*(11), 2066-2074.

Han, C.C., Borman, C., Osantowski, D., Wagnitz, J., Koehler-Cole, K., Korus, K., Sonderegger, E., Werle, R., Wood, T., Lindquist, J.L. (2012). Productivity of field pea (*Pisum sativum* L.) and spring oat (*Avena sativa* L.) grown as sole and intercrops under different nitrogen levels. *Journal of Agricultural Science*, *4*(11), 136-143.

Hauggaard-Nilsen, H., Ambus, P., Jensen, E.S. (2001). Interspecific competition, N use interference with weeds in pea-barley intercropping. *Field Crop Research*, *70*, 101-109. doi:10.1016/S0378-4290(01)00126-5

Ijoyah, M.O., Fanen, F.T. (2012). Effects of different cropping pattern on performance of maize-soybean mixture in Makurdi, Nigeria. *Scientific Journal of Crop Science*, *1*(2), 39-47.

Javanmard, A., Nasab, A.D.M., Javanshir, A., Moghaddam, M., Janmohammadi, H. (2009). Forage yield and quality in intercropping of maize with different legumes as double-cropped. *Journal of Food, Agriculture and Environment*, *7*, 163-166.

Jensen, E.S. (1996).Grain yield, symbiotic N_2 fixation and interspecific competition for inorganic N in pea-barley intercrops. *Plant and Soil*, *182*, 25-38. doi:10.1007/BF00010992

Jing, Y., Chin, Y.J. (2013). Effects of twin-screw extrusion on soluble dietary fibre and physicochemical properties of soybean residue. *Food Chemistry*, *138*, 884-889. doi:10.1016/j.foodchem.2012.12.003

Justino, G.C., Sodek, L. (2013).Recovery of nitrogen fixation after short-term flooding of the nodulated root system of soybean. *Journal of Plant Physiology*, *170*, 235-241. doi:10.1016/j.jplph.2012.10.006

Karimuna, L., Leomo, S., Indriyani, L. (2012). Improvement of maize and peanut production in intercropping system through the application of organic fertilizer and mulch in ultisolsoil. *Chiang Mai University of Natural Sciences*, *11*(1), 387-394.

Lauk, E., Lauk, R. (2005). The yields of legume-cereal mixed in years with high precipitation vegetation periods. *Latvian Journal of Agronomy*, *8*, 281-285.

Lauk, R., Lauk, E. (2008). Pea-oat intercrops are superior to pea-wheat and pea-barley intercrops. *Acta Agriculturae Scandinavica, Section B- Soil and Plant Science*, *58*, 139-144. doi:10.1080/09064710701412692

Lee, Y.J., Yoon, W.B. (2013). Effects of particle size and heating time on thiobarbituric acid (TBA) test of soybean powder. *Food Chemistry*, *138*, 841-850. doi:10.1016/j.foodchem.2012.11.113

Li, Y., Ran, W., Li, X., Zhang, R., Sun, S., Xu, G. (2009). Facilitated legume nodulation, phosphate uptake and nitrogen transfer by arbuscular inoculation in an upland rice and mung intercropping system. *Plant and Soil*, *315*, 285-296. doi:10.1007/s11104-008-9751-9

Lithourgidis, A.S., Vasilakoglou, I.B., Dhima, K.V., Dordas, C.A., Yiakoulaki, M.D. (2006). Forage yield and quality of common vetch mixtures with oat and triticale in two seedling ratios. *Field Crops Research*, *99*, 106-113. doi:10.1016/j.fcr.2006.03.008

Malezieus, E., Crozat, Y., Dupraz, C., Laurans, M., Makowski, D., Ozier-Lafontaine, H.,Rapidel, B., DE Tourdonnet, S., Valantin-Morison, M. (2009). Mixing plant species in cropping systems: concepts, tools and models. A review. *Agronomy for Sustainable Development*, *29*, 43-62. doi:10.1051/agro:2007057

Mariotti, M., Masoni, A., Ercoli, L., Arduini, I. (2009). Above-and below-ground competition between barley, wheat, lupin and vetch in a cereal and legume intercropping system. *Grass and Forage Science*, *64*, 401-412. doi:10.1111/j.1365-2494.2009.00705.x

Mazza, C.A., Gimenez, P.I., Kantolic, A.G., Ballare, C.L. (2013). Beneficial effects of solar UV-B

radiation on soybean yield mediated by reduced insect herbivory under field conditions. *Physiologia Plantarum*, *147*, 307-315. doi:10.1111/j.1399-3054.2012.01661.x

Naumann, A., Heine, G., Rauber, R. (2010). Efficient discrimination of oat and pea roots by cluster analysis of fourier transform infrared (FTIR) spectra. *Field Crops Research*, *119*, 78-84. doi:10.1016/j.fcr.2010.06.017

Pregl, F. (1945). Quantitative Organic Microanalysis. 4th ed. J. A. Churchill Ltd. London, p. 126-129.

Sharma, S., Kaur, A., Bansal, A. (2013). Positional effects on soybean seed composition during storage. *Journal of Food Science and Technology*, *50*(2), 353-359. doi:10.1007/s13197-011-0341-0

Soleymani, A., Shahrajabian, M.H.,Naranjani, L. (2011). The effects of nitrogen as starter fertilizer on ash percentage, important elements and solar radiation absorption of berseem clover cultivars intercropped by corn. *Journal of Food, Agriculture and Environment*, *9*(1), 342-344.

Soleymani, A., Shahrajabian, M.H. (2012). Forage yield and quality in intercropping of forage corn with different cultivars of berseem clover in different levels of nitrogen fertilizer. *Journal of Food, Agriculture and Environment*, *10*(1)*,* 602-604.

Soleymani, A., Shahrajabian, M.H., Naranjani, L. (2012). Evaluation the benefits of different clover cultivars and forage corn intercropping in different levels of nitrogen fertilizer. *Journal of Food, Agriculture and Environment*, *10*(1), 599-601.

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Effect of biofumigation by *Calligonum polygonoides*, dry olive leaves, and ash of olive leaves on chilli pepper growth and recovery of *Rhizoctonia solani*

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ABSTRACT

Rhizoctonia solani J.G. Kühn is a serious soilborne pathogen in chilli fields worldwide. This study examined the effect of biofumigation using arta (*Calligonum polygonoides* L.) and olive (*Olea europaea* L.) plant material on chilli growth and recovery of *R. solani* from chilli plants. The experiment was conducted under greenhouse conditions in potted soil amended with no plant material (control) or with plant material from *Calligonum and Olea* (olive leaves, or ash of olive leaves). Chilli was planted in the amended soils and inoculated with *R. solani*. Plant height, number of fruits , and frequency of recovery of *R. solani* from chilli were recorded. Soil amendment with *Calligonum* plant material resulted in the lowest frequency of recovery of *R. solani*. Moreover, *Calligonum* treatment increased dry mass and height of chilli plants compared to other treatments. Also, treatment with olive plant parts inhibited *R. solani* growth and enhanced growth of chilli compared to the control treatment. Thus, *C. polygonoides* and *O. europaea* are potental biofumigant plants to control *R. solani*.

Key words: chilli pepper; *Capsicum annuum*; ; biofumigation; *Calligonum polygonoides*; olive; *Rhizoctonia solani*; inoculation

IZVLEČEK

UČINKI BIOFUMIGACIJE Z VRSTO *Calligonum polygonoides*, SUHIMI LISTI OLJKE IN PEPELOM IZ OLJČNIH LISTOV NA RAST ČILIJEV IN REGENERACIJO GLIVE *Rhizoctonia solani*

Gliva *Rhizoctonia solani* J.G. Kühn je pomembne talni pathogen na poljih čilijev širom po svetu. V raziskavi je bil preučen učinek biofumigacije z vrsto dresnovk (*Calligonum polygonoides* L.) in pripravki iz oljke (*Olea europaea* L.) na rast čilijev in njihovega uspevanja po inokulaciji z glivo *R. solani* . Poskus je bil izveden v rastlinjaku kot lončni poskus, v katerem prsti niso dodali ničesar (kontrola) ali z dodatkom rastlinskih delov vrst *Calligonum* in *Olea* (oljčni listi ali pepel iz njih). Čiliji so bili posajeni v prst z dodatki in inokulirani z glivo *R. solani*. Zabeležena je bila višina rastlin, število plodov in frekvenca okrevanih rastlin po inokulaciji z glivo *R. solani*. Dodatki delov vrste *Calligonum* k prsti so rezultirali k najmanjši obnovi glive *R. solani*. Še več, dodatki delov te vrste so povečali višino in suho maso čilijev v primerjavi z drugimi obravnavanji. Tudi obravnavanja s pripravki iz oljke so zavrla rast glive *R. solani* in pospešila rast čilijev v primerjavi s kontrolo. Pripravki iz vrste *C. polygonoides* in oljke so lahko biofumigacijska sredstva za nadzor talne glive *R. solani*.

Ključne besede: čili; *Capsicum annuum*; biofumigacija; *Calligonum polygonoides*; oljka; *Rhizoctonia solani*; inokulacija

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1 INTRODUCTION

Chilli pepper (*Capsicum annuum* L.) hereafter referred to as 'chilli', is an important vegetable crop worldwide (Castillo et al., 2009; Scholberg et al., 2009). It is grown for several purposes such as fresh market, processing, extraction of food coloring, pharmaceuticals, and other products (Goldberg, 2001). Chilli yield has declined clearly during the last decade for a variety of reasons (Sanogo and Carpenter, 2006). Soilborne pathogens belonging to genera *Phytophthora*, *Verticillium*, *Fusarium*, and *Rhizoctonia* significantly reduced yield and quality of chilli (Sultana et al., 2008; Tariq et al., 2009). Plant pathogens such as *R. solani* J.G. Kühn are of great importance since they can attack plants throughout the cropping cycle, causing death of young seedlings early in the season and root rot in mature plants in the mid to late season (Woodhall et al., 2007).

Complete control of *R. solani* is not possible, but the activities of the pathogen can be minimized (Abeysinghe, 2009). Application of certain chemical fungicides could be the best solution to reduce the problems caused by *R. solani* and other soilborne plant pathogens (Bubici et al., 2006; Cimen et al., 2004; Jarvis, 1993). However, fungicides are not the most effective way to manage this pathogen. Moreover, fungicides has negative impact on the environment (Lazarovits et al., 2008; Powelson and Rowe, 1993). Therefore, environmental friendly control methods, such as biofumigation, are highly recommended and increasingly being used.

Biofumigation depends on the breakdown of plant metabolites in soil to produce volatile compounds that can suppress plant pathogens, nematodes, and weeds (Larkin et al., 2011). Many crops have been proven to work as natural biofumigants against several soilborne pests. Those plants can release glucosinolates and isothiocyanates through soil and inhibit soilborne pathogens (Aires et al., 2009; Kirkegaard et al., 2000; Laegdsmand et al., 2007; Matthiessen and Kirkegaard, 2006). Examples of biofumigant plants are *Brassica carinata* A. Braun (Porras et al., 2009; Snapp et al., 2007), *B. oleracea* L. (Fan et al., 2008), and *B. napus* L. (Laegdsmand et al., 2007). Those plants were intensively studied as biofumigants worldwide.

Other plants might have the potential to act as biofumigants and are still undiscovered and require intensive research work. Examples include *Calligonum* spp. *and O. europaea* L.. The *Calligonum* genus descended from the Polygonaceae plant family and it is known as "arta" (Abrahim et al., 2014). Around 80 species across the Mediterranean region, Asia and North America belong to the *Calligonum* genus (Halis, 2007). Most *Calligonum* plants are shrubs with diffuse and irregular woody branches. These plants have scale-like or filiform simple leaves with perfect flowers having 10-18 stamens with filaments connate at the base. Among *Calligonum* species is *C. polygonoides* L.*,* which is a small shrub, usually 10 cm to 15 cm high, but occasionally may reach 25 cm in height with a girth of 2.5 to 5 cm (Kaul, 1963). *Calligonum* contains several chemicals which encouraged researchers to investigate the potential of these plants for medical uses (Kamil et al., 2000). Examples of chemicals found in *Calligonum* plants include anthraquinones, flanovonids, and dehydrodicatechin (Farid et al., 2007; Ghazanfar 1994; Kamil et al., 2000). Anthraquinones found in *Calligonum* showed high antimicrobial potential (Alkhalifah, 2013; Zaki et al. 1984). Similarly, these plants might have some components that have potential to control certain oilborne plant pathogens. Also, it was found that *Calligonum* plant material inhibit growth of two plant fungal pathogens, *Alternaria* spp. and *Rhizopus* spp. (Abrahim et al., 2014).

Another promising biofumigant plant is *O. europea*. Olive leaves were studied widely for their extracts and for their medicinal properties and uses (Benavente-Garcia et al., 2000). Extracts from olive-mill wastes, for example, include organic matter, potassium, carbohydrates, fats, polyphenols, and polyalcohols (Romero et al., 2005). Our interest in olive leaves is based on bioassays that showed antifungal effect of olive leaves against some human pathogens at laboratory level (Pinelli et al., 2000). Moreover, production of olive oil becomes a very common industry (Nogales et al., 2006), and a huge number of olive trees are growing worldwide. Consequently, huge amounts of olive leaves are lost during pruning and harvesting of olive fruit as waste. Traditionally, many farmers used to burn the pruned twigs with leaves and the resulting ash is incorporated with soil. Also, olive waste ash was used as soil amendment (Nogales et al., 2006). However, little research, if any, had focused on the effect of fresh olive leaves or leaf ash on soilborne plant pathogens. Therefore, our long-term goals are to encourage farmers and agricultural practioners to use the widely available plants as biofumigants to inhibit soilborne pathogens and consequently minimize the use of chemicals in the agroecosystem. Our objectives were to study the effect of incorporating *C. polygonoides* above-ground plant parts, olive leaves or olive ash (leaves and twigs ash) into soil on the recovery of *R. solani* from roots of chilli plants and on dry mass, number of fruits, and plant height of chilli pepper.

2 MATERIALS AND METHODS

2.1 Inoculum preparation

The *R. solani* isolate used was recovered from infected tomato plants displaying typical symptoms of *Rhizoctonia* root rot. The isolate was grown on potato dextrose agar in petri plates.

2.2 Soils

A large composite sample of silty clay soil (calcareous), was collected from the top 15 cm of a field at the Hashemite University, Zarqa, Jordan. The soil was thoroughly mixed, and stored until use. Round plastic pots (10 cm upper radius, 7.5 cm lower radius, and depth of 18 cm) were used. Three openings at the bottom of each pot were made for drainage. The pots were filled with soil (4 kg) mixed with each type of biofumigant plant materials. The pots were irrigated with tap water as needed throughout the study period.

2.3 Biofumigant plant materials

The aboveground *Calligonum* parts were collected from fields near the Hashemite University during summer 2014. Plant parts were spread over sheets of cheesecloth and allowed to air dry at room temperature (20-25 °C). Then, dry plant materials were milled and homogenized with a plant grinder (MF 10 basic-Thomas No. 1203C62), and collected and stored in plastic bags until use. By the time of olive fruit harvesting (October 2014), olive twigs were collected from local olive orchards at the Hashemite University. Leaf blades and peduncles were removed from the collected twigs, dried at room temperature for two weeks, and grinded. Olive twigs and leaves were collected at the time of pruning. Then, they were burned and their ash was collected and stored in plastic bags until use.

2.4 Pot preparation and plant production

On March 2015 and 2016 in experiments 1 and 2, soil of each pot was mixed thoroughly with 150 ml of the grinded plant materials according to each treatment (*Calligonum*, olive leaves, and olive ash). No plant materials were added to pots in the control treatment. One chilli seedling ('AZ20') at the six to eight leaf growth stage was transplanted into each pot at a depth of 10 cm. The chilli seedlings were grown in germination trays containing a mixture of peat moss and perlite (1:1volume basis). The pots were irrigated immediately after transplanting and maintained in the greenhouse with a minimum air temperature of 20 °C and maximum air temperature of 32 °C (average greenhouse air temperature was 25 °C).

2.5 Inoculation

Shortly before inoculation, two holes were made to a depth of 2.5 cm and approximately 1 cm away from the seedling. On April 2015 and 2016, pots were inoculated simultaneously for each experiment. Seedlings in both experiments were at the 6-8 leaf growth stage. All pots were inoculated by placing one mycelium plug, 1 cm in diameter, of *R. solani* colony grown on potato dextrose agar into each hole. All holes were filled with soil after inoculation and the pots were irriagated with tap water.

2.6 *Rhizoctonia solani* assessment

Three and a half months after inoculation tap roots were collected and stored in a refrigerator until plating. Each tap root was cut into top, middle and bottom parts. The middle part of the tap root was then clipped into eight homogeneous segments under aseptic conditions, and four segments were plated on a 9 cm diameter petri plate containing potato dextrose agar. Two petri dishes were assigned to each treatment. All plates were then incubated at room temperature (22 to 25 °C). Two weeks after incubation, colonies of *R. solani* were counted to estimate the *R. solani* frequency based on 8 tap root segments.

2.7 Experimental design and data analysis

Each experiment was conducted in a randomized completly block design with four replicates per each of the four treatments: control, *Calligonum*, olive leaves, and olive ash. The measured variables were: (i) *R. solani* frequency of recovery from tap root segments, (ii) dry mass of the above-ground plant parts, (iii) plant height, (iv) and number of fruits. All data were subjected to an analysis of variance (ANOVA) using the Proc GLM procedure in SAS version 9.2 (SAS institute, Cary, NC). Proc GLM was used to calculate F statistics for the overall treatment effect. Least significant difference (LSD) was used to carry out pairwise comparisons to separate treatment means. All statistical tests were assessed at 5 % significance level.

3 RESULTS AND DISCUSSION

3.1 *Rhizoctonia solani* assessment

The overall F test for both expeiments ($P < 0.0001$ in experiment 1 and $P = 0.002$ in experiment 2) implies that the frequency of *R. solani* recovered from chilli tap root segments was significantly lower when soil was mixed with plant materials than that in control treatment (Fig. 1 & 2).

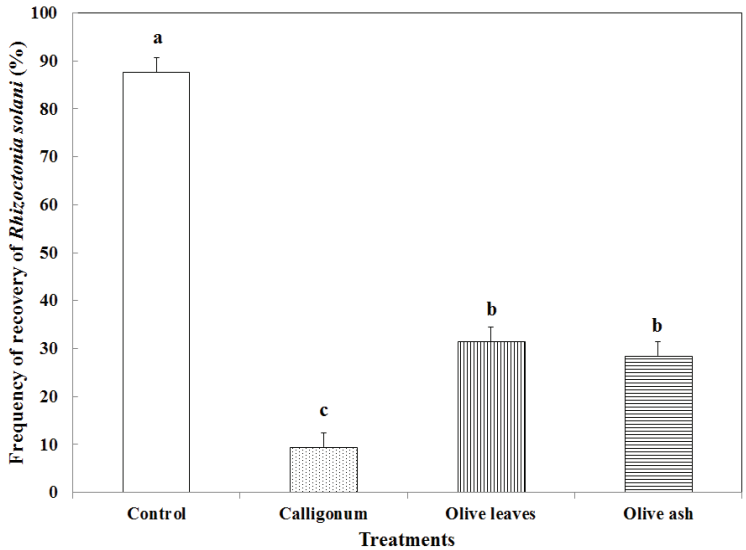

Figure 1: Frequency of recovery of *Rhizoctonia solani* from tap root segments of chilli in experiment 1. Each number is the mean of four individual plants, comprised of four replicates per treatment. Bars with the same letter are not significantly different based on the LSD at 5 % level of significance.

In experiment 1, the frequency of *R. solani* from root segments was significantly the lowest in the *Calligonum* treatment compared to all other treatments (Fig. 1). While in experiment 2 (Fig. 2), olive ash treatment resulted in the lowest frequency of *R. solani* compared to other treatments. Thus, mixing soil with *Calligonum*, olive leaves, and olive leaves ash reduced *R. solani* recovery from tap roots significantly.

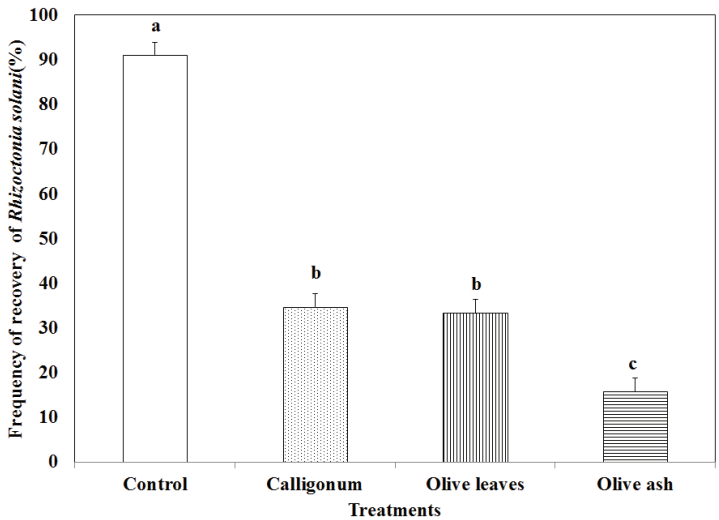

Figure 2: Frequency of recovery of *Rhizoctonia solani* from tap root segments of chilli in experiment 2. Each number is the mean of four individual plants, comprised of four replicates per treatment. Bars with the same letter are not significantly different based on the LSD at 5 % level of significance.

3.2 Plant growth

In experiment 1 and 2 (P = 0.002), dry mass was significantly higher for plants grown in soil mixed with *Calligonum* than plants grown in soil under all other treatments (Fig. 3 & 4).

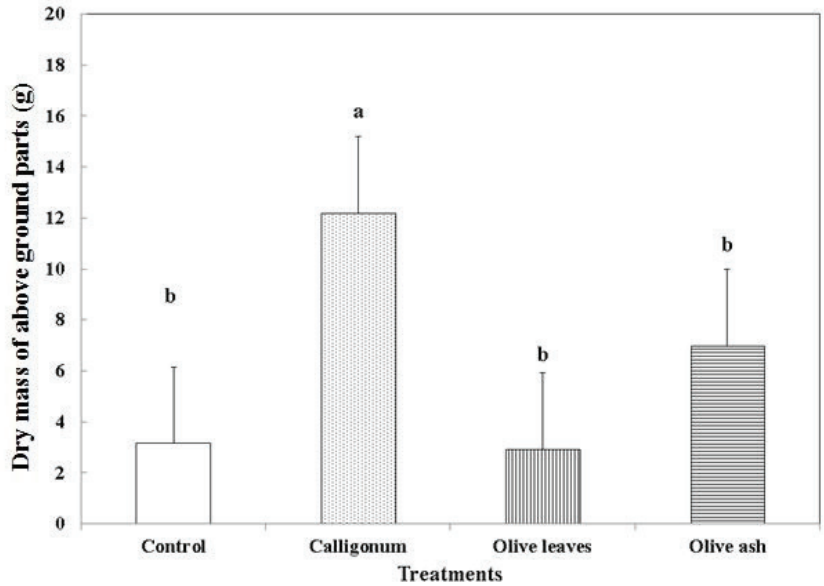

Figure 3: Dry mass of aboveground parts of chile in experiment 1. Each number is the mean of four individual plants, comprised of four replicates per treatment. Same letters on bars of the same parameter are not significantly different based on the LSD at 5 % level of significance

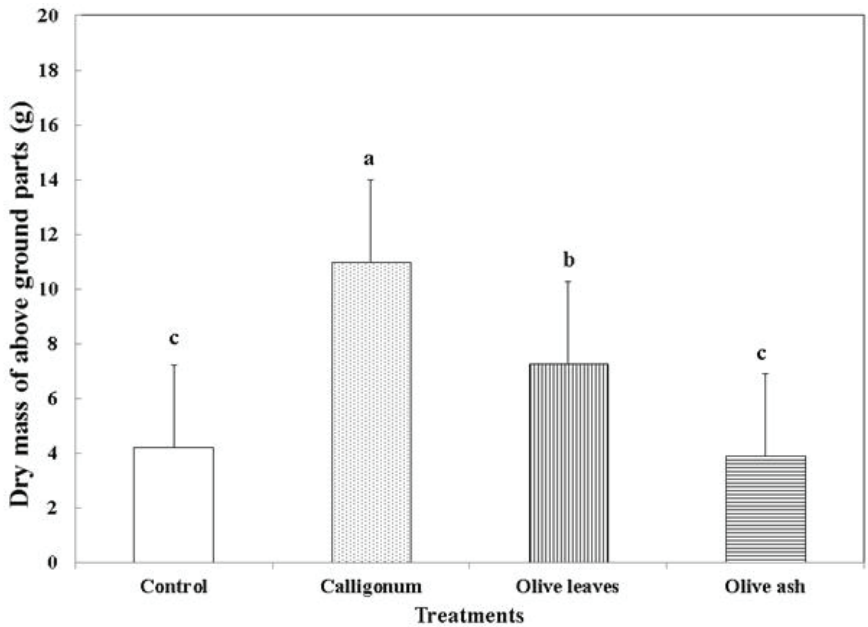

Figure 4: Dry mass of aboveground parts of chilli in experiment 2. Each number is the mean of four individual plants, comprised of four replicates per treatment. Bars with the same letter are not significantly different based on the LSD at 5 % level of significance.

In experiment 2, the dry mass of the above-ground parts of the chilli plants grown in soil mixed with olive leaves was significantly (P = 0.0035) higher than that of plants grown in soil mixed with olive ash. On the other hand, plant height was significantly different among treatments in experiment 1 (Fig. 5).

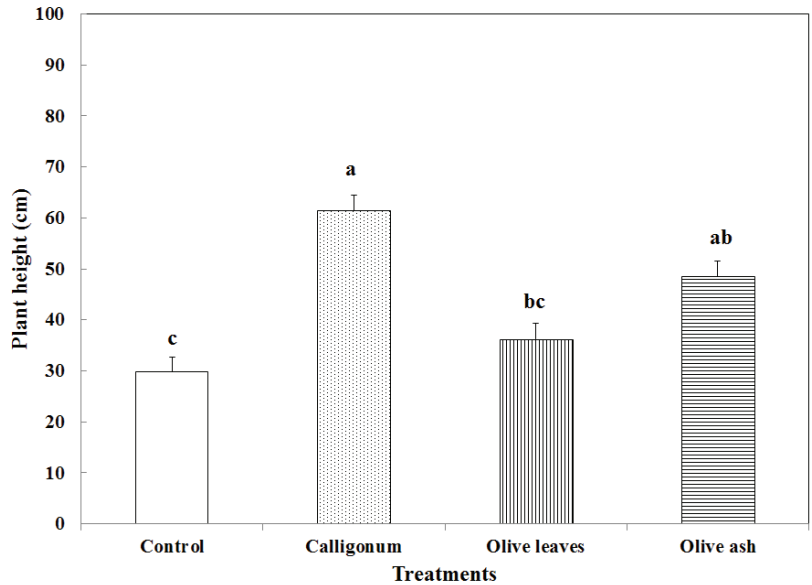

Figure 5: Plant height (cm) in experiment 1. Each number is the mean of four individual plants, comprised of four replicates per treatment. Bars with the same letters are not significantly different based on the LSD at 5 % level of significance

Plants grown in soil mixed with *Calligonum* or olive ash were significantly (P = 0.025) taller than plants grown in soil mixed with olive leaves or control soil. However, there were no significant differences among plants height under all treatments of experiment 2 (data not shown). Similarly, all comparisons indicated no significant effect of biofumigant plant materials on the number of chile fruits in both experiments.

Under field conditions, chilli plants are exposed to numerous soilborne pathogens such as *R. solani* (Skaggs et al., 2000; Sanogo, 2003). *Rhizoctonia solani* is well known as a potent pathogen that causes huge losses in yield of many crops (Siddiqui and Shaukat, 2005). Controlling soilborne fungal pathogens was achieved by several methods including the use of chemicals. On the other hand, alternative methods including biofumigation have been used. In this experiment, locally available plant residues were used. *Calligonum* and olive plant parts showed significant reduction in the frequency of *R. solani* recovered from tap roots of chile plants. Chilli was selected as an indicator crop since it is widely grown worldwide and has a great value .*Calligonum* plant material inhibited *R. solani* in both experiments significantly (Fig. 1 & 2). Incorporation of grinded *Calligonum* aboveground plant parts into soil was effective in reducing the recovery of *R. solani* from chilli tap roots. This result provides an evidence that *Calligonum* contains substances that are able to inhibit *R. solani*. In agreement with this evidence, Abrahim et al. (2014) concluded that *Calligonum* species have antifungal compounds effective in the treatment of plant diseases. Different factors might have affected the efficiency of *R. solani* recovery and chilli responses. These factors include the inoculum level of *R. solani* which was two mycelium plugs,diameter of 1 cm , of the colony grown on potato dextrose agar, and the amount, and timing and method of application of *Calligonum.* On the other hand, *Calligonum* plant material gave the highest dry mass of chilli aboveground parts (Fig. 3 & 4). It is possible that *Calligonum* plant material enhanced chilli crop to uptake water and nutrients since it inhibited growth of *R. solani*. Additionally, the use of *Calligonum* encouraged chilli roots growth which possibly had lead to better performance of chilli plants. *Calligonum* treatments produced the tallest chilli plants (Fig. 5). This could be attributed to the inhibition of *R. solani*, thus allowing chilli plant to grow well.

Similarly, olive leaves used as dry material or ash showed significant reduction in the frequency of *R. solani* from chilli tap roots. Plants grown in soil amended with olive plant material showed comparable increase in dry mass and plant height. The grinded, burned, or decomposed plant parts used in these

experiments might have also contributed to the growth and development rate of chilli not only as inhibitors of *R. solani*, but also as a source of nutrients. In agreement with our results, Solomon et al. (2015) concluded that olive leaves incorporated into soil increased soil fertility and increased plant growth. Similarly, it was found that olive waste ash increased dry mass of chilli stems and leaves significantly (Nogales et al., 2006). Also, it was found that adding dry olive-mill wastes to the soil increases phophorus and potassium contents of chilli leaves (Benitez et al., 2000). Furthermore, olive leaves contain many substances reported to have inhibitory effects on several fungi. It is assumed that *R. solani* was inhibited in our experiments because of the effects of such substances.

The primary active compounds in the raw olive leaves include oleuropein and hydroxytyrosol, as well as several other polyphenols and flavonoids such as oleocanthal and elenolic acid (Vivioli et al., 1998). It is also expected that plant materials added to the soil in our experiments encouraged microbial activity in the rhizosphere of chilli. Consequently, beneficial soil microorganisms could compete with *R. solani* and reduce the activities of this pathogen. Benitez et al. (2000) suggested that olive plant material increased microbial number and activity in chilli rhizosphere. Our results showed promising results of using olive leaves, dry or ash, to control *R. solani*. Additional research is needed in order to explore more aspects of using biofumigant plants to inhibit soilborne pathogen in major crops. Our current study may be the first research to investigate the use of *Calligonum*, olive leaves, and ash of olive leaves as biofumigants to control *R. solani* on chilli crop.

4 CONCLUSION

We showed that incorporating *C. polygonoides*, olive leaves, and ash of olive leaves in soil infested by *R. solani* is effective in reducing the frequency of recovery of the pathogen from chilli, and in increasing dry mass of chilli aboveground plant parts. The incorportated plant materials can also serve as a source of nutrients to support plant health and plant growth. Future work should be conducted with focus on the relationship between plant growth and plant health and the amount, and timing and method of application of the biofumigants, and inoculum levels of *R. solani*.

5 REFERENCES

Abeysinghe, S. (2009). Effect of combined use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on biological control or *Rhizoctonia solani* on *Solanum melongena* and *Capsicum annuum*. *Plant Pathology Journal*, *8*, 9-16. https://doi.org/10.3923/ppj.2009.9.16

Abrahim, J., Afrah, E., M., Mudawi, M., E. (2014). Assessment of in-vitro anti-fungal potential of ethanolic extract of *Calligonum comosum* against two fungal postharvest pathogens of fruit and vegetables in Saudi Arabia. *International Journal of Applied Biology and Pharmaceutical Technology (ijabpt)*, *5*,90-94.

Aires, A., Carvalho, R., Barbosa, M. D. C., Rosa, E. (2009). Suppressing potato cyst nematode, *Globodera rostochiensis*, with extracts of Brassicacea plants. *American Journal of Potato Research, 86*, 327-333. https://doi.org/10.1007/s12230-009-9086-y

Alkhalifah, DHM, (2013). In-vitro antibacterial activity of ethanol extract of *Calligonum comosum* plant against four human pathogens in Saudi Arabia. *International Journal of Plant, Animal and Environmental Sciences*, *3*, 170 -175.

Benavente-Garcia, O., Castillo, J., Lorente, J., Ortuno, A., Del Rio, J. (2000). Antioxidant activity of phenolics extracted from *Olea europa* L. leaves. *Food Chemistry, 68,* 457-462. https://doi.org/10.1016/S0308-8146(99)00221-6

Benitez, E; Melgar, R; Sainz, H; Gomez, M; Nogales, R. (2000). Enzyme activities in the rhizosphere of pepper (*Capsicum annuum*, L.) grown with olive cake mulches. *Soil Biology & Biochemistry*, *13*(32), 1829-1835. https://doi.org/10.1016/S0038-0717(00)00156-5

Bubici, G., Amenduni, M., Colella, C., D'Amico, M., Cirulli, M. (2006). Efficacy of acibenzolar-S-methyl and two strobilurins, azoxystrobin and trifloxystrobin, for the control of corky root of tomato and *Verticillium* wilt of eggplant. *Crop Protection*, *25*, 814-820. https://doi.org/10.1016/j.cropro.2005.06.008

Castillo, C. R., Sotomayor, L. S., Ortiz, C. O., Leonelli, G. C., Borie, F. B., Rubio, R. H. (2009). Effect of arbuscular mycorrhizal fungi on an ecological crop

of chile peppers (*Capsicum annuum* L.). *Chilean Journal of Agricultural Research*, *69*, 79-87. https://doi.org/10.4067/S0718-58392009000100010

Cimen, I., Basbag, S., Temiz, M., Sagir, A. (2004). The effect of paclobutrazol, growth retardant, on cotton growth and Verticillium wilt (*Verticillium dahliae* Kleb.). *Plant Pathology Journal, 3*, 35-39. https://doi.org/10.3923/ppj.2004.35.39

Fan, C. M., Isong, G. R., Qi, P., Ji, G. H., He, Y. Q. (2008). Potential biofumigation effects of *Brassica oleracea* var. *caulorapa* on growth of fungi. *Journal of Phytopathology*, *156*, 321-325. https://doi.org/10.1111/j.1439-0434.2007.01343.x

Farid, A. B., Madiha, A., Mohamed, R. A. (2007). Evaluation of cytotoxic compounds from *Calligonum comosum* L. growing in Egypt. *Zeitschrift für Naturforschung*, *62*, 656 – 660.

Ghazanfar, S. A. (1994). *Handbook of Arabian Medicinal Plants*. CRC Press, p.173.

Goldberg, N. P. (2001). *Chilli pepper diseases*. New Mexico State University, Colleg of Agricultural, Consumer and Environmental Sciences. Circular 549. Online publication. http://aces.nmsu.edu/pubs/_circulars/circ549.html. 6/27/2010.

Halis Y, Ed. (2007). *Plant Encyclopedia in area Oued Souf: desert plants common in the Big East race*, El Walid: 62-63.

Jarvis, W. R. (1993). *Managing diseases in greenhouse crops*. American Phytopathological Society Press. St Paul, MN.

Kamil M, Jayaraj AF, Ahmad F, Gunasekhar C, Samuel S, Habiballah M, Chan K, (2000). Pharmacognostic andphytochemical standardisation of *Calligonum comosum*. *Journal of Pharmacy and Pharmacology, 52* (Suppl), 262 p.

Kaul, R. N. (1963). Need for afforestation in the arid zones of India. *La-Yaaran*, *13*.

Kirkegaard, J. A., Sarwar, M., Wong, P. T. W., Mead, A., Howe, G., Newell, M. (2000). Field studies on the biofumigation of take-all by Brassica break crops. *Australian Journal of Agricultural Research*, *51*, 445-456. https://doi.org/10.1071/AR99106

Laegdsmand, M., Gimsing, A. L., Strobel, B. W., Sørensen, J. C., Jacobsen, O. H., Christian, H., Hansen, B. (2007). Leaching of isothiocyanates through intact soil following simulated biofumigation. *Plant Soil*, *291,* 81-92. https://doi.org/10.1007/s11104-006-9176-2

Larkin, R. P., Griffin, T. S. (2007). Control of soilborne potato diseases using *Brassica* green manures. *Crop Protection*, *26*, 1067-1077. https://doi.org/10.1016/j.cropro.2006.10.004

Lazarovits, G., Conn, K. L., Abbasi, P. A., Soltani, N., Kelly, W., McMillan, E., Peters, R. D., Drake, K. A. (2008). Reduction of potato tuber diseases with organic soil amendments in two Prince Edward Island fields. *Canadian Journal of Plant Pathology*, *30*, 37-45. https://doi.org/10.1080/07060660809507494

Matthiessen, J. N., Kirkegaard, J. A. (2006). Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. *Critical Reviews in Plant Sciences*, *25*, 235-265. https://doi.org/10.1080/07352680600611543

Nogales, R., Melgar, Benetiz, E. (2006). Potential use of olive-waste ash from congeneration plants as a soil amendment. *Journal of Environmental Science and Health Part B, 41*, 1405-1415. https://doi.org/10.1080/03601230600964282

Pinelli, P., Galardi, C., Mulinacci, N., Vincieri, F., Tattini, ., Romani, A .(2000). Quali-quantitative analysis and antioxidant activity of different polyphenolic extracts from *Olea europea* L. leaves. *Journal of Commodity Science*, 39(2), 71-83.

Porras, M., Barrau, C., Romero, E., Zurera, C., Romero, F. (2009). Effect of biofumigation with *Brassica carinata* and soil solarization on *Phytophthora* spp. and strawberry. Yield. Proc. VIth Internat. Strawberry Symposium; Huelva, Spain, March 3-7, 2008. *Acta Horticulturae*, *842*, 969-972. https://doi.org/10.17660/ActaHortic.2009.842.215

Powelson, M. L., Rowe, R. C. (1993). Biology and management of early dying of potatoes. *Annual Review of Phytopathology*, *31*, 111-126. https://doi.org/10.1146/annurev.py.31.090193.000551

Romero, E.; Benitez, E.; Nogales, R. (2005). Suitability of wastes from olive-oil industry for initial reclamation of a Pb/Zn mine tailing. *Water, Air, & Soil Pollution*, *165*, 153–165. https://doi.org/10.1007/s11270-005-4638-3

Sanogo, S. (2003). Chile pepper and the threat of wilt diseases. *Plant Health Progress*. https://doi.org/10.1094/PHP-2003-0430-01-RV

Sanogo, S., Carpenter, J. (2006). Incidence of *Phytophthora* blight and *Verticillium* wilt within chilli pepper fields in New Mexico. *Plant Diseases*, *90*, 291-296. https://doi.org/10.1094/PD-90-0291

Scholberg, J. M., Zotarelli, L., Tubbs, R. S., Dukes, M. D., Muñoz-Carpena, R. (2009). Nitrogen uptake efficiency and growth of bell pepper in relation to

time of exposure to fertilizer solution. *Communications in Soil Science and Plant Analysis*, *40*, 2111-2131. https://doi.org/10.1080/00103620902960625

Siddiqui, I. A., Shaukat, S. S. (2005). Phenylacetic acid-producing *Rhizoctonia solani* represses the biosynthesis of nematicidal compounds in vitro and influences biocontrol of *Meloidogyne incognita* in tomato by *Pseudomonas fluorescens* strain CHA0 and its GM derivatives. *Journal of Applied Microbiology*, *98*, 43-55. https://doi.org/10.1111/j.1365-2672.2004.02457.x

Skaggs, R., Decker, M., VanLeeuwen, D. (2000). *A survey of southern New Mexico chilli producers: Production practices and problems*. NM Agricultural Experiment Station Technology Bulletin., 782.

Snapp, S. S., Date, K. U., Kirk, W., O'Neil, K., Kermen, A., Bird, G. (2007). Root, shoot tissues of *Brassica juncea* and cereal secale promote potato health. *Plant Soil, 294*, 55-72. https://doi.org/10.1007/s11104-007-9228-2

Solomon, T. E., Lodolini, E. M., Neri, D. (2015). Effects of olive shoot residues on shoot and root growth of potted olive plantlets. *Scientia Horticulturae*, *182,* 31-40. https://doi.org/10.1016/j.scienta.2014.11.008

Sultana, V., Ara, J., Ehteshamul-Haque, S. (2008). Suppression of root rotting fungi and root knot nematode of chile by seaweed and *Pseudomonas aeruginosa*. *Journal of Phytopathology*, *156*, 390-359. https://doi.org/10.1111/j.1439-0434.2007.01369.x

Tariq, S., Khan, R., Sultana, V., Ara, J., Ehteshamul-Haque, S. (2009). Utilization of endo-root fluorescent *Pseudomonas* of chilli for the management of root diseases of chilli. *Pakistan Journal of Botany, 41*, 3191-3198.

Vivioli, F., Bellomo, G. Galli, C. (1998). Free radical-scavenging properties of olive oil polyphenols. *Biochemical and Biophysical Research Communications*, *247,* 60-64. https://doi.org/10.1006/bbrc.1998.8735

Woodhall, J. W., Lees, A. K., Edwards, S. G., Jenkinson, P. (2007). Characterization of *Rhizoctonia solani* from potato in Great Britain. *Plant Pathology*, *56*, 286-295. https://doi.org/10.1111/j.1365-3059.2006.01545.x

Zaki, D., Abd-El-Aziz, M., El-Gengeihy, S., Morsi, N. (1984). Antimicrobial potentialities of some Egyptian desert plants. *Herba Hungarica, 23*, 73 – 84.

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Effect of different concentrations of saffron corm and leaf residue on the early growth of arugula, chickpea and fenugreek under greenhouse conditions

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ABSTRACT

In this study the effect of different concentrations of leaf (LR) and corm residue (CR) of saffron on seedling growth of fenugreek (*Trigonella foenum-graecum* L.), chickpea (*Cicer arietinum* L.) and arugula (*Eruca sativa* Mill.) as three potential companion crops for saffron were investigated under greenhouse condition. The experimental treatments were four concentrations (0.5, 1.5, 3 and 6 %) together with no residue treatment of LR and CR were arranged in completely randomized design. Results showed that the highest emergence percentage of chickpea was obtained at LR concentration of 6 %. Chickpea shoot length and mass had an increasing trend with increase in concentrations of saffron CR. LR and CR of saffron only at concentration of 3 and 6 % reduced the emergence percentage and emergence rate of fenugreek, but all concentration of CR and LR had no negative effect on length and mass of shoot. Saffron residue imposed a slight inhibitory effect on emergence percent and emergence rate of arugula, while had it a positive effect on some seedling growth characters especially root dry mass. Hence, three tested crops can be recommended as potential candidates to be associated with the saffron, although, the growth response of selected plants was dose-dependent and somewhat different.

Key words: saffron residue; associated crop; germination; seedling growth

IZVLEČEK

UČINKI RAZLIČNIH KONCENTRACIJ OSTANKOV GOMOLJEV IN LISTOV ŽAFRANA NA ZGODNJO RAST RUKVICE, ČIČERKE IN SABLJASTEGA TRIPLATA V RASTLINJAKU

V raziskavi so bili preučevani učinki različnih koncentracij ostankov listov (LR) in čebulastih gomoljev (CR) žafrana na rast sejank sabljastega triplata (*Trigonella foenum-graecum* L.), čičerke (*Cicer arietinum* L.) in rukvice (*Eruca sativa* Mill.) kot treh potencialnih soposevkov žafrana v rastlinjaku. Poskusna obravnavanja so obsegala štiri koncentracije (0.5, 1.5, 3 in 6 %) ostankov listov in gomoljev žafrana in kontrolo brez teh dodatkov v popolnem naključnem poskusu. Rezultati so pokazali, da je bil največji vznik čičerke pri uporabi listnih ostankov žafrana v koncentraciji 6 %. Masa in dolžina pogankov čičerke sta se povečevali z naraščajočo koncentracijo ostankov gomoljev žafrana. Dodatek ostankov listov in gomoljev žafrana v koncentracijah med 3 in 6 % je zmanjšal odstotek vznika sabljastega triplata, vendar so imeli dodatli listnih in gomoljevih ostankov žafrana negativni učinek na dolžino in maso poganjkov te rastline. Ostanki žafrana so imeli rahel zaviralni učinek na vznik rukvice, a so imeli pozitivni učinek na nekatere rastne parameter te vrste, še posebej na suho maso korenin. Zaključimo lahko, da bi te polščine lahko priporočili kot potencialne kandidate za vmesne posevke v žafranu, čeprav je njihov rastnih odziv nekoliko različen in odvisen od koncentracije ostankov žafrana.

Ključne besede: ostanki žafrana; soposevki; kalitev; rast sejank

1 INTRODUCTION

Saffron (*Crocus sativus* L.) as the most expensive spice of the world is used in the food, dairy, dye, cosmetic and perfume industries and also has cooking and medical applications (Molina et al*.,* 2005; Kumar et al., 2009). This plant perhaps originated from Iran, Greece and Asia Minor with a long history of cultivation in Iran and currently being cultivated in Iran, Spain, India, Egypt, Turkey, Pakistan, Azerbaijan, Greece, France, Italy, Morocco, China, and some other countries around the world (Kumar et al*.,* 2009). Iran with about 336 tons

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dry stigma production per year is currently the world's largest producer and exporter of saffron so that, about 90 % of the global production of this valuable crop is belonged to Iran. Khorasan-Razavi and Southern Khorasan provinces, respectively, with 82700 and 14450 ha area under cultivation and annual production of 258 and 51 tons, have a significant share of saffron global production (Mohammad-Abadi et al., 2011; Koocheki et al., 2016). Due to the special canopy structure of saffron, some of the light and the space are not used appropriately. Furthermore, it's non-extensive and partially shallow root system causes that water and nutrient to be absorbed only from the topsoil. Therefore, the use of suitable intercropping systems can be a useful method for efficient use of these above and underground resources (Koocheki et al., 2016; Fallahi et al., 2014).So far, very few studies have been conducted on the feasibility of intercropping of saffron with catch and cover crops. In a study Khosravi (2005) reported that the use of saffron-black cumin (*Bunium persicum* (Boiss.) B. Fedtsch. Syn.. *Carum persicum* Boiss.) intercropping is a suitable way for filling the empty spaces, weeds control and increasing inputs use efficiencies. Also, results of Faravani et al. (2010) in an intercropping system of saffron with black cumin (black zira or black caraway) revealed that the maximum stigma yield of saffron and seed yield of black cumin were obtained in planting ratio of 67:33 for saffron and black cumin, respectively. Results of Koocheki et al. (2009) showed that application of some spring crops such as chickpea (*Cicer arietinum* L.), ajowan (*Carum copticum* (L.) Link.)) and green cumin (*Cuminum cyminum* L.) can be considered as a component crop in saffron cultivation. In another study it has been reported that the use of Persian clover (*Trifolium resupinatum* L.) and fodder pea (*Lathyrus annus* L.) as associated crops with saffron is an effective way for improving of growth and yield of saffron (Koocheki et al., 2016). Naderi-Darbaghshahi et al. (2012) also found that saffron intercropping with three species of daisy family, Asteraceae, (*Matricaria chamomilla* L., *Tanacetum parthenium* (L.) Sch. Bip. and *Anthemis nobilis* L.) is possible, without any competition between main crop and associated crops. Koocheki et al. (2009) focused on the cultivation of associated crops that a part of their life cycle was simultaneous with saffron dormancy period. They concluded that there was a negative relationship between the growth period length and irrigation times of associated crops with saffron yield. Therefore, it seems that application of winter associated crops with maximum matching to vegetative phase of saffron growth is a more appropriate strategy (Koocheki et al., 2016).

Saffron is a summer dormant and winter active, low water requirement and low input crop with partially shallow root system which has also allelopathic effects on some other crops (Eghbali et al., 2008; Rashed-Mohassel et al., 2009; Koocheki et al., 2009; Kumar et al., 2009). Therefore, it seems that the selected associated crops for use in saffron intercropping systems must have certain characteristics including: 1 - maximum coincidence between the growth of selected crop with saffron vegetative growth stage (Naderi-Darbaghshahi et al., 2012), 2 - shorter growth cycle for reducing the irrigation times of spring associated crop and consequently avoidance from the loss of saffron yield (Koocheki et al., 2009), 3 - the different root system compared with saffron, 4 - low nutritional demand, 5 - producing sufficient organic matter that would be incorporated into the soil, 6 - the ability for nitrogen fixation or improving soil aggregation, 7 - producing residue with high decomposability and suitable C:N ratio (Koocheki et al., 2016), 8 - resistance to cold for winter associated crops, 9 - low water demand and resistance to drought stress regarding the low water requirement of saffron and its distribution in dry regains of Iran (Eghbali et al., 2008; Koocheki et al., 2009) and 10 - the lack of strong negative allelopathic effects between saffron and associated crop, because the intercropping is possible if there is no allelopathic effect between intercropped crops (Abbasi & Jahani, 2007; Fallahi et al., 2014). Accordingly, in this research three associated crops were selected and as the first step the possible inhibitory effects of saffron on these plants was explored.

Allelopathy is defined as the inhibitory or stimulatory biochemical interactions within plants using secondary metabolites (allelochemicals) especially phenolic acids and the terpenoid compounds (Chengxu et al., 2011; El-Darier & El-Dien, 2011). In some studies the inhibiting or sometimes stimulating effects of saffron petal, leaf and corm residue on germination and seedling growth criteria of some crops including redroot pigweed (*Amaranthus retroflexus* L.), lambs quarter (*Chenopodium album* L.), common vetch (*Vicia sativa* L.), common bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* (L.) Merr.), common wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), common barley (*Hordeum vulgare* L.), corn (*Zea mays* L.), canola (*Brassica napus* L. ssp. *napus*), Mexican cotton (*Gossypium hirsutum* L.), alfalfa (*Medicago sativa* L.), and arugula (*Eruca sativa* Mill.) has been reported (Eskandari- Torbaghan et al., 2007; Abbasi & Jahani, 2007; Eghbali et al., 2008; Rashed-Mohassel et al., 2009; Fallahi et al., 2014).

Investigations on the feasibility of saffron intercropping with other crops that can provide the more favorable growth condition for main crop and also increase the farmer income, has high importance. In this regards the first step is to ensure from the absence of strong inhibitory effects between the saffron and selected

associated crops. It must be also noted that the chemical composition of saffron leaves and corms are some different. For example, the corms have essential amino acids, glucose, glutamic acid, glycine, alanine, lysine, histidine, proline, leucine and secondary metabolites such as anthraquinones, while phenolic and flavonoid compounds has been identified in leaves (Behdani & Fallahi, 2015). Accordingly, the allelopathic effects of leaves and corms may be different, as well as the response of received crop of allelochemicals may also be dose dependent. Therefore, the aim of this study was to find out the possible allelopathic effects of different concentrations of saffron corms and leaves residue on early growth of fenugreek, chickpea and arugula. We chose these crops due partially to their growth cycle, relatively low competitiveness, low nutrient demand, the ability to biologically fix atmospheric nitrogen and possible crop establishment in autumn.

2 MATERIALS AND METHODS

In order to investigate the effect of saffron (land race of Sarayan) leaf (LR) and corm (CR) residue on seedling growth of fenugreek, chickpea and arugula three separate experiments were conducted based on a completely randomized design with three replications (a one-way ANOVA analysis) in the greenhouse of Sarayan Faculty of Agriculture, University of Birjand in 2014. In the first experiment the effects of four concentrations of LR (0.5, 1.5, 3 and 6 %) and four concentrations of CR (0.5, 1.5, 3 and 6 %) along with the control treatment (no-residue application) was evaluated on emergence and initial growth indices of chickpea. Similarly, in the second and the third experiments the effects of mentioned CR and LR levels were studied on emergence and seedling growth of fenugreek and arugula, respectively. The concentrations were selected based on previous studies that have been conducted about allelopathic effects of saffron on similar plants such as canola (Brassicaceae), soybean, vetch and bean (Leguminosae) (Abbasi & Jahani, 2007; Eghbali et al., 2008). These crops were they of the same botanical groupings as the test crop used in this experiment (fenugreek and chickpea from Leguminosae and arugula from Brassicaceae) to have justified the choice of concentration used.

The leaves and corms of saffron were harvested and dried separately at 75 ^{o}C for 72 hours and then were milled. After that, appropriate values of milled leaf and corm residue were mixed separately with soil (0 as control treatment as well as 5, 15, 30 and 60 g kg^{-1} soil for concentrations of 0, 0.5, 1.5, 3 and 6 %, respectively) and then mixed samples were poured into the cotton bags and were kept in greenhouse for 35 days (Eghbali et al., 2008). The aim of this period of time was to ensure the decomposition of plant residue (ambient temperature was 20-25 ^{o}C). During this period, the moisture of samples was adjusted about in field capacity (FC) point (by weighing two days among the samples and adding water lost) for appropriate decomposition and also avoiding of saffron residue leaching from the soil (Eghbali et al*.,* 2008). Finally, the prepared soil samples were used in planting trays for conducting the experiments. For each treatment, one row of planting trays (including 7 planting hole) was considered as an experimental unit (one replication) and then in each planting hole 5, 2 and 3 seeds were planted for arugula, chickpea and fenugreek (all landrace), respectively (35, 14 and 21 seeds in each experimental unit for mentioned plants, respectively). The difference in planted seed number between tested crops was due to difference in their canopy and root system size as well as differences in their dormancy and viability. All emerged plants were used at the end of experiment for destructive sampling. Soil mass in each experimental unit was about 100 g and the planting depth was 1-1.5 cm. The greenhouse temperature for germination of arugula, chickpea and fenugreek was kept at 17 (Jalilian & Khalili Aqdam, 2014), 26 (Ganjeali et al., 2011) and 21 ˚C (Mehrafarin et al*.,* 2011b), which are the optimum temperatures for them, respectively.

After sowing, the irrigation of planting trays was done daily with distillated water. The amount of used water was so low that it would be avoided from leaching of plant residues used in the soil (~ 60 ml for each experimental unit). The emerged seedlings were counted daily in the morning for ten days. At the end of tenth day, in each treatment the seedlings were removed from the soil and after washing, the shoot and seminal root lengths were determined. After that, all emerged seedlings in each treatment, were dried at 75 ^{o}C for 48 hours and then the average mass of seminal root and shoot were measured. Moreover, emergence rate (equivalent to germination rate in Petri Dish studies) was calculated using the below formula which has been developed by Maguire (1962).

$$Rs = \sum_{i=1}^{n} \frac{S_i}{D_i}$$

In which Rs is emergence rate, Si is daily seed emergence, Di is number of day to n computation and n is number of days computation.

Finally, for each of the variate, normality test (Kolmogorov-Smirnov) was done (Marsaglia et al., 2003) and data transformation (Arc-sin) was applied about emergence percent. Then, data analysis was carried out using ANOVA one-way analysis and calculations were done with SAS 9.1. In addition, means were compared by the Duncan's multiple ranges test at the 5 % level of probability.

3 RESULTS AND DISCUSSION

3.1 Effect of saffron residue on chickpea early growth

Saffron leaf and corm residue had significant effect on all the growth variables except root length (Table 1). There was no significant difference between leaf residue concentrations in terms of emergence percentage and emergence rate. Low levels of saffron corm residue (up to 1.5 %) imposed an increasing effect on emergence percentage and emergence rate, while higher levels of corm residue had a decreasing effect on these indices. However, it should be noted that emergence percentage of chickpea at 6 % concentration of corm residue was 12 % more than its 3 % concentration (Table 2). Allelochemicals have a threshold concentration which their effects below or over it can be different depending upon the sensitivity of the receiving species (Chon & Nelson, 2010). Considering appropriate growth of chickpea even at the highest corm residue concentration, it seems that the corm concentrations of more than 6 % is a threshold point for chickpea as receiving species. Plant growth can be increased below the allelopathic threshold, but severe growth reductions occur above the threshold concentration (Chon & Nelson, 2010).

There was statistically no significant different between control and all levels of leaf residue in terms of seminal root and shoot length. The effect of corm residue on shoot length was different from what was observed about leaf residue, so that, this index increased considerably in all levels of corm residues. For example, the value of shoot length at 6 % corm residue concentration was 2.31 times more than control (5.33 vs. 12.33 cm) (Table 2). Root dry mass of chickpea at leaf and corm concentration of 0.5 % had no significant difference with the control treatment, but more increase in residue concentration reduced this index. Increasing concentration of corm residue significantly increased shoot dry mass, while there was no significant difference between leaves residue levels with the control treatment in terms of shoot mass. In addition, all levels of corm residue decreased the root to shoot ratio in terms of length and mass compared with the control treatment (Table 2). In this regard, Farooq et al. (2013) reported that allelochemicals promote plant growth at certain concentration, which may be a reason for our findings, if we assume that the exudates from the saffron residues have allelopathic effect. They stated that allelochemicals secreted into rhizosphere improved nutrients availability through the processes of solubility, biological nitrification inhibition, chelation and selected retention. Understanding the possible reason of our observation required more research on precise identification of chemical compounds exudates by saffron residues into soil.

Table 1: Mean of square for the effects of experimental treatments (different concentrations of saffron corm and leaf residue) on emergence and seedling growth criteria of chickpea

Sources of variation	df	Emergence percentage	Emergence rate	Root length	Shoot length	Root to shoot length ratio	Root dry mass	Shoot dry mass	Root to shoot mass ratio
Treatment	8	323.1^{*}	1.01^{**}	3.56^{ns}	26.34^{**}	0.49^{**}	0.00017^{**}	0.00019^{**}	1.23^{**}
Error	18	122.7	0.11	2.27	3.02	0.11	0.00004	0.00001	0.099
C.V	-	12.3	14.3	11.54	12.49	11.95	19.24	14.56	13.61

**, * and ns, significant in 1 % and 5 % levels and not significant, respectively.
df and CV are degree of freedom and coefficient of variation, respectively.

Table 2: Mean comparisons of the effects of different levels of saffron corm and leaf residue on emergence and seedling growth criteria of chickpea

Treatment (percentage of residue)	Emergence percentage	Emergence rate (seedling per day)	Root length (cm)	Shoot length (cm)	Root to shoot length ratio	Root dry mass (g)	Shoot dry mass (g)	Root to shoot mass ratio
Control (0.0 %)	95.3^{a}	2.47^{b}	7.63^{ab}	5.33^{c}	1.46^{ab}	0.038^{ab}	0.016^{cd}	2.31^{a}
L* 0.5 %	90.6^{a}	2.24^{bc}	5.96^{ab}	5.33^{c}	1.23^{a-d}	0.049^{a}	0.019^{cd}	2.60^{a}
L1.5 %	80.6^{ab}	1.75^{cd}	7.53^{ab}	4.33^{c}	1.73^{a}	0.028^{bc}	0.014^{d}	2.04^{a}
L3.0 %	97.6^{a}	2.34^{bc}	7.00^{ab}	5.16^{c}	1.40^{abc}	0.038^{ab}	0.015^{d}	2.50^{a}
L6.0 %	100.0^{a}	2.75^{b}	5.36^{b}	6.46^{bc}	0.86^{bcd}	0.029^{bc}	0.014^{d}	2.01^{a}
C0.5 %	93.0^{a}	3.46^{a}	7.56^{ab}	10.10^{a}	0.766^{cd}	0.040^{ab}	0.028^{b}	1.41^{b}
C1.5 %	97.6^{a}	2.51^{b}	8.56^{a}	11.03^{a}	0.780^{cd}	0.032^{bc}	0.029^{b}	1.07^{b}
C3.0 %	69.0^{b}	1.41^{d}	5.66^{ab}	9.23^{ab}	0.610^{d}	0.024^{c}	0.022^{c}	1.09^{b}
C6.0 %	81.0^{ab}	2.28^{bc}	7.73^{ab}	12.33^{a}	0.666^{d}	0.037^{ab}	0.037^{a}	1.02^{b}

*L = leaf residue and C = corm residue. Means with the same letters in each column are not significantly different at the 0.05 level of probability.

On average there was no considerable difference between leaf and corm residue of saffron in terms of emergence percentage and emergence rate of chickpea. Mean data obtained from all levels of saffron residue revealed that emergence percent of chickpea for leaf and corm residue were 92.2 % and 85.2 %, and emergence rate were 2.27 and 2.42 seedling per day, respectively. In addition, mean root length in all corm residue treatments was 7.38 cm and in leaf residue was 6.46 cm. This difference was higher for shoot length, where mean of this index for all levels of corm and leaf residue was 5.32 and 10.67 cm, respectively. Considering the germination percentage, saffron leaf residue until 6 % and corm residue up to 1.5 % were the most appropriate concentrations for chickpea growth without any considerable deterrent allelopathic effect (Table 2). In similar study the results of Eghbali et al. (2008) showed that with increasing the amounts of saffron leaf residue added to the soil, the values of chlorophyll content, plant height, leaf area, shoot and root biomass of wheat, rye, vetch and bean increased, while with increasing the amount of saffron corm residue, all mentioned traits of the crops decreased compared with control treatment. Also, results of Yasmin et al. (1999) revealed that chickpea aqueous extract had an inhibitory effect on all germination and seedling growth indices of wheat, while, there was no negative effect of wheat on chickpea growth. Inhibiting effect of allelochemicals on germination and seedling growth is due to the alterations in some primary mechanisms such as cell ultra-structures, molecular biology as well as biochemical and physiological properties (Tanveer et al., 2012). Moreover, allelochemicals can alter the ions absorption rate and reduce macro and micronutrients concentrations in the plants (El-Darier & El-Dien, 2011). In our study there was no considerable inhibiting effect of saffron residue on chickpea growth, however, understanding the mechanisms of inhibiting effects require to more biochemical and physiological studies with higher concentrations of saffron residue.

The life cycle of planted companion crops in saffron field must be as much as possible similar to saffron growth cycle, in terms of time, because irrigation is harmful to saffron after its leaf senescence stage in mid-May (Koocheki et al., 2009). Chickpea is a cool season grain legume which reaches maturity stage approximately one month after saffron leaves senescence (Valimohammadi et al., 2007; Sharafzadeh, 2011). This plant is usually grown without irrigation and survives until harvesting despite progressively increasing drought (Krouma, 2010). Therefore, it could be an appropriate choice to be intercropped with saffron. This conclusion is due to the possible autumnal planting of chickpea, considerable synchronization of chickpea growth cycle with saffron and good resistance of chickpea against drought which is according to low water requirement of saffron.

3.2 Effect of saffron residue on fenugreek early growth

All measured seedling growth characters except for root dry mass were affected significantly by saffron residue

(Table 3). Percentage of emergence increased up to the middle levels of leaf residue (1.5 %) and then decreased, so that, the amount of this index at the highest level of leaf residue (6 % leaf crude powder) was 17 % lower than control treatment (Table 4). Emergence percentage was similar to control treatment up to the high levels of soil application of corm residue but then decreased about 12 % in the highest level (6 %) of corm residue compared with the control treatment. The average value of this index for leaf residue levels was 81.5 % and for all corm residue concentrations was 83.3 % (Table 4). Emergence rate of fenugreek showed some reduction by increasing in concentration of saffron leaf residue and the value of this criterion at 6 % leaf concentration decreased about 22 % compared with the control treatment. Similarly, the amount of this index decreased significantly with increase in corm residue concentrations above 3 %, so that the value of this index in the highest level of corm residue application was 35 % lower than control treatment (Table 4).

Root length of fenugreek seedlings decreased by soil application of saffron corm and leaf residue. In addition, there was no significant difference between control and leaf and corm residue levels in terms of shoot length. Root to shoot length ratio decreased in response to saffron leaf and corm residue application (Table 4). It seems that this phenomenon is because of roots are directly exposed to possible allelopathic compounds which can more affect their growth. This finding is similar to results of Eghbali et al. (2008) on wheat and bean but different with those obtained on rye and vetch. They reported that this variation is due to the differences in plant genetic nature. In addition, they stated that reduction in root to shoot ratio will probably lead to more plant sensitivity to environmental stress at later growth phases.

Table 3: Mean of square for the effects of experimental treatments (different concentrations of saffron corm and leaf residue) on emergence and seedling growth characters of fenugreek

Sources of variation	df	Emergence percentage	Emergence rate	Root length	Shoot length	Root to shoot length ratio	Root dry mass	Shoot dry mass	Root to shoot mass ratio
Treatment	8	155.3^{*}	1.60^{**}	4.26^{**}	0.60^{*}	0.112^{**}	2.65^{ns}	0.0000009^{ns}	26.01^{ns}
Error	18	51.0	0.25	0.85	0.37	0.016	2.28	0.0000006	0.65
C.V	-	8.61	13.10	13.21	8.98	12.28	13.23	13.43	8.96

**, * and ns, significant in 1 % and 5 % levels and not significant, respectively.
df and CV are degree of freedom and coefficient of variation, respectively.

Table 4: Mean comparison of the effects of different levels of saffron corm and leaf residues on emergence and seedling growth criteria of fenugreek

Treatment (percentage of residue)	Emergence percentage	Emergence rate (seeding per day)	Root length (cm)	Shoot length (cm)	Root to shoot length ratio	Root dry mass (g)	Shoot dry mass (g)	Root to shootmass ratio
Control	87.0^{ab}	4.21^{bc}	9.56^{a}	6.63^{ab}	1.45^{a}	0.0019^{a}	0.0059^{a}	0.32^{a}
L* 0.5 %	88.6^{a}	4.13^{bc}	6.10^{c}	6.56^{ab}	0.93^{bc}	0.0019^{a}	0.0056^{a}	0.34^{a}
L1.5 %	90.3^{a}	4.10^{bc}	7.26^{bc}	6.66^{ab}	1.08^{b}	0.0025^{a}	0.0062^{a}	0.39^{a}
L3.0 %	77.33^{abc}	3.56b^{cd}	6.66^{bc}	6.63^{ab}	1.00^{b}	0.0024^{a}	0.0061^{a}	0.41^{a}
L6.0 %	69.6^{c}	3.27^{cd}	6.16^{bc}	6.00^{b}	1.03^{b}	0.0017^{a}	0.0058^{a}	0.30^{a}
C0.5 %	87.3^{ab}	5.19^{a}	7.40^{bc}	6.76^{ab}	1.10^{b}	0.0017^{a}	0.0051^{a}	0.33^{a}
C1.5 %	85.6^{ab}	4.26^{b}	7.90^{b}	7.20^{a}	1.11^{b}	0.0021^{a}	0.0070^{a}	0.30^{a}
C3.0 %	85.6^{ab}	3.26^{cd}	6.33^{bc}	7.16^{ab}	0.88^{bc}	0.0022^{a}	0.0056^{a}	0.39^{a}
C6.0 %	74.6^{c}	2.71^{d}	5.70^{c}	7.53^{a}	0.75^{c}	0.0018^{a}	0.0056^{a}	0.32^{a}

*L = leaf residue and C = corm residue. Means with the same letters in each column are not significantly different at the 0.05 level of probability.

Overall regards to emergence percentage and rate, 1.5 and 3 % concentrations of saffron leaf and corm residue were the most appropriate treatment for fenugreek growth, but higher increase in residue resulted to occurrence of deterrent allelopathic effect (Table 4). In similar study it has been reported that the allelopathic effects of saffron corm residue on the early growth characters of wheat, rye, vetch and bean was inhibiting, while the allelopathic effect of saffron leaves residue was stimulating (Eghbali et al*.,* 2008). Edrisi & Farahbakhsh (2011) also reported that application of different concentrations of leaf extract of flixweed (*Descurainia sophia* (L.) Webb ex Prantl) exerted a positive effect on common wheat primary growth only up to the concentration of 4 %. In addition, results of Yasmin et al. (1999) showed that chickpea and wheat extracts are containing some compounds which had inhibitory or stimulatory effect on each other.

Our results showed that fenugreek root was relatively more sensitive to saffron corm and leaf powder than shoot. This result is in agreement with earlier study of Teerarak et al. (2010) and can be explained by this reason that roots are the first organ which emerge and are in direct contact with allelochemicals (Teerarak et al., 2010; Tanveer et al., 2012). Results of Eghbali et al. (2008) showed that soil application of saffron corm residue had more increasing effect on root biomass of rye and vetch, while its effect on wheat and bean was more effective on aerial part, because of differences in site and mode of action of allelochemicals in different crops.

Fenugreek is a vegetable, spice, medicinal, food and feed crop that can be a useful legume crop for incorporation into short-term rotation (Mehrafarin et al*.,* 2011a). This nitrogen fixing crop is fairly drought resistant and generally grown as a winter crop in areas with mild winter and as spring crop in areas with soil that keeps moisture in the summer. The plant is partially a short living annual crop that its growth period is between 80 to 140 days, depend on ecotype, so that for spring and autumnal sowing, ripening take place usually 3–5 and about 7 months after sowing, respectively (Mehrafarin et al*.,* 2011a). Therefore, considering the mentioned characteristics and the large coincidence of its growth cycle with saffron growth season, it could be used as a suitable associated crop in saffron cultivation especially as a green manure.

3.3 Effect of saffron residue on arugula early growth

Results of analysis of variance revealed that the effect of soil application of different levels of saffron leaf and corm residue was significant on all early growth indices of arugula (Table 5). Increase in concentration of saffron leaf residue caused a reduction in emergence percentage and rate, so that the amounts of these criteria in the highest leaf residue concentration decreased about 13 and 19 % compared with the control treatment, respectively. However, the negative effects of corm residue on arugula emergence was observed at the lower concentrations of corm, but more increase in corm residue imposed a positive effect, where the highest

emergence percentage among all experimental treatments was obtained at corm concentration of 6 % (Table 6).

There were no significant differences in the mean root length with increase in the concentration of saffron leaf residue. However, saffron corm residue up to the concentration of 3 % had a stimulatory effect on root length, but the higher concentrations of corm residue decreased the root growth. The positive effects of corm residue on root growth of arugula shows the lack of inhibiting compounds or even the nutritional values of saffron residue for its better growth (Eghbali et al. 2008). Root to shoot length ratio also was dose-dependent, where leaf residue up to the concentration of 1.5 % and corm residue up to the concentration of 3 %, increased the amount of this index. In addition, root mass of arugula showed a positive response to application of saffron leaf and corm residue. Its highest value obtained in the highest level of leaf residue, which was 42 % more than control treatment. By increase in leaf residue concentration the shoot dry mass of arugula increased, while corm residue at concentrations above 1.5 % had a deterrent effect on shoot mass (Table 6). Totally, the effect of saffron corm and leaf residue even until 6 % concentration wasn't so inhibiting on early growth of arugula, therefore, this plant can be a good choice for intercropping with saffron.

In similar work, it has been reported that the aqueous extract of saffron petals had a positive effect on early growth indices of cotton (*Gossypium hirsutum* L.) in low and medium concentrations, whereas in high concentrations it decreased all measured indices (Eskandari- Torbaghan et al*.,* 2007). A number of plant species have chemicals that cause allelopathic activity on the growth of other plants and under certain conditions, these compounds are released into the environment from living plants or residues in sufficient quantities to affect neighboring or successional plants (Kato-Noguchi et al., 2010). It has been reported that some allelochemicals reduce shoot and root expansion and plant growth by blocking of nutrient reserve, altering cell division and differentiation as well as prevention from ion and water uptake, phytohormon metabolism, respiration, photosynthesis, enzyme function, signal transduction and gene expression (El-Darier & El-Dien, 2011).

Mixed cropping is one of the methods for increasing resource use efficiency in saffron cultivation. This strategy is only possible if there are no negative allelopathic interactions between the intercropped crops (Eskandari- Torbaghan et al*.,* 2007). In a study it has been reported that saffron leaf and corm residue exerted different effects on growth criteria of bean and vetch, as its leaf tissue increased and corm tissue decreased seedling growth indices of the plants in compared with control (Eskandari- Torbaghan et al*.,* 2007). Arugula is a fast growing, cool season crop with a short growth cycle (Jakše et al*.,* 2013). It is an important oilseed crop of the rapeseed-mustard group that grown on marginal lands with poor fertility. Moreover, due to its drought tolerance and adaptability to adverse environmental conditions, it is preferred over *Brassica* species under water scarce conditions (Jakhar et al., 2010). Therefore, considering the mentioned characteristics combined with current experimental results it seems that arugula is a good choice to be intercropped with saffron.

Table 5: Mean of square for the effects of experimental treatments (different concentrations of saffron corm and leaf residue) on emergence and seedling growth characters of arugula

Sources of variation	df	Emergence percentage	Emergence rate	Root length	Shoot length	Root to shoot length ratio	Root dry mass	Shoot dry mass	Root to shoot mass ratio
Treatment	8	167.8**	1.70**	2.05**	0.283*	0.215*	1.46*	1.20*	0.47*
Error	18	23.8	0.44	0.25	0.167	0.100	9.85	8.05	0.16
C.V	-	6.56	10.69	9.53	11.19	12.56	16.84	15.30	11.18

**, * and ns, significant in 1 % and 5 % levels and not significant, respectively.
df and CV are degree of freedom and coefficient of variation, respectively.

Table 6: Mean comparison of the effects of different levels of saffron corm and leaf residue on emergence and seedling growth characters of arugula

Treatment (percentage of residue)	Emergence percentage	Emergence rate (seedling per day)	Root length (cm)	Shoot length (cm)	Root to shoot length ratio	Root dry mass (g)	Shoot dry mass (g)	Root to shoot mass ratio
Control (0.0	83.0^{a}	7.66^{a}	5.56^{bc}	4.00^{a}	1.39^{abc}	0.0008^{b}	0.0013^{b}	0.58^{b}
L* 0.5 %	84.0^{a}	6.90^{ab}	5.10^{bc}	3.66^{ab}	1.41^{abc}	0.0011^{ab}	0.0015^{ab}	0.73^{b}
L1.5 %	73.6^{b}	5.53^{c}	5.63^{bc}	3.20^{b}	1.79^{a}	0.0010^{ab}	0.0015^{ab}	0.68^{b}
L3.0 %	64.0^{c}	5.66^{bc}	5.00^{cd}	3.63^{ab}	1.37^{abc}	0.0014^{a}	0.0016^{ab}	0.91^{b}
L6.0 %	73.3^{b}	6.60^{abc}	5.43^{bc}	3.46^{ab}	1.03^{c}	0.0014^{a}	0.0019^{a}	0.76^{b}
C0.5 %	64.6^{bc}	5.56^{c}	4.13^{d}	3.96^{ab}	1.05^{bc}	0.0011^{ab}	0.0007^{c}	1.75^{a}
C1.5 %	71.6^{bc}	5.46^{c}	6.01^{ab}	3.60^{ab}	1.68^{a}	0.0012^{ab}	0.0016^{ab}	0.83^{b}
C3.0 %	73.3^{b}	6.46^{abc}	6.66^{a}	4.05^{a}	1.66^{ab}	0.0011^{ab}	0.0009^{c}	1.38^{ab}
C6.0 %	85.6^{a}	6.13^{bc}	4.10^{d}	3.28^{ab}	1.25^{abc}	0.0009^{ab}	0.0007^{c}	1.33^{ab}

*L = leaf residue and C = corm residue. Means with the same letters in each column are not significantly different at the 0.05 level of probability.

4 CONCLUSIONS

This research was a preliminary greenhouse study towards possibility of simultaneous saffron and some associated crop cultivation (fenugreek, chickpea and arugula). Generally, the saffron corm and leaf residue not only did not considerably decrease the growth of tested crops, which in some cases increased their growth parameters. Therefore, these crops are potentially good candidates to be used in intercropping systems with saffron. However, it is also a need to be investigated the effects of proposed crops residue on saffron growth. Moreover, further studies under field conditions are necessary to evaluate the possible use of mentioned associated crops in saffron field.

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6 REFERENCES

Abbassi, F. & Jahani, M. (2007). Allelopathic effects of saffron corms on seed germination of several important crops. *Acta Horticulture*, *739*, 269-273. https://doi.org/10.17660/ActaHortic.2007.739.33

Behdani, M.A., Fallahi, H.R. 2015. *Saffron: Technical Knowledge Based on Research Approaches*. University of Birjand Press. [In Persian].

Chengxu, C., Mingxing, Z., Xuhui, C. & Bo, Q. (2011). Review on allelopathy of exotic invasive plants. *Procedia Engineering*, *18*, 240-246. https://doi.org/10.1016/j.proeng.2011.11.038

Chon, C. U. & Nelson, C. J. (2010). Allelopathy in compositae plants. A review. *Agronomy for Sustainable Development*, *30*, 349-358. https://doi.org/10.1051/agro/2009027

Edrisi, S. & Farahbakhsh, A. (2011). Allelopathic Effects of *Sisymbrium irio* L. and *Descurainia sophia* (L.) Schur on the Germination of Wheat (*Triticum aestivum* L.). *World Academy of Science, Engineering and Technology*, *50*, 638-640.

Eghbali, S., Rashed Mohassel, M. H., Nassiri Mahallati, M. & Kazerooni Monfared, E. (2008). Allelopathic potential of shoot and corm of saffron residues on

wheat, rye, vetch and bean. *Journal of Field Crops Researches*, *6*(2), 227-234.(In Persian with English Summary).

El-Darier, S. M. & El-Dien, M. H. Z. (2011). Biological activity of *Medicago sativa* L. (alfalfa) residues on germination efficiency, growth and nutrient uptake of *Lycopersicon esculentum* L. (tomato) seedlings. *Journal of Taibah University for Science*, *5*, 7-13. https://doi.org/10.1016/S1658-3655(12)60033-8

Eskandari-Torbaghan, M., Abbasi, R. & Astaraei, A. (2007). Effect of saffron (*Crocus sativus* L.) petals on germination and primary growth of cotton (*Gossypium hirsutum* L.). *Acta Horticulture*, *739*, 87-91. https://doi.org/10.17660/ActaHortic.2007.739.10

Fallahi, H. R., Paravar, A., Behdani, M. A., Aghhavani-Shajari, M. & Fallahi, M. J. (2014). Effects of Saffron corm and leaf extracts on early growth of some plants to investigate the possibility of using them as associated crop. *Notulae Scientia Biologicae*, *6*(3), 282-287. https://doi.org/10.15835/nsb.6.3.9259

Fallahi, H. R., Fadaeian, G., Gholami, M., Daneshkhah, O., Hosseini, F.S., Aghhavani-Shajari, M. & Samadzadeh, A. (2015). Germination response of grass pea (*Lathyrus sativus* L.) and arugula (*Eruca sativa* L.) to osmotic and salinity stresses. *Plant Breeding and Seed Science*, *71*, 97-108. https://doi.org/10.1515/plass-2015-0025

Faravani, M., Behshtii, A. & Khanizadeh, H. (2010). Yield performance of black zira and saffron intercropping system in different plant distances and replacement series. *International Journal of Science and Nature, 1*(2), 195-197.

Farooq, M., Bajwa, A. A., Cheema, S., Cheema, Z. A. (2013). Application of allelopathy in crop production. *International Journal of Agriculture and Biology*, *15*, 1367-1378.

Ganjeali, A., Parsa, M. & Amiri-Deh-Ahmadi, S.R. (2011). Determination of cardinal temperatures and thermal time requirement during germination and emergence of chickpea genotypes (*Cicer arietinum* L.). *Iranian Journal of Pulses Research*, *2*(2), 97-108. In Persian with English Summary).

Jakhar, M. L., Jajoria, R. N., Sharma, K. C., & Ram, S. (2010). Genetic divergence in Taramira (*Eruca sativa* Mill.). *Journal of Oilseed Brassica*, *1*(2), 79-83.

Jakše, M., Hacin, J. & Kacjan Maršić, N. (2013). Production of rocket (*Eruca sativa* Mill.) on plug trays and on a floating system in relation to reduced nitrate content. *Acta Agriculturae Slovenica*, *101*(1), 59- 68. https://doi.org/10.2478/acas-2013-0007

Jalilian, J. & Khalili-Aqdam, N. (2014). Effects of alternative temperatures on germination rate of rocket seed (*Eruca sativa*). *Iranian Journal of Seed Research*, *2*(1), 127-133. In Persian with English Summary).

Kato-Noguchi, H., Seki, T. & Shigemori, H. (2010). Allelopathy and allelopathic substance in the moss *Rhynchostegium pallidifolium*. *Journal of Plant Physiology*, *167*, 468-471. https://doi.org/10.1016/j.jplph.2009.10.018

Khosravi, M. (2005). *Intercropping black zira (Bonium persicum) with saffron and annual crops: Agroecological and economic perspectives*. PhD Thesis, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran. (In Persian with English Summary).

Koocheki, A., Najibnia, S. & Lalehgani, B. (2009). Evaluation of saffron yield (*Crocus sativus* L.) in intercropping with cereals, pulses and medicinal plants. *Iranian Journal of Field Crops Research*, *7*(1), 163-172. (In Persian with English Summary).

Koocheki, A., Rezvani Moghaddam, P. & Fallahi, H. R., (2016). Effects of planting dates, irrigation management and cover crops on growth and yield of saffron (*Crocus sativa* L.). *Journal of Agroecology*, *8*(1), 435-451. (In Persian with English Summary).

Krouma, A. (2010). Plant water relations and photosynthetic activity in three Tunisian chickpea (*Cicer arietinum* L.) genotypes subjected to drought. *Turkish Journal of Agriculture and Forestry*, *34*, 257-264.

Kumar, R., Virendra, S., Kiran, D., Sharma, M., Singh, M. K. & Ahuja, P. S. (2009). State of art of saffron (*Crocus sativus* L.) agronomy: A comprehensive review. *Food Reviews International*, *25*, 44-85. https://doi.org/10.1080/87559120802458503

Maguire, J. D. (1962). Speed of germination aid in selection and evaluation for seedling emergence and vigor. *Crop Science*, *2*, 176-177. https://doi.org/10.2135/cropsci1962.0011183X000200020033x

Marsaglia, G., Tsang, W.W. & Wang, J. (2003). Evaluating Kolmogorov's distribution. *Journal of Statistical Software*, *8*(18), 1-4. https://doi.org/10.18637/jss.v008.i18

Mehrafarin, A., Rezazadeh, S., Naghdi Badi, H., Noormohammadi, G., Zand, E. & Qaderi, A. (2011a). A review on biology, cultivation and biotechnology of Fenugreek (*Trigonella foenum-*

graecum L.) as a valuable medicinal plant and multipurpose. *Journal of Medicinal Plants*, *37*, 6-24.

Mehrafarin, A., Naghdi Badi, H., Noormohammadi, G., Zand, E., Rezazadeh, S. & Qaderi, A. (2011b). Effects of environmental factors and methanol on germination and emergence of Persian Fenugreek (*Trigonella foenum-graecum* L.). *African Journal of Agricultural Research*, *6*(19), 4631-4641.

Mohammad-Abadi, A. A., Rezvani-Moghaddam, P. & Fallahi, H. R. (2011). Effects of planting pattern and the first irrigation date on growth and yield of saffron. *Journal of Agroecology*, *3*(1), 84-93. (In Persian with English Summary).

Molina, R. V., Valero, M., Navarro, Y., Guardiola, J. L. & Garcıa-Luis, A. (2005). Temperature effects on flower formation in saffron (*Crocus sativus* L.). *Scientia Horticulturae*, *103*, 361-379. https://doi.org/10.1016/j.scienta.2004.06.005

Naderi Darbaghshahi, M. R., Banitab, A. R. & Bahari, B. (2012). Evaluating the possibility of saffron and chamomile mixed culture. *African Journal of Agricultural Research*, *7*(20), 3060-3065. https://doi.org/10.5897/AJAR11.999

Rashed Mohassel, M. H., Gherekhloo, J. & Rastgoo, M. (2009). Allelopathic effects of saffron (*Crocus sativus*) leaves and corms on seedling growth of redroot pigweed (*Amaranthus retroflexus*) and lambsquarter (*Chenopodium album*). *Iranian Journal of Field Crop Research*, *7*(1), 53-61.

Sharafzadeh, S. (2011). Saffron: A concise review of researches *Advances in Environmental Biology*, *5*(7), 1617-1621.

Tanveer, A., Jabbar, M. K., Kahliq, A., Matloob, A., Abbas, R. N. & Javaid, M. M. (2012). Allelopathic effects of aqueous organic fractions of *Euphorbia dracunculoides* Lam on germination and seedling growth of chickpea and wheat. *Chilean Journal of Agricultural Research*, *72*(4), 495-501. https://doi.org/10.4067/S0718-58392012000400006

Teerarak, M., Laosinwattana, C. & Charoenying, P. (2010). Evaluation of allelopathic, decomposition and cytogenetic activities of *Jasminum officinale* L. f. var. *grandiflorum* (L.) Kob. on bioassay plants. *Bioresource Technology*, *101*, 5677-5684. https://doi.org/10.1016/j.biortech.2010.02.038

Valimohammadi, F., Tajbakhsh, M. & Saied, A. (2007). Comparison winter and spring sowing dates and effect of plant density on yield, yield components and some quality, moephological traits of chickpea (*Cicer arietinum* L.) under environmental condition of Urmia, Iran. *Journal of Agronomy*, *6*(4), 571-575. https://doi.org/10.3923/ja.2007.571.575

Yasmin, S., Saleem, B. & Irshad, A. (1999). Allelopathic effects of aqueous extract of chickpea (*Cicer arietinum*) and wheat (*Triticum aestivum* L.) on each other's growth and quality. *International Journal of Agriculture and Biology*, *3*, 110-111.

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Physiological and biochemical changes in *Matricaria chamomilla* induced by *Pseudomonas fluorescens* and water deficit stress

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ABSTRACT

Environmental stresses and rhizosphere microorganisms affect growth parameters and accumulation of active ingredients especially in plants with medicinal properties. The present study examined the effects of chamomile (*Matricaria chamomilla* L.) seedling inoculation with *Pseudomonas fluorescens* PF-135 strain on its growth parameters, photosynthetic pigments, proline, malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) content, and essential oil concentration at both regular watering and water deficit experiments. Based on the obtained results, water deficit stress reduced root dry mass, and flower fresh and dry mass as well. However, amount of H_2O_2 and MDA in root and shoot tissues were considerably lower in inoculated plants compared to non-inoculated ones under both normal watering and water deficit regimes. It indicates that lipid peroxidation and production of reactive oxygen species has been diminished in inoculated plants. Also, essential oil content in inoculated plants significantly increased compared with that of non-inoculated ones under water deficit stress condition. It can be concluded that *P. fluorescens* PF-135 strain has an outstanding potential to alleviate adverse effects of water deficit on plant growth, and hence can be used as an excellent PGPR in order to boost chamomile productivity especially under water deficit stress condition.

Key words: PGPR; chamomile; essential oil; injury indices; water deficit stress

IZVLEČEK

FIZIOLOŠKE IN BIOKEMIČNE SPREMEMBE PRAVE KAMILICE (*Matricaria chamomilla* L.) VZPODBUJENE Z BAKTERIJO *Pseudomonas fluorescens* IN POMANKANJEM VODE

Okoljski stresi in mikroorganizmi v rizosferi vplivajo na rastne parametre in na kopičenje aktivnih snovi, še posebej v rastlinah z zdravilnimi lastnostmi. V raziskavi so bili preučevani učinki inokulacije sejank kamilice (*Matricaria chamomilla* L.) s sevom bakterije *Pseudomonas fluorescens* PF-135 na njene rastne parametre, vsebnost fotosinteznih barvil, prolina, malondialdehida (MDA), vodikovegan peroksida (H_2O_2) in eteričnih olj v razmerah rednega zalivanja in ob pomanjkanju vode. Rezultati so pokazali, da je pomanjkanje vode zmanjšalo suho maso korenin in svežo ter suho maso cvetov. Količini H_2O_2 in MDA v tkivih korenin in poganjkov sta bili manjši pri inokuliranih rastlina v primerjavi z neinokuliranimi pri obeh vodnih režimih. To kaže, da je bila peroksidacija lipidov in tvorba reaktivnih zvrsti kisika manjša pri inokuliranih rastlinah. Tudi vsebnost eteričnioh olj se je pri inokuliranih rastlinah značilno povečala v primerjavi z neinokuliranimi v razmerah pomanjkanja vode. Zaključili bi lahko, da ima sev bakterije *P. fluorescens* PF-135 izjemen potencial za odpravljanje škodljivih učinkov pomanjkanja vode na rast rastlin in bi bil lahko uporabljen kot odlična PGPR snov za povečanje pridelka kamilice, še posebej v razmerah pomanjkanja vode.

Ključne besede: PGPR; kamilica; eterična olja; indeksi poškodovanosti; sušni stres

1 INTRODUCTION

Chamomile (*Matricaria chamomilla* L.) is a well-known herbaceous annual medicinal plant belonging to Asteraceae family. It is widely used in traditional medicine and pharmaceutical and cosmetic industries around the globe (Frank and Schilcher, 2005). Chamomile possesses antimicrobial and antioxidant properties and significant antiplatelet and anticancer activities (Srivastava et al., 2010; Charousaei et al., 2011; Roby et al., 2013). Flower of chamomile contains essential oil and flavonoids, which contribute to its medicinal properties (Srivastava and Gupta, 2015).

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Water stress adversely affects physiological aspects of plants (Farooq et al., 2009). One of the inevitable consequences of water deficit is emergence of various reactive oxygen species (ROSs) in different cellular compartments (Cruz de Carvalho, 2008). If amount of ROSs inside the cell exceed the certain threshold, they become extremely deleterious, initiating uncontrolled oxidative cascades that damage photosynthetic pigments, enzymes and other cell constituents (Gill and Tuteja, 2010; Mittler, 2002). ROSs may also attack cellular membrane phospholipids, leading to production of malondialdehyde (MDA). Since MDA is one of the resultants of cellular lipid peroxidation, less amount of MDA production is a sign of more cell membrane integrity. Generally, plants employ various strategies to manage ROSs, including increase in antioxidant activity and production of various compatible organic solutes such as proline (Ashraf and Foolad, 2007).

Plant growth promoting rhizobacterias (PGPRs) are sort of beneficial bacteria predominantly living in the soil around the plant root surface (rhizosphere). These kinds of bacteria stimulate growth and development of the plants via production and exudation of several regulatory chemicals (Ghorbanpour et al., 2015). PGPRs increase plant yield and can improve plant tolerance to abiotic stresses as well, and hence are considered as an excellent alternative for chemical fertilizers which threaten environment and human health (Vessey, 2003). The effects of PGPRs on plant performance are complex. They make many positive morphological and physiological changes in plants (Sánchez-Blanco et al., 2004; Glick et al., 2007 and 1995). For example, PGPRs induce synthesis of osmo-protectants such as proline and help the plants to maintain cell membrane integrity especially under stress conditions. Therefore, PGPRs can alleviate adverse effects of environmental stresses on the plants (Christians et al., 2009; Glick et al., 2007; Chauhan et al., 2015).

Among the PGPRs, *Pseudomonas fluorescens* (Flügge 1886) Migula, 1895 is a dominant Gram-negative, rod-shaped bacterium in the soil that contributes to stimulation of plant growth as well as inhibiting growth of pathogens (Mehrabi et al., 2016). There is little information on physiological responses of chamomile to inoculation with these bacteria especially under water deficit stress condition. Therefore, the main objective of present investigation was to evaluate changes in stress related physiological parameters, biomass and essential oil content of chamomile (*Matricaria chamomilla* L.) in response to *P. fluorescens* inoculation under both water deficit stress and non-stress conditions.

2 MATERIALS AND METHODS

2.1 Plant Growing condition, Treatments and Sampling

Chamomile (*Matricaria chamomilla* L.) seeds were purchased from Research Institute of Forests and Rangelands, Karaj, Iran. Sterilized seeds with 1 % sodium hypochlorite were washed three times with distilled water and put on filter paper (Whatman no. 1) in Petri dishes for initiating seed germination.

In order to identify the best *fluorescent pseudomonads* strain, 20 PGPR strains (belonging to the fluorescent pseudomonads group) were obtained from Department of Plant Protection, Azarbaijan Shahid Madani University, Tabriz, Iran. A single colony of each PGPR strain transferred to 100-ml flasks containing 25 ml of tryptone soybean broth (TSB) and grown aerobically in the flasks on a rotating shaker (120 rpm) for 72 h at 28 ºC. The bacterial suspension centrifuged (6000 rpm for 15 min) and then washed and diluted in sterile 0.85 % NaCl (saline solution) to attain a final concentration of 109 CFU.ml^{-1}. The PGPR strains then were evaluated based on their ability to increase chamomile seedling growth index in a sand culture assay (data are not shown). Based on the results of this assay, the *Pseudomonas fluorescens*-135 (PF-135) was determined as the best *pseudomonads* strain (Mohammadi et al., 2017). Plant growth promoting activities of this strain are shown in Table 1.

Table 1: Multiple plant growth promoting activities of *Pseudomonas fluorescens*-135 (PF-135).

Pseudomonas strains	PGPR activities					Ecological site of strains isolation (rhizosphere type)
	P solubility (µg ml^{-1})	Siderophore production	IAA production (mg l^{-1})	ACC deaminase activity	*PhlD* gene	
PF-135	328.35	0.42	2.46	1.02	active	Wheat (cv. Azar2)

PGPR: Plant growth promoting rhizobacteria, P: phosphorus, IAA: Indole-3-acetic acid (without presence of tryptophan), ACC: 1-aminocyclopropane-1-carboxylic acid.

Part of germinated seeds was inoculated with the inoculums (10^9 CFU.ml^{-1}) of the promising selected strain (i.e. *P. fluorescens* PF-135). Then, all of the seeds (inoculated or non-inoculated) were sown in pots containing sandy loam soil. Pots were placed in greenhouse with 16 h light period per day and 28/18 ˚C day/night temperatures. Supplementary light (250 µmol m^{-2} s^{-1}) was provided if necessary.

A factorial experiment in randomized complete block design with three replications was performed. Factors were inoculation (i.e. inoculation with *P. fluorescens* PF-135 strain and non-inoculation) and watering regime (i.e. well-watered and watering up to 50 % of soil field capacity, representing non-stress and water deficit stress treatments, respectively). Water deficit stress treatment exerted on the corresponding pots at the initiation of chamomile flowering stage and continued afterward. However, non-stressed pots were continually irrigated normally through the entire experimental period. Plant samples from each treatment were taken at complete flowering stage and kept at -70 ˚C for measuring physiological parameters. Also, part of samples was shade dried for a week and used for extraction of essential oils. Plant morphological traits including shoot and root dry mass and flower fresh and dry mass were assessed at full flowering stage.

2.2 Plant Physiological Parameters Assays

2.2.1 Plastid pigment measurements

Fifty milligram of fresh shoots were grounded in 0.5 ml of acetone (80 % V/V) in order to extract photosynthetic pigments (i.e. chlorophyll (Chl.) a, Chl. b and carotenoids). The absorbance of the samples was measured at 645, 663, and 470 nm in a T80$^+$ UV–Vis spectrophotometer (PG Instrument Ltd., UK). Photosynthetic pigment contents were calculated using the following equations as described by Lichtenthaler and Wellburn (1983):

Chl a (mg/g FM) = 11.75 × A663 - 2.35 × A645
Chl b (mg/g FM) = 18.61 × A645 - 3.96 × A663
Carotenoids (mg g^{-1} FM) = 4.69 × A470 - 0.268 × (20.2 × A645 + 8.02 × A663)

2.2.2 H_2O_2 content determination

In order to determine H_2O_2 content in shoot and root of chamomile plants, 0.5 g fresh tissues were homogenized with 5 ml of 0.1 % w/v trichloroacetic acid (TCA) and centrifuged (12,000×g for 15 min). Then supernatant (0.5 ml) was supplemented to 0.5 ml of potassium phosphate ($KHPO_4$) buffer (10 mM, pH 7.0) and 1 ml of potassium iodide (1 M). The upper phase was aliquoted to read its absorbance at 390 nm. H_2O_2 was used for graphing calibration curve in order to calculate H_2O_2 concentration (Velikova et al., 2000). The content of H_2O_2 was expressed as µmol g^{-1} FM based on the standard curve.

2.2.3 Assessment of MDA content

Shoot and root fresh tissues (0.5 g each) were crushed and blended in 5 ml of TCA solution (0.1 % w/v) and centrifuged (12,000×g for 15 min). Two milliliters of supernatant was added to 2 ml of TBA (0.6 % w/v). The mixture incubated at 95 °C for 30 min; cooled down on ice and the samples were centrifuged (4,000 × g for 20 min). Absorbance of supernatant was measured at 532 nm. The amount of MDA calculated based on Heath and Packer (1968). The MDA content was calculated using a correction factor of 155 mM^{-1} cm^{-1} and expressed in terms of nmole g^{-1} FM.

2.2.4 Determination of proline content

In order to determine amount of proline free amino acid content in shoot and root, 0.5 g of fresh tissues were homogenized with 10 ml of 3 % aqueous sulfosalicylic acid and briefly centrifuged. Two milliliters of the supernatant was blended with acid ninhydrin and glacial acetic acid (two milliliters of each). The mixture in test tube was put in a water bath for 1 h at 100 °C. The reaction mixture was extracted with toluene (four milliliters). Absorbance of the mixture determined at 520 nm after being cooled down to room temperature. Standard calibration curve was graphed using appropriate proline concentrations (Bates et al., 1973). Finally, based on the standard curve obtained from different concentrations of proline in terms of µmole g-^{1} FM was calculated.

2.2.5 Determination of essential oil yield

Plant aerial parts (15 g) were shade-dried for a week. In order to extract their essential oils, the dried samples were hydro-distilled in Clevenger apparatus for 4 hours (Letchamo, 1993). The obtained aqueous essential oil was dehydrated by sodium sulfate, then its value was calculated according to its volume to the dry mass of the plant sample (v/w %).

2.3 Statistical Analysis

The obtained data were subjected to analysis of variance by SAS statistical software. Means were compared by Duncan's Multiple Range Test at 0.01 probability level ($P \leq 0.01$).

3 RESULTS

3.1 Plant morphological traits

Significant differences were observed between treatments in the case of root and shoot dry mass and flower fresh/dry mass ($p < 0.01$) (Table 2). Compared to well-watered condition, all of the morphological traits were reduced significantly under water deficit stress condition (Table 3). Compared to non-inoculated plants, inoculated ones possessed highest amount of foregoing traits under both watering conditions (Table 3).

Table 2: Analysis of variance (ANOVA) for different studied traits. Mean squares are shown for main factors and their interactions

Traits	Mean squares for sources of variations				
	block	Factor a (Inoculation)	Factor b (Watering regime)	Interaction a×b	Error
Root dry mass (g)	0.00298^{**}	0.00019^{*}	0.0072^{**}	0.01599^{**}	0.00002
Shoot dry mass (g)	0.2856^{**}	0.7793^{**}	1.6339^{**}	0.0025^{ns}	0.0019
Flower fresh mass (g)	0.3295^{**}	1.1439^{**}	2.2995^{**}	0.0647^{**}	0.0028
Flower dry mass (g)	0.0315^{**}	0.1863^{**}	0.1938^{**}	0.0137^{**}	0.0003
Essential oil yield (v/w) %	0.0008^{ns}	1.235^{**}	0.261^{**}	0.126^{**}	0.0016
Chlorophyll *a*	0.0034^{ns}	0.1145^{**}	0.228^{**}	0.0114^{ns}	0.0026
Chlorophyll *b*	0.00124^{ns}	0.01628^{*}	0.0678^{**}	0.00002^{ns}	0.00142
Total chlorophyll	0.0073^{ns}	0.2174^{**}	0.5449^{**}	0.0104^{ns}	0.0068
Carotenoids	0.0015^{ns}	0.049^{**}	0.09^{**}	0.0027^{ns}	0.0023
Root MDA contents	0.601^{ns}	26.883^{**}	28.296^{**}	14.434^{**}	0.566
Shoot MDA contents	0.081^{ns}	22.509^{**}	35.271^{**}	15.872^{**}	0.367
Root H_2O_2 contents	0.005^{ns}	1.3068^{**}	2.5595^{**}	1.0585^{**}	0.0032
Shoot H_2O_2 contents	0.0022^{*}	1.261^{**}	2.3039^{**}	0.7752^{**}	0.0003
Root proline contents	212.861^{*}	4170.178^{**}	36259.641^{**}	1652.076^{**}	21.021
Shoot proline contents	21.923^{ns}	3078.596^{*}	28419.581^{**}	5344.461^{**}	384.557

*: Significant at the 0.05 probability level, **: Significant at the 0.01 probability level, and ^{ns}: Not significant.

Table 3: Mean comparison of morphological traits in non-inoculated and inoculated chamomile plants with PGPR under different watering regimes

Traits	Non-inoculated		Inoculated	
	Well-watered	Water deficit stress	Well-watered	Water deficit stress
Root dry mass (g)	$0.308^{c} \pm 0.04$	$0.267^{d} \pm 0.02$	$0.389^{a} \pm 0.05$	$0.332^{b} \pm 0.04$
Flower fresh mass (g)	$3.448^{b} \pm 0.05$	$2.719^{d} \pm 0.04$	$4.212^{a} \pm 0.06$	$3.19^{c} \pm 0.03$
Flower dry mass (g)	$1.016^{b} \pm 0.04$	$0.829^{c} \pm 0.03$	$1.333^{a} \pm 0.05$	$1.011^{b} \pm 0.06$

Values with the same letters in each row are not significantly different at $P < 0.05$.

3.2 Plant physiological traits

The photosynthetic pigments were significantly affected by watering regimes and inoculation factors, and there were not significant interaction among levels of the two factors in the case of these traits (Table 2). As shown in table 4, these traits were decreased in response to water deficit stress treatment. Maximum content of photosynthetic pigments were observed in the inoculated plants (Table 4). In the other hand, amount of H_2O_2 in inoculated plants was lower than that of non-inoculated plants in both watering conditions. Significant differences were also observed among different treatments in the case of root and shoot MDA contents (Table 1 and Figure 1). The minimum and maximum amount of MDA in both shoot and root tissues were observed in well-watered inoculated plants and water-stressed non-inoculated plants, respectively (Figure 1). Under water deficit stress condition, proline content of root and shoot were significantly ($P < 0.01$) augmented in bacterial-inoculated chamomiles (Figure 1).

Table 4: Mean comparison of some traits in chamomile plants under water deficit stress conditions

Irrigation	Shoot dry mass (g)	Chlorophyll a (mg g^{-1} FM)	Chlorophyll b (mg g^{-1} FM)	Total chlorophyll	Carotenoids (mg g^{-1} FM)
Well-watered	$2.904^{a} \pm 0.15$	$0.629^{a} \pm 0.04$	$0.406^{a} \pm 0.03$	$1.035^{a} \pm 0.06$	$0.583^{a} \pm 0.03$
Water deficit stress	$2.79^{b} \pm 0.12$	$0.589^{b} \pm 0.05$	$0.368^{b} \pm 0.04$	$0.957^{b} \pm 0.05$	$0.561^{b} \pm 0.02$
Non-inoculated	$3.413^{b} \pm 0.05$	$0.824^{b} \pm 0.03$	$0.48^{b} \pm 0.02$	$1.305^{b} \pm 0.05$	$0.711^{b} \pm 0.04$
Inoculated	$3.528^{a} \pm 0.06$	$0.865^{a} \pm 0.04$	$0.519^{a} \pm 0.03$	$1.384^{a} \pm 0.04$	$0.734^{a} \pm 0.03$

Values with the same letters in each row are not significantly different at $P < 0.05$.

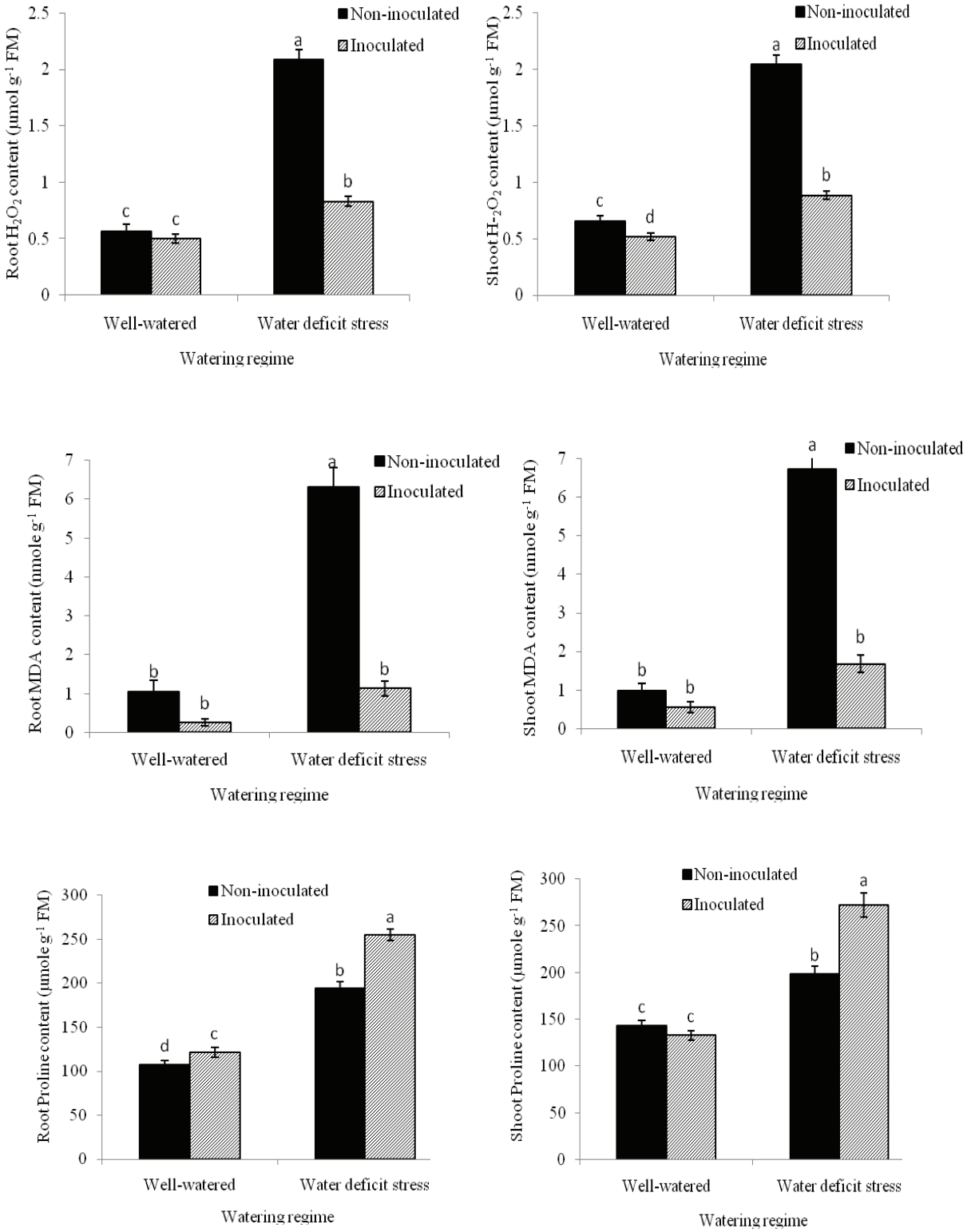

Figure 1: The content of root and shoot H_2O_2, MDA and proline in non-inoculated and inoculated chamomile plants with PGPR under well-watered and water deficit stress conditions. Columns with the same letters are not significantly different at $P < 0.05$. Vertical bars are ± SD of three independent replicates

3.3 Essential oils yield

The results showed that there were considerable variations among treatments in the case of essential oil yield (Table 1). Generally, water-stressed plants had more essential oil than well watered plants regardless of inoculation status. In the other hand, inoculated plants had more essential oil than non-inoculated ones in both watering conditions (Figure 2).

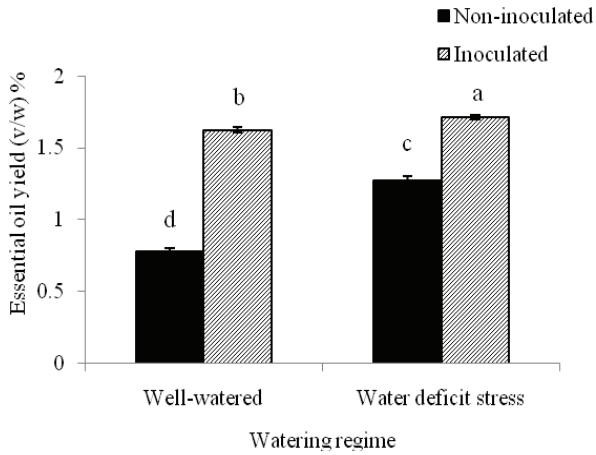

Figure 2: Essential oil yield in non-inoculated and inoculated chamomile plants with PF-135 under well-watered and water deficit stress conditions

4 DISCUSSIONS

It is well accepted that PGPRs ameliorate plant growth and productivity by numerous diverse mechanisms. Generally, these mechanisms include: a) producing various plant hormones; b) asymbiotic nitrogen fixation; c) having antagonistic activity against many plant pathogens; d) converting the nutrients into the more accessible forms to be readily absorbed by plant roots, and e) inhibiting synthesis of ethylene precursor in root cells (Yasmin et al., 2007).

In the present study, inoculation of chamomile with *P. fluorescens* PF-135 strain increased flower fresh and dry mass and root dry mass under both water deficit stress and non-stress conditions (Table 3). Mohammadi et al. (2017) reported that inoculation of *Satureja hortensis* L. with *P. fluorescens* (PF-135) not only significantly increased the plant biomass and essential oil yields under water deficit stress condition but also improved the activity of antioxidant enzymes and synthesis of proline. Moreover, an investigation on corn inoculated with *Pseudomonas* spp. under water deficit stress condition also revealed that inoculated plants had higher plant biomass, relative moisture content, proline, sugar, and amino acids compared to that of non-inoculated plants. Besides, inoculated plants had lower electrolytic leakage and higher antioxidant enzymes activity (ascorbate peroxidase, catalase, and glutathione peroxidase) under drought stress condition (Sandhya et al., 2010). These observations showed that inoculated plants compared to non-inoculated ones are less susceptible to stress. It can be attributed to improved plant performance which brought about by *P. fluorescens* inoculation.

Fluorescent pseudomonads are important rhizobacteria which can considerably enhance plant productivity especially under stressful environment. It has been reported that fluorescent pseudomonas are able to synthesis ACC-deaminase enzyme (Saravanakumar and Samiyappan, 2007). This enzyme play a vital role in ameliorating environmental stresses through minimizing ethylene production and stimulating plant root and shoot growth (Glick, 2014; Saleem et al., 2007). These bacteria also inhibit growth of plant pathogenic microorganisms through producing antibiotics. In addition, *P. fluorescens* sustain plant growth via increasing phosphorus solubility, and secreting iron chelating siderophores and indole-3-acetic acid in the soil (Mehrabi et al., 2016).

In this study, leaf MDA and H_2O_2 content (as classical markers of oxidative stress) were decreased more than 2-folds in inoculated plants in comparison with non-inoculated ones under water deficit stress condition.

Inoculated plants also synthesized much more amount of proline compared to non-inoculated plants in response to water deficit condition (Figure 1). It has been reported that plants accumulate nitrogen bearing compounds such as amino acids to be able to cope with drought stress (Amunda and Balasubramani, 2011). In addition, proline is considered to be a compatible solute serve to conserve macromolecular structures and cellular membrane integrity as well as tuning osmotic adjustment especially in plants grown under stressful environment (Ashraf and Foolad, 2007; Maggio et al., 2002). Therefore, it can be extrapolated that application of *P. fluorescens* PF-135 strain decreased lipid peroxidation probably through improving plant enzymatic and/or non-enzymatic antioxidant activities.

Plants secondary compounds have variety of ecological functions such as assisting plants to cope better with harsh environments. It has been demonstrated that when plants exposed to water deficit, they produce and accumulate much more secondary compounds compared to normal watering (Selmar and Kleinwachter, 2013). Under water deficit stress condition, plants produce higher amount of terpenes, since photo-assimilates are less allocated to current plant growth demand, resulting in considerable accumulation of essential oils (Turtola et al., 2003). In the other hand, biotic elicitors which are produced and secreted by microorganisms such as bacteria can induce production of plant secondary metabolites. They altogether give details why the plants inoculated with the fluorescent pseudomonads synthesis much more amount of essential oils compared to non-inoculated plants especially under water deficit stress condition (Figure 2). Thus, biological elicitors which are also found in *P. fluorescens* can be used for inducing synthesis of secondary metabolites especially in medicinal plants (Ghorbanpour et al., 2016).

5 CONCLUSIONS

Based on the results of the present research, inoculation of chamomile with *P. fluorescens* PF-135 strain significantly boosted essential oil content and plant biomass especially under water deficit condition. Inoculation with *P. fluorescens* PF-135 not only decreased cellular lipid peroxidation but also induced accumulation of proline. Therefore, inoculation of chamomile with *P. fluorescens* alleviated adverse effects of water deficit stress through inducing antioxidant activity, decreasing amount of MDA and H_2O_2 as well as increasing proline content. It can be concluded that *P. fluorescens* PF-135 strain is as an excellent PGPR for improving chamomile drought tolerance and boosting its growth and essential oil content especially under water deficit stress condition. Since chemical fertilizers are seriously threatening environment and human health in the globe, therefore PGPRs such as *P. fluorescens* (PF-135 strain) can be considered as one of the outstanding alternative for chemical fertilizers in order to develop sustainable farming especially in dry regions of the world where agricultural ecosystems are fragile.

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7 REFERENCES

Ashraf, M., & Foolad, M.R. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, *59*, 206-216. https://doi.org/10.1016/j.envexpbot.2005.12.006

Bates, L., Waldren, R. & Teare, I. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil, 39*, 205-207. https://doi.org/10.1007/BF00018060

Charousaei, F., Dabirian, A. & Mojab, F. (2011). Using chamomile solution or a 1% topical hydrocortisone ointment in the management of peristomal skin lesions in colostomy patients: results of a controlled clinical study. *Ostomy-Wound Manage*ment, *57*, 28-36.

Chauhan, H., Bagyaraj, D.J., Selvakumar, G. & Sundaram, S.P. (2015). Novel plant growth promoting rhizobacteria—Prospects and potential. *Applied Soil Ecology, 95,* 38–53. https://doi.org/10.1016/j.apsoil.2015.05.011

Christians, M.J., Gingerich, D.J., Hansen, M., Binder, B.M., Kieber, J.J. & Vierstra, R.D. (2009). The

BTB ubiquitin ligases ETO_1, EOL_1 and EOL_2 act collectively to regulate ethylene biosynthesis in Arabidopsis by controlling type-2 ACC synthase levels. *The Plant Journal, 57,* 332-345. https://doi.org/10.1111/j.1365-313X.2008.03693.x

Cruz de Carvalho, M.H. (2008). Drought stress and reactive oxygen species: production, scavenging and signaling. *Plant Signaling & Behavior, 3,* 156-165. https://doi.org/10.4161/psb.3.3.5536

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. & Basra, S. (2009). Plant drought stress: effects, mechanisms and management. *Agronomy for Sustainable Development, 29*, 185-212. https://doi.org/10.1051/agro:2008021

Gill, S.S. & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry, 48*, 909-930. https://doi.org/10.1016/j.plaphy.2010.08.016

Glick, B.R. (1995). The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology, 41*, 109-117. https://doi.org/10.1139/m95-015

Glick, W.H., Miller, C.C. & Cardinal, L.B. (2007). Making a life in the field of organization science. *Journal of Organizational Behavior, 28*, 817-835. https://doi.org/10.1002/job.455

Glick, B.R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiological Research, 169*(1), 30-39. https://doi.org/10.1016/j.micres.2013.09.009

Ghorbanpour, M., Hatami, M., Kariman, K. & Khavazi, K. (2015). Enhanced Efficiency of Medicinal and Aromatic Plants by PGPRs. *In: Plant-Growth-Promoting Rhizobacteria (PGPR) and Medicinal Plants*, pp. 43-70. E. Dilfuza, Sh. Smriti, V. Ajit (eds.). Springer International Publishing. Switzerland. https://doi.org/10.1007/978-3-319-13401-7_3

Ghorbanpour, M., Hatami, M., Kariman, K. & Abbaszadeh, P. (2016). Phytochemical variations and enhanced efficiency of antioxidant and antimicrobial ingredients in *Salvia officinalis* as inoculated with different rhizobacteria. *Chemistry & Biodiversity, 13*, 319-330. https://doi.org/10.1002/cbdv.201500082

Heath, R.L. & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics, 125*, 189-198. https://doi.org/10.1016/0003-9861(68)90654-1

Letchamo, W. (1993). *Nitrogen application affects yield and content of the active substances in chamomile genotypes. New Crops.* Willey. New York, 636-639.

Lichtenthaler, H.K., Wellburn, A.R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochemical Society Transactions, 11,* 591-592. https://doi.org/10.1042/bst0110591

Maggio, A., Miyazaki, S., Veronese, P., Fujita, T., Ibeas, J.I., Damsz, B., Narasimhan, M.L., Hasegawa, P.M., Joly, R.J. & Bressan, R.A. (2002). Does proline accumulation play an active role in stress-induced growth reduction? *The Plant Journal, 31*, 699-712. https://doi.org/10.1046/j.1365-313X.2002.01389.x

Mehrabi, Z., McMillan, V.E., Clark, I.M., Canning, G., Hammond-Kosack, K.E., Preston, G., Hirsch, P.R. & Mauchline, T.H. (2016). *Pseudomonas* spp. diversity is negatively associated with suppression of the wheat take-all pathogen. *Scientific. Reports, 6*, 1-9. https://doi.org/10.1038/srep29905

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science, 7*, 405-410. https://doi.org/10.1016/S1360-1385(02)02312-9

Mohammadi, H., Dashi, R., Farzaneh, M., Parviz, L., & Hashempour, H. (2017). Effects of beneficial root pseudomonas on morphological, physiological, and phytochemical characteristics of *Satureja hortensis* (Lamiaceae) under water stress. *Brazilian Journal of Botany*, *40*(1), 41-48. https://doi.org/10.1007/s40415-016-0319-2

Roby, M.H.H., Sarhan, M.A., Selim, K.A.H. & Khalel, K.I. (2013). Antioxidant and antimicrobial activities of essential oil and extracts of fennel (*Foeniculum vulgare* L.) and chamomile (*Matricaria chamomilla* L.). *Industrial Crops and Products*, *44*, 437-445. https://doi.org/10.1016/j.indcrop.2012.10.012

Saleem, M., Arshad, M., Hussain, S. & Bhatti, A.S. (2007). Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. *Journal of Industrial Microbiology & Biotechnology, 34*, 635–648. https://doi.org/10.1007/s10295-007-0240-6

Sánchez-Blanco, M.J., Ferrández, T., Morales, M.A., Morte, A. & Alarcón, J.J. (2004). Variations in water status, gas exchange, and growth in *Rosmarinus officinalis* plants infected with *Glomus deserticola* under drought conditions. *Journal of Plant Physiology, 161*, 675-682. https://doi.org/10.1078/0176-1617-01191

Sandhya, V. S. K. Z., Ali, S. Z., Grover, M., Reddy, G., & Venkateswarlu, B. (2010). Effect of plant growth promoting Pseudomonas spp. on compatible solutes, antioxidant status and plant growth of maize under drought stress. *Plant Growth Regulation*, *62*(1), 21-30. https://doi.org/10.1007/s10725-010-9479-4

Saravanakumar, D. & Samiyappan, R. (2007). ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. *Journal of Applied Microbiology, 102*, 1283-1292. https://doi.org/10.1111/j.1365-2672.2006.03179.x

Selmar, D. & Kleinwächter, M. (2013). Stress enhances the synthesis of secondary plant products: the impact of stress-related over-reduction on the accumulation of natural products. *Plant and Cell Physiology, 54*, 817-26. https://doi.org/10.1093/pcp/pct054

Srivastava, J.K., Shankar, E. & Gupta, S. (2010). Chamomile: A herbal medicine of the past with a bright future (Review). *Molecular Medicine Reports, 3*, 895-901.

Srivastava, J.K. & Gupta, S. (2015). Chamomile: A herbal agent for treatment of diseases of the elderly. In *Foods and Dietary Supplements in the Prevention and Treatment of Disease in Older Adults*, pp. 171-183. R. R. Watson (ed.). Academic Press, Tokyo, Japan. https://doi.org/10.1016/B978-0-12-418680-4.00018-X

Turtola, S., Manninen, A.M., Rikala, R. & Kainulainen, P. (2003). Drought stress alters the concentration of wood terpenoids in Scots pine and Norway spruce seedlings. *Journal of Chemical Ecology, 29*, 1981-1985. https://doi.org/10.1023/A:1025674116183

Velikova, V., Yordanov, I. & Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science*, *151*, 59-66. https://doi.org/10.1016/S0168-9452(99)00197-1

Vessey, J.K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil, 255*, 571-586. https://doi.org/10.1023/A:1026037216893

Yasmin, F., Radziah, O., Mohd, S.S. & Kamaruzaman, S. (2007). Screening for beneficial properties of rhizobacteria isolated from sweetpotato rhizosphere. *Biotechnology, 6*, 49-52. https://doi.org/10.3923/biotech.2007.49.52

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Effects of zinc, boron and sulfur on grain yield, activity of some antioxidant enzymes and fatty acid composition of rapeseed (*Brassica napus* L.)

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ABSTRACT

A field experiment was conducted to study the effects of elements zinc (Zn), boron (B) and sulfur (S) and their interactions on quantitative and qualitative agronomic characteristics of rapeseed. Minimum grain oil and seed yield were obtained from control treatments and the highest seed yield were obtained from S + B + Zn treatments. The maximum of oleic acid (229.6 mg g^{-1}) and linolenic acid (27.14 mg g^{-1}) were obtained from B + Zn + S treatment. Maximum of linoleic acid (55.55 mg g^{-1}) were obtained from B + Zn treatment. However, the highest superoxide dismutase activity was obtained from S + B + Zn treatments 10.24 unit mg^{-1} and the highest peroxidase activity were obtained from Zn treatment 0.87 µmol g^{-1} FM min. Regard to this experiment results, application of B, S and Zn fertilizers with NPK fertilizer can help to increase the yield and yield components in rapeseed. Also fatty acids composition of rapeseed are influenced by nutrients and since quality of edible oils depends on unsaturated fatty acids, especially linoleic and linolenic acids and these acids are essential fatty acids for the human body that must be supplied through diet. Therefore this research showed that we are not only able only to increase oil yield but also oil quality with desired fatty acid composition.

Key words: erucic acid; grain oil; linoleic acid; superoxide dismutase; rapeseed

IZVLEČEK

UČINKI CINKA, BORA IN ŽVEPLA NA PRIDELEK ZRNJA, AKTIVNOST NEKATERIH ANTIOKSIDACIJSKIH ENCIMOV IN SESTAVO MAŠČOBNIH KISLIN OLJNE OGRŠČICE (*Brassica napus* L.)

Izveden je bil poljski poskus za preučevanje učinkov Zn, B in S ter njihovih interakcij na količinske in kakovostne agronomske lastnosti oljne ogrščice. Najmanjša pridelka zrnja in olja sta bila dobljena v kontrolnem obravnavanju in največja pri obravnavanju S + B + Zn. Največ oleinske (229.6 mg g^{-1}) in linolenične kisline (27.14 mg g^{-1}) je bilo pri obravnavanju B + Zn + S. Največ linolenične kisline (55.55 mg g^{-1}) je bilo doseženo pri B + Zn obravnavi. Največja aktivnost superoksid dizmutase je bila pri S + B + Zn obravnavi (10.24 enot mg^{-1}) in največja aktivnost peroksidaze pri obravnavi samo s cinkom (0.87 µmol g^{-1} FM min). Glede na rezultate raziskave sklepamo, da uporaba B, S in Zn gnojil s NPK gnojili lahko pomaga povečati pridelek in njegove dele pri oljni ogrščici. Gnojila vplivajo tudi na sestavo maščobnih kislin v olju, kar vpliva na kakovost jedilnega olja, ki je odvisna od vsebnosti nezasičenih maščobnih kislin, predvsem linoleične in linolenične, ki sta za človeka esencialni in jih mora dobiti s hrano.V tem pogledu je raziskava pokazala, da nismo sposobni le povečati pridelka olja ampak tudi dosegati željeno sestavo maščobnih kislin v njem.

Ključne besede: erucična kislina; olje v zrnu; linoleična kislina; superoksid dismutaza; oljna ogrščica

1 INTRODUCTION

Rapeseed (*Brassica napus* L. ssp. *napus*) is grown in different agro-climatic zones of the world, differing in soil nutrient status (Bybordi & Mamedov, 2010). Canola is an important agricultural crop, grown primarily for oil production, but also as a valuable break-crop in cereal crop rotations (Gammelvind et al., 1996).

Zinc is a structural part of several enzymes or is necessary for enzyme activation; thus Zn deficiency also affects carbohydrate metabolism, damages pollen

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structure, and decreases the yield (Das et al., 2005; Pandy et al., 2006; Fang et al., 2008). Applying zinc to a Zn-deficient soil could also advance the seed yield of rapeseed (Singh Grewal et al., 1997; Singh Grewal & Grahma, 1999). Zinc is a cofactor of over 300 enzymes and proteins and has an early and effect on cell division and protein synthesis (Marschner, 1986). Low solubility of zinc in soils rather than low total amount of Zn is the main reason for the general occurrence of Zn deficiency problem in crop plants (Cakmak, 2008). High seed- Zn has very vital physiological roles during germination and seedling growth (Cakmak, 2008). The review by Cakmak (2008) provides further reasons and relevant research for profit of high seed Zn on plant growth.

Rapeseed, one of the main oil crops in Iran as well as in the world, is sensitive to B deficiency (Chu et al., 1996). Boron plays roles in structure and cell wall synthesis, and possibly membrane stability (Matoh 1997; Goldbach et al., 2001; Brown et al., 2002; Iwai et al., 2006). It promotes the strength and rigidity of cell wall structure and therefore, supports the figure and power of the plant cell (Brown et al., 2002). Furthermore, boron is maybe concerned in the integrity of the plasma membrane (Brown et al., 2002: Cara et al., 2002; Dordas & Brown, 2005). B deficiency causes irregular development of reproductive organs (Dell & Huang, 1997; Huang et al., 2000), and reduces plant yield (Chen et al., 2005; Nabi et al., 2006). Boron is involved in carbohydrates metabolism and it is basically necessary for protein synthesis, pollen germination and seed and cell wall development.

Boron and Zn deficiencies are more possible early in the season for the reason that the translocation of elements from the root to the aboveground part may not be sufficient before leaf expansion (Neilsen et al., 2004). Applications Zn and B have been experimental to have a positive effect on chlorophyll contents in B - and Zn deficient plants (Kaya & Higgs, 2002). Sinha et al. (2000) noted a synergistic interaction among Zn and B in black mustard (*Brassica nigra* L.) when both nutrients were also in small or excess supply.

Sulfur is main nutrient in crop production. Deficiency of S affected all crop, from forage to oilseed, but the clearest effects have been seen in canola for the cause that of its high S required (Malhi et al., 2005). Rapeseed has a high protein concentration with a high proportion of the amino acids as cysteine and methionine (Anderson 1975; Clandinin, 1981; Grant & Bailey, 1993). It has been experimental proved that rapeseed requires 3-10 times more S than barley (Bole & Pitman, 1984). Rapeseed has a high requirement for S to optimize yield (Grant & Bailey, 1993), i.e., about 1.5 kg of S to manufacture 100 kg of grain (Nyborg et al., 1974). For maximum seed yield rapeseed, the S requirement is greater than that for cereals (Hamm 1967; Bole & Pitman, 1984). Therefore, rapeseed is more likely to respond to S fertilization. Sulphate-S application is reported to increase concentration of oil in rapeseed seed (Nuttal et al., 1987; Malhi & Gill 2002; Grant et al., 2003), but in some reported a decrease (Wetter et al., 1970) or no alter (Ridley, 1973) was found. Rapeseed oil has a lower level of saturated fats (only 6 %) than any other edible oil plant and also has a high amount of un-saturated fat containing a good combine of both poly and monounsaturated fats. Relative proportion of different fatty acid determines the quality of edible oil. Higher percentage of polyunsaturated fatty acid is considered beneficial for lowering cholesterol in human body (Cunnae, 1995). Sulfur increases the percentage of oil content of the seed (Chaudhry et al., 1992), glucosinolate content and erucic acid (Marschner, 1986).

Most researchers have studied the effect of a single element fertilizer on the crop yield, whereas few have payed attention on the function of the combined applications of nutrients in improving the yield. In this present study, the effects of Zn, B, S and their interactions on the quality and seed yield rapeseed were examined.

2 MATERIALS AND METHODS

The field experiment was conducted at University of Guilan, Guilan Province, Rasht, Iran (37º 16´ 21" N, 51 º 3´ 36" E), during 2014 cropping seasons. Prior to the beginning of experiment, soil samples were taken to determine the chemical and physical properties. A composite soil samples were collected at a depth of 0 - 30 cm. The chemical properties of the clay loam were: pH (1:2 soil:H_2O) 6.9, total nitrogen 0.12 %, available P 14.9 mg kg^{-1}, available K 202 mg kg^{-1}, available Zn 0.94 mg kg^{-1}, available B 0.02 mg kg^{-1}, soluble SO_4 0.64 meq l^{-1}, EC 0.255 dS m^{-1}.

After ploughing and disk harrowing twice, the land was flatted by leveler and then plots were prepared. The experiment with completely randomized block design was performed with eight treatments in three replicates. Treatment consisted control, Zn, B, S, Zn + S, Zn + B, B + S, and S + B + Zn. Sulfur treatment added as sulfur flower at the rate 100 kg ha^{-1} in plots and mixed with

surface soil before seed sowing. Boron was added as H_3BO_3 at the rate of 8 kg ha^{-1}, zinc was added as $ZnSO_4.7H_2O$ at the rate of 30 kg ha^{-1} were applied in plots.

The plots had 5 m length and 2.25 m width consisted of 8 rows. Between all plots, 1.5 m distance was kept to eliminate all influenced of lateral water movement. According to results of soil analysis 150 kg ha^{-1} ammonium phosphate, 150 kg ha^{-1} potassium, and 120 kg ha^{-1} urea was used. All of ammonium phosphate, potassium and one third of urea were distributed in plots and mixed with surface soil before seed sowing. Side dressings of nitrogen fertilizer were applied at bolting and flowering stages.

'Hyola 401' a double low (low erucic acid and low glucosinolate) rapeseed (*B. napus* L.) cultivar was used.

When rapeseed commenced flowering, twelve plants in each plot were randomly marked with tags for analysis of yield components, including thousand-seed mass, seeds number per silique and siliques number per plant. The remaining plants in each plot were harvested to measure the plots seed yield at maturity.

At 80 % flowering (BBCH, 67), five upper leaves were collected for determined of Zn, B and S content of leaves. Leaves were washed with distilled water, dried at 70 °C for 48 h, ground to pass 1 mm sieve, stored in bags before analyses, and analyzed. Zinc was determined by atomic absorption spectrophotometry (AAS) (Perkin Elmer model 3030). Boron was measured with the curcumin spectrophotometric method (Lieten, 2002). Sulfur was measured with the turbidimetric method (Benton, 2001).

Oil seed amount was measured by soxhlet (Soxtec system HT 1043) method. The fatty acid compositions of the canola seed oils were determined by gas chromatography (model, Unicam 4600).

2.1 Enzymes Assay

Superoxide dismutase activity was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium according to the methods of Beauchamp and Fridovich (1971).

Peroxidase activity was assayed in leaves by the oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance was recorded at 470 nm (Chance & Maehly, 1955).

2.2 Statistical Analysis

A completely block randomized design in three replications was used. The statistical analyses of data were performed by ANOVA procedure from SAS 9.1 (SAS Institute, United States). Differences between means were evaluated by the least significant difference methods. The 0.05 and 0.01 probability values were used to determine significant difference.

3 RESULTS AND DISCUSSION

3.1 Effects of B, Zn, S and their interactions on seed yield

Statistical analysis of the data on seed yield revealed significant difference among treatments ($p < 0.01$) (Table 1). The highest seed yield was produced from B + Zn + S treatment. The minimum seed yield was recorded for control (Table 2). Results showed in the three single nutrient treatments the application of B, Zn and S significantly increased the seed yield over that of the control (Table 2). This indicated that B, Zn and S fertilizer played a very important role in promoting the seed yield of 'Hyola 401'. The application of B with S or Zn fertilizer increased the seed yield by a future 25 % and 19.7 %, respectively, compared to that of the control. Elements of Zn + S without B increased the seed yield for 23 % (Table 2). The effect of B + Zn + S was the biggest, indicating that the combined application of the three nutrients was beneficial for the seed yield of rapeseed.

Table 1: Mean squares from analysis of variance of yield, yield components, seed oil, S, Zn and B contents of rapeseed

S.O.V	df	Mean Squares							
		Seed yield	1000 seed mass	Seed number per Silique	Siliques number per plant	Seed oil	S content	Zn content	B content
Block	2	26117.41	0.005	0.29	7777.54	7.73	4726.52	25.04	87.16
Treatment	7	418462.67**	0.39**	9.18**	4685.8**	27.53**	115889.71**	110.77**	54.59**
Error	14	30539.34	0.0028	0.29	259.54	0.34	1391.08	1.56	2.89
Cv (%)	-	18.8	16.1	13.9	16.1	15.5	6.5	13.1	10.4

*ns and *, **: non-significant and significant at 5 % and 1 % probability levels, respectively*

Mei et al., (2009) showed that seed yield of the B + Mo + Zn treatment was the highest in all treatment, 68.1 % above the control. Fang et al. (2008) showed that zinc increased seed yield because it can give an optimum effect on photosynthesis rate. The increase in yield with Zn might have been the results of increased branch number per plant, siliques number per plant, seed number per silique and thousand-seed mass. In proceed to the previous studies (Cakmak et al., 1999), all methods of Zn application for plants significantly increased grain yield. Micronutrients increase photosynthesis rate and improves leaf area duration thus seed yield will be increased. Zinc plays important role in tryptophan biosynthesis, later in its role as precursor of auxin. Zinc is also found in phosphoenolpyruvate carboxylase structure.

Zhang (2001) also showed that the critical range of the B concentration corresponding to 90 % of the maximum oilseed rape yield was 0.04-0.52 mg kg^{-1}. Thus it is not unexpected that the plants showed B deficiency symptoms at the lack of B even though a small amount of basal B was added to prevent complete loss of reproductive yield. One of the essential physiological roles of B in plants is to improve pollen tube growth and fertilization in reproductive growth (Dell & Huang, 1997). Thus, B deficiency results in a typical symptom called "flowering without seed setting" (Liu, 1999). In the study, use of B increased the number of siliques per plant and seeds per silique, therefore resulting in a significant increase in the seed yield. This confirmed the important role of B in pollen development and fertilization reported earlier (Dell and Huang, 1997; Huang et al., 2000). Rapeseed has a high requirement for S to optimize yield (Grant & Bailey, 1993), i.e., about 1.5 kg of S to produce 100 kg of seed (Nyborg et al., 1974). For high seed yield, the S requirement for rapeseed is greater than that for cereals (Hamm, 1967; Bole and Pitman, 1984). Rapeseed responded to S fertilization through increasing number of siliques per plantand so increased yield (Table 2).

3.2 Effects of B, Zn, S and their interactions on yield components:

The components of seed yield were the number of siliques per plant, seeds per silique, and the thousand seed mass. As seen in Table 1 analysis of variance showed effects of fertilizer treatment on siliques number per plant, seeds number per silique, and the thousand seed mass were significant ($p<0.01$) (Table 1). The highest siliques number per plant (319.7) was produced from B + Zn + S treatment and minimum (199.7) was recorded for control (Table 2).

Table 2: Effects of boron (B), zinc (Zn), sulfur (S), and their interactions on the seed yield, yield components, seed oil and S, Zn and B content in leaf of rapeseed

Treatment	Seed yield (kg ha^{-1})	1000 seed mass (g)	Seed number per silique	Siliqua number per plant	Seed oil (%)	S content (mg kg^{-1})	Zn content (mg kg^{-1})	B content (mg kg^{-1})
Control	3860.6^{d}	4.82^{c}	24.3^{d}	199.7^{c}	33.65^{f}	223.97^{f}	30.31^{g}	10.21^{f}
B	4377.9^{bc}	4.86^{c}	27^{bc}	227.7^{de}	37.89^{d}	391.13^{c}	34.99^{f}	17.35^{bc}
Zn	4091.6^{cd}	4.94^{dc}	25.7^{cd}	250.3^{cd}	35.47^{e}	490.53^{d}	41.1^{e}	13. 3de
S	4496.1^{a-c}	5.04^{cd}	27.3^{b}	264^{b-d}	39.37^{cd}	730.5^{b}	38.81^{de}	12.87^{ef}
B+S	4808.7^{ab}	5.57^{b}	27. 7^{b}	282.3^{a-c}	40.02^{bc}	609.79^{c}	37.6^{e}	19.29^{b}
B+Zn	4620.8^{ab}	5.17^{c}	27.3^{b}	272. 7^{b-d}	41.26^{ab}	559.96^{c}	40.37^{cd}	16.64^{bc}
S+Zn	4744.9^{ab}	5.58^{b}	29.3^{a}	306.3^{ab}	40.54^{bc}	745.55^{ab}	49.49^{a}	16.01^{cd}
B+S+Zn	4965^{a}	5.74^{a}	29.7^{a}	319.7^{a}	42.66^{a}	802. 6^{a}	46. 4^{b}	24^{a}

* Means followed by the same letter(s) in each column are not significantly different (P = 0.05).

The application of B with Zn or S fertilizer increased the siliques number per plant by a further 41.4 % and 37 % respectively, compared to that of the control. The addition of S + Zn without B also increased the siliques number per plant for 53.4 % (Table 2). The effect of B + Zn + S was the biggest (60 %), indicating that the combined application of the three nutrients was beneficial for the siliques number per plant of rapeseed. This beneficial effect might be due to interaction effect of sulphur, zinc, boron and their role in synthesis of IAA, metabolism of auxin and formation of chlorophyll synthesis.

Sulfur fertilizer effect on the plant height was increased due to more light penetration into plant canopy and increased the number of branches per plant and silique number per plant. Rathinavel et al. (2000) reported for flax (*Linum usitatissium* L.) that zinc transfered assimilates more effectively and directly affected flax capsule mass. Zinc is involved in synthesis of indole-3-acetic acid. This hormone is the main factor preventing loss in the number of flax capsula, and Zn can also be used for preventing the losses of rapeseed siliquas. Also, the reason for that the potential loss of silique per plant, may be a poor pollination (Azizi et al., 2006). Based on the results of previous research the cause of decreased formation of male and female sexual organs and the lack of pollination processes is due to zinc deficiency. They attributed this to a decrease in the production of indole acetic acid (Brown et al., 1993). The researchers also stated that boron is essential element needed for pollen germination and pollen tube growth (Marschner, 1995). They believe that the low absorption of boron in the soil impairs the plant's pollination, resulting in plant sterility (Vitosh et al., 1997).

3.3 Seed number per silique

Seed number per silique is an important yield component, since the seeds are produced as storage organs. It seems that the number of seeds per silique is larger sink to store more materials there. The highest seed number per silique (29.7) was produced from B + Zn + S treatment and minimum (24.3) was recorded for control (Table 2).

Increased seed number per silique as effect of zinc and boron fertilizer might be because of boron key role in translocation water and nutrients from the roots to the shoots (Rehem et al., 1998). Production of more chlorophyll and IAA which delayed plant senescence and thus prolonged the period of photosynthesis. This improves carbohydrate production and their transfer it to the growing seeds (Vitosh et al., 1997). Mc-Grath and Zhao (1996) and Roe et al. (1997) in their research showed that sulfur ratio of reproductive organs to the total dry matter increased. Sulfur deficiency inhibits the growth of reproductive organs and even leads to sterility of siliques.

Table 2 showes the effects of the single nutrient treatments of B, Zn, S as the increased seed number per silique, 12.3, 6 and 11.1 %, respectively. Also, all combined application of two nutrients resulted in a significant increase in the seed number per silique in comparison with the single nutrient application. The maximum seed number per siliques in comparison with the control indicates that the combined application of the three nutrients gives the best results (22.2 %) (Table 2). According to Brown et al. (1993) formation of male and female reproductive organs and pollination process are disturbed in Zn deficiency, which results in a severe

reduction in plant yield, this is due to reduction of indol acetic acid (IAA) synthesis. Rehem et al. (1998) stated that B plays a key role in water and nutrients transportation from root to shoot and they believed that B shortage can causes barren and small stalks in corn and soybean.

3.4 1000 seed mass

The highest 1000 seed mass was observed in B + Zn + S treatment (Table 2). Zinc is necessary for the biosynthesis of the plant growth regulator such as IAA and for carbohydrate and N metabolism which leads to high yield and yield components. This may be due to provision of macro and micro nutrients at latter stages which might have improved accumulation of assimilate in seeds and thus resulting in heavier seed. The results of this investigation are in consonance with the findings of Mei et al. (2009) that to effects of B + Mo + Zn treatment increased 1000 seed mass in rapeseed plant.

These results were in agreement with those reported by Brown et al. (1993), Cakmak et al. (1996), Grewal et al. (1998) and Hosseini et al. (2007) that Zinc application increased thousand grain mass in corn plant. Hemantaranjan and Gray (1988) observed that optimum utilization of Zn and Fe significantly increased thousand grain mass in wheat. They declared that total content of carbohydrates, starch, IAA, chlorophyll and seed protein were significantly increased by consumption of these two nutrients. They believe that more production of chlorophyll and IAA can causes delay in plant senescence and prolong the period of photosynthesis. This event improves the production of carbohydrates and their transportation to the growing seeds.

3.5 Seed oil content

Analysis of variance showed significant difference among treatments ($p < 0.01$) (Table 1). B + Zn + S treatment produced the highest seed oil content. The lowest seed oil content was obtained from control (Table 2). Application of B, Zn and S single or together increased the seed oil content compared with the control, and the increase in oil content with combined nutrients application was higher than that with single application (Table 2). Singh and Sinha (2005) reported the decline in oil concentration may be due to oxidation of some polyunsaturated fatty acids.

The results showed that zinc deficiency would prevent the activity of antioxidant enzymes, leading to widespread and severe damage to lipid membranes, Therefore the lack of zinc can reduce the oil content of seeds (Cakmak, 1997). In the study of sulfur effects on rapeseed have been reported in India that the use of different sulphur sources before flowering increased grain yield and oil content (Sharma et al., 1991). Ahmad et al. (2007) found that sulfur treatment increased the rapeseed oil yield for 20 kg ha^{-1}. Malhi et al. (2007) showed that sulfur treatment increased oil and protein content.

This might be due to role of sulfur in oil synthesis; sulfur played an important role in the formation of glucosides and glucosinolates (mustard oil). This confirms the findings of Mishra & Agarwal (1994) in soybean, Ravi et al. (2008) in safflower and Gangadhara et al. (1990) in sunflower.

3.6 B, Zn and S content in leaves

F value and level of significance from ANOVA on content of plant nutrients in rapeseed plant traits are shown in Table 1. B, Zn and S contents in plant leaves as affected by B, Zn and S application are shown in Table 2.

Table 3: Mean squares from analysis of variance of antioxidant enzyme activity and fatty acid of rapeseed

S.O.V	df	Mean Squares							
		POD	SOD	Stearic acid	Palmitic acid	Oleic acid	Linoleic acid	Linolenic acid	Erucic acid
Block	2	0.038	0.13	4.39	1.23	240.7	21.11	11.37	0.106
Treatment	7	0.058**	0.64**	1.48*	3.37**	1585.2**	53.06**	28.91**	0.064**
Error	14	0.0049	0.072	0.83	0.318	88.82	1.94	3.59	0.0015
Cv (%)	-	10.6	9.7	16.6	4.1	4.7	12.8	8.1	5.7

*ns and *, **: non-significant and significant at 5 % and 1 % probability levels, respectively*

POD: Peroxidase SOD: Superoxide dismutase.

The highest leaf sulphur content (802.6 mg kg^{-1}) was produced from B + Zn + S treatment and minimum (223.97 mg kg^{-1}) was recorded for control (Table 2). The application of B, Zn and S single treatments increased the leaf sulphur content by a further 226.119 % and 74.6 % compared to the control and the highest leaf sulphur content (802.6 mg kg^{-1}) was obtained from B + Zn +S treatment. S and Zn had a grater effect than the application of B.

The highest zinc content was observed in S + Zn treatment (49.49 mg kg^{-1}). The smallest zinc content was observed in control (30.31 mg kg^{-1}) (Table 2). Grawel & Graham (1999) have reported that zinc application increases zinc concentration in seed, roots and leaves. These results were in agreement with those reported by El-Gazzar et al (1979) and Foregoni et al. (1984). Zinc plays an important role in auxine and protein synthesis and it is essential for seed setting (Bybordi & Mamedov, 2010).

Table 4: Effects of boron (B), zinc (Zn), sulfur (S), and their interactions on antioxidant enzyme activity and fatty acid of rapeseed

Treatment	POD (μmol g^{-1} FM min)	SOD (Unit mg^{-1})	Stearic acid (mg g^{-1})	Palmitic acid (mg g^{-1})	Oleic acid (mg g^{-1})	Linoleic acid (mg g^{-1})	Linolenic acid (mg g^{-1})	Erucic acid (mg g^{-1})
Control	0.47^{f}	8.8^{e}	6.112^{ab}	13.27^{c}	158.3^{e}	41.41^{e}	17.11^{d}	0.878^{a}
B	0.53^{ef}	9.15^{dc}	4.542^{b}	13.18^{c}	197.1^{cd}	48.03^{cd}	23.25^{bc}	0.641^{cd}
Zn	0.87^{a}	9.57^{b-d}	5.078^{ab}	13.77^{bc}	193.6^{d}	47.03^{cd}	22.51^{c}	0.75^{b}
S	0.64^{e-e}	9.47^{cd}	6.096^{ab}	12.88^{c}	$188.\ 3^{d}$	46.06^{d}	22.05^{c}	0.815^{ab}
B+S	0.56^{d-f}	9.54^{cd}	$4.\ 833^{ab}$	12.74^{c}	211.8^{bc}	52.32^{b}	23.65^{a-c}	0.579^{de}
B+Zn	0.74^{bc}	9.82^{a-c}	6.527^{a}	$14.\ 67^{ab}$	$227.\ 6^{ab}$	55.55^{a}	$26.\ 58^{ab}$	$0.\ 432^{f}$
S+Zn	0.68^{cd}	10.03^{ab}	5.151^{ab}	15.47^{a}	203.2^{cd}	48.58^{cd}	24.45^{a-c}	0.661^{c}
B+S+Zn	0.82^{ab}	10.24^{a}	5.514^{ab}	15.14^{a}	$229.\ 6^{a}$	$49.\ 48^{c}$	27.14^{a}	0.557^{e}

* Means followed by the same letter(s) in each column are not significantly different (P = 0.05).
POD: Peroxidase SOD: Superoxide dismutase.

The application of single B, Zn and S increased the leaf zinc content by a further 28, 36 and 15.4 % compared to the control and the highest leaf zinc content (53 %) compared to the control was obtained from B + Zn + S treatment (Table 2). At leaf zinc content, sulphur and zinc had a greater impact than the single boron.

The highest boron content was observed in B + Zn + S treatment (24 mg kg^{-1}). The lowest boron content was observed in control (10.21 mg kg^{-1}) (Table 2). These results were in agreement with those reported by Bybordi & Mamedov (2010) who made the evaluation of application of zinc and iron in oilseed rape.

The application of single B, Zn and S increased the leaf B content by a further 26, 30.3 and 70 % compared to the control and the highest leaf boron content (135 %) was obtained by the B + Zn + S treatment (Table 2). Regarding leaf boron content, the effects of boron and zinc were more comparable than that of the treatment sulphur.

3.7 Antioxidant enzyme activity

Analysis of variance showed that effects of fertilizer treatment on POD and SOD were significant ($p < 0.01$) (Table 3). The highest POD activity was observed in Zn treatment (0.87 μmol g^{-1} FM.min) while the lowest activity was related to control treatment (0.47 μmol g^{-1} FM.min) (Table 4). It seems that Zn causes the increase of POD activity. Our finding was in agreement with the results reported by Jiang & Huang (2001) and Habibi et al. (2004).

The role of zinc is known in the effects on the activity of many enzymes (Grotz & Guerinot, 2006). It seems that zinc enhances the activity of the enzyme POD. The results are consistent with the findings of Jiang & Huang (2001) and Bybordi & Mamedov (2010).

The highest POD activity in comparison to the control was observed in Zn treatment (85.5 %). The application of S, Zn and B singly increased POD activity by a further 36.1, 85.5 and 12.8 % compared to the control.

The highest POD activity (74.5 %) compared to the control was produced from B + Zn + S treatment (Table 4).

The results showed that B + Zn + S treatment (10.24 unit mg^{-1}) increased significantly SOD activity. The lowest activity was observed in control (8.8 unit mg^{-1}) (Table 4). Cacmak (2000) reported that Zn deficiency may inhibit the activities of a number of antioxidant enzymes.

It has been demonstrated that environment stress induces oxidative stress in plant tissues. Exposition of chloroplasts to excessive excitation energy may increases generation of reactive oxygen species and induces the oxidative stress. To overcome the effects of oxidative stress, plants make use of a complex antioxidant system. Relatively higher activity of reactive oxygen species scavenger enzymes have been reported in many stressed plants, which suggests that the antioxidant system plays an important role in plants against environmental stresses (Habibi et al., 2004).

Superoxide dismutase may function as a reactive oxygen species scavenger, by converting O_2 to H_2O_2 (Baily et al., 2000). Even thought high SOD activity protects plants against superoxide radicals, it can not be considered solely responsible for membrane protection against peroxidation. In general, nutrients application and different effects on antioxidant enzymes activity, in some case increase and in some cases decrease were observed.

The application of single B, Zn and S increased SOD activity to the 7.6, 8.7 and 4 % compared to the control and combined application of two nutrients further enhanced activity of SOD than single nutrient. The highest SOD activity (16.4 %) compared to the control was produced by B + Zn + S treatment (Table 4). These results agree with the findings of Bybordi and Mamedov (2010).

3.8 Fatty acids

Analysis of variance showed that effects of fertilizer treatment on fatty acids were significant ($p < 0.01$) (Table 3). Rapeseed oil consists of different types of saturated and unsaturated fatty acids (palmitic acid, oleic acid, linoleic acid, linolenic acid, erucic acid, etc.). The palmitic acid and stearic acid are the major saturated fatty acids, whereas oleic and linoleic, linolenic acids are unsaturated. Fatty acid composition of rapeseed in particular and other oil seed crops in general, are influenced by fertilizing management. The findings of present study show that fatty acid composition is affected by B + Zn + S application, for example Bybordi & Mamedov (2010) found that application zinc and iron increased the percentage of unsaturated fatty acids and decreased saturated fatty acids of rapeseed.

Applications of B, Zn, and S resulted in a significant decrease in the erucic acid content compared with the control (Table 4). Analysis of fatty acids by GC showed that, the application of B, Zn, and S increased the fatty acids compared to the control (Table 4). The maximum of oleic acid (229.6 mg g^{-1}) and linolenic acid (27.14 mg g^{-1}) were obtained from B + Zn + S treatment (Table 4). Maximum of linoleic acid (55.55 mg g^{-1}) were obtained from B + Zn treatment. The highest contents of stearic and palmitic acid were obtained from B + Zn and S + Zn treatments which were 6.527 and 15.47 mg g^{-1}, respectively. The highest erucic acid content (0.878 mg g^{-1}) was found at control treatment (Table 4).

The increased monounsaturated fatty acid composition associated with the application of B, Zn, and S may offer several health benefits. Rapeseed oil with higher levels of monounsaturated fatty acids reduced blood cholesterol levels, thereby reducing the incidence of cardiovascular diseases (Weaver et al., 2000). Such rapeseed oil is also more chemically stable than conventional rapeseed oil because it is less susceptible to oxidation. The use of rapeseed oil with a greater content of monounsaturated fatty acids may improve the food quality of meat (Brown et al., 2000; Cromwell, 2000). Higher levels of monounsaturated fatty acids used in animal feed result in meat products that remain fresher longer, with less oxidation.

4 CONCLUSIONS

Regarding to this experiment results, application of Zn, B and S fertilizers with NPK fertilizer can help to increase the yield and yield components in rapeseed. Also fatty acids composition of rapeseed are influenced by nutrients and since quality of edible oils depends on unsaturated fatty acids, especially linoleic and linolenic acids and these acids are essential fatty acids for the human body that must be supplied through diet. Therefore this research showed that we are not only able to increase oil yield with these treatments but we can also increase oil quality with increasing fatty acid content and changed composition.

5 REFERENCES

Ahmad, G., Jan, A., Arif, M., Khattak, R. A. (2007). Influence of nitrogen and sulfur fertilization on quality of canola (*Brassica napus* L.) under rainfed conditions. *Journal of Zhejiang University Science, 8*, 731-737. https://doi.org/10.1631/jzus.2007.B0731

Anderson, J. W. (1975). *The function of sulphur in plant growth and metabolism*. Pages 87-97 In K. D. McLachlan, (Eds.), *Sulphur in Australian agriculture*. University Press, Sydney, Australia.

Bailly, C., Benamar, A., Corbineau, F., Come, D. (2000). Antioxidant systems in canola seeds as affected by priming. *Seed Science Research*, *10(2),* 35-42. https://doi.org/10.1017/S0960258500000040

Beauchamp, C. & Fridovich, J. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Annual Biochemistry*, *44*, 276-287. https://doi.org/10.1016/0003-2697(71)90370-8

Benton, J. J. (2001). *Laboratory guide for conducting soil tests and plant analysis.* CRC Press. 363 P. https://doi.org/10.1201/9781420025293

Bole, J. B., & Pittman, U. J. (1984). Availability of subsoil sulphate to barley and rapeseed. *Canadian Journal of Soil Science*, *64*, 301-312. https://doi.org/10.4141/cjss84-032

Brown, P. H., Cakmak, I., Zhang, Q. (1993). *Form and function of zinc in plants*. (PP. 93-106). In: A. D. Robson (Eds.), *Zinc in soil and plants*. Kluwar Academic Publishers. Dordecht, The Netherlands.

Brown, P. H., Bellaloui, N., Wimmer, M. A., Bassil, E. S., Ruiz, J., Hu, H., Pfeffer, H., Dannel, F., Römheld, V. (2002). Boron in plant biology, *Plant Biology, 4*, 205-223. https://doi.org/10.1055/s-2002-25740

Bybordi, A. & Mamedov, G. (2010). Evaluation of application methods efficiency of zinc and iron for canola (*Brassica napus* L.). *Notulae Scientia Biologicae, 2(1)*, 94-103.

Cakmak, I. (1997). Effect of different zinc application methods on grain yield and zinc concentration in wheat cultivars grown on zinc-deficient calcareous soils. *Journal of Plant Nutrition*, *20(4/5),* 461-471.

Cakmak, I. (2008). Enrichment of cereal grains with zinc: Agronomic or genetic biofortification? *Plant and Soil*, *302*, 1-17. https://doi.org/10.1007/s11104-007-9466-3

Cakmak, I., Kalayci, M., Ekis, H., Brauni, J., Kilinc, Y., Yilmaz, A. (1999). Zn Deficiency as a practical problem in plant and human nutrition in Turkey: a NATO-science for stability project. *Field Crop Research*, *60*, 175-188. https://doi.org/10.1016/S0378-4290(98)00139-7

Cakmak, I., Yilmaz, A., Kalayli, E., Torun, B., Ernoglu, B., Brown, H. J. (1996). Zinc deficiency as a critical problem in wheat production in central Anatolia. *Plant and Soil*, *180*, 165-172. https://doi.org/10.1007/BF00015299

Cara, A. F., Sanchez, E., Ruiz, J. M., Rornero, L. (2002). Is phenol oxidation responsible for the short-term effects of boron deficiency on plasma membrane permeability and function in squash roots? *Plant Physiology and Biochemistry*, *40*, 853-858. https://doi.org/10.1016/S0981-9428(02)01447-X

Chance, B. & Maehly, S. K. (1955). Assay of catalase and peroxidase. *Methods in Enzymology*, *2*, 764-775. https://doi.org/10.1016/S0076-6879(55)02300-8

Chen, G., Nian, F. Z., Xu, F. S., Wang, Y. H. (2005). Effect of boron and molybdenum on yield and quality of two rapeseed cultivars. *Plant Nutrition Fertility Science*, (In Chinese), *11*, 243–247.

Chu, T. D., Chen, J. J., Liu, C. Z., Xu, G. B. (1996). Study on reason of floral sterility of rape under boron deficient condition. *Plant Nutrition Fertility Science*, (In Chinese), *2(1),* 23-31.

Clandinin, D. (1981). *Canola meal for livestock and poultry*. Publication No. 59. Canola Council of Canada, Winnipeg, MB. 25 pp.

Cormwell, G. L. (2000). *An animal nutritionalist's view*. P. 57-82. In C. F. Murphy and D. M. Peterson (Eds.). *Desinging crops for added value*. Agron, Monogr. 40. ASA, CSSA, and SSSA, Madison, WI.

Cunnane, S.C. (1995). Metabolism and function of linolenic acid in humans. In: Cunnane, S. C., and L. U. Thompson (Eds.). *Flax seed in human nutrition*. Pp:99-127. AOCS, Press, Champaign, Illinois, USA.

Das, K., Dang, R., Shivananda, T. N., Sur, P. (2005). Interaction between phosphorus and zinc on the biomass yield and yield attributes of the medicinal plant stevia (*Stevia rebaudiana*). *Science World Journal*, *5,* 390–395. https://doi.org/10.1100/tsw.2005.49

Dell, B., & Huang, L. B. (1997). Physiological response of plants to low boron. *Plant and Soil*, *193*, 103–120. https://doi.org/10.1023/A:1004264009230

Dordas, C., & Brown, P. H. (2005). Boron deficiency affects cell viability, phenolic leakage and oxidative burst in rose cell cultures. *Plant Cell Physiology*, *268*, 293-301. https://doi.org/10.1007/s11104-004-0309-1

Fang, Y., Wang, L., Xin, Z., Zhao, L., An, X., Hu, Q. (2008). Effect of foliar application of zinc, selenium, and iron fertilizers on nutrients concentration and yield of rice grain in China. *Journal of Agriculture and Food Chemistry*, *56*, 2079–2084. https://doi.org/10.1021/jf800150z

Foregoni, M., Boselli, M., Bartoletti, C., Dorotea, G. (1984). Effect of iron deficiency or an excess of leaf-applied iron on the mineral and biochemical composition and on the production parameters of chlorotic grapevines. *Horticulture Abstract*, *54*, 95-110.

Habibi, D., Boojar, M. M., Mahmoudi, A., Ardekani, M. R., Taleghani, D. (2004). Antioxidative enzymes in sunflower subjected to drought stress. 4th International Crop Sci. Congress, Australia.

Hamm, J.W. (1967). *Sulfur on rapeseed and cereals*. Papers presented at the Eleventh Annual Manitoba Soil Science Meeting. University of Manitoba, Winnipeg, MB. pp. 91-108.

Hemantaranjan, A., & Grag, O. K. (1998). Iron and zinc fertilization with reference to the grain quality of *Triticum aestivum*. *Journal of Plant Nutrition*, *11*(6-11), 1439-1450. https://doi.org/10.1080/01904168809363900

Hosseni, S. M., Maftoun, M., Karimian, N., Rounaghi, A., Emam, Y. (2007). Effect of zinc×boron interaction on plant growth and tissue nutrient concentration of corn. *Journal of Plant Nutrition*, *30*, 773-781. https://doi.org/10.1080/01904160701289974

Huang, L. B., Pant, J., Dell, B., Bell, R. W. (2000). Effects of boron deficiency on anther development and floret fertility in wheat (*Triticum aestivum 'Wilgoyne'*). *Annals of Botany*, *85*, 493–500. https://doi.org/10.1006/anbo.1999.1095

Gammelvind, L. H., Schjoerring, J. K., Mogensen, V. O., Jensen, C. R., Bock, J. G. H. (1996). Photosynthesis in leaves and siliques of winter oilseed rape (*Brassica napus* L.). *Plant and Soil*, *186*, 227-236.

Gangadhara, G. A., Manjunathiah, H. M., Satyanarayana, T. (1990). Effect of sulphur on yield, oil content of sunflower and uptake of micronutrient by plants. *Journal of the Indian Society of Soil Science*, *38*, 692-694.

Goldbach, H. E., Yu, Q., Wingender, R., Schulz, M., Wimmer, M., Findeklee, P., Baluska, F. (2001). Rapid response reaction of roots to boron deprivation. *Journal of Plant Nutrition and Soil Science*, *164,* 173–181. https://doi.org/10.1002/1522-2624(200104)164:2<173::AID-JPLN173>3.0.CO;2-F

Grant, C., Clayton, G. W., Johnston, A. M. (2003). Sulphur fertilizer and tillage effects on canola seed quality in the Black soil zones of western Canada. *Canadian Journal of Plant Science*, *83*, 745-758. https://doi.org/10.4141/P02-107

Grant, C. A. & Bailey, L. D. (1993). Fertility management in canola production. *Canadian Journal of Plant Science*, *73*, 651-670. https://doi.org/10.4141/cjps93-087

Grewal, H. S., Graham, R. D., Stangoulis, J. (1998). Zinc-boron interaction effects in oilseed rape. *Journal of Plant Nutrition*, *21*(10), 2231-2243. https://doi.org/10.1080/01904169809365557

Grotz, N., & Guerinot, M. L. (2006). Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochimica et Biophysica Acta*, *1763,* 595- 607. https://doi.org/10.1016/j.bbamcr.2006.05.014

Iwai, H., Hokura, A., Oishi, M., Chida, H., Ishii, T., Sakai, S., Satoh, S. (2006). The gene responsible for borate cross-linking of pectin rhamnogalacturonan-II is required for plant reproductive tissue development and fertilization. *Proceedings of the National Academy of Sciences of the United States of America.. 103*, 16592-16597. https://doi.org/10.1073/pnas.0605141103

Jiang, Y. & Huang, B. (2001). Drought and heat stress injury to two cool-season turf grasses in relation to antioxidant metabolism and lipid peroxidation. *Crop Science*, *41*, 436-442. https://doi.org/10.2135/cropsci2001.412436x

Kaya, C., & Higgs, D. (2002). Response of tomato (*Lycopersicon esculentum* L.) cultivars to foliar application of zinc when grown in sand culture at low zinc. *Scientia Horticulturae*, *93*, 53-64. https://doi.org/10.1016/S0304-4238(01)00310-7

Lieten, P. (2002). Boron deficiency of strawberries grown in subtract culture. *Acta Horticulturae*, *567*, 451-454. https://doi.org/10.17660/ActaHortic.2002.567.94

Liu, W. D. (1999). *Microelements nutrition and application of micro-fertilizers* (in Chinese). China Agricultural Press. Beijing.

Mc-Grath, S. P., & Zhao, F. J. (1996). Sulphur uptake, yield response and the interaction between nitrogen

and sulphur in winter oilseed rape (*Brassica napus* L.). *The Journal of Agricultural Sciences*, *126*, 53-62. https://doi.org/10.1017/S0021859600088808

Malhi, S. S., Schoenau, J. J., Grant, C. A. (2005). A review of sulphur fertilizer management for optimum yield and quality of canola in the Canadian Great Plains. *Canadian Journal of Plant Science*, *85*, 297-307. https://doi.org/10.4141/P04-140

Malhi, S. S., & Gil, K. S. (2002). Effectiveness of sulphate-S fertilization at different growth stages for yield, seed quality and S uptake of canola. *Canadian Journal of Plant Science*, *82*, 665-674. https://doi.org/10.4141/P01-184

Malhi, S. S., Gan, Y., Raney, J. P. (2007). Yield, seed quality and sulfur uptake of Brassica oilseed crops in response to sulfur fertilization. *Agronomy Journal*, *99*, 570-577. https://doi.org/10.2134/agronj2006.0269

Marschner, H. 1995. *Mineral nutrition of higher plants*. Academic Press. New York.

Marschner, H. 1986. *Function of mineral nutrients*; Macronutrients, p. 195-267. In: R. J. Haynes (Eds.). Mineral nutrition of higher plants. Academic Press. Orlando, FL.

Mishra, A. K. & Agarwal, H. P. (1994). Effect of sulphur on growth yield, protein and oil content of soybean. *Journal of Oilseeds Research*, *11*, 99-102.

Matoh, T. (1997). Boron in plant cell walls. *Plant and Soil*, *193*, 59-70. https://doi.org/10.1023/A:1004207824251

Mei, Y., Lei, S. H., Fang-Sen, X., Jian-wei, L.U., Yun-Hua, W. (2009). Effect of B, Mo, Zn, and Their Interactions on Seed Yield of Rapseed (*Brassica napus* L.). *Pedosphere, 19*(1), 53-59. https://doi.org/10.1016/S1002-0160(08)60083-1

Nabi, G., Rafique, E., Salim, M. (2006). Boron nutrition of four sweet pepper cultivars grown in boron-deficient soil. *Journal of Plant Nutrition*, *29*, 717–725. https://doi.org/10.1080/01904160600564543

Neilsen, G. H., Neilsen, D., Hogue, E. J., Herbert, L.C. (2004). Zinc and boron nutrition management in fertigated high density apple orchards. *Canadian Journal of Plant Science*, *84*, 823-828. https://doi.org/10.4141/P03-153

Nuttall, W. F., Ukrainetz, H., Stewart, J. W., Spurr, D. T. (1987). The effect of nitrogen, sulphur and boron on yield and qulity of rapeseed (*Brassica napus* L. and *Brassica compestris* L.). *Canadian Journal of Soil Science*, *67*, 545-559. https://doi.org/10.4141/cjss87-051

Nyborg, M., Bentley, C. F., Hoyt, P. B. (1974). Effect of sulphur deficiency. *Sulphur Institute Journal, 10*, 14-15.

Pandey, N., Pathak, G. C., Sharma, C. P. (2006). Zinc is critically required for pollen function and fertilization in lentil. *Journal of Trace Element in Medicine and Biology*, *20*, 89–96. https://doi.org/10.1016/j.jtemb.2005.09.006

Rathinavel, K., Dharmalingam, C., Paneersel vam, S. (2000). Effect of micronutrient on the productivity and quality of cotton seed cv. TCB 209 (*Gossypium barbadense* L.). *The Madras Agricultural Journal*, *86*, 313-316.

Ravi, S., Channal, H. T., Hebsur, N. S., Patil, B. N., Dharmatti, P. R. (2008). Effect of sulphur, zinc and iron nutrition on growth, yield, nutrient uptake and quality of safflower (*Carthamus tinctorius* L.). *Karnataka Journal of Agricultural Sciences*, *21*(3), 382-385.

Rehem, G. W., Fendter, W. E., Overdahi, C. J. (1998). *Boron for Minnesota soils*. University of Minnesota Ridley, A. O. (1973). *Effect of nitrogen and sulfur fertilizers on yield and quality of rapeseed*. Papers presented at the 17th Annual Manitoba Soil Science Meeting, University of Manitoba, Winnipeg, MB. pp. 182-187.

Roe, N. E., Stoffella, P. J., Greatz, D. (1997). Comparison of canola (*Brassica campestris* L.) and (*Brassica napus* L.) oilseed rape using different growth regulators plant population densities and irrigation treatments. *Journal of Agricultural Sciences*, *77*, 177-187.

Sharma, D. N., Khadar, V. K., Sharma, R. A., Singh, D. (1991). Effect of different doses and sources of sulphur on the quality and yield of mustard (*Brassica juncea* L.). *Journal of the Indian Society of Soil Science*, *39*, 197-200.

Singh Grewal, H., & Grahma, R. D. (1999). Residual effects of subsoil zinc and oilseed rape genotype on the grain yield and distribution of zinc in wheat. *Plant and Soil*, *207*, 29-36. https://doi.org/10.1023/A:1004479911399

Singh Grewal, H., Lu, Z. G., Graham, R. D. (1997). Influence of subsoil zinc on dry matter production, seed yield and distribution of zinc in oilseed rape genotypes differing in zinc efficiency. *Plant and Soil*, *192*, 181-189. https://doi.org/10.1023/A:1004228610138

Singh, S., & Sinha, S. (2005). Accumulation of metals and its effects in (*Brassica juncea* L.) Czern. (cv. Rohini) grown on various amendments of tannery waste, *Ecotoxicolgy and Environmental Safety*, *62*,

118-127. https://doi.org/10.1016/j.ecoenv.2004.12.026

Sinha, P., Jain, R., Chatterjee, C. (2000). Interactive effect of boron and zinc on growth and metabolism of mustard commun. *Communications in Soil Science and Plant Analysis*, *31*, 41-49. https://doi.org/10.1080/00103620009370419

Vitosh, M. L., Warnek, D. D., Lucas, R. E. (1997). *Boron*. Mishigan State University Extension Soil and Management Fertilizer. Available on the http://www.msue.msu.EDV

Zhang, W. Y. (2001). Critical range of soil boron for prognosis of boron deficiency in oilseed rape. *Pedosphere*, *11*(3), 283-288.

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Sugar beet profits from intercropping with wheat both under optimum and deficient phosphorus supply

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ABSTRACT

An experiment was conducted with wheat and sugar beet as monocrop and intercrop under low or adequate phosphorus supply. Dry matter production of shoot and roots were decreased in wheat while increased in sugar beet under intercrop conditions. Photosynthesis rate was diminished under intercrop conditions in wheat while elevated in sugar beet concomitant with reduction of transpiration rate and higher water use efficiency in the latter species. Phosphorus, potassium and iron contents were also lower in intercrop wheat while increased in sugar beet. The same effect of intercropping on biomass and nutrients uptake was observed in the short term hydroponic experiment. Interestingly, three root parameters including length, soluble carbohydrates and activity of secretory acid phosphatase that are characteristics for phosphorus-deficient plants were enhanced in both species by intercropping irrespective the phosphorus supply level. These data suggested an interspecific interaction beyond the different nutrient acquisition capacity in the intercrop pots.

Key words: intercrop; monocrop; sugar beet; wheat; phosphorus deficiency; acid phosphatase

IZVLEČEK

SLADKORNA PESA USPEVA BOLJE KOT PŠENICA V MEDSETVI V RAZMERAH OPTIMALNE IN POMANKLJIVE PRESKRBE S FOSFORJEM

V raziskavi je bil opravljen poskus s pšenico in sladkorno peso v čisti kulturi in medsetvi v razmerah primerne in pomankljive preskrbe s fosforjem. V razmerah medsetve sta se biomasi korenin in poganjkov zmanšali pri pšenici a povečali pri sladkorni pesi. Podobno se je pri pšenici v medsetvi zmanjšala fotosinteza in povečala pri sladkorni pesi s hkratnim zmanjšanjem transpiracije in večjo učinkovitostjo izrabe vode. Pravtako so bile vsebnost fosforja, kalija in železa manjše pri pšenici v medsetvi in večje pri sladkorni pesi. Podoben učinek medsetve na biomaso in vsebnost hranil je bil opazen v kratkotrajnem hidroponskem poskusu. Zanimivo je, da so se neglede na preskrbo s fosforjem v medsetvi pri obeh vrstah povečali parametri korenin kot so njihova dolžina, vsebnost topnih ogljikovih hidratov in aktivnost izločenih kislih fosfataz, kar je značilnost rastlin, ki rastejo v pomanjkanju fosforja. Podatki nakazujejo na medvrstne interakcije, ki presegajo razlike v sposobnosti privzema hranil v lončnem poskusu z medsetvijo.

Ključne besede: medsetev; čista kultura; sladkorna pesa; pšenica; pomanjkanje fosforja; kisle fosfataze

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1 INTRODUCTION

Crop plants are cultivated mainly as monocultures. Monocropping facilitates agricultural practices such as weed removing and harvest. However, this system maintains crop productivity through heavy chemical inputs including fertilizers and pesticides and reduces the plant and microorganism diversity (Brooker et al., 2015). Intercropping, in which at least two crop species are grown on the same plot of land simultaneously, results in higher yield under different environmental and soil conditions (Brooker et al., 2015; Li et al., 2014). Indeed, in intercropping systems both negative interaction (competition) and positive interaction (facilitation) can occur simultaneously. Competition prevents crop growth by sharing the limited resources or allelopathy, whereas facilitation promotes crop performance by improving the micro-environment for utilizing resources (Zhang & Li, 2003; Li et al., 2014; Brooker et al., 2015).

Phosphorus (P) deficiency is a widespread nutritional disorder in crop plants (Hawkesford et al., 2012). In soil solution, P mostly exists as $H_2PO_4^-$ but its fixation to the soil particles and minerals is very important factor determining its availability for plant roots. Thus, spatial availability of roots to soil P resources is an important criterion for plants P uptake capacity. Every factor that changes root morphology in favor of higher spatial exploration of the soil profile can facilitates P acquisition capacity in plants (Hawkesford et al., 2012).

Interspecific interactions consist of both above and below-ground interactions (Zhang et al., 2001). Increasing evidence from studies on intercropping systems indicate that root interactions are more important than shoot interactions for determining crop productivity and intercropping advantages (Wu et al., 2012). In low-input agroecosystems, the productivity of cultivated land will primarily depend on the availability of soil resources. Thus, facilitative root interactions in mixed cropping systems are important for the nutritional improvement of crops in nutrient-poor soils (Zhang et al., 2010; Wu et al., 2012). Understanding interspecific below-ground interactions between crops is crucial to promote sustainable production (Brooker et al., 2008; Wu et al., 2012).

Rhizosphere effects contribute significantly to the improved P and Fe uptake under field conditions (Neumann & Römheld, 2012). It has been shown that intercropping legumes and cereals improves the Fe nutrition of both species because of higher ferric chelate reductase activity in the rizosphere of legumes and excretion of phytosiderophores into the rhizosphere of Gramineae that are particularly effective in non-calcareous and calcareous conditions, respectively (Zuo & Zhang, 2009; Li et al., 2014). Legumes when intercropped with cereals facilitate the P uptake through acidification of the rhizosphere, exudation of carboxylates and flavonoids, and root secretion of acid phosphatase in P-deficient soils (Li et al., 2007; Li et al., 2014; Dissanayaka et al., 2015). Most of the works on interspecific interactions between crops have focused on cereal–cereal and particularly on legume-based intercropping systems. Knowledge is lacking on interspecific interactions in the intercropping cereals with other dicotyledonous species.

In order to study the physiological interactions in an intercropping system, a sugar beet-wheat intercropping experiment was conducted at low and adequate P supply levels under growth chamber conditions. In addition of dry matter production and photosynthesis, nutrients uptake capacity and rhizosphere properties were studied under monocropping and intercropping systems. In order to evaluate the effect of spatial availability and a better interpretation of uptake data, a short-term hydroponic experiment was also conducted as monocrop and intercrop cultures.

2 MATERIALS AND METHODS

Seeds of wheat (*Triticum aestivum* 'Homa') plants were provided by Dryland Agricultural Research Institute (DARI) (Maragheh, Iran), and of sugar beet (*Beta vulgaris* 'IC') by Agricultural Research Institute, West-Azarbaijan Province, Iran.

2.1 Plants culture and treatments

Seeds were surface-sterilized with 1 % active hypochlorite and germinated in dark. Young seedlings were transferred to the light. Five-day-old sugar beet seedlings were pre-cultured in 50 % Hoagland nutrient solution for one month. Thereafter, 35-day-old sugar beet together with seven-day-old wheat seedlings with similar size of shoot and roots were transferred to two liter plastic pots filled with washed perlite and irrigated with 100 % Hoagland nutrient solution at field capacity after daily weighing. Wheat and sugar beet were cultivated either as monocrop (MC) or intercrop (IC). Four plants were cultivated in each MC pot and two plants of each species in IC pots. The amount of nutrient solution used for irrigation was 100 ml $week^{-1}$ l^{-1} at the first 4 weeks and increased to the 200 ml $week^{-1}$ l^{-1}

during further growth stage. Irrigation was performed after daily weighing and water was used as interval if necessary. Two different P supply levels including adequate (2 mM) and low (0.2 mM) P were applied immediately after transferring plants to the pots. Different P levels in the nutrient solution were provided through reduction of $NH_4H_2PO_4$ in the nutrient solution. In order to equilibrate NH_4^+ concentration between two P treatments, 1.8 mM NH_4Cl was added to the low P nutrient solution.

Plants were grown under growth chamber conditions with a day/night temperature regime of 25-28/15-17 °C, a relative humidity of 70/80 % and a photoperiod of 17/7 h at a photon flux density of about 300 µmol m^{-2} s^{-1} provided by fluorescent lamps. Before harvest, net photosynthesis rate were determined in the attached leaves using a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) during the light period between 9:00 and 13:00 under a photon flux density of about 300 µmol m^{-2} s^{-1}.

2.2 Plants harvest and determination of elements

Ten weeks after transferring to the pots and starting different P treatments, plants were harvested. Shoot and roots were separated, roots were washed with distilled water and blotted dry on filter paper and their fresh mass (FM) were determined. Plants dry mass (DM) was determined after drying in 70 °C for 48 h. Subsamples from leaves and roots were taken for biochemical analyses before drying.

For determination of P, K and Fe concentrations, oven dried leaf samples were ashed in a muffle furnace at 550 ºC for eight hours, resolved in HCl, and made up to volume by distilled water. Concentration of P was determined using ammonium molybdate-vanadate colorimetric method (Jaiswal, 2004). Concentrations of K and Fe were determined by flame photometry (PFP7, Jenway, UK) and atomic absorption spectrometry (AA-6300, Shimadzu, Japan), respectively.

In addition of elements concentration in shoot and root, the proportion (%) of elements taken up by plant, i.e. the ratio of plants element content to that added to the medium throughout the experiment, was calculated for P and K.

2.3 Assay of rhizosphere pH and root acid phosphatase activity

At harvest, roots were shaken gently to collect loosely attached perlite particles defined as rhizosphere (Bagayoko et al., 2000). These perlite samples with a volume of one ml were suspended immediately in the same volume of distilled water and shaken vigorously for 30 min. After centrifugation, pH was determined in the supernatant.

For determination of secretory acid phosphatase (APase) activity, root segments (2 cm) were excised from apical root zone (without root tips) of the first-order laterals and washed twice with distilled water for 5 min. Root segments were treated with incubation medium containing 0.5 ml distilled water, 0.4 ml Na-acetate buffer (200 mM, pH 5.2) and 0.1 ml *p*-nitrophenyl phosphate (NPP, 150 mM) for one min at 25–30 °C. Thereafter, 0.8 ml of the reaction media was mixed with 0.4 ml of 500 mM NaOH to terminate the reaction. The absorption of the dephosphorylation product, *p*-nitrophenol (PNP), was determined spectrophotometrically at 405 nm (Wang et al., 2015).

2.4 Biochemical determinations

Leaf concentration of chlorophylls (Chl) a, b and carotenoids (Car) were determined according to Lichtenthaler and Wellburn (1985). For determination of non-structural carbohydrates, samples were homogenized in 100 mM phosphate buffer (pH 7.5) at 4 ^oC, after centrifugation at 12000 g for 15 min, supernatant was used for determination of total soluble sugars whereas the pellets were kept for starch analysis (Yemm & Willis, 1954). Soluble protein concentration was determined using a commercial reagent (Bradford reagent, Sigma, St. Louis, USA) and bovine albumin serum (BSA, Merck, Darmstadt, Germany) as standard. Content of total free α-amino acids was assayed using a ninhydrin colorimetric method (Yemm & Cocking, 1955) with glycine (Merck, Darmstadt, Germany) as standard.

2.5 Hydroponic experiment

Thirty five-day-old sugar beet seedlings together with seven-day-old wheat seedlings were transferred to one liter plastic pots (4 plants in each pot; 2-2 in intercrop) filled with aerated 100 % Hoagland nutrient solution and grown under growth chamber conditions. One group of plants were harvested one week after transferring to the pots and analyzed for biomass and nutrients content as described above. The second group was grown for ten weeks before harvest, and during this time nutrient solutions were replaced every 5 days.

2.6 Experimental design and statistical analyses

The experiments were undertaken in randomized block design with four replications as four independent pots. Differences between the means were detected according to Tukey's test ($p < 0.05$) using Sigma Stat 2.03 software.

3 RESULTS

Growth parameters were influenced significantly under IC conditions in both species. In wheat, shoot biomass was decreased by IC while increased in sugar beet at both P supply levels significantly. In wheat, root mass decreased by intercropping only in low P plants while root length was significantly increased under these conditions. In sugar beet, similar to shoot growth, root mass and length both were higher in IC than MC, irrespective the P supply level (Fig. 1).

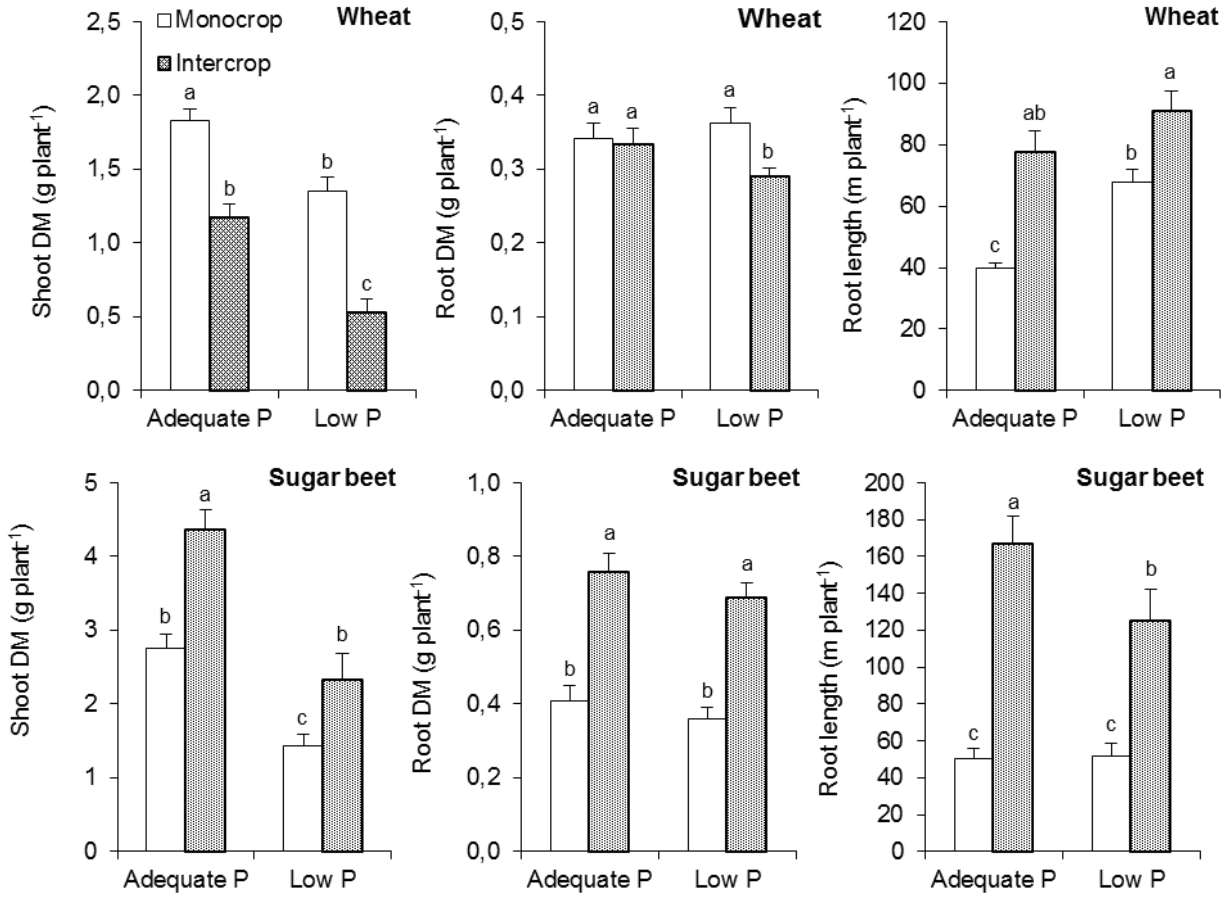

Figure 1: Shoot and root dry mass (DM) and root length in wheat and sugar beet plants in monocropping and intercropping under adequate or low P supply. Bars indicated by the same letter are not statistically different ($p < 0.05$).

Leaf concentrations of Chls and Car decreased under IC conditions in wheat under both P supply levels, while these parameters were not affected in sugar beet, except Car in P-deficient plants. P deficiency, however, caused a significant reduction of Chl a in sugar beet and Chl b in wheat under both cropping systems (Table 1). Net photosynthesis rate was expectedly lower under P deficiency conditions in both species. Intercropping, however, increased photosynthesis in sugar beet under both P supply levels while decreased it in wheat. A statistically significant reduction of the transpiration rate due to P deficiency was only observed in sugar beet in MC. Intercropping tended to decrease the transpiration rates in both species regardless the P supply, but a statistically significant influence was only observed in sugar beet under adequate P supply. Instant water use efficiency (WUE) was not influenced in wheat plants either by P supply or IC, while in sugar beet it was significantly higher in IC plants irrespective the P supply level (Table 1).

Table 1: Leaf concentration of chlorophyll a, b and carotenoids (mg g^{-1} FM), net photosynthesis rate (µmol CO_2 m^{-2} s^{-1}), transpiration rate (mmol H_2O m^{-2} s^{-1}) and instant water use efficiency (iWUE, µmol $mmol^{-1}$) in wheat and sugar beet plants in monocropping (MC) or intercropping (IC) in perlite at adequate or low P supply for 10 weeks under controlled environmental conditions.

Plant species	Culture mode	**Chl *a***		**Chl *b***		**Car**	
		Adequate P	Low P	Adequate P	Low P	Adequate P	Low P
Wheat	MC	1.98±0.22 [a]	1.85±0.29 [a]	1.18±0.13 [a]	0.99±0.17 [b]	0.19±0.04 [a]	0.22±0.05 [a]
	IC	1.49±0.10 [b]	1.20±0.16 [b]	0.62±0.05 [c]	0.49±0.06 [c]	0.09±0.02 [b]	0.12±0.01 [b]
Sugar beet	MC	1.54±0.32 [a]	0.89±0.02 [b]	0.68±0.11 [a]	0.69±0.50 [a]	0.25±0.06 [a]	0.21±0.01 [ab]
	IC	1.72±0.11 [a]	0.90±0.09 [b]	0.62±0.02 [a]	1.06±0.08 [a]	0.26±0.04 [a]	0.12±0.06 [b]
		Photosynthesis rate		**Transpiration rate**		***iWUE***	
		Adequate P	Low P	Adequate P	Low P	Adequate P	Low P
Wheat	MC	7.27±0.40 [a]	6.17±0.57 [b]	2.04±0.34 [a]	2.12±0.30 [a]	3.39±0.51 [a]	2.96±0.20 [a]
	IC	5.50±0.59 [b]	4.12±0.41 [c]	1.51±0.54 [a]	1.52±0.18 [a]	3.48±0.75 [a]	2.73±0.59 [a]
Sugar beet	MC	4.58±0.70 [b]	3.53±0.16 [c]	2.53±0.29 [a]	1.75±0.41 [b]	1.81±0.15 [b]	1.21±0.47 [b]
	IC	5.65±0.37 [a]	4.48±0.18 [b]	1.59±0.23 [b]	1.23±0.10 [b]	3.48±0.44 [a]	3.44±0.60 [a]

Data of each parameter within each plant species indicated by the same letter are not statistically different ($p < 0.05$).

Leaf concentrations of soluble sugars were expectedly higher in P deficient plants. This effect was significant for both species except for sugar beet under IC conditions. Intercropping increased the concentrations of soluble sugars in both species regardless the P level except for sugar beet under low P conditions. In wheat, soluble sugar concentration of roots was lower in MC plants at low P conditions. Intercropped plants had higher root soluble sugars in sugar beet under both P nutritional conditions, this effect was observed in wheat only under low supply of P (Table 2). Starch concentrations in the leaves and roots of sugar beet were affected by neither P nutrition nor IC. In wheat, starch increased in the leaves by IC under sufficient P supply (Table 2).

Table 2: Concentration (mg g^{-1} FM) of soluble sugars and starch in wheat and sugar beet plants in monocropping (MC) or intercropping (IC) in perlite at adequate or low P supply for 10 weeks under controlled environmental conditions.

Plant species	Culture mode	**Soluble sugars**		**Starch**	
		Adequate P	Low P	Adequate P	Low P
		Shoot			
Wheat	MC	7.9±2.62 [c]	19.4±5.23 [b]	0.17±0.01 [b]	0.15±0.01 [b]
	IC	14.5±3.47 [b]	26.4±1.82 [a]	0.23±0.01 [a]	0.15±0.02 [b]
Sugar beet	MC	5.43±0.61 [b]	12.9±2.79 [a]	0.46±0.04 [a]	0.58±0.19 [a]
	IC	14.1±1.65 [a]	14.4±1.89 [a]	0.41±0.04 [a]	0.59±0.13 [a]
		Root			
Wheat	MC	7.52±1.54 [a]	4.51±1.47 [b]	0.09±0.03 [a]	0.03±0.00 [b]
	IC	7.17±0.99 [a]	9.05±0.97 [a]	0.08±0.01 [a]	0.05±0.01 [b]
Sugar beet	MC	5.90±0.95 [c]	4.77±0.44 [c]	0.24±0.15 [a]	0.36±0.01 [a]
	IC	8.32±0.87 [b]	11.15±0.10 [a]	0.24±0.12 [a]	0.28±0.03 [a]

Data of each parameter within each plant species indicated by the same letter are not statistically different ($p < 0.05$).

In the leaves, total leaf amino acid concentrations were lower in IC wheat at both P supply levels, while in sugar beet it increased by IC in P-deficient plants. Soluble protein concentrations in the leaves were not affected by IC in sugar beet, while they decreased in wheat at low P level. Low P supply resulted in higher leaf amino acid concentrations while in sugar beet rather a decrease was observed under these conditions (Table 3). In the roots, amino acid concentrations were not affected by P supply, but protein concentrations decreased significantly in both species under MC conditions. Intercropping decreased significantly amino acids and protein concentrations in wheat. Negative effects of IC on these parameters were not observed in sugar beet and a significant increase of soluble proteins upon IC was found in low P plants (Table 3).

Table 3: Concentration of total free amino acids (µmol g^{-1} FM) and soluble proteins (mg g^{-1} FM) in wheat and sugar beet plants in monocropping (MC) or intercropping (IC) in perlite at adequate or low P supply for 10 weeks under controlled environmental conditions.

Plant species	Culture mode	**Amino acids**		**Soluble proteins**	
		Adequate P	Low P	Adequate P	Low P
		Shoot			
Wheat	MC	10.0±1.90 [b]	20.9±2.63 [a]	2.04±0.59 [a]	1.73±0.38 [a]
	IC	7.6±1.48 [b]	8.9±1.10 [b]	1.53±0.18 [ab]	0.91±0.40 [b]
Sugar beet	MC	5.0±0.54 [a]	2.3±0.23 [b]	2.13±0.78 [a]	1.99±0.14 [a]
	IC	3.9±0.72 [a]	5.9±1.06 [a]	2.62±0.07 [a]	2.13±0.23 [a]
		Root			
Wheat	MC	4.7±1.24 [a]	4.62±1.24 [a]	0.74±0.16 [a]	0.36±0.03 [b]
	IC	3.0±0.68 [b]	2.90±0.35 [b]	0.13±0.01 [c]	0.16±0.02 [c]
Sugar beet	MC	1.1±0.24 [a]	1.08±0.10 [a]	0.91±0.21 [a]	0.13±0.01 [b]
	IC	1.1±0.26 [a]	1.56±0.46 [a]	0.71±0.33 [a]	0.77±0.02 [a]

Data of each parameter within each plant species indicated by the same letter are not statistically different ($p < 0.05$).

Shoot and root P content decreased by IC in wheat, while increased in sugar beet. This effect, however, was not statistically significant in P-deficient sugar beet. P content was expectedly lower in low P plants in both species irrespective the cultivation system (Fig. 2).

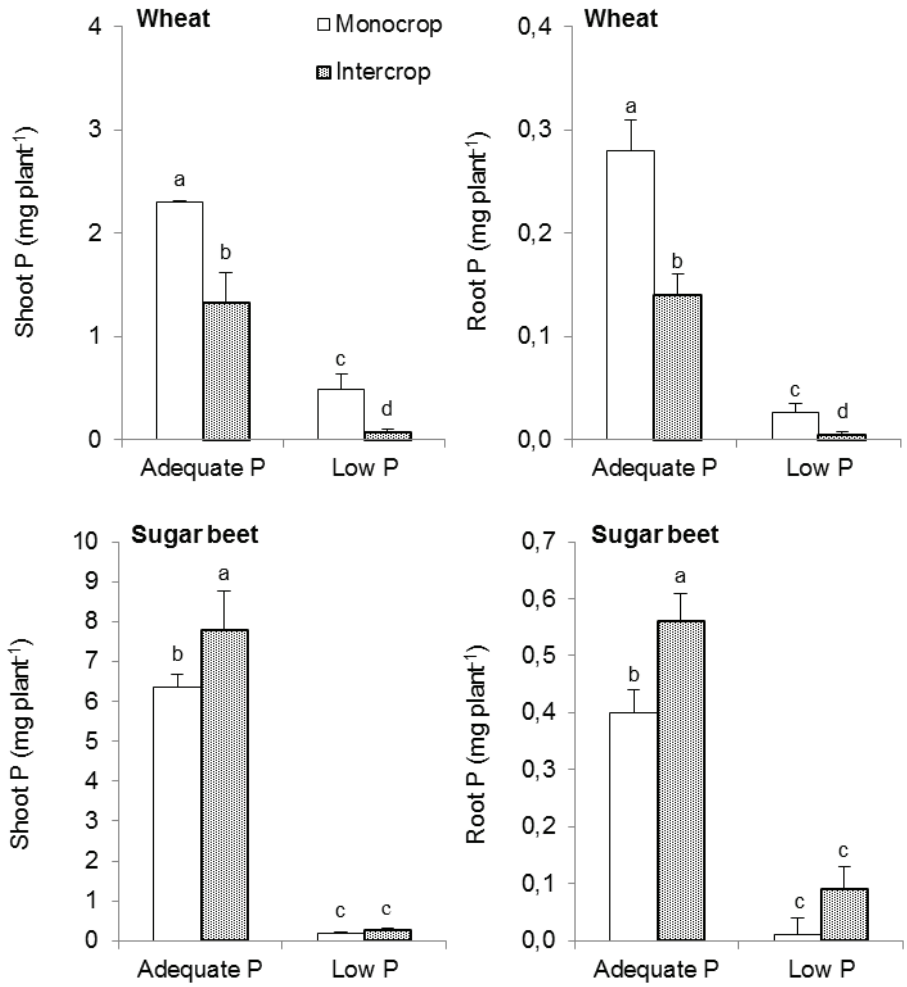

Figure 2: Phosphorus content in the shoot and roots of wheat and sugar beet plants in monocropping and intercropping under adequate or low P supply. Bars indicated by the same letter are not statistically different ($p < 0.05$).

Similar to P, K contents were decreased in IC wheat while increased in sugar beet in both shoot and roots (Fig. 3). In both species P-deficient plants had significantly lower shoot K content under both MC and IC conditions. In the roots, this effect was significant only in sugar beet under IC conditions (Fig. 3).

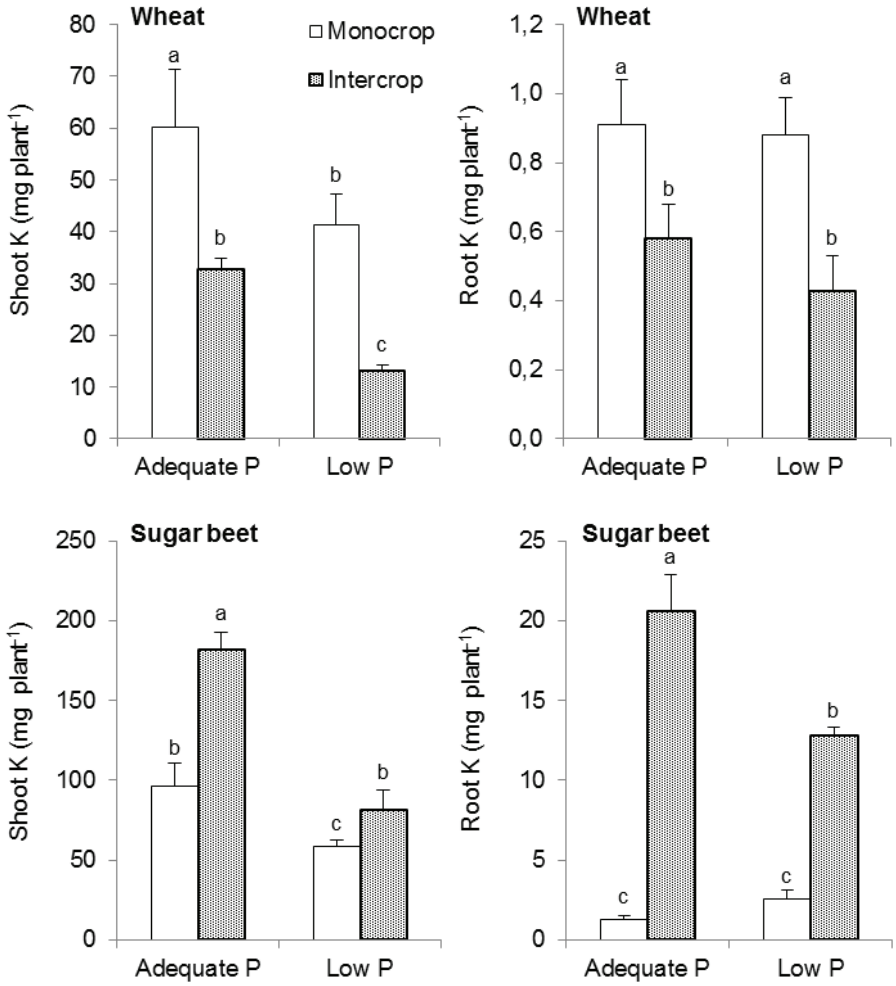

Figure 3: Potassium content in the shoot and roots of wheat and sugar beet plants in monocropping and intercropping under adequate or low P supply. Bars indicated by the same letter are not statistically different ($p < 0.05$).

P deficiency decreased shoot Fe content in both species under MC conditions (Fig. 4). A similar effect of low P was observed on root Fe only in MC wheat, while in MC sugar beet root Fe contents were significantly higher in low P plants. Intercropping influenced Fe content differently depending on P nutrition and plant species. In wheat, IC plants had lower Fe contents under adequate but not under low P supply. Contrastingly, P-deficient, but not P-sufficient sugar beet plants had significantly higher Fe contents in both shoot and root when intercropped with wheat. Significant reduction of root Fe was also found in sugar beet upon IC (Fig. 4).

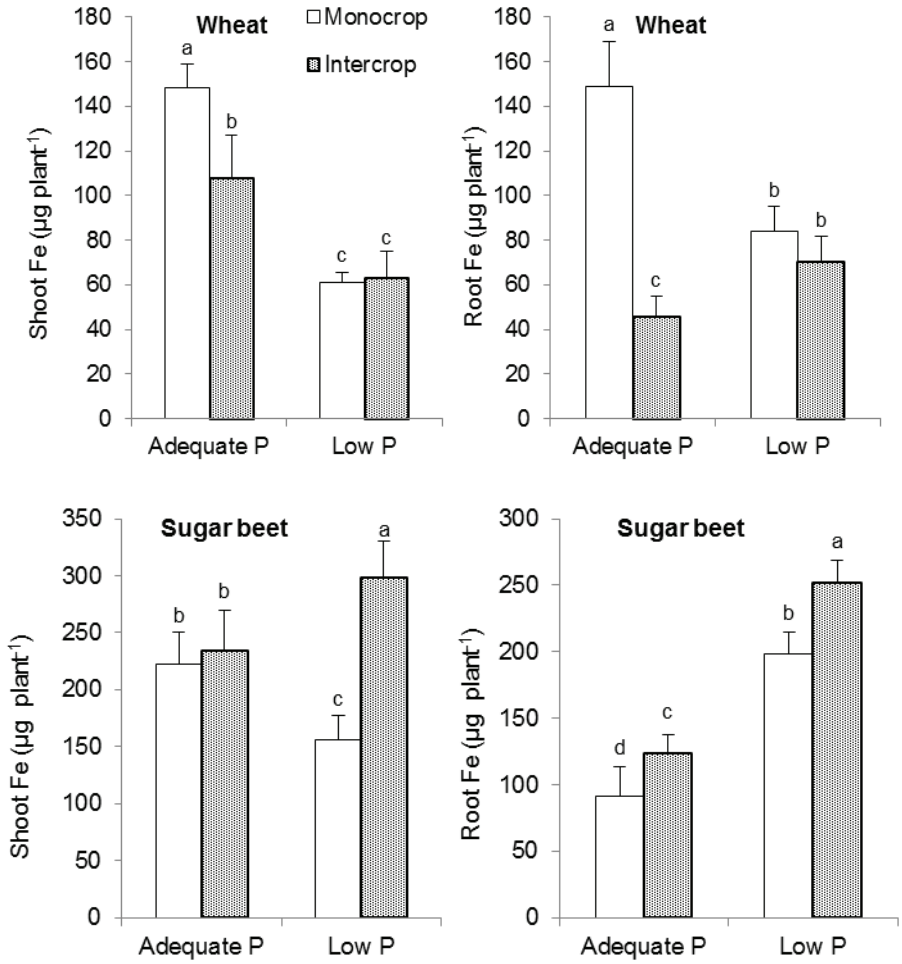

Figure 4: Iron content in the shoot and roots of wheat and sugar beet plants in monocropping and intercropping under adequate or low P supply. Bars indicated by the same letter are not statistically different ($p < 0.05$).

Rhizosphere pH was decreased under P deficiency conditions in sugar beet in both MC and IC systems, while in wheat this effect was observed only in IC pots. Intercropping increased the rhizosphere pH in sugar beet while decreased it in wheat (Table 4). Root activity of acid phosphatase was expectedly higher in low P plants. However, this effect was not significant in wheat under MC (Table 4). Intercropped plants had significantly higher ACPase activity under both P supply levels, the highest activity was observed in IC sugar beet under low P conditions (Table 4).

Table 4: Rhizosphere pH and root activity of acid phosphatase (APase, µmol PNP min^{-1} g^{-1} root DM) in wheat and sugar beet plants in monocropping (MC) or intercropping (IC) in perlite at adequate or low P supply for 10 weeks under controlled environmental conditions.

Plant species	Culture mode	**Rhizosphere pH**		**APase activity**	
		Adequate P	Low P	Adequate P	Low P
Wheat	MC	7.50±0.01 [a]	7.47±0.14 [a]	3.35±0.56 [c]	3.94±0.69 [c]
	IC	7.15±0.03 [b]	6.89±0.02 [c]	7.35±1.75 [b]	12.13±1.44 [a]
Sugar beet	MC	6.70±0.17 [b]	6.48±0.05 [c]	8.59±1.07 [c]	16.44±3.21 [b]
	IC	7.19±0.01 [a]	6.82±0.04 [b]	10.6±0.42 [c]	24.97±4.57 [a]

Data of each parameter within each plant species indicated by the same letter are not statistically different ($p < 0.05$).

Extraction of nutrients calculated as the total nutrient content of plants in each pot showed different capacity of species and its modification by IC. Under adequate P supply, P extraction ability was higher in sugar beet than wheat and P extraction from IC pots stood between wheat and sugar beet MC pots. At low P supply, in contrast, P extraction in MC wheat pots was higher than both MC sugar beet and IC pots (Table 5). K extraction was higher in sugar beet than wheat under MC conditions and P supply level did not change it. Plants in IC pots had significantly higher P extraction at adequate but not low P supply. Higher Fe extraction was observed in sugar beet than wheat at both P supply levels, while effect of IC depended on P supply level. Slightly higher Fe extraction in IC pots than both MC pots was observed at low but not adequate P supply level (Table 5).

Table 5: Extraction of P (mg pot^{-1}), K (mg pot^{-1}) and Fe (µg pot^{-1}) from the growth medium in wheat and sugar beet plants in monocropping (MC) or intercropping (IC) in perlite at adequate or low P supply for 10 weeks under controlled environmental conditions.

	P		**K**		**Fe**	
	Adequate P	Low P	Adequate P	Low P	Adequate P	Low P
Wheat MC	9.47±1.28 [c]	2.02±0.59 [a]	243±65 [b]	170±22 [b]	993±43 [b]	706±77 [b]
Sugar beet MC	26.89±4.22 [a]	0.92±0.22 [b]	393±59 [a]	246±10 [a]	1497±171 [a]	922±120 [a]
Intercropping	19.65±4.72 [b]	0.80±0.14 [b]	457±22 [a]	172±50 [b]	850±162 [b]	1091±181 [a]

Data of each column indicated by the same letter are not statistically different ($p < 0.05$).

Similar effects of IC on biomass and nutrients uptake were observed in plants grown for one week under hydroponic conditions. Fresh and dry biomass as well as P and K content were decreased in wheat by IC, while that of sugar beet increased (Table 6). Ten weeks after growth in hydroponic medium, biomass of wheat was less affected by culture mode while in sugar beet it was higher in IC compared to MC pots (Table 6). In general, effect of culture mode was less expressed in this experiment compared to the long term experiment (2.5 months) on perlite substrate.

Table 6: Shoot and root fresh (g plant^{-1}) and dry (mg plant^{-1}) mass and content of P (µg plant^{-1}) and K (mg plant^{-1}) in wheat and sugar beet plants grown hydroponically in monocropping (MC) or intercropping (IC) system for one or 10 weeks under controlled environmental conditions.

Plant species	Culture mode	**One week**			
		Fresh mass		**Dry mass**	
		Shoot	Root	Shoot	Root
Wheat	MC	0.50±0.08 [a]	0.20±0.02 [a]	59±7 [a]	14±1.2 [a]
	IC	0.32±0.05 [b]	0.23±0.06 [a]	40±4 [b]	12±3.5 [a]
Sugar beet	MC	10.2±2.5 [b]	1.38±0.42 [b]	413±159 [a]	83±8 [b]
	IC	16.0±2.6 [a]	2.52±0.14 [a]	538±193 [a]	126±28 [a]
		P		**K**	
		Shoot	Root	Shoot	Root
Wheat	MC	63±9.0 [a]	15±1.8 [a]	4.39±0.53 [a]	0.50±0.10 [a]
	IC	44±0.5 [b]	6±1.4 [b]	2.07±0.34 [b]	0.15±0.01 [b]
Sugar beet	MC	345±65 [b]	55±18 [a]	26±9 [b]	1.9±0.40 [a]
	IC	709±168 [a]	70±21 [a]	55±16 [a]	2.5±0.43 [a]
		10 weeks			
		Fresh mass		**Dry mass**	
		Shoot	Root	Shoot	Root
Wheat	MC	2.95±0.34 [a]	1.78±0.23 [a]	362±59 [a]	203±32 [a]
	IC	2.01±0.22 [a]	1.27±0.11 [a]	265±33 [a]	139±44 [a]
Sugar beet	MC	104±9.8 [b]	16.9±2.67 [b]	5552±840 [b]	1024±168 [b]
	IC	127±2.8 [a]	23.7±1.65 [a]	7019±719 [a]	2287±244 [a]

Significant difference between MC and IC plants were indicated by different letters (t-test, $p < 0.05$).

4 DISCUSSION

Research works on the dicotyledonous-cereals intercropping systems are focused on legume-cereals and to the best of our knowledge no study has been published on sugar beet-based intercropping and its comparison with legume-based systems. In this work intercropping system had distinctly different effect on dry matter production of wheat and sugar beet in parallel with differences in nutrient uptake and rhizosphere characteristics. In contrast to legume-cereals intercropping systems in which both species benefit from the interaction (Li et al., 2007; Zuo & Zhang, 2009; Dissanayaka et al., 2015), in sugar beet-wheat intercropping the growth of sugar beet was increased at the expense of wheat growth. Reduction of wheat growth in IC compared to MC pots was associated with changes in several parameters that may be either the cause or the consequence for the growth impairment in wheat. Although photosynthetic area in sugar beet was greater than wheat, the latter plants were held upright throughout the experiment using holders ensuring that sufficient light was captured in the growth chamber by wheat. Thus, it is likely that the observed intercropping effects are mainly caused by belowground interactions. To our best knowledge any allelopathic compound has not been isolated or identified in sugar beet.

4.1 P and K uptake under MC and IC conditions

Competition for nutrients is responsible, at least in part, for the observed differences in the nutrient contents

between sugar beet and wheat in IC pots. The data of P and K extraction from the medium in MC pots indicate that sugar beet had greater capacity for the uptake of P and K under both P supply levels (except for P at low P supply). It is thus likely that in IC pots, P and K were depleted faster by sugar beet in the time intervals of nutrient solution addition to the pots. This, accordingly, resulted in higher P and K contents in sugar beet in IC pots at the end of experiment. However, there are reasons against competition as the main cause for the observed difference between sugar beet and wheat in IC pots. P and K extraction rates were not higher in IC than MC pots implying that the nutrients limitation was not intensified upon IC. Under low P supply, in addition, total P extracted from the MC wheat pots was greater than MC sugar beet pots (Table 5) an indication of higher competition ability in wheat than sugar beet under P deficiency conditions. In agreement with this, and considering shoot biomass production in MC pots, wheat was more efficient under P deficiency conditions (25 % reduction) compared to sugar beet (48 % reduction). The enhancement of root length in response to P deficiency observed, but not in sugar beet, may account for this. It has been observed that under field conditions wheat has a large root system that compensates for low P influx when P availability is low, whereas sugar beet is able to achieve high P influx despite low P availability in soil (Bhadoria et al., 2002) mainly because of its ability for root release of citramalic acid and salicylic acid that solubilize and mobilize P in the soil (Khorassani et al., 2011). As related to other intercropping systems, it has been reported that in low P soils intercropping maize/faba bean and maize/white lupin resulted in an over yielding of both species through rhizosphere facilitation mechanisms (Li et al., 2007; Dissanayaka et al., 2015). An enhancement in P availability in the rhizosphere of both species in the legumes-cereals intercropping in low P soils has been attributed to an interspecific rhizosphere effect including acidification, exudation of carboxylates and greater phosphatase activity in the rhizosphere which mobilize insoluble soil P, increase the availability of insoluble inorganic P and decompose soil organic P into an inorganic form which can be used by both species (Li et al., 2007). However, in our work we could not evaluate the effect of chemical modification of the rhizosphere by root exudates because of using soluble P in the medium and the observed differences could be only attributed to differences in the spatial availability and/or root uptake efficiency. Higher root length in sugar beet (130-170 m plant^{-1}) than wheat (80-90 m plant^{-1}) in IC pots in contrast to the similar amounts in MC pots (40-70 m plant^{-1}) may be considered one mechanism for higher nutrients content in sugar beet in IC pots. However, the same differences between two species observed in IC pots in the short term hydroponic experiment indicated that the role of root length and higher spatial availability could not be the sole mechanism for the higher P and K uptake capacity of sugar beet in IC pots.

4.2 Iron uptake under MC and IC conditions

Fe uptake capacity as evaluated by total Fe extracted from MC pots was higher in sugar beet than in wheat. This further implies higher competition ability of sugar beet for Fe acquisition from the medium. Greater Fe uptake in legume-gramineae intercropping has been reported under field conditions (Zuo & Zhang, 2009). Different response mechanisms to Fe-deficiency stress between graminaceous and dicotyledonous species, i.e. strategy I (reduction strategy) in dicots and II (chelation strategy) in gramineae, has been considered the reason for greater Fe uptake in intercropping dicot/graminae systems in a wide range of soil pH values (Zuo & Zhang, 2009; Li et al., 2014). Because no Fe deficiency was imposed to the plants in this experiment, we could not evaluate the effect of these mechanisms for Fe uptake. Shoot and root Fe content in wheat decreased under IC conditions under adequate, but not under low P supply. This result suggests a positive effect of P deficiency on the Fe acquisition capacity in wheat alleviating their reduction of Fe uptake caused by IC in this species. The decrease of rhizosphere pH, an important mechanisms for facilitation of Fe mobilization and uptake (Neumann & Römheld, 2012), that was observed more at low P (pH 6.89) compared to adequate P (pH 7.15) conditions in wheat grown in IC pots could be a probable reason for the different effects of IC under low and adequate P conditions.

4.3 Biochemical parameters under MC and IC conditions

Leaf pigments decreased in IC wheat while remained unchanged in sugar beet. Reduction of Fe content (or concentration, data not shown) could explain partly reduction of Chl in IC compared to MC wheat plants under adequate, but not under low P supply. Net assimilation rate was consistently lower in IC wheat while increased in IC sugar beet compared with their corresponding MC plants. Although effects of IC on P, K and Fe on the corresponding changes in the photosynthesis rate could not be ruled out, other interspecific interactions were also likely important players in this regard. Interestingly, transpiration rate was lower in both species under IC conditions suggesting the influence of IC on stomatal behavior. Greater leaf area and transpiration surface in IC pots because of the presence of sugar beet may be the reason for faster water depletion in IC compared to MC wheat pots. Nonetheless, more closed stomata and a similar decrease in transpiration rate of sugar beet in IC pots suggests that water status of the substrate was only partly involved in reduction of transpiration rate in IC

plants. Reduction of transpiration rate concomitant with higher photosynthesis rate resulted in a significantly higher WUE in IC sugar beet that may be of high importance under field conditions during a long growth season. Effect of intercropping on plants photosynthesis rate has not yet been reported. However, there are reports on both higher (Yang et al., 2011) and lower (Gao et al., 2009) WUE in intercropping systems compared to monocropping under field conditions. This implies a complex interaction between rooting depth, water extraction capacity and transpiration surface area in intercropping systems.

Unexpectedly, IC-mediated changes in the soluble sugars concentrations were not correlated with the photosynthesis rate and they were consistently higher in IC conditions in both species. Accumulation of soluble sugars in IC wheat though reduction of photosynthesis rate may be a sign for sink limitation in this species. In addition, higher soluble carbohydrates in the roots may be the reason for higher root length of IC plants. Higher soluble sugars allocation to the roots has been observed under P limitation and P availability is a key regulator of many aspects of root growth and development, including root hair length and density, elongation, secondary development, branching and adventitious rooting (Niu et al., 2013). The preferential allocation of C to the root system, and the resulting increased root/shoot biomass ratio, appears to be a direct consequence of altered shoot metabolism and is mediated by increased translocation of sucrose to the root (Hammond & White, 2008; Chiou & Lin, 2011). In addition, the sucrose delivered to the root acts as a systemic signal (indicating low shoot P status) that can initiate changes in gene expression to alter root biochemistry and remodel root morphology (Niu et al., 2013; Lin et al., 2014). Increased root sucrose concentrations appear to upregulate genes encoding phosphatases and other metabolic enzymes in combination with the PHR1 transcriptional cascade, whilst its effects on lateral rooting occur through modulation of auxin transport (Hammond & White, 2008; Rouached et al., 2010). This mechanism could be responsible for higher carbohydrates associated with greater root length in –P compared with +P wheat (but not sugar beet) irrespective the cultivation mode. However, higher carbohydrate allocation to the roots and greater root length under IC conditions is unlikely to have occurred through triggering P deficiency in both species because P extraction rate was not enhanced upon IC conditions. It is likely that interspecific interactions under IC conditions are mediated by the same signaling pathway of P deficiency that in turn triggered similar responses in both species.

Reduction of amino acids and soluble proteins in IC wheat compared to MC indicated lower N uptake and/or assimilation under these conditions. Nitrogen was not analyzed in this work, but the same mechanisms for P and K as the important anion and cation was also expected for NO_3^- and NH_4^+ in the medium. Correlation between N nutrition and plant growth response to IC may indicate an important role of N in determining plants growth under IC but it does not indicate necessarily a competition between sugar beet and wheat in IC conditions and the contribution of an interspecific interaction like for other nutrients could not be ruled out.

4.4 Rhizosphere properties under MC and IC conditions

Decrease of rhizosphere pH in IC wheat and its increase in IC sugar beet compared with their corresponded MC pots was expected because of a known difference between monocot and dicot species in the cation/anion uptake ratio and frequently reported difference between these plants in the ability for modification of rhizosphere pH. Nutrient uptake is closely coupled to uptake or release of protons and therefore frequently associated with root-induced changes in rhizosphere pH. Due to differences in plant requirements and also in the availability of nutrients, uptake of cations and anions is often not balanced. Excess uptake of cations is balanced by a net release of protons and consequently leads to rhizosphere acidification (Neumann & Römheld, 2012). It has been observed that faba bean acidified its rhizosphere intensivelly, in contrast, maize alkalized its rhizosphere (Li et al., 2007). Effect of modifications in the rhizosphere pH on Fe and P uptake capacity in IC conditions was discussed above.

Root activity of secreted acid phosphatase was higher in IC plants of both species irrespective the P nutritional status. A well-documented component of the plant Pi stress response is the up-regulation of both intracellular and secreted acid phosphatases (APases; E.C. 3.1.3.2) that catalyze the hydrolysis of Pi from various phosphate monoesters and anhydrides in the acidic pH range (Rouached et al., 2010; Chiou & Lin, 2011). It has been shown that a decrease of local, external Pi availability is sufficient to induce *AtPAP10* transcription, one of the secreted ACPase in roots in the presence of sucrose as a systemic signal from shoots and only the magnitude of the induction is affected by the Pi status of the whole plant (Zhang et al., 2014). Although we did not determine Pi in the substrate in this work, the P extraction rate during whole growth period as an indication of P depletion in the pots was not higher in IC compared to MC pots, excepting for wheat under adequate P. However, it is likely that, a temporary P deficiency has been induced in IC pots in the time intervals between application of nutrient solution due to a greater uptake capacity of sugar beet. Nevertheless, regarding an immediate effect of Pi in down regulation

of ACPase (Rouached et al., 2010; Chiou & Lin, 2011) and the absence of a significant ACPase induction in the P-deficient wheat plants in MC pots, it seems unlikely that IC conditions induced P deficiency in our experiment. Molecular analyses of transcripts and proteins have hinted at complex control of plant APase gene expression. It has been demonstrated that Pi deprivation induces temporal and tissue-specific expression of –Pi-inducible APase isozymes and the concomitant downregulation of other APases (Misson et al., 2005). The purple APases (PAPs) as the largest class of nonspecific plant APases have a complex structure variation and expression regulation, so that gene transcription of two PAPs (*AtPAP11* and *AtPAP12*) was induced and increased, respectively, whereas that of the remaining five AtPAPs was not affected by phosphate deprivation (Li et al., 2002). It is plausible that IC conditions induced some of PAPs that are not responsive to P nutritional status or Pi in the medium and is triggered by other factors that in turn were modified by intercropping and particular yet unknown plant-plant interactions. Greater ACPase activity in both species may have important consequences on low P soils under field conditions for plants grown as IC.

5 CONCLUSION

Reduction of P, K and Fe content, leaf pigments, photosynthesis rate, amino acids and protein contents was observed in IC wheat while these parameters were improved or remained stable in IC sugar beet. Indeed under IC conditions facilitative interaction for sugar beet was occurred at the expense of wheat and an increase in the ability for more efficient use of resources for both species was not observed in this work. The existence of three characteristic responses of plant to P deficiency in both species even at adequate P supply (higher root length, accumulation of carbohydrates and upregulation of secretory ACPase activity) implied likely that a signaling pathway similar with the response induced by Pi starvation, is triggered by intercropping in both species. Our results present an example of direct interspecific interaction, but unfortunately, we fail to fully explore the nature of it and further investigations are needed for unraveling the involving mechanisms.

6 REFERENCES

Bagayoko, M., Alvey, S., Neumann, G., Buerkert, A. (2000). Root-induced increases in soil pH and nutrient availability to field-grown cereals and legumes on acid sandy soils of Sudano-Sahelian West Africa. *Plant and Soil, 225*, 117–127. doi:10.1023/A:1026570406777

Bhadoria, P. B. S., Steingrobe, B., Claassen, N., Leibersbach, H. (2002). Phosphorus efficiency of wheat and sugar beet seedlings grown in soils with mainly calcium, or iron and aluminium phosphate. *Plant and Soil, 264*, 41–52. doi:10.1023/A:1021567331637

Brooker, R. W., Maestre, F. T., Callaway, R. M., Lortie, C. T., Cavieres, L. A., Kunstler, G., Liancourt, P., Tielbbörger, K., Travis, J. M. J., Anthelme, F., Armas, C., Coll, L., Corcket, E., Delzon, S., Forey, E., Kikvidze, E., Olofsson, J., Pugnaire, F., Quiroz, C. L., Saccone, P., Schiffers, K., Seifan, M., Touzard, B., Michalet, B. (2008). Facilitation in plant communities: the past, the present, and the future. *Journal of Ecology, 96*, 18–34.

Brooker, R. W., Bennett, A. E., Cong, W. F., Daniell, T. J., George, T. S., Hallett, P. D., Hawes, C., Iannetta, P. P. M., Jones, H. G., Karley, A. J., Li, L., Mc Kenzie, B. M., Pakeman, R. J., Paterson, E., Schöb, C., Shen, J., Squire, G., Watson, C. A., Zhang, C., Zhang, F., Zhang, J., White, P. J. (2015). Improving intercropping: a synthesis of research in agronomy, plant physiology and ecology. *New Phytologists, 206*, 107–117. doi:10.1111/nph.13132

Chiou, T. J., Lin, S. I. (2011). Signaling network in sensing phosphate availability in plants. *Annual Review of Plant Biology, 62*, 185–206. doi:10.1146/annurev-arplant-042110-103849

Dissanayaka, D. M. S. B., Maruyama, H., Masuda, G., Wasaki, J. (2015). Interspecific facilitation of P acquisition in intercropping of maize with white lupin in two contrasting soils as influenced by different rates and forms of P supply. *Plant and Soil, 390*, 223–236. doi:10.1007/s11104-015-2392-x

Gao, Y., Duan, A., Sun, J., Li, F., Liu, Z., Liu, H., Liu, Z. (2009). Crop coefficient and water-use efficiency of winter wheat/spring maize strip intercropping. *Field Crops Research, 111*, 65–73. doi:10.1016/j.fcr.2008.10.007

Hammond, J. P., White, P. J. (2008). Sucrose transport in the phloem: integrating root responses to phosphorus starvation. *Journal of Experimental Botany, 59*, 93–109. doi:10.1093/jxb/erm221

Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J., Skrumsager, I., White, P. (2012). Function of macronutrients. In Marschner, P. (Ed.), *Marschner's Mineral Nutrition of Higher Plants* (pp. 135–472). London, UK: Academic Press. doi:10.1016/B978-0-12-384905-2.00006-6

Jaiswal, P. C. (2003). *Soil, Plant and Water Analysis*. Ludhiana, India: Kalyani Publishers.

Khorassani, R., Hettwer, U., Ratzinger, A., Steingrobe, B., Karlovsky, P., Claassen, N. (2011). Citramalic acid and salicylic acid in sugar beet root exudates solubilize soil phosphorus. *BMC Plant Biol*ogy, 11, 1. doi:10.1186/1471-2229-11-121

Li, L., Li, S. M., Sun, J. H., Zhou, L. L., Bao, X. G., Zhang, H. G., Zhang, F. S. (2007). Diversity enhances agricultural productivity via rhizosphere phosphorus facilitation on phosphorus-deficient soils. *Proceedings of the National Academy of Sciences (USA), 104*, 11192–11196. doi:10.1073/pnas.0704591104

Li, L., Tilman, D., Lambers, H., Zhang, F. S. (2014). Plant diversity and overyielding: insights from below-ground facilitation of intercropping in agriculture. *New Phytologists, 203*, 63–69. doi:10.1111/nph.12778

Li, D., Zhu, H., Liu, K., Liu, X., Leggewie, G., Udvardi, M., Wang, D. (2002). Purple acid phosphatases of Arabidopsis thaliana comparative analysis and differential regulation by phosphate deprivation. *Journal of Biological Chemistry, 277*, 27772–27781. doi:10.1074/jbc.M204183200

Lichtenthaler, H. K., Wellburn, A. R. (1985). Determination of total carotenoids and chlorophylls a and b of leaf in different solvents. *Biochemical Society Transactions, 11,* 591–592. doi:10.1042/bst0110591

Lin, W. Y, Huang, T. K., Leong, S. J., Chiou, T. J. (2014). Long-distance call from phosphate: systemic regulation of phosphate starvation responses. *Journal of Experimental Botany, 65*, 1817–1827. doi:10.1093/jxb/ert431

Misson, J., Raghothama, K. G., Jain, A., Jouhet, J., Block, M. A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N., Doumas, P., Nacry, P., Herrerra-Estrella, L., Nussaume, L., Thibaud, M. C. (2005). A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. *Proceedings of the National Academy of Sciences (USA), 102*, 11934–11939. doi:10.1073/pnas.0505266102

Neumann, G., Römheld, V. (2012). Rhizosphere chemistry in relation to plant nutrition. In: Marschner, P. (Ed.), *Marschner's Mineral Nutrition of Higher Plants* (pp. 347–368). London, UK: Academic Press. doi:10.1016/B978-0-12-384905-2.00014-5

Niu, Y. F., Chai, R. S., Jin, G. L., Wang, H., Tang, C. X., Zhang, Y. S. (2013). Responses of root architecture development to low phosphorus availability: a review. *Annals of Botany, 112*, 391–40. doi:10.1093/aob/mcs285

Rouached, H., Arpat, A. B., Poirier, Y. (2010). Regulation of phosphate starvation responses in plants: signaling players and cross-talks. *Molecular Plant, 3*, 288–299. doi:10.1093/mp/ssp120

Wang, Z., Shen, J., Ludewig, U., Neumann, G. (2015). A re-assessment of sucrose signaling involved in cluster-root formation and function in phosphate-deficient white lupin (*Lupinus albus*). *Physiologia Plantarum, 154*, 407–419. doi:10.1111/ppl.12311

Wu, K., Fullen, M. A., An, T., Fan, Z., Zhou, F., Xue, G., Wu, B. (2012). Above-and below-ground interspecific interaction in intercropped maize and potato: A field study using the 'target' technique. *Field Crops Research, 139*, 63–70. doi:10.1016/j.fcr.2012.10.002

Yang, C., Huang, G., Chai, Q., Luo, Z. (2011). Water use and yield of wheat/maize intercropping under alternate irrigation in the oasis field of northwest China. *Field Crops Research, 124*, 426–432. doi:10.1016/j.fcr.2011.07.013

Yemm, E. W., Cocking, E. C. (1955). The determination of amino acids with ninhydrin. *Analyst, 80*, 209–213. doi:10.1039/an9558000209

Yemm, E. W., Willis, A. J. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochemistry Journal, 57*, 508–514. doi:10.1042/bj0570508

Zhang, Y., Wang, X., Lu, S., Liu, D. (2014). A major root-associated acid phosphatase in *Arabidopsis*, AtPAP10, is regulated by both local and systemic signals under phosphate starvation. *Journal of Experimental Botany, 65*, 6577–6588. doi:10.1093/jxb/eru377

Zhang, F., Li, L. (2003). Using competitive and facilitative interactions in intercropping systems enhances crop productivity and nutrient-use efficiency. *Plant and Soil, 248*, 305–312. doi:10.1023/A:1022352229863

Zhang, F., Shen, J., Zhang, J., Zuo, Y., Li, L., Chen, X. (2010). Rhizosphere processes and management for improving nutrient use efficiency and crop productivity: implications for China. *Advances in*

Agronomy, 107, 1–32. doi:10.1016/S0065-2113(10)07001-X

Zhang, F. S., Li, L., Sun, J. H. (2001). Contribution of above- and below-ground interactions to intercropping. In Horst, W. J., Schenk, M. K., Bürkert, A., Claassen, N., Flessa, H., Frommer, W. B., Goldbach, H., Olfs, H. W., Römheld, V., Sattelmacher, B., Schmidhalter, U., Schubert, S., Wirén, N. v., Wittenmayer, L., (Eds.), *Plant Nutrition, Volume 92, Developments in Plant and Soil Sciences* (pp. 978–979). Netherlands: Springer.

Zuo, Y., Zhang, F. (2009). Iron and zinc biofortification strategies in dicot plants by intercropping with gramineous species: a review. *Agronomy for Sustainable Development, 29*, 63–71. doi:10.1051/agro:2008055

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CUPRAC assay-guided profiling of antioxidant compounds in methanol extract of *Lentinus squarrosulus* Mont. mycelium

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ABSTRACT

A cupric reducing antioxidant capacity (CUPRAC)-guided purification approach was performed on a methanol extract of *Lentinus squarrosulus* (LsqMeOH) by using reversed phase-high performance liquid chromatography. Using reversed phase-high performance liquid chromatography, three fractions were separated arbitrarily named FR1, FR2 and FR3. Results showed that FR2 exhibited the highest antioxidant activity in CUPRAC assay (A_{450}, 0.86) but not significantly different from LsqMeOH (A_{450}, 0.84). FR1 and FR3 showed much lower absorbance, with values (A_{450}, 0.21) and (A_{450}, 0.36) respectively at 1 mg ml^{-1}. The most active fraction (F3) was further subjected to LC-MS/MS to obtain its detailed chemical profile. Uridine, ganoderic acid derivative, and flavonoids were the first time being found in *L. squarrosulus* antioxidative fractions. The present results indicate that the fraction extracts of *L. squarrosulus* possess antioxidant properties and can be used as free radical inhibitors. Therefore, this research suggested the potentials of *L. squarrosulus* as a source of antioxidant extract to be used in food industries (functional food).

Key words: medicinal mushroom; active compounds; *Lentinus squarrosulus*

IZVLEČEK

CUPRAC TEST ZA LOČITEV ANTIOKSIDACIJSKIH SPOJIN V METANOLNEM IZVLČEKU MICELIJA GLIVE *Lentinus squarrosulus* Mont.

Izveden je bil CUPRAC ločitveni postopek metanolnega izvlečka micelija glive *Lentinus squarrosulus* (LsqMeOH) z uporabo vzratne visoko zmogljive tekočinske kromatografije, kar je dalo tri frakcije, poimenovane FR1, FR2 in FR3. Rezultati so pokazali, da je največjo antioksidacijsko aktivnost v CUPRAC preiskusu pokazala frakcija FR2 (A_{450}, 0.86), vendar ne značilno različno od LsqMeOH (A_{450}, 0.84). Frakciji FR1 in FR3 sta imeli veliko manjšo absorbanco z vrednostmi A_{450}, 0.21 in A_{450}, 0.36 pri 1 mg ml^{-1}. Antioksidacijsko najaktivnejša frakcija (F3) je bila v nadaljevanju analizirana z LC-MS/MS za pridobitev podrobnejše kemijske sestave. Uridin, derivat ganodermične kisline in flavonoidi so bili prvič najdeni v antioksidacijskih izvlečkih glive *L. squarrosulus*. Pridobljeni rezulati kažejo, da imajo frakcijski izvlečki glive *L. squarrosulus* antioksidacijske lastnosti in, da bi lahko bili uporabljeni kot lovilci prostih radikalov. Raziskava nakazuje, da je lahko gliva *L. squarrosulus* vir za antioksidacijske izvlečke za uporabo v prehrambeni industriji pri pripravi funkcionalne hrane.

Ključne besede: medicinske gobe; aktivne sestavine; *Lentinus squarrosulus*

1 INTRODUCTION

Oxidative stress is involved in the pathogenesis of lifestyle-related diseases, including atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, and malignancies (Yoshikawa and Naito, 2002). Reiter reported the increased oxidative damage observed during aging is probably due to the deficiency of antioxidants (Reiter, 1995). Even though enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase and catalase or nonenzymatic antioxidants such as vitamin E (α-tocopherol), vitamin

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C (ascorbic acid), thiol antioxidants (glutathione), carotenoids and flavonoid compounds are useful for protection against free-radical damage they are still insufficient to prevent damage entirely (Rahman, 2007). Therefore, external sources of antioxidants from the diet are needed to compensate this unbalance situation. In most countries, strict governmental rules regarding the use of synthetic antioxidants has been imposed due to safety concerns associated with the toxicity studies (Wanasundara and Shahidi, 2005). Thus, exploring for natural antioxidant is necessary.

From previous studies, it is apparent that mushrooms have great potential as a source of novel compounds (Deiana et al., 2004; Lee and Yun, 2006; Chen et al., 2012). For instance, ergothionine was isolated from *Pleurotus citrinopileatus* Singer*, P. ostreatus* (Jacq. ex Fr.) P.Kumm and *Pleurotus salmoneostramineus* L.Vass.*,* lovastatin from *Pleurotus ostreatus* and *Agaricus bisporus* (J.E.Lange) Imbach (Chen et al., 2012) and schizophyllan from *Schizophyllum commune* Fries (Bae et al., 2004). Some of these compounds have been developed as drugs as well as an ingredient in nutraceuticals. Mycelia of mushrooms serve as a convenient alternative to fruiting bodies due to the shorter incubation time, uniform and ease for higher production of biomass (Lee et al., 2004; Halpern, 2007). Moreover, the mycelial biomass can be produced in large quantities by using submerged fermentation technique.

Lentinus squarrosulus Mont. was amongst the commonly encountered Polyporales in Peninsular Malaysia (Bolhassan et al., 2012). The nutrient content of *L. squarrosulus* mycelia has been analyzed. The authors (Omar et al., 2011) reported that the mycelia of *L. squarrosulus* is high on protein (57.6 %) and low on total fat (0.5 %) content, are rich in magnesium (0.4 %), potassium (3.8 %) and vitamin B_3 (0.2 %). Furthermore, they also investigated on anti-ulcerogenic activity of *L. squarrosulus* mycelia extract and proven that this extract was able to prevent and heal ulcer that is associated with the attenuation of proinflammatory cytokines IL-1*β* and the inhibition of NF-*k*B in ulcerated rats.

The cupric reducing antioxidant capacity (CUPRAC) is based on utilizing the copper (II)-neocuproine reagent as the chromogenic oxidant. This assay is based on the redox reaction with antioxidants producing the cuprous–neocuproine chelate (Cu(I)–Nc) stable complex showing maximum light absorption wavelength at 450 nm (Apak et al., 2004). As yet, no research has been established on the purification and identification of antioxidant compounds from *L. squarrosulus* mycelium grown by liquid fermentation. Hence, the objective of this study is to carry out CUPRAC-assay fractionation of antioxidant compounds from methanol extract of *L. squarrosulus* (LsqMeOH) mycelium using reversed-phase high-performance liquid chromatography (RP-HPLC). Confirmatory analysis using liquid chromatography (LC) combined with tandem (MS/MS) mass spectrometry approach is used to identify antioxidant compounds.

2 MATERIALS AND METHODS

2.1 Sample preparation

Mycelial culture of *L. squarrosulus* (KUM 50016) was obtained from Mycology Laboratory, Institute of Biological Sciences, University of Malaya and maintained on Glucose-Yeast-Malt-Peptone (GYMP) agar consisting of $MgSO_4.7H_2O$ (0.40 g l^{-1}), KH_2PO_4, (0.40 g l^{-1}) K_2HPO_4, (0.40 g l^{-1}) glucose (4.00 g l^{-1}), peptone, (2.00 g l^{-1}) malt-extract (2.00 g l^{-1}), agar (7.20 g l^{-1}). Seven days old *L. squarrosulus* mycelium grown on GYMP agar media at 25 ± 2 ºC was used as inoculum. Ten 7 mm diameter plugs of *L. squarrosulus* mycelium were inoculated into the 500 ml Erlenmeyer flasks containing 100 ml GYMP liquid medium and then incubated for 14 days at 25 ± 2 ºC under static conditions. After 14 days, mycelial biomass obtained were harvested and freeze-dried before proceeded with extraction process. Dried mycelial biomass of *L. squarrosulus* was extracted by soaking in methanol for two days at room temperature. The filtrate was concentrated under reduced pressure using a rotary evaporator (BüchiRotavapor R-114, Flawil, Switzerland) under 45 ºC.

2.2 Fractionation of *Lentinus squarrosulus* methanol extract by RP-HPLC

Distilled water was pumped to remove air bubbles before use. All eluents except water were passed through a 0.2 µm cellulose nitrate membrane (Whatman No. 1, Maidstone, England) filtration apparatus immediately before use. Distilled water was passed through a 0.45 µm cellulose membrane filtration. The methanol extract of *L. squarrosulus* was applied onto an analytical reverse phase (250 mm × 4.6 mm, 5 µm) C18 ODS Hypersil column (Thermo Fisher Scientific, MA, USA) and analyzed by LC UV/VIS photodiode array detector SPD-M10 AVP (Shimadzu, Kyoto, Japan). The mobile phase consisted of 100 % distilled water (solvent A) and 100 % acetonitrile (Merck, Darmstadt,

Germany) (solvent B) at a flow rate of 3.5 ml.min^{-1}. The gradient system started at 0.01 min (40 % B), 3.00 min (60 % B), 4.00 min (80 % B), 7.00 min (10 % B) and stopped at 8.00 min. These various eluted fractions were collected and each fraction was assayed for CUPRAC.

2.3 Cupric ion reducing antioxidant capacity (CUPRAC)

The Cupric ion reducing antioxidant capacity (CUPRAC) was determined according to the method of Apak et al. (2004) based on the principle of utilizing copper (II)-neocuproine reagent as the chromogenic oxidizing agent. In this assay, 1 ml of extracts were mixed with 1 ml of Copper (II), 1 ml of ammonium acetate (NH_4Ac) buffer solution (1 mM, pH 7.0) and 1 ml of neocuproine (7.5 mM). The reaction mixture (total of 4 ml) then was mixed thoroughly and allowed to stand for 30 minutes before the absorbance was measured at 450 nm against a reagent blank. The assay was carried out in triplicate and the results expressed as means values ± standard deviations.

2.4 Profiling of chemical constituent by LC-MS/MS

The constituents of the most active fraction were analyzed by HPLC tandem mass spectrometry (LC-MS/MS). This procedure was done on an AB Sciex 3200QTrap® LC-MS/MS (Applied Biosystems, Toronto, Canada) triple quadropol mass spectrometer equipped with a Turbospray Ionization source. HPLC separation was carried out on a Phenomenex Aqua C18 column (5 µm, 50 mm × 2.0 mm i.d.; Phenomenex, Torrance, California, US). A mobile phase gradient of water with 0.1 % formic acid, 5 mm ammonium formate as eluent A and acetonitrile with 0.1 % formic acid and 5 mm ammonium formate as eluent B. Samples were diluted with 1.0 ml methanol and then filtered through 0.22 µm nylon filter. Injection volume was 20 µl. A gradient program was used as follows: 10 % A to 90 % B from 0.01 min to 8.00 min, held for 3.00 min and back to 10 % A in 0.10 min and re-equilibrated for 4.00 min, the flow rate was changing between 250 ml min^{-1} to 400 ml min^{-1}. The negative ion mode for MS/MS analysis was selected. Data analysis was carried out by using AB Sciex Analyst software and Advanced Chemistry Development, Inc (ACD/Labs, Toronto, Canada) software. Identification of constituents present in FR2 was performed by comparing the retention time and MS spectra of the peaks in the samples with those authentic reference samples or data reported in the literature.

2.5 Statistical analysis

For each antioxidant fraction (FR1, FR2 and FR3), assays were carried out in triplicates. The results were expressed as mean values and standard deviation (SD) or standard errors (SE). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test. Comparisons were carried out to detect significant difference ($p < 0.05$) between the mean values that had more than two groups. This treatment was carried out using SPSS v.16.0 software. The ANOVA results were classified using letters (different letters mean significant differences among results).

3 RESULTS AND DISCUSSION

3.1 CUPRAC analysis of RP-HPLC isolates

Separation by RP-HPLC resulted in three fractions collected at retention times of 2.8, 4.9 and 6.5 min. The profile obtained at the detection wavelength of 254 nm. The gradient system started at 0.01 min (40 % B), 3.00 min (60 % B), 4.00 min (80 % B), 7.00 min (10 % B) and stopped at 8.00 min. Figure 1 shows the absorbance values of fraction 1 (FR1), fraction 2 (FR2), fraction 3 (FR3) and the crude extract (LsqMeOH). CUPRAC activity revealed FR1and FR3 having lower absorbance than FR2 and LsqMeOH. From the result obtained, FR2 exhibited the highest absorbance of (A_{450}, 0.86) but not significantly different ($p < 0.05$) from LsqMeOH with an absorbance of (A_{450}, 0.84). FR1 and FR3 showed much lower absorbance, with values (A_{450}, 0.21) and (A_{450}, 0.36) respectively. The antioxidant capacities showed that the LsqMeOH (crude) was higher than its active fraction (FR1and FR3) and comparable high to FR2. FR2 was subjected to LC-MS/MS analyses to profile the compounds present. Table 1 shows the masses of peaks and ion fragment productions detected by the LC-MS/MS analysis of this extracts.

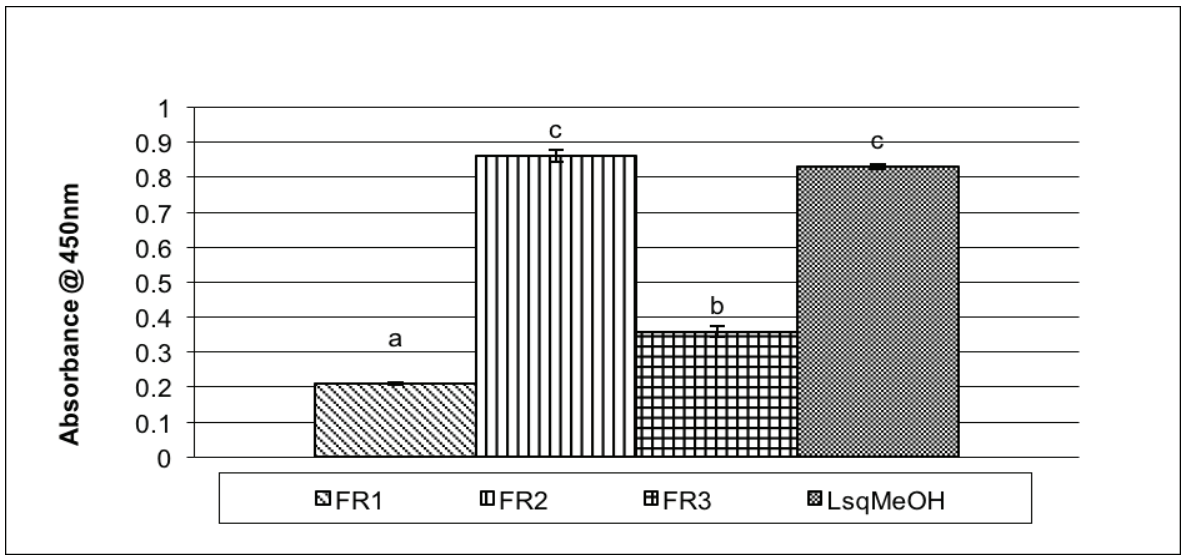

Figure 1: Reducing power of fraction RP-HPLC (FR1 to FR3) and LsqMeOH extracts on cupric ion. Concentration tested at 1mg ml^{-1}. Each value is expressed as mean ± standard deviation (n = 3). Average values with different letters are significant at $p < 0.05$

3.2 Profiling of selected potent antioxidative extract constituent by LC-MS/MS

From the LC-MS/MS data obtained (Table 1), Peak 1 was identified as uridine with mass fragments at *m/z* (mass to charge ratio) 225, 200 and 140. From the fragmentation scheme (Figure 2), uridine produces *m/z* ion at 200 with the loss of $-C_2H_3O$ (-43) due to hemolytic cleavage at the side chain. Further fragment ions unique to uridine were also observed at *m/z* 225 formed by a loss of a H_2O molecule and at *m/z* 140 due to further hydrogen rearrangements and hemolytic cleavage. Uridine is known to be one of the four basic components of ribonucleic acid (RNA) and it plays a role in the glycolysis pathway of galactose (Berg et al., 2002). Nucleosides including adenosine, cytidine, uridine, guanosine, inosine and thymidine had been isolated from *Cordyceps* (Li et al., 2006). A study by Yu et al. (2006) showed that uridine has antioxidant activities. They reported that uridine from *Cordyceps sinensis* (Berk.) Sacc. and *Cordyceps militaris* (L.) Fr. extracts have Trolox Equivalent Antioxidant Capacity value of 2.0 ± 0.9 µg/ml.

Table 1: Masses of peaks and ion fragments productions detected by LC-MS/MS of FR2

Peak no.	Rt (min)	MW	MS, M/Z [M-H]-	Fragment	Detected compound
1	0.80	244	243	225, 200, 140	Uridine
2	1.28	228	227	183,181, 155, 140, 127, 82	Phenolic acid
3	1.93	299	298	254, 236, 223, 193, 179, 167	Phenolic acid
4	2.25	242	241	197, 168, 141, 130, 82	Phenolic acid
5	3.21	276	275	231, 147, 127, 109	Unknown
6	3.69	498	497	451, 433	Ganoderic acid derivative
7	4.17	725	724	724, 678	Unknown
8	8.50	312	311	293, 225, 183	Flavonoids
9	8.82	326	325	225, 197, 183	Flavonoids
10	9.14	340	339	293, 197, 183	Flavonoids
11	9.62	874	873	647, 607	Unknown

Peaks 2, 3, and 4 had masses corresponding to phenolic acid group of compounds. This is due to the fact that all these peaks have a common loss of carbon dioxide molecule (-CO_2, - 44), which is characteristic of carboxylic-based compounds. Phenolic acid received considerable attention in the literature, specifically because of their biological and physiological importance. In a study of phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species, Barros et al. (2009) reported that various phenolic acids such as protocatechuic, *p*-hydroxybenzoic and p-coumaric acids, and two vanillic acid isomers were detected. *p*-Hydroxybenzoic acid exhibited in the majority of the samples tested and also being the most abundant compound in *Agaricus silvicola* (Vittad.) Peck (238.7 mg kg^{-1}). In the same study, *Ramaria botrytis* (Pers.) Ricken showed the highest phenolic acids concentrations (356.7 $mg.kg^{-1}$) due to the significant contribution of protocatechuic acid (342.7 mg kg^{-1}).

A type of ganoderic acid derivative was also determined from the FR2 extract. This finding contradicts Xu et al. (2010) who claimed that *Ganoderma* spps. are the only known producer for ganoderic acid. Peak number 6 (Table 1) had a mass corresponding to ganoderic acid $[M-H]^-$ion at *m/z* 497 produced a fragment ion with *m/z* 451 and 433. When applied to a collision energy spread of 35eV (+/-15), the ganoderic acid compound produces ion *m/z* 451 formed by sequential loss of a water molecule (-H_2O, -18), followed by carbon dioxide (-CO_2, -44) followed by re-arrangement process at the side chain, as shown in the schematic diagram below (Figure 3). A further loss of an H_2O molecule formed the next fragment ion at *m/z* 433. Ganoderic acid was proven to exhibit significant pharmacological activities by means of anti-histamine, anti-hypercholesterolemic, antibacterial and capacity to scavenge free radicals (Kohda et al., 1985; Komoda et al., 1989; Zhu et al., 1999; Wang and Liu, 2008). Other research has implicated that ganoderic acid DM isolated from *Ganoderma lucidum* (Curtis) P. Karst. inhibits cell proliferation and colony formation in MCF7, human breast cancer cells (Wu et al., 2012). Triterpene, a type of ganoderic acid, from *G. lucidum* was antiviral, specifically towards HIV (El-Mekkawy et al., 1998). Terpene and polysaccharide fractions were obtained from *G. lucidum* in a study on antioxidative activity by bioassay guided isolation (Zhu et al., 1999). Results showed that terpene fraction possesses the highest activity at 40 µg ml^{-1} against iron (II)-ascorbic acid induced lipid peroxidation, chemical isolation of the terpene fraction resulted in the detection of ganoderic acid A, B, C and D, lucidenic acid B and ganodermanontriol.

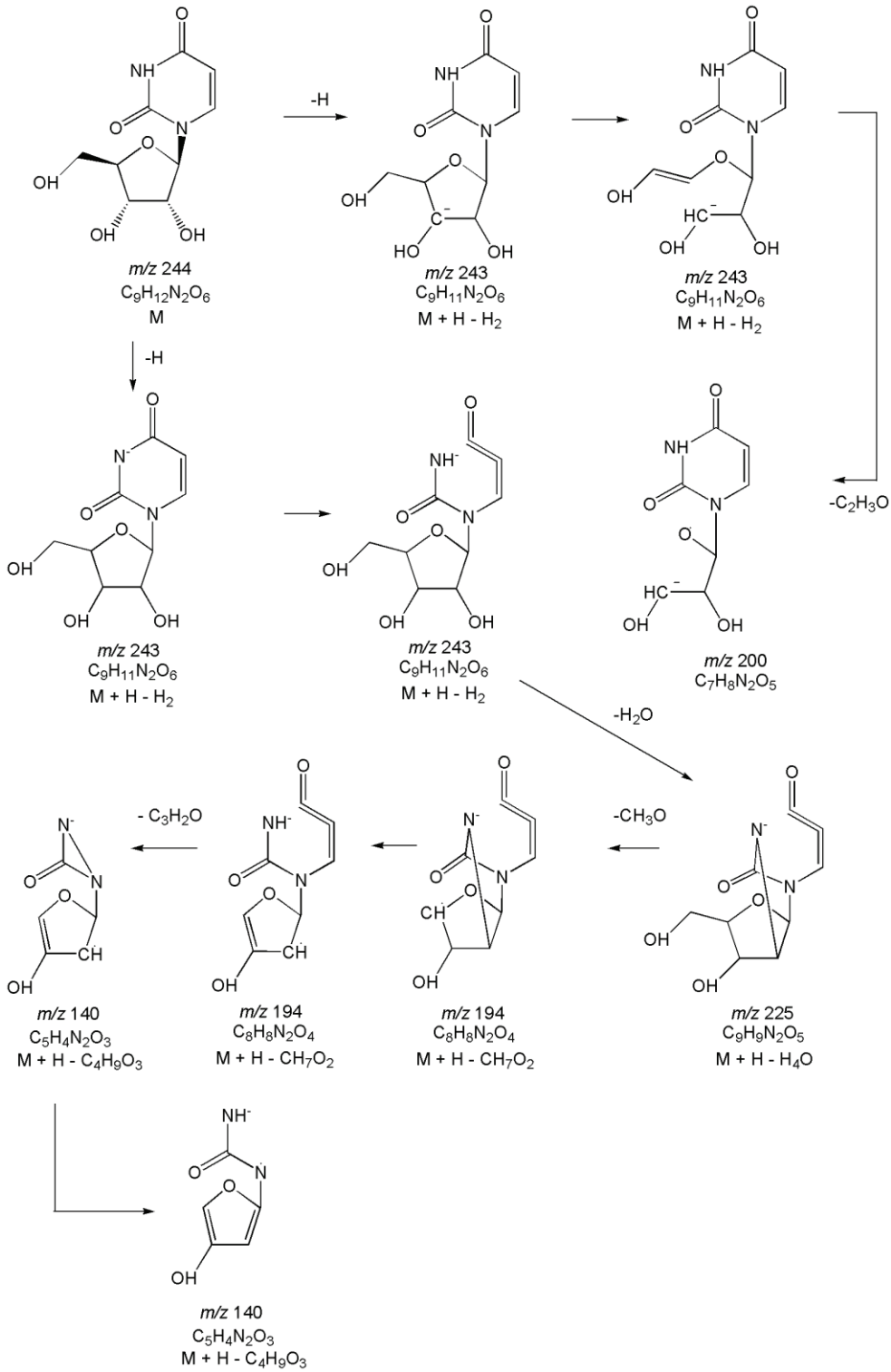

Figure 2: Uridine fragmentation pathway scheme

Peaks 8, 9 and 10 for FR2 (Table 1) have masses corresponding to flavonoid compounds. For peak 8 with *m/z* parent mass of 311 (M), the fragment ion observed was 293, which correspond to a loss of H_2O and other fragment ions were *m/z* 225 and 183. The fragment ion at *m/z* 225 was most likely the loss of M-H-C_2H_2O-CO_2, and further loss of C_2H_2O that corresponds to the ion *m/z* 183. This fragmentation pathway usually corresponds to compounds from the class flavonoids, probably for apigenin, as reported by Wu et al. (2004)

which showed such a fragmentation pathway. Peaks 9 and 10, which correspond to *m/z* 325 and 339 respectively, also shared similar fingerprint fragment ions (Table 1). Both the masses differ by a mass of 14 sequentially. This showed most likely to be carbon and hydrogen rearrangements of CH_2 to form both compounds that are derivatives from compound Peak 8 (Table 1). Flavonoids can be subdivided into six main subclasses namely flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins. Other flavonoid groups, which quantitatively are in comparison, minor components of the diet, are dihydroflavonols, flavan-3, 4-diols, coumarins, chalcones, dihydrochalcones and aurones (Crozier et al., 2006). Methanol extracts from cultivated fruiting bodies of endophytic fungus *Xylaria* sp. YX-28, are rich in phenolics and flavonoids with 54.51 mg GAE g^{-1} dry mass and 86.76 mg RE g^{-1} dry mass respectively (Liu et al., 2007). The total phenols and among them flavonoids were the major components found in the mushroom extract of *Lactarius deliciosus* (L.) Gray*, Sarcodon imbricatus* (L.) P. Karst., and *Tricholoma portentosum* (Fr.) Quèl (Barros et al., 2007). Flavonoids have been considered as important antioxidants for health and were reported to play an important role in the prevention of lipid peroxidation and cardiovascular disease (Peng and Kuo, 2003; Ding et al., 2006). Several unknown compounds were also detected in this study. Three unknown compounds for FR2 that were represented by peak numbers 5, 7 and 11 (Table 1). A match for the mass spectrum of unknown compounds could not be found from both the database and literature.

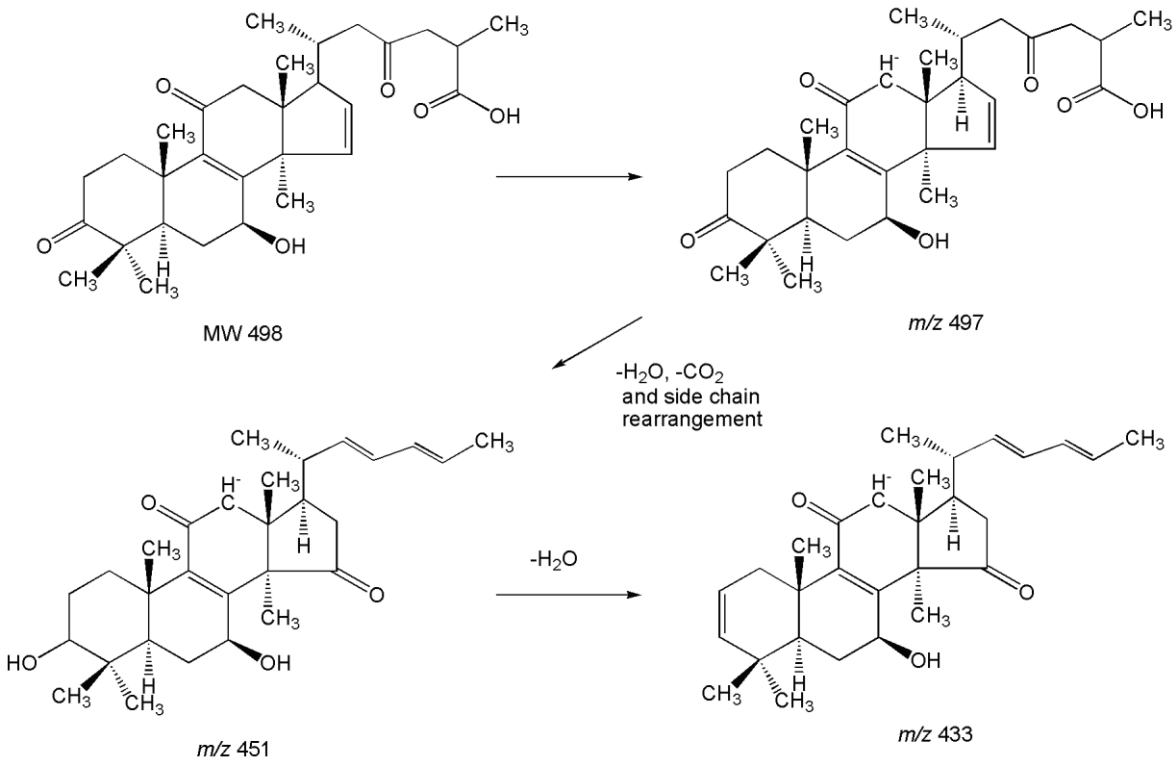

Figure 3: Ganoderic acid derivative fragmentation pathway scheme

4 CONCLUSIONS

Compounds contributing to antioxidant capacities from *Lentinus squarrosulus* Mont. are varied and between them in the complex system such as food sources and foodstuff there exist interactions and synergistic effect, therefore rather than a single compound it is preferred to use them as a whole. Chang and Buswell (2003) reported that the combination of several individual components contributed to the overall medicinal effect of the mushroom-based nutraceutical products. In this study, the potent antioxidant fraction from *L. squarrosulus* has been standardized by HPLC to contain phenolic-based compound, ganoderic acid derivative, uridine and flavonoids. The use of this extract as nutraceutical ingredient must conform to this standardized component to ensure quality and potency. Furthermore, *L. squarrosulus* mycelial antioxidant

extract is non toxic based on our previous study in experimental rats. It contains absorbable antioxidants that enter the circulating plasma and cause a significant acute increase in plasma antioxidant capacity (Omar et al., 2015). Therefore, this study validates the potential use of *L. squarrosulus* Mont. as an alternative source of natural antioxidant for nutraceuticals and pharmaceuticals industries.

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6 REFERENCES

Apak, R., Güclü, K., Özyürek, M. & Karademir, S. E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamin C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *Journal of Agriculture and Food Chemistry, 52,* 7970-7981. doi:10.1021/jf048741x

Bae, A. H., Lee, S.W., Ikeda, M., Sano, M., Shinkai, S., Sakurai, K. (2004). Rod-like architecture and helicity of the poly(C)/schizophyllan complex observed by AFM and SEM. *Carbohydrate Research*, 339, 251-258. doi:10.1016/j.carres.2003.09.032

Barros, L., Calhelha, R. C., Vaz, J. A., Ferreira, I. C. F. R., Baptista, P. & Estevinho, L. M. (2007). Antimicrobial activity and bioactive compounds of Portuguese wild edible mushrooms methanolic extracts. *European Food Research and Technology, 225,* 151-156. doi:10.1007/s00217-006-0394-x

Barros, L., Duenas, M., Ferreira, I. C. F. R., Baptista, P., Santos-Buelga, C. (2009). Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species. *Food and Chemical Toxicology, 47*, 1076-1079. doi:10.1016/j.fct.2009.01.039

Berg, J. M., Tymoczko, J. L. & Stryer L. (2002). *Biochemistry* (5th ed.). New York, NY: W H Freeman.

Bolhassan, M. H., Abdullah, N., Sabaratnam, V., Tsutomu, H., Abdullah, S., Rashid, N. M. N. & Musa, M. Y. (2012). Diversity and distribution of Polyporales in Peninsular Malaysia. *Sains Malaysiana, 41,* 155-161.

Chang, S. T. & Buswell, J. A. (2003). Medicinal mushrooms- A prominent source of nutriceuticals for the 21st century. *Current Topics in Nutraceutical Research, 1,* 257-280.

Chen, S. Y., Ho, K. J., Hsieh, Y. J., Wang, L. T., Mau, J. L. (2012). Contents of lovastatin, γ-aminobutyric acid and ergothioneine in mushroom fruiting bodies and mycelia. *LWT- Food Science Technology, 47*, 274-278. doi:10.1016/j.lwt.2012.01.019

Crozier, A., Jaganath I. B. & Clifford M. N. (2006). Phenols, polyphenols and Tannins: An overview. In A. Crozier, M. N. Clifford and H. Ashihara (Eds.), *Plant secondary metabolites: Occurrence, structure and role in the human diet* (pp. 2-3). Oxford, UK: Blackwell Publishing Ltd. doi:10.1002/9780470988558.ch1

Deiana, M., Rosa, A., Casu, V., Piga, R., Dessí, M. A., & Aruoma, O. I. (2004). L-ergothioneine modulates oxidative damage in the kidney and liver of rats *in vivo*: Studies upon the profile of polyunsaturated fatty acids. *Clinical Nutrition, 23,* 183-193. doi:10.1016/S0261-5614(03)00108-0

Ding, E. L, Hutfless, S. M., Ding, X., & Girotra, S. (2006). Chocolate and prevention of cardiovascular disease: A systematic review. *Nutrition & Metabolism, 3*. Retrieved from doi:10.1186/1743-7075-3-2 doi:10.1186/1743-7075-3-2

El-Mekkawy, S., Meselhy, M. R., Nakamura, N., Tezuka, Y., Hattori, M., Kakiuchi, N., Shimotohno, K., Kawahata, T., & Otake, T. (1998). Anti-HIV-1 and anti-HIV-1- protease substances from *Ganoderma lucidum. Phytochemistry, 49,* 1651-1657. doi:10.1016/S0031-9422(98)00254-4

Halpern, G. M. (2007). Mushroom cultivation. In G. M. Halpern (Ed.), *Healing mushrooms: Effective treatments for today's illnesses* (pp. 121-123). Garden City Park, New York, NY: Squares One Publishers.

Kohda, H., Tokumoto, W., Sakamoto, K., Fujii, M., Hirai, Y., Yamasaki, K., Komoda, Y., Nakamura, H., Ishihara, S., & Uchida, M. (1985). The biologically active constituents of *Ganoderma lucidum* (Fr.) Karst. Histamine release-inhibitory

triterpenes. *Chemical and Pharmaceutical Bulletin, 33,* 1367-1374. doi:10.1248/cpb.33.1367

Komoda, Y., Shimizu, M., Sonoda, Y., & Sato, Y. (1989). Ganoderic acid and its derivatives as cholesterol synthesis inhibitors. *Chemical and Pharmaceutical Bulletin, 37,* 531-533. doi:10.1248/cpb.37.531

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang, H. J., & Yun, J. W. (2004). Submerged culture conditions for the production of mycelia biomass and exopolysaccharides by the edible Basidiomycete *Grifola frondosa*. *Enzyme and Microbiological Technology, 35,* 369-376. doi:10.1016/j.enzmictec.2003.12.015

Lee I. K., & Yun B. S. (2006). Hispidin analogs from the mushroom *Inonotus xeranticus* and their free radical scavenging activity. *Bioorganic and Medical Chemistry Letters, 16,* 2376-2379. doi:10.1016/j.bmcl.2006.01.121

Li, S. P., Yang, F. Q., & Tsim, K. W. (2006). Quality control of *Cordyceps sinensis*, a valued traditional Chinese medicine. *Journal of Pharmaceutical and Biomedical analysis, 41,* 1571-1584. doi:10.1016/j.jpba.2006.01.046

Liu, X., Dong, M., Chen, X., Jiang, M., Lv, X., & Yan, G. (2007). Antioxidant activity and phenolics of an endophytic *Xylaria* sp. from *Ginkgo biloba*. *Food Chemistry, 105,* 548-554. doi:10.1016/j.foodchem.2007.04.008

Omar, N. A. M., Abdullah, N., Kuppusamy, U. R., Abdulla, M. A., & Sabaratnam, V. (2011). Nutritional composition, antioxidant capacity and antiulcer potential of *Lentinus squarrosulus* (Mont.) mycelia extract. *Evidenced-Based Complementary and Alternative Medicine.* doi:10.1155/2011/539356

Omar, N. A. M., Abdullah, S., Abdullah, N., Kuppusamy, U. R., Abdulla, M. A., & Sabaratnam, V. (2015). *Lentinus squarrosulus* (Mont.) mycelium enhanced antioxidant status in rat model. *Drug Design, Development and Therapy, 9,* 5957-5964.

Peng, I. W., & Kuo, S. M. (2003). Flavanoid structure affects the inhibition of lipid peroxidation in Caco-2 intestinal cells at physiological concentration. *The Journal of Nutrition, 133,* 2184-2187. doi:10.1093/jn/133.7.2184

Rahman, K. (2007). Studies on free radicals, antioxidants, and co-factors. *Clinical Interventions in Aging, 2,* 219-236.

Reiter, R. J. (1995). Oxidative process and antioxidative defense mechanism in aging brain. *FASEB Journal, 9,* 526-533. doi:10.1096/fasebj.9.7.7737461

Wanasundara, P. K. J. P. D., & Shahidi, F. (2005). Antioxidants: Science, technology, and applications. In F. Shahidi (Ed.), *Bailey's industrial oil and fat products* (6th ed.), (pp. 431-489). New York, NY: John Wiley & Sons, Inc. doi:10.1002/047167849X.bio002

Wang, X. L., & Liu, Z. T. (2008). *In vitro* bacteriostasis of the intracellular ganoderic acids from the mycelium of *Ganoderma lucidum*. *Food Science Technology, 10,* 184-186.

Wu, G. S., Lu, J. J., Guo, J. J., Li, Y. B., Tan, W., Dang,Y. Y., Zhong, Z. F., Xu, Z. T., Chen, X. P., & Wang, Y. T. (2012) Ganodermic acid DM, a natural triterpenoid, induces DNA damage, G1 cell cycle arrest and apoptosis in human breast cancer cells. *Fitoterapia, 83,* 408-414. doi:10.1016/j.fitote.2011.12.004

Wu, W., Yan, C., Li, L., Liu, Z., & Liu, S. (2004). Studies on the flavones using liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Chromatography A, 1047,* 213-220. doi:10.1016/S0021-9673(04)01135-5

Xu, J. W., Zhao, W., & Zhong, J. J. (2010). Biotechnological production and application of ganoderic acids. *Applied Microbiology and Biotechnology, 87,* 457-466. doi:10.1007/s00253-010-2576-5

Yoshikawa, T., & Naito, Y. (2002). What is oxidative stress? *Japan Medical Association Journal, 45,* 271-276.

Yu, H. M., Wang, B. S., Huang, S. C., & Duh, P. D. (2006). Comparison of protective effects between *Cordyceps militaris* and natural *Cordyceps sinensis* against oxidative damage. *Journal of Agriculture and Food Chemistry, 54,* 3132-3138. doi:10.1021/jf053111w

Zhu, M., Chang, Q., Wong, L. K., Chong, F. S., & Li, R. C. (1999) Triterpene antioxidants from *Ganoderma lucidum*. *Phytotherapy Research, 13,* 529-531. doi:10.1002/(SICI)1099-1573(199909)13:6<529::AID-PTR481>3.0.CO;2-X

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Problematika določanja vsebnosti prehranske vlaknine – vpliv frakcije mletja in načina mešanja vzorca

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IZVLEČEK

Prehranska vlaknina je pomembna sestavina zdrave prehrane, predstavlja neprebavljive ogljikove hidrate in lignin. V zadnjih desetletjih je zaradi dokazanih ugodnih vplivov na zdravje pridobila na pomenu za humano prehrano. Poleg klasičnih encimsko-gravimetričnih metod so bile v zadnjem času razvite nove metode za določanje skupne, netopne in topne prehranske vlaknine v živilih, vendar še niso popolnoma vpeljane v uporabo. Za namen oblikovanja podatkovnih baz o sestavi živil in za označevanje živil se še vedno uporabljata klasični metodi AOAC 985.29 in 991.43. Metodi sta encimsko-gravimetrični in zaradi tega občutljivi na encimsko kinetiko. Cilj študije je bil preveriti vpliv frakcije mletja vzorca in mešanja vzorca na določitev vsebnosti prehranske vlaknine z metodo AOAC 991.43. Rezultati so pokazali, da mletje pomembno vpliva na določitev prehranske vlaknine, še posebej, če določamo prehransko vlaknino v nepredelanih ali malo predelanih žitih. Kot najbolj primerno se je izkazalo mletje vzorca med 200 in 500 µm, mešanje pa je delovalo sinergistično z mletjem. Za natančno določitev vsebnosti prehranske vlaknine je potrebno pravilno pripraviti vzorec, saj je metoda AOAC 991.43 kljub svoji robustnosti občutljiva v fazi priprave vzorca.

Ključne besede: prehranska vlaknina, AOAC uradne metode, žita za zajtrk, mletje, frakcioniranje, mešanje

ABSTRACT

DETERMINATION OF DIETARY FIBRE – THE INFLUENCE OF MILLING FRACTION AND MIXING PROCESS

Dietary fibre is an important constituent of a healthy diet, composed of non-digestible carbohydrates and lignin. Over the last decades dietary fibre has gained importance for human nutrition, due to its beneficial effects on health. In addition to classical enzyme-gravimetric methods, new methods for the determination of total, insoluble and soluble dietary fibres in foods have recently been developed, but have not yet been fully implemented for use. For the purpose of creating food composition databases and for food labelling, the classical AOAC 985.29 and 991.43 methods are still widely used. The methods are enzyme-gravimetric and therefore sensitive to enzyme kinetics. The aim of the study was to investigate the effect of milling fraction and mixing of the sample on dietary fibre content determined with the AOAC method 991.43. The results showed that milling fraction significantly influences the content of dietary fibre, especially in unprocessed or slightly processed cereals, the mixing acts synergistically with milling. According to the results it is proposed to mill the sample between 200 and 500 µm. For accurate determination of dietary fibre content, it is necessary to prepare the sample correctly, since the AOAC 991.43 method is, despite its robustness, sensitive during the sample preparation step.

Key words: dietary fibre, AOAC official methods, breakfast cereals, milling, fractionation, mixing

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1 UVOD

Prehranska vlaknina je pomemben del uravnotežene prehrane in za človeka predstavlja pomembne varovalne snovi, ker ugodno vpliva na nekatere funkcije v prebavnem traktu in ima številne pozitivne učinke na preprečevanje pojava kroničnih nenalezljivih bolezni. Prehransko vlaknino predstavljajo ogljikovi hidrati ali ogljikovim hidratom podobne spojine, ki so odporne na prebavo in absorpcijo v tankem črevesu človeka, se pa popolno ali delno fermentirajo v debelem črevesu pod vplivom črevesne mikrobiote (Kendall in sod., 2011; Fuller in sod., 2016). Konvencionalna razdelitev prehranske vlaknine je glede na topnost v vodi na topno prehransko vlaknino (TPV), kamor prištevamo necelulozne polisaharide, oligosaharide, topne pektine, β-glukane in gume, ter netopno prehransko vlaknino (NPV), ki jo predstavljajo celuloza, hemiceluloza in lignin (Dai in Chau, 2017; Li in Komarek, 2017). Povečan vnos prehranske vlaknine pripomore k rednemu odvajanju ter zmanjšuje tveganje za pojav srčno-žilnih obolenj, debelosti, sladkorne bolezni tipa 2, bolezni prebavil in rakavih obolenj (Dahl in Stewart, 2015; Perry in Ying, 2016; Tarcea in sod., 2017; Fernstrand in sod., 2017). Priporočila za vnos prehranske vlaknine se nekoliko razlikujejo. Referenčne vrednosti za vnos hranil navajajo kot priporočen dnevni vnos vsaj 30 g vlaknine dnevno (Referenčne vrednosti…, 2016). Evropska agencija za varnost hrane (EFSA) pa za odrasle priporoča vnos vsaj 25 g prehranske vlaknine dnevno za normalno odvajanje, za zmanjšanje tveganja za razvoj kroničnih nenalezljivih bolezni pa je potreben večji vnos (EFSA, 2010; EFSA, 2017). Ocenjeni vnos pri prebivalcih razvitih držav je precej manjši od navedenih priporočil (Li in Komarek, 2017; Stephen in sod., 2017). Prehranska vlaknina je pretežno v živilih rastlinskega izvora, predvsem v žitih, stročnicah, sadju, zelenjavi, oreških ter izdelkih iz njih. V žitnih zrnih se nahaja v perikarpu in alevronski plasti. Na voljo so tudi živila, obogatena s prehransko vlaknino, in prehranska dopolnila (Golob in sod., 2012). Od leta 2009 je s strani komisije Codex Alimentarius prehranska vlaknina definirana kot polimerni ogljikovi hidrati z desetimi ali več monomernimi enotami in lignin, ki se ne hidrolizirajo z endogenimi encimi v človeškem tankem črevesu in spadajo v naslednje kategorije: i.) užitni polimerni ogljikovi hidrati in lignin, ki so naravno prisotni v živilih, ii.) polimerni ogljikovi hidrati, ki so bili pridobljeni iz surovega živila z uporabo fizikalnih, kemijskih ali encimskih metod in za katere je bilo dokazano, da imajo fiziološki učinek ali pripomorejo k zdravju in iii.) sintetični polimerni ogljikovi hidrati, za katere je dokazan fiziološki učinek ali ugoden vpliv na zdravje. Nacionalne institucije pa se odločijo, ali vključijo v definicijo tudi neprebavljive ogljikove hidrate s 3 do 9 monomernimi enotami (CAC, 2017; Dai in Chau, 2017). Konsenz, ki so ga sprejeli z novo definicijo prehranske vlaknine, je razrešil polemiko o vključevanju lignina in sintetično pridobljenih neprebavljivih ogljikovih hidratov v skupino prehranske vlaknine (Westenbrink in sod., 2013). Nova definicija je tudi prekinila tradicijo definiranja prehranske vlaknine preko analitskih postopkov za njeno določanje, saj je bila oblikovana na podlagi fizioloških učinkov (McCleary, 2013). V Uredbi (EU) št. 1169/2011 Evropskega parlamenta in Sveta o zagotavljanju informacij o živilih potrošnikom pa je skladno z Direktivo Komisije 2008/100/ES prehranska vlaknina opredeljena kot polimerni ogljikovi hidrati s tremi ali več monomernimi enotami, ki se ne prebavijo niti absorbirajo v tankem črevesu človeka, ter vključuje naravno prisotne užitne polimerne ogljikove hidrate in tudi tiste, ki so bili pridobljeni iz surovine za živilo s fizikalnimi, kemijskimi ali encimskimi sredstvi ter užitne sintetične polimere ogljikovih hidratov.

Za določanje vsebnosti prehranske vlaknine je razvitih in uradno predpisanih več metod, tako za določanje skupne prehranske vlaknine (SPV), TPV in NPV, kot tudi za določanje posameznih komponent vlaknine (Preglednica 1). Metode so se spreminjale in posodabljale skladno s spreminjanjem definicije za prehransko vlaknino. Metode na splošno delimo na encimsko-gravimetrične in encimsko-kemijske metode. Slednje vključujejo kvantitativno določanje vsebnosti vlaknine s pomočjo kolorimetrije, plinske kromatografije (GC) ali tekočinske kromatografije visoke ločljivosti (HPLC). Encimsko-gravimetrične metode so relativno enostavne, cenovno dostopne, dokaj hitre in dovolj robustne za rutinsko analizo, vendar ne zagotavljajo podrobnega profila za različne komponente vlaknine in ne vključujejo nizkomolekularnih komponent (oligosaharidov). Kolorimetrične analize večinoma zahtevajo referenčno metodo za zanesljivo interpretacijo rezultatov zaradi nespecifičnih barvnih reakcij reducirajočih sladkorjev. Kvantitativna določitev vsebnosti vlaknine z uporabo HPLC ali GC, ki sta vključeni v novejše metode določanja vlaknine (metodi AOAC 2009.01 in AOAC 2011.25), omogoča določanje vseh komponent prehranske vlaknine, tudi nizkomolekularnih polimernih ogljikovih hidratov (s 3 do 9 monomernimi enotami), in je idealna, kadar obravnavano živilo vsebuje neznano količino in vrsto vlaknine. Vendar pa so te metode dolgotrajne in zelo drage ter zahtevajo tudi ekonomsko veliko začetno investicijo in zelo usposobljeno osebje (Monro, 2015; Li in Komarek, 2017). Tradicionalni metodi za določanje prehranske vlaknine sta encimsko-gravimetrični metodi AOAC 985.29 in 991.43 za določanje SPV oz. ločeno TPV in NPV. Metodi AOAC 2009.01 in 2011.25 pa sta zasnovani tako, da zajemata vse komponente prehranske vlaknine, kot je navedeno v trenutno veljavni definiciji,

nista pa še široko uporabljeni za oblikovanje podatkovnih baz o sestavi živil (Zielinski in Rozema, 2013; Fuller in sod., 2016).

Preglednica 1: Uradne AOAC metode za določanje prehranske vlaknine in njenih komponent (DeVries, 2010; Li in Komarek, 2017)
Table 1: Official AOAC methods for determination of dietary fibre and individual specific components (DeVries, 2010; Li and Komarek, 2017)

Vrsta prehranske vlaknine/komponenta	AOAC metoda	Princip določanja
Skupna prehranska vlaknina	985.29	encimsko-gravimetrična metoda
Skupna, netopna in topna prehranska vlaknina	991.43	encimsko-gravimetrična metoda
Netopna prehranska vlaknina	991.42	encimsko-gravimetrična metoda
Topna prehranska vlaknina	993.19	encimsko-gravimetrična metoda
Skupna prehranska vlaknina (kot uronska kislina in Klason lignin)	994.13	encimsko-kemijska metoda
Skupna prehranska vlaknina, vključno z rezistentnim škrobom in nizkomolekularnimi komponentami PV	2009.01	encimsko-gravimetrična metoda/HPLC
Skupna, netopna in topna prehranska vlaknina, vključno z rezistentnim škrobom in nizkomolekularnimi komponentami PV	2011.25	encimsko-gravimetrična metoda/HPLC
Rezistentni škrob (retrogradiran škrob)	2002.02	encimska metoda
Fruktani (inulin, fruktooligosaharidi, hidroliziran inulin, polifruktoze)	999.03	encimsko-kolorimetrična metoda
Fruktani (inulin, fruktooligosaharidi, hidroliziran inulin, polifruktoze)	997.08	encimska metoda/ionsko-izmenjalna kromatografija
Trans-galaktooligosaharidi	2001.02	anionsko-izmenjalna kromatografija
β-glukani (1-3, 1-4-β-D-glukani iz žit)	995.16	encimska metoda
Rezistentni maltodekstrini	2001.03	encimsko-gravimetrična metoda/HPLC
Polidekstroza	2000.11	anionsko-izmenjalna kromatografija

Metoda AOAC 991.43 je encimsko-gravimetrična metoda pri kateri s pomočjo encimov, termostabilne α-amilaze, proteaze in amiloglukozidaze, vzorec in vitro razgradimo, ostanek, prehransko vlaknino, pa gravimetrično določimo. Postopek nam omogoča določitev netopne in topne frakcije prehranske vlaknine ter del rezistentnega škroba, ne moremo pa določiti nizkomolekularnih komponent prehranske vlaknine (AOAC, 1995). Za metodo je pomembna encimska razgradnja škroba in beljakovin v analiziranem vzorcu. Delovanje α-amilaze v žitih je omejeno z velikostjo delcev. V delcih, večjih od 500 µm, je škrob lahko ujet v žitnem zrnu in α-amilaza ne prodre do škrobnega zrna, encimska aktivnost α-amilaze na delcih zrn, manjših od 250 µm, pa se približuje konstanti (Al-Rabadi in sod., 2009). De la Hera in sod. (2013) so ugotovili, da je hitrost encimske razgradnje škroba v riževem zrnu obratno sorazmerna velikosti delcev, vendar se ne spreminja pri delcih manjših od 80 µm. Velikost in oblika škrobnih zrn je odločilen, vendar ne edini dejavnik hitrosti delovanja α-amilaze. Upoštevati je potrebno še kristalizacijo škroba in tip glikozidne vezi (Tester in sod., 2006). Na encimsko razgradnjo škroba pomembno vpliva tudi matriks živila, na katerega vpliva tudi tehnološka obdelava živila, kot je na primer kuhanje, ekstruzija ipd. ter interakcija škroba z lipidi in beljakovinami v živilu (Singh in sod., 2010). Lipidi v kombinaciji s škrobom tvorijo amorfno strukturo, ki ima enako hidrolitsko kinetiko kot kristaliziran škrob, ki se počasneje hidrolizira. Podobno učinkujejo tudi beljakovine, ki tvorijo gosto strukturno mrežo okrog škroba in preprečujejo dostop encimom (Zhang in sod., 2015).

Velikost delcev pri mletju običajno določamo s siti, kar povzroči nastanek mlevskih frakcij, ki se med seboj

razlikujejo po vsebnosti prehranske vlaknine. V grobih frakcijah je vsebnost prehranske vlaknine večja, saj pri mletju žitnih zrn v grobe frakcije prehajajo zunanji deli zrna, medtem ko fine mlevske frakcije zajemajo predvsem notranjost žitnega zrna, ki je bolj bogata s škrobom (Frølich in Nyman, 1988; Steadman in sod., 2001; Tosi in sod., 2001).

Namen raziskave je bil preveriti vpliv frakcije mletja vzorca (velikosti delcev), ki jo pridobimo pri presejanju vzorca, in mešanja na določitev vsebnosti netopne in topne prehranske vlaknine v izbranih vzorcih žit za zajtrk z metodo AOAC 991.43.

2 MATERIAL IN METODE

2.1 Vzorci žit za zajtrk

V raziskavo smo vključili 3 vzorce žit za zajtrk, ekstrudirane pšenične otrobe in valjane ovsene kosmiče, oba izdelka sta dostopna na slovenskem tržišču, ter referenčni vzorec suhih žit za zajtrk (ERM-BD518, vzorec 0578). Vzorca ekstrudiranih pšeničnih otrobov in valjanih ovsenih kosmičev sta bila zmrznjena s tekočim dušikom in zmleta v mlinčku. Izjema je bil referenčni vzorec suhih žit, ki je bil že predhodno zmlet. Mletje vzorcev je trajalo 15 s. Iz celote zmletih vzorcev je bil odvzet reprezentativen del vzorca, ki je predstavljal standardno mletje (izvorni vzorec). Preostanek vzorca je bil ločen s pomočjo 250 µm sita. Frakcija, ki je prešla sito, je bila frakcija 1 (del vzorca, mletega 15 s, ki je vseboval delce, manjše ali enake 250 µm), ostanek pa je predstavljal grobo mleto frakcijo oziroma frakcijo 2 (samo delci, večji od 250 µm, nekateri večji od 500 µm). Velikosti delcev smo izbrali glede na priporočila v postopku določanja prehranske vlaknine. Vzorci so bili do analize zaprti v PVC vrečkah in shranjeni na -20 ºC.

2.2 Metode

Vsebnost prehranske vlaknine v vzorcih žit za zajtrk smo določili v štirih ponovitvah z encimsko-gravimetrično metodo AOAC 991.43 (AOAC, 1995), ki smo jo v manjši meri modificirali za potrebe našega poskusa. Metoda AOAC 991.43 je encimsko gravimetrična metoda, pri kateri škrob in beljakovine v razmaščenem vzorcu razgradimo z encimi α-amilazo, proteazo in amiloglukozidazo. Ostanek korigiramo na vsebnost pepela in beljakovin ter gravimetrično določimo topno prehransko vlaknino (TPV) in netopno prehransko vlaknino (NPV). Skupno prehransko vlaknino (SPV) izračunamo kot vsoto TPV in NPV. Namesto v 400 ml erlenmajericah smo encimsko razgradnjo izvedli v 50 ml centrifugirkah. Glede na navodila za določanje prehranske vlaknine smo spreminjali način mešanja. Določanje prehranske vlaknine po metodi AOAC 991.43 predvideva encimsko hidrolizo v erlenmajericah, inkubiranih v stresalni kopeli, kjer valovanje pufra nenehno suspendira vzorec. Vzorce žit različnih mlevskih frakcij smo mešali na dva načina. Za simulacijo slabega mešanja (pokončno mešanje) smo pri inkubaciji vzorcev z α-amilazo vzorce premešali vsakih 10 min, v inkubaciji s proteazo in amiloglukozidazo, ki smo jo izvajali v stresalni kopeli, pa so bile centrifugirke postavljene pokončno, tako da valovanje vzorca ni doseglo dna. V simulaciji dobrega mešanja (ležeče mešanje) smo med inkubacijo z α-amilazo vzorce mešali vsakih 5 min, ob delovanju proteaze in amiloglukozidaze pa so bili v stresalno kopel položeni ležeče in vzdolžno z stresanjem, da je valovanje nenehno suspendiralo vzorec. Poleg vzorcev smo analizirali tudi slepi vzorec (vpliv dodanih reagentov in encimov).

2.3 Statistična obdelava rezultatov

Rezultate smo obdelali s pomočjo statističnega programa R. Rezultati določanja prehranske vlaknine v pšeničnih otrobih in referenčnem vzorcu so bili primerni za ANOVA analizo z definiranimi kontrasti med različnimi obravnavami, saj so bili parametrično razporejeni in so ustrezali kriteriju homogenih varianc. V primeru vzorca ovsenih kosmičev, kjer rezultati niso ustrezali standardni ANOVA analizi, ki predpostavlja homogenost varianc, smo izvedli prilagojeno ANOVA analizo, ki ne predpostavlja homogenih varianc. Nadalje smo analizirali tudi morebitne interakcije med vplivom mletja in mešanja. Interakcija med mletjem in mešanjem pomeni, da sprememba enega faktorja vpliva na drugi faktor, kar pomeni, da v analizi vpliva obeh faktorjev na odvisno spremenljivko, govorimo o sovplivu.

3 REZULTATI IN DISKUSIJA

V okviru študije smo želeli preveriti, ali frakcioniranje vzorca ter mešanje vplivata na določitev prehranske vlaknine po metodi AOAC 991.43. V preglednici 2 so predstavljeni rezultati vsebnosti NPV, TPV in SPV v treh vzorcih žit za zajtrk ter njihovih frakcijah mletja. Ko govorimo o vplivu frakcije mletja so v primerjavo vključeni izvorni vzorec in obe frakciji.

Preglednica 2: Vsebnost prehranske vlaknine (povprečna vrednost ± standardni odklon, n = 4) v vzorcih različnih frakcij mletja in z različnim načinom mešanja

Table 2: Dietary fibre content (mean value ± standard deviation, n = 4) in samples of different milling fractions and different mixing methods

Vzorec	Frakcija mletja	Mešanje	NPV (g 100 g^{-1})	TPV (g 100 g^{-1})	SPV (g 100 g^{-1})
Pšenični otrobi	izvorni vzorec	ležeče	$20,15 \pm 0,83^{b}$	/	$20,15 \pm 0,83^{b}$
	1		$18,25 \pm 0,51^{a}$	/	$18,25 \pm 0,51^{a}$
	2		$21,57 \pm 1,06^{b}$	/	$21,57 \pm 1,06^{b}$
	izvorni vzorec	pokončno	$26,40 \pm 0,30^{c}$	/	$26,40 \pm 0, 30^{c}$
	1		$18,16 \pm 0,53^{a}$	/	$18,16 \pm 0,53^{a}$
	2		$20,54 \pm 0,37^{b}$	/	$20,54 \pm 0,37^{b}$
Ovseni kosmiči	izvorni vzorec	ležeče	$5,34 \pm 0,22^{b}$	$3,33 \pm 0,13^{bc}$	$8,67 \pm 0,22^{b}$
	1		$1,77 \pm 0,31^{a}$	$2,54 \pm 0,42^{ab}$	$4,32 \pm 0,18^{a}$
	2		$4,86 \pm 0,70^{b}$	$4,27 \pm 0,18^{c}$	$8,06 \pm 2,65^{b}$
	izvorni vzorec	pokončno	$4,81 \pm 0,98^{b}$	$3,97 \pm 0,26^{c}$	$8,78 \pm 0,77^{b}$
	1		$1,99 \pm 0,22^{a}$	$2,20 \pm 0,68^{a}$	$3,64 \pm 1,16^{a}$
	2		$6,26 \pm 1,05^{b}$	$4,00 \pm 0,26^{b}$	$10,26 \pm 1,07^{b}$
Referenčni vzorec	izvorni vzorec	ležeče	$25,98 \pm 0,56^{ab}$	$3,70 \pm 0,19^{b}$	$29,68 \pm 0,41^{a}$
	1		$25,41 \pm 0,68^{a}$	$5,53 \pm 0,39^{c}$	$30,94 \pm 0,64^{a}$
	2		$26,78 \pm 0,12^{abc}$	$3,70 \pm 0,39^{b}$	$30,48 \pm 0,49^{a}$
	izvorni vzorec	pokončno	$25,6 \pm 0,74^{a}$	$3,67 \pm 0,08^{b}$	$29,27 \pm 0,75^{a}$
	1		$27,63 \pm 1,3^{bc}$	$2,70 \pm 0,44^{a}$	$30,34 \pm 1,35^{a}$
	2		$28,13 \pm 0,58^{c}$	$2,70 \pm 0,12^{a}$	$30,84 \pm 0,60^{a}$

povprečne vrednosti z isto črko v stolpcu, znotraj vzorca se ne razlikujejo ($p \leq 0,05$); /: pod mejo zanesljivosti
NPV: netopna prehranska vlaknina; TPV: topna prehranska vlaknina; SPV: skupna prehranska vlaknina
izvorni vzorec: standardno mletje; 1: ≤250 µm frakcija; 2: grobo mleta frakcija

3.1 Netopna prehranska vlaknina

Vsebnosti NPV v ekstrudiranih pšeničnih otrobih se med seboj razlikujejo, kot je razvidno iz preglednice 2. Na določeno vrednost NPV je pomembno vplivalo mešanje ($p < 0,001$), kar je najbolj opazno pri izvornem vzorcu, kjer je pri simulaciji dobrega mešanja (ležeče mešanje) določena vsebnost NPV $20,15 \pm 0,83$ g 100 g^{1}, pri slabem mešanju (pokončno mešanje) pa je določena vsebnost NPV 31 % večja in znaša $26,40 \pm 0,30$ g 100 g^{-1}. Na določitev prehranske vlaknine vpliva tudi frakcija mletja vzorca ($p < 0,001$) in kombinacija obeh dejavnikov skupaj ($p < 0,001$). Rezultati pri standardnem mletju in pri grobi frakciji mletja se glede na mešanje značilno razlikujejo ($p < 0,001$ in $p = 0,040$). Vrednosti se pomembno razlikujejo tudi če primerjamo samo frakcije mletja, brez mešanja ($p < 0,001$).

Vsebnost NPV v vzorcih ovsenih kosmičev, ki smo jih različno mešali, se statistično značilno razlikuje med ≤250 µm frakcijo mletja in ostalimi frakcijami mletja (Preglednica 2). Določena vsebnost prehranske vlaknine v frakciji 1 (≤250 µm) je $1,77 \pm 0,31$ g 100 g^{-1} pri dobrem mešanju in $1,99 \pm 0,22$ g 100 g^{-1} pri slabem mešanju, med seboj se ne razlikujeta značilno. Majhne vrednosti določene NPV so posledica izgube PV zaradi frakcioniranja vzorca. Za izvorni vzorec in frakcijo 2 pa določena vsebnost NPV variira med $4,81 \pm 0,98$ g 100 g^{1} in $6,26 \pm 1,05$ g 100 g^{-1}. Značilnih razlik glede na mešanje ni ($p = 0,110$), prav tako ni značilne interakcije med mešanjem in frakcijo mletja.

Rezultati vsebnosti NPV v referenčnem vzorcu suhih žit za zajtrk se razlikujejo glede na različno obravnavanje. Značilen vpliv imata mešanje ($p = 0,003$) in Frakcija mletja ($p = 0,001$). Med frakcijo mletja in mešanjem je tudi značilna interakcija ($p = 0,011$). Razlik med izvornim vzorcem in frakcijo 1 ni.

3.2 Topna prehranska vlaknina

Vsebnost TPV je bila pri ekstrudiranih pšeničnih otrobih pod pragom zanesljive določitve. Določena vsebnost TPV v valjanih ovsenih kosmičih se značilno razlikuje glede na frakcijo mletja ($p < 0,001$). Določene vrednosti prehranske vlaknine za frakcijo 1 dosežejo $2,54 \pm 0,42$ g 100 g^{-1} in $2,20 \pm 0,68$ g 100 g^{-1}. V izvornem vzorcu in frakciji 2 je bila določena vsebnost TPV med $3,33 \pm 0,13$ g 100 g^{-1} in $4,27 \pm 0,18$ g 100 g^{-1}. Značilna je interakcija med mešanjem in frakcijo mletja ($p = 0,034$). Statistično pomembna razlika v določenih vsebnostih TPV se pokaže pri standardno mletem vzorcu, glede na način mešanja. Mešanje ne povzroči pomembnih razlik pri drugih frakcijah mletja.

Vsebnost TPV, določena v referenčnih vzorcih suhih žit, se glede na različno obravnavo vzorca med seboj

značilno razlikuje (preglednica 2). Razlike se ne pojavijo pri analizi izvornega, standardno mletega vzorca, glede na mešanje. Na razlike vpliva mešanje ($p < 0,001$) in frakcija mletja ($p < 0,001$). Značilna je tudi interakcija med mešanjem in frakcijo mletja ($p < 0,001$).

3.3 Skupna prehranska vlaknina

Izračunana vsebnost SPV v valjanih ovsenih kosmičih se razlikuje glede na obravnavanje ($p < 0,001$). Vsebnost SPV v frakciji velikosti ≤250 µm (frakcija 1) je značilno manjša v primerjavi z vsebnostjo SPV v izvornem vzorcu in frakciji 2. Na razlike v vsebnostih SPV značilno vpliva frakcija mletja ($p < 0,001$), mešanje pa nima vpliva. Interakcije med mešanjem in mlevsko frakcijo ni.

Pri vsebnostih SPV v referenčnih vzorcih suhih žit se kaže značilen vpliv obravnave na rezultat ($p = 0,036$). Razlike v rezultatih so posledica mlevske frakcije ($p = 0,005$). Interakcije med mlevsko frakcijo in mešanjem ni. Samo mešanje vzorca med encimsko razgradnjo ne vpliva na določeno vsebnost SPV.

Povzamemo lahko, da na določanje prehranske vlaknine v različnih žitih za zajtrk vpliva frakcija mletja, to je velikost delcev v vzorcu. Ta pojav je bil opažen že v raziskavi, ki jo je izvedel Ehle (1984), kjer so se pokazale razlike v vsebnosti surove vlaknine, glede na velikost delcev vzorca. V vzorcih z večjimi delci je bila vsebnost surove vlaknine večja. V naši raziskavi so razlike v določeni vsebnosti prehranske vlaknine še posebej izrazite v primeru valjanih ovsenih kosmičev. Valjani ovseni kosmiči, ki so predstavljali naš vzorec, so blanširana in valjana zrna ovsa. Z mletjem in sejanjem skozi sita za pridobivanje mlevske frakcije, smo posnemali proces mletja. Pri referenčnem vzorcu lahko opazimo večjo vsebnost TPV, določene v frakciji 1, v kombinaciji s simulacijo dobrega mešanja. Pojav lahko pripišemo spremembi topnosti prehranske vlaknine (pretvorba dela NPV v TPV), ki je posledica delovanja fizične sile, ki mehansko poškoduje dolgoverižne komponente netopne prehranske vlaknine (Gualberto in sod., 1997). Različno vsebnost prehranske vlaknine v ajdovem zrnu ter v mlevskih izdelkih (moka, zdrob, otrobi…), glede na frakcijo mletja ter glede na izvorno surovino, so določili tudi Steadman in sod. (2001). Otrobi so vsebovali največ prehranske vlaknine, medtem ko jo je bela moka vsebovala najmanj. Tosi in sod. (2001) so proučevali vpliv frakcij mletja vzorca na vsebnost prehranske vlaknine v amarantu in ugotovili, da večji kot so bili delci, večja je bila določena vsebnost prehranske vlaknine (Tosi in sod., 2001). Škrabanja in sod. (2004) pa so proučevali hranilno vrednost mlevskih frakcij ajde in v primeru prehranske vlaknine ugotovili, da je delež topne prehranske vlaknine v skupni prehranski vlaknini večji v mokah, kot v zdrobu in otrobih. Razlike v določeni vsebnosti prehranske vlaknine se kažejo tudi med sortami istega žita ter med moko in ekstrudati (Djurle in sod., 2016). Navedene študije so uporabljale primerljive metode, saj imajo vse vključeno encimsko razgradnjo vzorcev. Poleg vpliva frakcij je potrebno upoštevati tudi hitrost hidrolize škroba, saj se večji delci škroba hidrolizirajo počasneje, zato je možno, da se v času inkubacije z encimom α-amilaza ne razgradijo in ga lahko določimo kot prehransko vlaknino. Upoštevati pa je potrebno tudi, da zaradi večjega razmerja volumen/površina encimi ne morejo delovati na škrobna zrna, ali pa delujejo le delno (Mahasukhonthachat in sod., 2010). Večji vpliv ima verjetno nedostopnost škrobnih zrn za encime, saj so škrobna zrna načeloma majhna in dosežejo velikost do okrog 100 µm (Dhital in sod., 2010). Na določitev vsebnosti prehranske vlaknine lahko vpliva tudi različna encimska aktivnost (McCleary, 2001), zato je, za primerjavo vzorcev med seboj, priporočeno uporabljati enak encimski kit ali pa dodatno meriti encimsko aktivnost uporabljenih encimov.

Analitika vsebnosti prehranske vlaknine v živilih je potrebna zaradi njene vloge v številnih fizioloških procesih in pri zmanjševanju tveganja za razvoj bolezni. V zadnjem času je prehranska vlaknina pridobila še dodaten pomen, povezan z njeno uporabo kot funkcionalne in tehnološko pomembne sestavine. Natančna analitika vsebnosti prehranske vlaknine je pomembna tudi za pravilno označevanje živil ter za presojanje živil z namenom možnosti uporabe dveh prehranskih trditev na označbi živila, saj je glede na vsebnost prehranske vlaknine možno pridobiti 2 trditvi in sicer: i.) trditev *vir prehranske vlaknine*, kadar izdelek vsebuje vsaj 3 g prehranske vlaknine na 100 g živila ali 1,5 g prehranske vlaknine na 100 kcal in ii.) trditev *velika vsebnost prehranske vlaknine*, ki jo lahko pridobi živilo, ki vsebuje vsaj 6 g prehranske vlaknine na 100 g ali 3 g prehranske vlaknine na 100 kcal (Uredba 1924/2006). Morebitne zdravstvene trditve pa ne veljajo za prehransko vlaknino na splošno, ampak se morajo nanašati na točno določeno komponento oz. vrsto prehranske vlaknine, za katero je dokazan fiziološki učinek (Salobir in sod., 2015; Stephen in sod., 2017).

4 SKLEPI

V raziskavi smo ugotovili, da frakcioniranje vzorca in način mešanja vplivata na določitev vsebnosti prehranske vlaknine v vzorcih žit za zajtrk. Posebej velik vpliv frakcioniranja vzorca se kaže na nepredelanih ali malo predelanih žitih, kjer z mletjem in presejanjem ločimo različne dele zrnja in s tem vplivamo na vsebnost prehranske vlaknine. Mešanje preprečuje posedanje vzorca in s tem nastanek mehanske prepreke za delovanje encimov. Zaključimo lahko, da je priporočeno vzorce mleti na velikost med 200 µm in 500 µm, kar je zgornja meja navedena v protokolu, ter pri tem vzorca ne frakcionirati skozi sita. V primeru, da steklovina ne omogoča dobrega valovanja vzorca v pufru z encimi, je potrebno prilagoditi inkubacijo, da se vzorec lahko meša. Metoda za določanje prehranske vlaknine AOAC 991.43 je robustna metoda, ki daje, znotraj svojih analitskih okvirov, natančne in ponovljive rezultate, vendar mora biti vzorec ustrezno pripravljen.

5 VIRI

Al-Rabadi, G. J. S., Gilbert, R. G., Gidley, M. J. (2009). Effect of particle size on kinetics of starch digestion in milled barley and sorghum grains by porcine *alpha*-amylase. *Journal of Cereal Science, 50*(2), 198-204. doi:10.1016/j.jcs.2009.05.001

AOAC (1995). *Official methods of analysis* (16th ed.). Washington: DC. Association of Official Analytical Chemists.

CAC (2017). Codex Alimentarius Commission. *Guidelines on nutrition labeling CAC/GL 2–1985 as last amended 2017*. Joint FAO/WHO Food Standards Programme, Secretariat of the Codex Alimentarius Commission. Rome, FAO: 10 str. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/es/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCAC%2BGL%2B2-1985%252FCXG_002e.pdf

Dahl, W. J., Stewart. M. L. (2015). Position of the Academy of Nutrition and Dietetics: Health implications of dietary fiber. *Journal of Academy of Nutrition and Dietetics, 115*(11), 1861-1870. doi:10.1016/j.jand.2015.09.003

Dai, F. J., Chau, C. F. (2017). Classification and regulatory perspectives of dietary fiber. *Journal of Food and Drug Analysis, 25*(1), 37-42. doi:10.1016/j.jfda.2016.09.006

de la Hera, E., Gomez, M. Rosell, C. M. (2013). Particle size distribution of rice flour affecting the starch enzymatic hydrolysis and hydration properties. *Carbohydrate Polymers, 98*(1), 421-427. doi:10.1016/j.carbpol.2013.06.002

DeVries, J.W. (2010). Validating official methodology commensurate with dietary fibre research and definitions. In: J. W. van der Kamp, J. Jones, B. McCleary & D. Topping (Eds.), *Dietary fibre: new frontiers for food and health* (pp. 29-49).Wageningen: Wageningen Academic Publishers. doi:10.3920/978-90-8686-692-2

Dhital, S., Shrestha, A. K., Gidley, M. J. (2010). Relationship between granule size and in vitro digestibility of maize and potato starches. *Carbohydrate Polymers, 82*(2), 480-488. doi:10.1016/j.carbpol.2010.05.018

Djurle, S., Andersson, A. A. M., Andersson, R. (2016). Milling and extrusion of six barley varieties, effects on dietary fibre and starch content and composition. *Journal of Cereal Science, 72*, 146-152. doi:10.1016/j.jcs.2016.09.017

EFSA (2010). Scientific opinion on dietary reference values for carbohydrates and dietary fibre. European Food Safety Authority, *EFSA Journal*, *8*(3), 1-77. doi:10.2903/j.efsa.2010.1462

EFSA (2017). Dietary reference values for nutrients: Summary report. European Food Safety Authority: 92 str. doi:10.2903/sp.efsa.2017.e15121

Ehle, F. R. (1984). Influence of particle size on determination of fibrous feed components. *Journal of Dairy Science, 67*(7),1482-1488. doi:10.3168/jds.S0022-0302(84)81465-4

Fernstrand, A. M., Burya, D., Garssen, J., Verster, J. C. (2017). Dietary intake of fibers: differential effects in men and women on perceived general health and immune functioning. *Food & Nutrition Research, 61*(1), 1-7. doi:10.1080/16546628.2017.1297053

Frølich, W., Nyman, M. (1988). Minerals, phytate and dietary fibre in different fractions of oat-grain. *Journal of Cereal Science, 7*(1), 73-82. doi:10.1016/S0733-5210(88)80061-4

Fuller, S., Beck, E., Salman, H., Tapsell, L. (2016). New horizons for the study of dietary fibre and health: a review. *Plant Foods for Human Nutrition*, *71*(1), 1-12. doi:10.1007/s11130-016-0529-6

Golob, T., Bertoncelj, J., Korošec, M. (2012). Pomen prehranske vlaknine v prehrani človeka. *Acta Agriculturae Slovenica, 99*(2), 201-211.

Gualberto, D. G., Bergman, C. J., Kazemzadeh, M., Weber, C.W. (1997). Effect of extrusion processing on the soluble and insoluble fiber and phytic acid contents of cereal brans. *Plant Foods for Human Nutrition, 51*(3),187-198. doi:10.1023/A:1007941032726

Kendall, C. W. C., Esfahani, A., Jenkins D. J. A. (2010). The link between dietary fibre and human health. *Food Hydrocolloids, 24*(1): 42-48. doi:10.1016/j.foodhyd.2009.08.002

Li, Y. O., Komarek, A. R. (2017). Dietary fibre basics: Health, nutrition, analysis, and applications. *Food Quality and Safety, 1*, 47-59. doi:10.1093/fqs/fyx007

Mahasukhonthachat, K., Sopade, P. A., Gidley, M. J. (2010). Kinetics of starch digestion in sorghum as affected by particle size. *Journal of Food Engineering, 96*(1): 18-28. doi:10.1016/j.jfoodeng.2009.06.051

McCleary, B. V. (2001). Measurement of dietary fibre components: the importance of enzyme purity, activity and specificity. In: B. V. McCleary & L. Prosky (Eds.), *Advanced dietary fibre technology* (pp. 89-106). Oxford: Blackwell Science.

McCleary, B. V., Sloane, N., Draga, A., Lazewska, I. (2013). Measurement of total dietary fiber using AOAC method 2009.01 (AACC International approved method 32-45.01): Evaluation and updates. *Cereal Chemistry, 90*(4), 396-414. doi:10.1094/CCHEM-10-12-0135-FI

Monro, J. A. (2015). Dietary fibre. In: L. M. L. Nollet & F. Toldrá (Eds.), *Handbook of food analysis, Third edition* (pp. 755-767). Boca Raton: Taylor & Francis Group.

Perry, J. R., Ying, W. (2016). A review of physiological effects of soluble and insoluble dietary fibers. *Journal of Nutrition & Food Sciences, 6*(2), 1-6. doi:10.4172/2155-9600.1000476

Referenčne vrednosti za energijski vnos ter vnos hranil: tabelarična priporočila za otroke (od 1. leta starosti naprej), mladostnike, odrasle, starejše, nosečnice ter doječe matere. 2016. Ljubljana, Nacionalni inštitut za javno zdravje: 8 str. http://www.mz.gov.si/fileadmin/mz.gov.si/pageuploads/javno_zdravje_2015/foto_DJZ/prehrana/2016_referencne_vrednosti_za_energijski_vnos_ter_vnos_hranil_17022016.pdf

Salobir, J., Levart, A., Korošec, M. (2015). Prehranske in zdravstvene trditve v primeru maščob in prehranske vlaknine. V: M. Korošec, T. Polak & L. Demšar (ur.). *Hrana, prehrana in mediji. 28. Bitenčevi živilski dnevi.* (pp. 81-97). Ljubljana, Biotehniška fakulteta, Oddelek za živilstvo.

Singh, J., Dartois, A., Kaur, L. (2010). Starch digestibility in food matrix: a review. *Trends in Food Science & Technology, 21*(4), 168-180. doi:10.1016/j.tifs.2009.12.001

Steadman, K. J., Burgoon, M. S., Lewis, B. A. Edwardson, S. E., Obendorf, R. L. (2001). Buckwheat seed milling fractions: description, macronutrient composition and dietary fibre. *Journal of Cereal Science, 33*(3), 271-278. doi:10.1006/jcrs.2001.0366

Stephen, A. M., Champ, M. M. J., Cloran, S. J., Fleith, M., van Lieshout, L., Mejborn, H., Burley, V. J. (2017). Dietary fibre in Europe: current state of knowledge on definitions, sources, recommendations, intakes and relationships to health. *Nutrition Research Reviews*, *30*(2), 149-190. doi:10.1017/S095442241700004X

Škrabanja, V., Kreft, I., Golob, T., Modic, M., Ikeda, S., Ikeda, K., Kreft, S., Bonafaccia, G., Knapp, M., Košmelj, K. (2004). Nutrient content in buckwheat milling fractions. *Cereal Chemistry, 81*(2), 172-176. doi:10.1094/CCHEM.2004.81.2.172

Tarcea, M., Rus, V., Zita, F. (2017). Insight of dietary fibers consumption and obesity prevention. *Journal of Obesity & Eating Disorders, 3*(1), 1-3. doi:10.21767/2471-8203.100033

Tester, R. F., Qi, X., Karkalas, J. (2006). Hydrolysis of native starches with amylases. *Animal Feed Science and Technology, 130*(1-2), 39-54. doi:10.1016/j.anifeedsci.2006.01.016

Tosi, E. A., Re, E., Lucero, H., Masciarelli, R. (2001). Dietary fiber obtained from amaranth (*Amaranthus Cruentus*) grain by differential milling. *Food Chemistry, 73*(4), 441-443. doi:10.1016/S0308-8146(00)00326-5

Uredba (EU) št. 1169/2011 evropskega parlamenta in sveta z dne 25. oktobra 2011 o zagotavljanju informacij o živilih potrošnikom (2011). *Uradni list Evropske unije, 304*, 18-63 http://eur-lex.europa.eu/legal-content/SL/TXT/PDF/?uri=CELEX:32011R1169&from=sl

Uredba (EU) št. 1924/2006 evropskega parlamenta in sveta z dne 20. decembra 2006 o prehranskih in zdravstvenih trditvah na živilih (2006). *Uradni list Evropske unije, 404*: 9-25 http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:404:0009:0025:SL:PDF

Westenbrink, S., Brunt, K., van der Kamp, J. W. (2013). Dietary fibre: challenges in production and use of food composition data. *Food Chemistry, 140*(3), 562-567. doi:10.1016/j.foodchem.2012.09.029

Zhang, B., Dhital, S., Gidley, M. J. (2015). Densely packed matrices as rate determining features in starch hydrolysis. *Trends in Food Science & Technology, 43*(1), 18-31. doi:10.1016/j.tifs.2015.01.004

Zielinski, G., Rozema, B. (2013). Review of fiber methods and applicability to fortified foods and supplements: choosing the correct method and interpreting results. *Analytical and Bioanalytical Chemistry, 405*(13), 4359-4372. doi:10.1007/s00216-013-6711-x

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Izprana tla v Sloveniji: pedološke lastnosti, prostorska razporeditev in klasifikacija

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IZVLEČEK

Eluvialno-iluvialni procesi so ključni pri pedogenezi. Po podatkih pedološke karte Slovenije 1 : 25.000 izprana tla pokrivajo 2,3 % površine. Pojavljajo se na različnih matičnih podlagah, večinoma na uravnanem površju, kjer so zavarovana pred erozijskimi in koluvialnimi procesi. Namen naše raziskave je ovrednotenje njihovih morfoloških, fizikalnih in kemičnih lastnosti, prostorske razporeditve in vpliva tlotvornih dejavnikov, predvsem matične podlage, na njihov nastanek. Pedološke lastnosti smo ovrednostili na podlagi analitskih in opisnih podatkov 49 izpranih tal iz pedološke baze Talnega informacijskega sistema Slovenije. Očitne znake procesov izpiranja smo prepoznali v večini profilov izpranih tal. Eluvialni horizont je imel v primerjavi z iluvialnim običajno nižji pH, v povprečju za 0,2 enote, svetlejšo barvo, manjšo vsebnost bazičnih kationov (v povprečju za 16,6 %) in manjšo kationsko izmenjalno kapaciteto (v povprečju za 5,5 $mmol_c$ 100 g^{-1} tal). Povprečno razmerje v vsebnosti gline med iluvialnim in eluvialnim horizontom je 1,63. 75 % izpranih tal ima razmerje v vsebnosti gline nad 1,38. Na podlagi kriterijev WRB klasifikacije smo argični horizont določili 40 profilom, medtem ko ostalih 9 ni ustrezalo kriterijem ali pa ni bilo na voljo podatkov za klasifikacijo. Podroben pregled WRB kriterijev (kationska izmenjalna kapaciteta preračunana na vsebnost gline in delež bazičnih kationov v B_t horizontih) je pokazal, da se pojavljata dve referenčni skupini: luvisoli in alisoli. Proti pričakovanju in dosedanjim znanstvenim objavam nismo evidentirali akrisolov v bazi pedoloških profilov Pedološke karte Slovenije.

Ključne besede: tla; eluvialno-iluvialno procesi; pedološke lastnosti; klasifikacija tal

ABSTRACT

LEACHED SOILS IN SLOVENIA: PEDOLOGICAL PROPERTIES, SPATIAL DISTRIBUTION AND CLASSIFICATION

Eluvial-illuvial processes plays key role in pedogenesis, especially in the development of leached soils. As reported in Slovenian soil map 1 : 25.000 leached soils cover 2,3 % of Slovenian territory. They occur on different parent materials, mostly on flat relief preserved from erosion and colluvial processes. The aim of our study is the evaluation of their morpohological, physical and chemical properties, spatial distribution and dependency on soil forming factors, especially on parent material. Pedological properties are demonstrated according to analytical and descriptive data of 49 leached soils from the pedological base of Soil Information System of Slovenia. Obvious leaching processes are clearly recognized in almost all profiles of leached soils. Eluvial horizon in comparison to illuvial horizon has lower pH value, which is in average 4,4 and 4,6 for E and Bt horizon respectively, brighter color, lower base saturation (in average for 16,6 %) and lower CEC (in average for 5,5 $mmol_c$ 100 g^{-1} soil). On average ratio of clay content between illuvial and eluvial horizon is 1,63. In the 75 % of all studied leached soils this ratio is above 1,38. After evaluation, according to WRB classification, an argic horizon is identified only in 40 soil profiles, while other 9 profiles do not match criteria of sufficient textural differentiation or there is not enough data to classify them. Detailed overview of the WRB criteria for argic horizons (cation exchange capacity of clay fraction and base saturation in argic horizons) reveals that Luvisols and Alisols are the most widespread groups in Slovenia among leached soil. Against expectations based on different references, we do not determined Acrisols within Soil Map Database.

Key words: soil; eluvial-illuvial processes; pedological properties; soil classification

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1 UVOD

Eluvialno-iluvialni procesi imajo pomembno vlogo pri razvoju tal, predvsem v okoljih z izrazitimi procesi izpiranja (Vidic in Lobnik, 1997; Quénard in sod., 2011), ki se odvijajo na območjih z zadostno letno količino efektivnih padavin. O njihovem pojavljanju poročajo iz Kanade (Lavkulich in Arocena, 2011), ZDA (Bockheim in Hartemink, 2013), Evrope (Kühn in sod., 2006; Quénard in sod., 2011; Costantini in sod., 2013; Carballas in sod., 2016, Fedoroff, 1997) in drugje. Tal, za katera so eluvialno-iluvialni procesi ključni, je po oceni IUUS Working group WRB (2015) na svetu približno 25,7 milijonov km^2. Med najbolj razširjenimi referenčnimi talnimi skupinami so akrisoli, sledijo luvisoli, lixisoli, retisoli in alisoli. V Evropi so najbolj razširjeni retisoli in luvisoli (21 %), v manj kot odstotku se pojavljajo akrisoli in alisoli (Soil Atlas of Europe, 2005). Tla opredelimo kot izprana, če imajo izraženo teksturno diferenciacijo med horizonti ob sočasni prisotnosti diagnostičnega eluvialnega (E) horizonta. Iz eluvialnega horizonta se poleg glinenih mineralov izpirajo tudi bazični kationi, topna organska snov ter druge komponente tal (Blume in sod., 2016). Teksturno razliko v talnem profilu je običajno mogoče zaznati s prstnim poskusom. Kot znak premeščanja gline se v B_t horizontih na stenah por in strukturnih agregatov pojavljajo glinene prevleke. Izraz »izpiranje« (ang.: *leaching*) se v tuji literaturi uporablja za poimenovanje izpiranja v talni raztopini raztopljenih snovi iz profila v podtalje (Shaetzl in Anderson, 2005). Pri nas je uporaba izraza vezana tako na proces izpiranja iz tal v podtalje kot tudi na proces premeščanja snovi znotraj tal (iz zgornjih plasti v globlje dele tal). V pedološki literaturi za proces premeščanja gline po profilu navzdol običajno uporabljajo francoski izraz *lessivage*, ki je prisoten tudi v starejši domači literaturi (Stritar 1972, 1984, 1990; Sušin, 1964), kjer izprana tla imenujejo lesivirana tla, proces izpiranja gline lesivaža oziroma lesivacija. Kot sinonima pogosto zasledimo tudi izraza ilimerizacija in argiluviacija. Po Slovenski klasifikaciji tal (Prus in sod., 2015) izprana tla uvrščamo v razred eluvialno-iluvialnih tal. Po podatkih pedološke karte 1 : 25.000 izprana tla pokrivajo 2,3 % slovenskega ozemlja (TIS/ICPVO, 2017). Najdemo jih na različnih matičnih podlagah, največkrat na uravnanem površju, kjer ni delovanja erozijskih in koluvialnih procesov, v kombinaciji z rjavimi pokarbonatnimi tlemi na apnencih in dolomitih.

V prispevku želimo predstaviti morfološke, fizikalne in kemične lastnosti izpranih tal v Sloveniji, njihovo prostorsko razširjenost, odvisnost od tlotvornih dejavnikov, predvsem od matične podlage, ter primerjavo z izpranimi tlemi v Evropi. Preveriti želimo razvrstitev izpranih tal po WRB klasifikaciji.

2 MATERIALI IN METODE

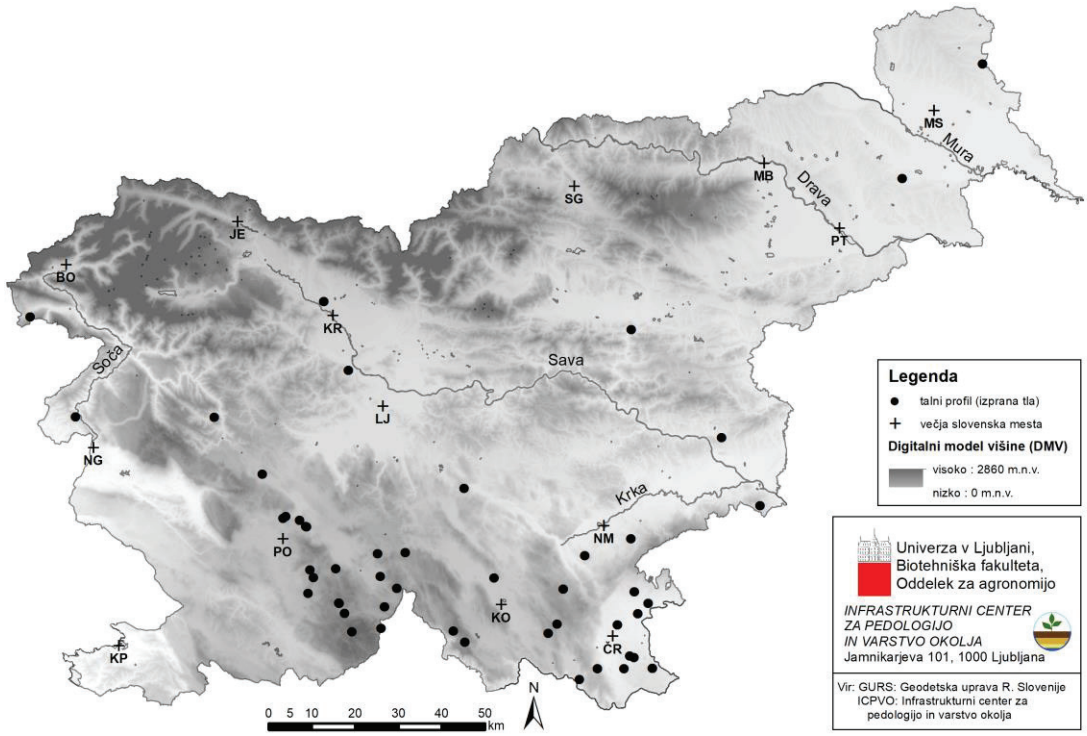
2.1 Vir in priprava podatkov

Podatki so bili pridobljeni iz baze Talnega informacijskega sistema Slovenije (TIS/ICPVO, 2017). Pedokartografske enote, ki so vključevale izprana tla, smo izvozili iz atributne tabele Digitalne pedološke karte Slovenije 1 : 25.000 in združili v nov podatkovni sloj (TIS/ICPVO, 2017). Za vsako pedokartografsko enoto smo izračunali površino izpranih tal ter izrisali prostorsko razporeditev. Izračunali smo delež izpranih tal glede na matično podlago in izrisali karto matičnih podlag izpranih tal v Sloveniji (Slika 3). Statistično analizo pedoloških lastnosti smo izvedli na 49 profilih izpranih tal, ki so bili izkopani v sklopu pedološkega kartiranja Slovenije ali drugih raziskav tal v obdobju od 1974 do 1998, za katere so bili na voljo potrebni analitski in opisni morfološki podatki po horizontih in prostorska opredelitev. Lokacije talnih profilov izpranih tal so prikazane na sliki 1. Za potrebe statističnih analiz smo imena talnih horizontov generalizirali v 5 skupin (O_h/A_h, A, E, B_t, BC); kriteriji za razvrščanje so prikazani v preglednici 1. Za grafični prikaz prostorskih podatkov smo uporabili program ArcGIS 10.2.2 for Desktop (ESRI, 2014) in program R (R Development Core Team, 2016). Izris talnih profilov in analizo barv smo izvedli s pomočjo paketa »aqp« (Beaudette in sod., 2013).

Preglednica 1: Skupine posplošenih horizontov
Table 1: Groups of generalized horizons

Horizonti	Značilnosti
O_h/A_h	Organski horizonti z več kot 35 % organske snovi in humusno-akumulativni horizonti z več kot 15 % organske snovi.
A	Humusno-akumulativni horizonti z manj kot 15 % organske snovi.
E	Eluvialni horizonti: v primerjavi z B_t so svetlejše barve, lažje teksture, s slabše izraženo strukturo in z manjšim deležem bazičnih kationov.
B_t	Iluvialni horizonti – akumulacija gline, glinene prevleke.
BC	Prehodni horizonti – mineralni del tal v kombinaciji s C horizontom.

Prehodni horizonti so bili uvrščeni v skupino glede na prevladujoče lastnosti (primera: AC v A ali EB v E)



Slika 1: Lokacije talnih profilov izpranih tal (TIS/ICPVO, 2017)
Figure 1: Locations of soil profiles (TIS/ICPVO, 2017)

2.2 Laboratorijske metode

Analize fizikalnih in kemičnih lastnosti talnih vzorcev (n = 213) so bile izvedene v pedološkem laboratoriju Katedre za pedologijo in varstvo okolja. Vzorci so bili zračno posušeni in presejani skozi sito z 2 mm odprtinami. pH tal je bil izmerjen elektrometrično v suspenziji tla : ekstrakcijska raztopina (0,1 M KCl ali 0,01 M $CaCl_2$) v razmerju 1 : 2,5 (v/v). Organska snov je bila določena z mokrim sežigom po modificirani Walkley-Black metodi, tekstura tal (razen v organskih horizontih) je bila določena s sedimentacijsko pipetno metodo po ameriški teksturni klasifikaciji. Rastlinam dostopni fosfor in kalij sta bila izmerjena po ekstrakciji z amonlaktatom. Skupni dušik je bil analiziran po Kjeldahlovem postopku s TiO_2 kot katalizatorjem. Kationska izmenjalana kapaciteta je bila določena kot vsota bazičnih kationov določenih po ekstrakciji z 1 M amonacetatom (pH 7) in kislih kationov določenih po modificirani Melichovi metodi (Soil Survey Staff, 1992).

2.3 Statistična analiza

Lastnosti 49 talnih profilov smo predstavili z opisnimi statistikami. Za preverjanje razlik v pH in deležu bazičnih kationov med rabami tal smo izvedli analizo variance. Statistične analize smo izvedli v programu R

(R Development Core Team, 2016), ternarni diagram smo izrisali z orodjem GCDkit 4.0 (Janousek in sod., 2006). Preglednica 2 prikazuje opisne statistike vseh obravnavanih pedoloških spremenljivk.

Preglednica 2: Opisne statistike pedoloških spremenljivk (49 talnih profilov, 213 horizontov)
Table 2: Descriptive statistics of pedologic variables (49 soil profiles, 213 horizons)

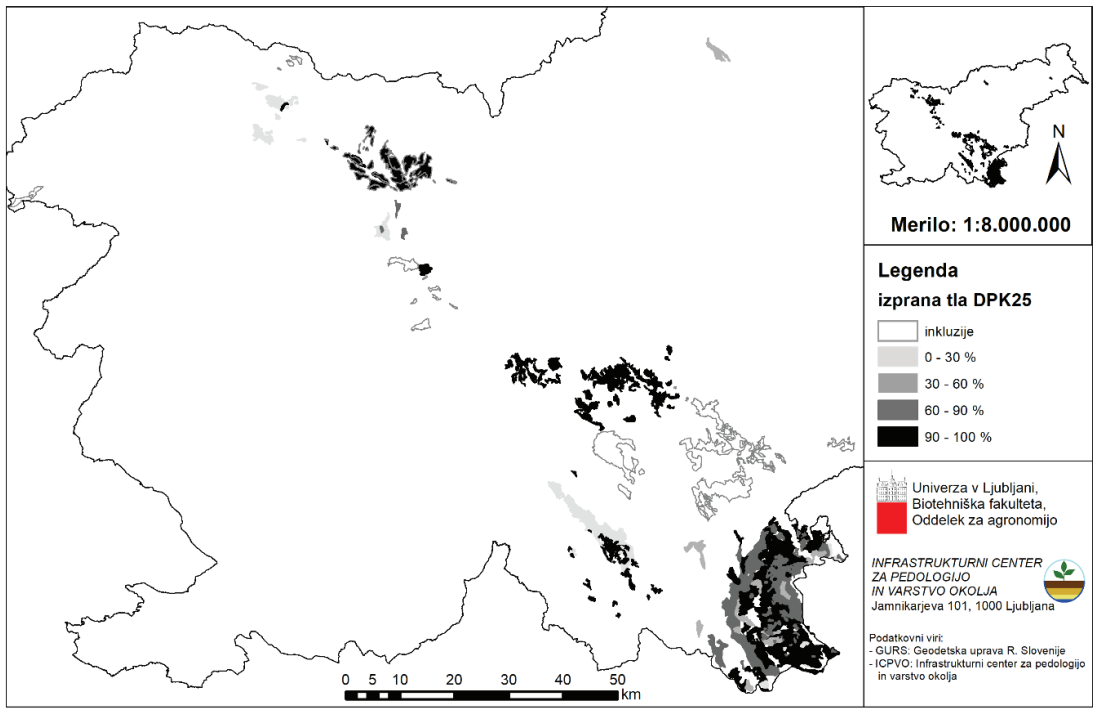
Spremenljivka	Enota	n	Povprečje	SD	KV	Mediana	Min	Max
pesek	%	199	12,2	9,1	0,75	9,4	0,5	59,3
melj	%	199	53,1	15,6	0,29	56,5	5,2	78,9
glina	%	199	34,8	16,0	0,46	30,5	3,5	92,0
pH_{KCl}		207	4,6	0,8	0,18	4,3	2,9	6,8
organska snov	%	184	6,0	10,2	1,70	2,0	0,2	70,8
C_{org}	%	184	3,5	5,9	1,70	1,5	0,1	41,0
P_2O_5	mg/100g	86	4,4	6,9	1,54	2,2	0,1	44,5
K_2O	mg/100g	101	11,3	10,9	0,97	7,5	1,2	71,1
N	%	161	0,2	0,3	1,21	0,1	< 0,1	100,2
CN		161	13,9	9,1	0,66	13,2	0,6	1,7
izmen. Ca^{2+}	$mmol_c$/100g	199	8,5	8,5	0,99	5,8	0,1	62,3
izmen. Mg^{2+}	$mmol_c$/100g	199	1,6	1,8	1,11	0,9	< 0,1	9,2
izmen. K^+	$mmol_c$/100g	199	0,2	0,2	0,78	0,2	< 0,1	0,9
izmen. Na^+	$mmol_c$/100g	199	0,1	0,1	0,73	0,1	< 0,1	0,6
Vsota bazičnih kationov	$mmol_c$/100g	199	10,4	9,3	0,89	8,1	0,3	65,8
KIK	$mmol_c$/100g	199	24,9	11,5	0,46	22,5	17,3	99,9
KIK_{glina}*	$mmol_c$/100g	80	52,4	14,9	0,28	48,7	26,6	112,0
KIK_{glina}**	$mmol_c$/100g	80	45,8	12,1	0,26	42,9	19,9	98,7
V***	%	199	38,6	22,7	0,59	39,2	1,8	83,5

SD – standardna deviacija, KV – koeficient variacije, Min – minimum, Max – maksimum
* KIK_{glina}: (KIK / %gline) * 100 (vključeni le iluvialni horizonti)
** KIK_{glina}: (KIK – (%C_{org} * 4,5) * 100) / %gline (po Camargo in sod., 1986) (vključeni le iluvialni horizonti)
** V – delež bazičnih kationov na sorptivnem delu tal

3 REZULTATI IN DISKUSIJA

3.1 Razširjenost izpranih tal v Sloveniji

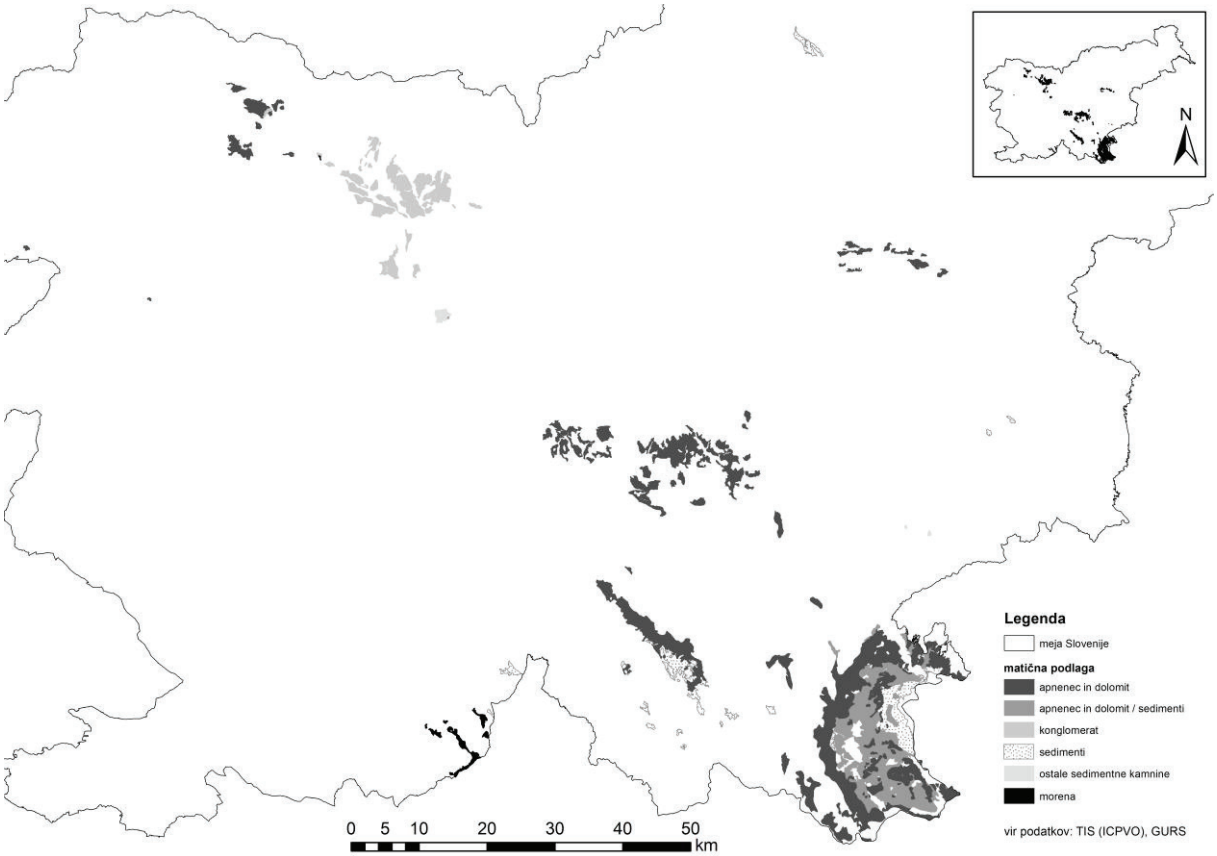
Izprana tla pokrivajo 2,3 % Slovenije. Najbolj so razširjena v jugovzhodnem delu države, še posebej v Beli krajini, kjer leži 60 % vseh izpranih tal. Izprana tla najdemo tudi v Dolenjskem podolju (15,3 %), na Gorenjskem (14,1 %), v Ribniško-Kočevskem podolju (6,4 %) in v manjšem obsegu na nekaterih drugih območjih Slovenije (Slika 2). Večina pedokartografskih enot izpranih tal se pojavlja med Belo krajino in Jesenicami (Slika 2), medtem ko je bilo največ profilov izpranih tal izkopanih v južnem delu Slovenije (Notranjska, Dolenjska). Neujemanje med lokacijami pedokartografskih enot izpranih tal (Slika 3) in številom dokumentiranih profilov izpranih tal (Slika 2), je posledica večjega števila raziskav na območju južnega dela Slovenije, kjer se izprana tla pojavljajo v združbi z rjavimi pokarbonatnimi tlemi in rendzinami. Ker prevladujejo mlajše razvojne oblike tal, izprana tla niso navedena v imenu pedokartografskih enot. V splošnem velja, da so procesi premeščanja gline in drugih v vodi topnih snovi v tleh zelo odvisni od količine padavin, vendar na območju Alp, kljub ekstremnim količinam padavin, izpranih tal ne zasledimo, saj na razvoj tal bistveno vplivajo erozijski in koluvialni procesi, zaradi česar tam prevladujejo mlada tla (litosoli, rendzine). Znaki izpiranja se pri nas v goratih območjih z veliko padavinami izrazijo le v globokih tleh na uravnanih platojih (na primer Pokljuka).



Slika 2: Zastopanost izpranih tal v Sloveniji po posameznih pedokartografskih enotah pedološke karte 1 : 25.000 (TIS/ICPVO, 2017)

Figure 2: Spatial distribution of leached soils in Slovenia according to percentage of their occurrence in pedocartographic unit (TIS/ICPVO, 2017)

Izprana tla se v večini pojavljajo na apnencu in dolomitu (60,7 %) v združbi z rendzinami in kambičnimi tlemi. Pojavljajo se tudi na sedimentih (25,6 %) in konglomeratu (12,3 %), v majhnem deležu na nekarbonatnih sedimentnih kamninah (0,7 %), moreni (0,4 %) in flišnih kamninah (0,3 %). Slika 3 prikazuje matične podlage, na katerih se pojavljajo izprana tla. Pri izpranih tleh na klastičnih sedimentnih kamninah lahko k teksturni razliki med horizonti prispeva tudi razlika v granulometrijski sestavi posameznih plasti sedimentnih kamnin oziroma kamninska nezveznost. Obstaja možnost, da nekateri profili na takih kamninah ne ustrezajo kriteriju izpranih tal, vendar tega nismo mogli preveriti, ker večinoma v bazi pedološke karte ni na voljo podatka o pojavljanju glinenih prevlek na strukturnih agregatih, ki nedvoumno dokazujejo eluvialno-iluvialne procese v tleh.



Slika 3: Matične podlage na katerih se pojavljajo izprana tla v Sloveniji; Digitalna pedološka karta Slovenije (TIS/ICPVO, 2017)

Figure 3: Different parent materials upon which leached soils appear; Digital soil map of Slovenia (TIS/ICPVO, 2017)

3.2 Klasifikacija izpranih tal

Osnovni diagnostični kriterij Slovenske klasifikacije tal (Prus in sod., 2015) za uvrstitev tal v eluvialno-iluvialni razred je prisotnost eluvialnega horizonta, ki ima v primerjavi z iluvialnim svetlejšo barvo, lažjo teksturo, slabšo strukturno obstojnost in manjši delež bazičnih kationov. Navodilo zahteva 20 % več gline v B_t horizontu glede na E, a ne opredeljuje ali gre za relativno ali absolutno razliko. Večina profilov izpranih tal v bazi pedološke karte ne izkazuje tako velike absolutne razlike v teksturi. Izprana tla so bila v času intenzivnega pedološkega kartiranja opredeljena na osnovi identifikacije teksturne razlike med B_t in E horizontom s prstnim poskusom, kot je bilo zapisano v takrat veljavni Jugoslovanski klasifikaciji tal (Škorič in sod., 1985). Statistična analiza profilov izpranih tal je pokazala, da imajo v povprečju B_t horizonti, ki ležijo neposredno pod E horizonti, za 1,6-krat več gline. Kar 75 % izpranih tal dosega količnik večji od 1,38, kar se približa pogojem WRB klasifikacije (IUUS Working group WRB, 2015). WRB kriterij je različen za različno količino gline v E horizontu (Preglednica 3). Pri vsebnosti gline med 10 in 50 %, kar je najpogosteje v Sloveniji, je faktor med B_t in E 1,4. Preverili smo tudi kriterije nemške, avstrijske in ameriške klasifikacije (Preglednica 3) in ugotovili, da večina proučevanih talnih profilov po teksturi ustreza kriterijem za izprana tla.

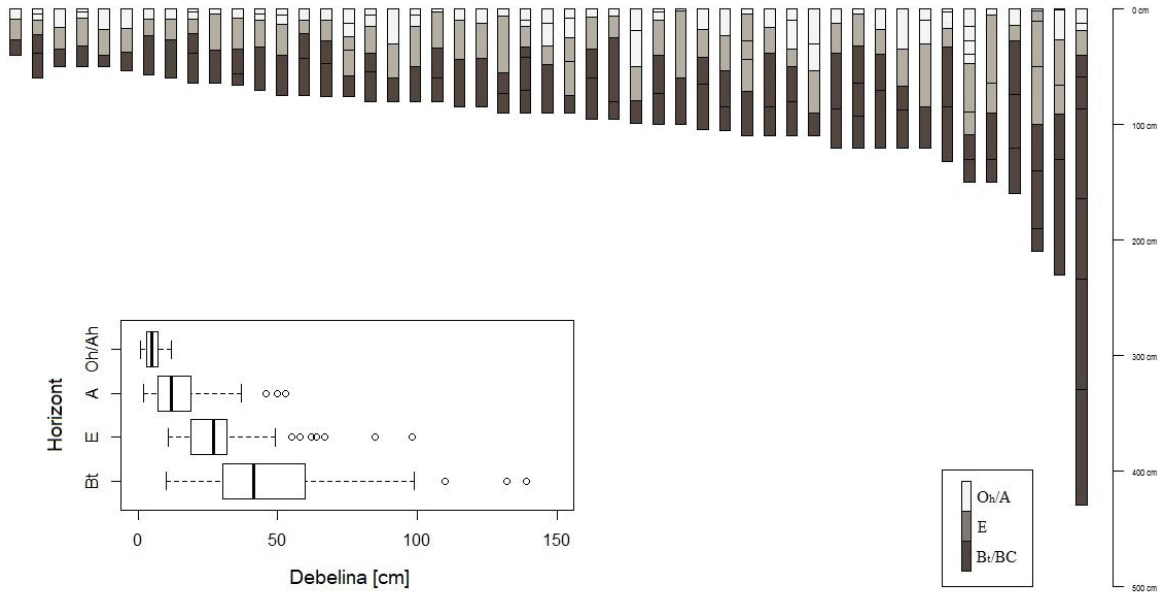
Preglednica 3: Diagnostični kriteriji za argični (B_t) horizont (Turniški, 2016)
Table 3: Diagnostic criteria for argic (B_t) horizons (Turniški, 2016)

Klasifikacija	Glavni kriterij (teksturna diferenciacija)	Vir
Jugoslovanska klasifikacija tal	Razlika v vsebnosti gline med horizontoma mora biti s prstnim poskusom zaznavna v prid B_t horizonta.	Škorič in sod., 1985
Slovenska klasifikacija tal	V iluvialnem horizontu mora biti najmanj 20 % več gline kot v E horizontu.	Prus in sod., 2015
Mednarodna klasifikacija tal (WRB)	Če ima E horizont manj kot 10 % gline, jo mora imeti argični horizont vsaj 4 % več. Če ima E horizont med 10 in 50 % gline, mora imeti argični horizont vsaj za 1,4x več gline. Če ima E horizont več kot 50 % gline, mora imeti argični horizont vsaj 20 % več gline.	IUUS Working group WRB, 2015
Nemška klasifikacija tal	Če je v B_t horizontu manj kot 17 % gline in manj kot 50 % melja, mora biti razlika med B_t in E vsaj 3 % v prid B_t. Če je v B_t horizontu manj kot 17 % gline in več kot 50 % melja ali pa je gline med 17 in 45 %, mora biti razlika med B_t in E vsaj 5 % v prid B_t. Če je v B_t horizontu več kot 45 % gline, mora biti razlika med B_t in E vsaj 8 % v prid B_t.	Arbeitskreis für Bodensystematik … , 1998
Avstrijska klasifikacija tal	B_t horizont mora vsebovati vsaj 25 % gline in za najmanj 15 % (relativno) več gline od E horizonta.	Nestroy in sod. 2011
Ameriška klasifikacija tal (USDA Soil Taxonomy)	Če ima E horizont manj kot 15 % gline, jo mora imeti argični (B_t) horizont vsaj 3 % več. Če ima E horizont med 15 in 40 % gline, mora imeti argični (B_t) horizont vsaj za 1,2x več gline. Če ima E horizont več kot 40 % gline, mora imeti argični (B_t) horizont vsaj 8 % več gline.	Keys to Soil …, 2014

Proučevana tla smo klasificirali tudi po WRB klasifikaciji (IUUS Working group WRB, 2015). Pregled je pokazal, da diagnostične zahteve za argični horizont dosega le 40 od 49 profilov izpranih tal. Izprana tla smo uvrstili v dve Referenčni talni skupini, med luvisole (n = 22) in alisole (n = 18). Luvisoli se običajno pojavljajo na apnencu in dolomitu in so se razvili iz evtričnih rjavih ali rjavih pokarbonatnih tal, ki so pogosta v Sloveniji. Argični horizonti v luvisolih so evtrični (zasičenost z bazičnimi kationi > 50 %) in imajo veliko vsebnost visoko aktivnih glin, ki prispevajo k večji kationski izmenjalni kapaciteti (KIK_{glina} > 24 $mmol_c$/100 g gline). V razmerah intenzivnega izpiranja se lahko luvisoli razvijejo v alisole. Slednji lahko nastanejo tudi iz distričnih rjavih tal. Za razliko od luvisolov imajo argični horizonti v alisolih majhno zasičenost z bazičnimi kationi (< 50 %). Glede na navedbe v literaturi, ki že povzema podatke Pedološke karte 1 : 25.000 (Urbančič in sod., 2005; Vidic in sod., 2015; Vrščaj in sod., 2017), smo pričakovali, da se bo del proučevanih tal uvrstilo med akrisole, vendar kationska izmenjalna kapaciteta preračunana na delež gline in delež bazičnih kationov ne dokazujejo prisotnost akrisolov. Prav tako se noben profil ni uvrstil med liksisole. Liksisoli in akrisoli sta referenčni talni skupini katerih argični horizonti so za razliko od luvisolov in alisolov močneje prepereli, kar se kaže tudi v večji vsebnosti kaolinita (Blume in sod., 2016). Glede na kriterij WRB bi morali imeti argični horizonti kationsko izmenjalno kapaciteto manjšo od 24 $mmol_c$/100 g gline (KIK_{glina}). Takšna tla se v Sloveniji pojavljajo v omejenem obsegu, liksisoli na območju Snežnika (Kobal, 2011; Turniški, 2016) in akrisoli na Dolenjskem (Kralj, 2008), vendar o njihovem razvoju in prostorski razporeditvi vemo bolj malo. Naše ugotovitve glede pojavnosti luvisolov, alisolov, akrisolov in lixisolov so primerljive z ugotvitvami raziskovalcev v Italiji, kjer se najpogosteje pojavljajo luvisoli (12,8 %) in alisoli (0,17 %), medtem ko se akrisoli (0,017 %) in lixisoli (0,004 %) pojavljajo zelo redko (Costantini in sod., 2013). Po Soil Atlas of Europe (2005) se akrisoli in alisoli v omejenem obsegu (< 1 %) pojavljajo v vlažnejših delih Mediterana v združbi z luvisoli, vendar sta njihov razvoj in prostorska razporejenost slabo poznana, zato so potrebne nadaljnje raziskave.

3.3 Morfološke lastnosti izpranih tal

Izprana tla so starejše razvojne oblike tal, zato smo pričakovali globoke talne profile. Statistični pregled podatkov je pokazal velik razpon v globini tal (Slika 4). Najbolj plitva tla merijo 40 cm, najgloblja 429 cm. 75 % tal je globljih od 75 cm, zato jih po kriterijih Slovenske klasifikacije tal (Prus in sod., 2015) uvrščamo med globoka ali zelo globoka tla. Trije talni profili so globlji od 210 cm. Pri globini tal je potrebno upoštevati, da večina profilov ni bilo izkopanih do matične podlage, zato spodnje globine brez C ali R horizonta niso zanesljiv podatek. Pri globokih tleh se je za potrebe klasifikacije običajno izkopalo profil le od 100 do 150 cm globine. V večini izpranih tal si horizonti sledijo v tipičnem (poenostavljenem) zaporedju A-E-B_t, pogosto se znotraj profila nahaja več horizontov s pedogenetskimi znaki izpiranja, ki se razlikujejo po stopnji izraženosti (npr.: A-E_1-E_2-B_{t1}-B_{t2}). Najdebelejši so B_t horizonti (mediana = 41,5 cm), sledijo E (mediana = 27 cm), A (mediana = 12 cm) in O_h/A_h horizonti (mediana = 5 cm) (Slika 4). Velika debelina B_t horizontov in skupna globina tal nakazujejo, da gre za stara tla (Vidic, 1989).



Slika 4: Prikaz talnih profilov izpranih tal razporejenih po globini in okvir z ročaji (odebeljena črta = mediana; okvir = kvartil 25 %, 75 %; sp. in zg. ročaj = min, max; krožec = osamelec) za skupno debelino istovrstnih horizontov znotraj talnega profila (brez vrednosti za B_t horizont globine 389 cm)

Figure 4: Soil profiles of leached soils arranged by depth and boxplots (black thickened line = median; rectangles = quartiles 25 %, 75 %; ranges = min, max; circles = outliers) for horizon thickness within soil profile (horizons of the same kind within soil profile were united; the thickest B_t horizon (389 cm) is not included)

Za eluvialne horizonte sta značilni sferična in poliedrična struktura. Talni agregati v E horizontih so v večini primerov slabše izraženi in manj obstojni, predvsem na račun izpiranja bazičnih kationov v spodnje plasti. V B_t in BC horizontih prevladuje poliedrična struktura, ki je značilna za kambične horizonte tal, nastalih na apnencih in dolomitih (Vidic in sod., 2015). V O_h/A_h horizontih prevladuje mrvičasta struktura, v A horizontih grudičasta in oreškasta. Spodnji B_t horizonti so običajno gostejši in/ali bolj zbiti, medtem ko so E horizonti drobljivi in manj gosti. Humusno akumulativni horizont je rahel in/ali drobljiv, organski horizont v večini rahel. Novotvorbe v obliki glinenih prevlek se pojavljajo v iluvialnih horizontih, kar je znak za premeščanje gline. Kot posledica kratkotrajnega zastajanja vode se v tleh v B_t horizontih tvorijo Fe in Mn prevleke in konkrecije. Barvni odtenki iluvialnih horizontov so v razponu od 2.5YR do 7.5YR, medtem ko v zgornjih horizontih prevladuje odtenek 10YR. Povprečna barva B_t horizontov je 5YR 4/5, 5YR 4/4 v BC, 10YR 5/5 v E, 10YR 4/3 v A in 10YR 3/2 v O_h/A_h horizontih (Preglednica 4). Diagnostični horizont izpranih tal je glede na kriterije Slovenske klasifikacije tal (Prus in sod., 2015) eluvialni (E) horizont, ki ga prepoznamo po svetlejši barvi. Analiza barv horizontov je potrdila, da so E horizonti svetlejši od ostalih. V B_t horizontih prevladujejo rdečerjavi barvni odtenki, kar lahko razlagamo s prisotnostjo Fe oksidov, ki se kopičijo v iluvialnih horizontih. Skeleta je v izpranih tleh malo. Večinoma se pojavlja le v spodnjih B_t horizontih tal na apnencu in dolomitu, na konglomeratu in na flišnih kamninah.

Preglednica 4: Morfološke lastnosti po skupinah horizontov
Table 4: Morphological characteristics of soil by groups of horizon

Horizont (skupina)	n	Barva*	Struktura	Konzistenca	Novotvorbe
$O_h + A_h$	21	10YR 3/2	mrvičasta	rahla	-
A	50	10YR 4/3	grudičasta, oreškasta	rahla, zelo drobljiva	-
E	58	10YR 5/5	poliedrična, oreškasta	drobljiva, gosta	-
B_t	78	5YR 4/5	poliedrična	gosta, zbita	Fe, Mn prevleke in konkrecije, glinene prevleke
BC	6	5YR 4/4	poliedrična	gosta	

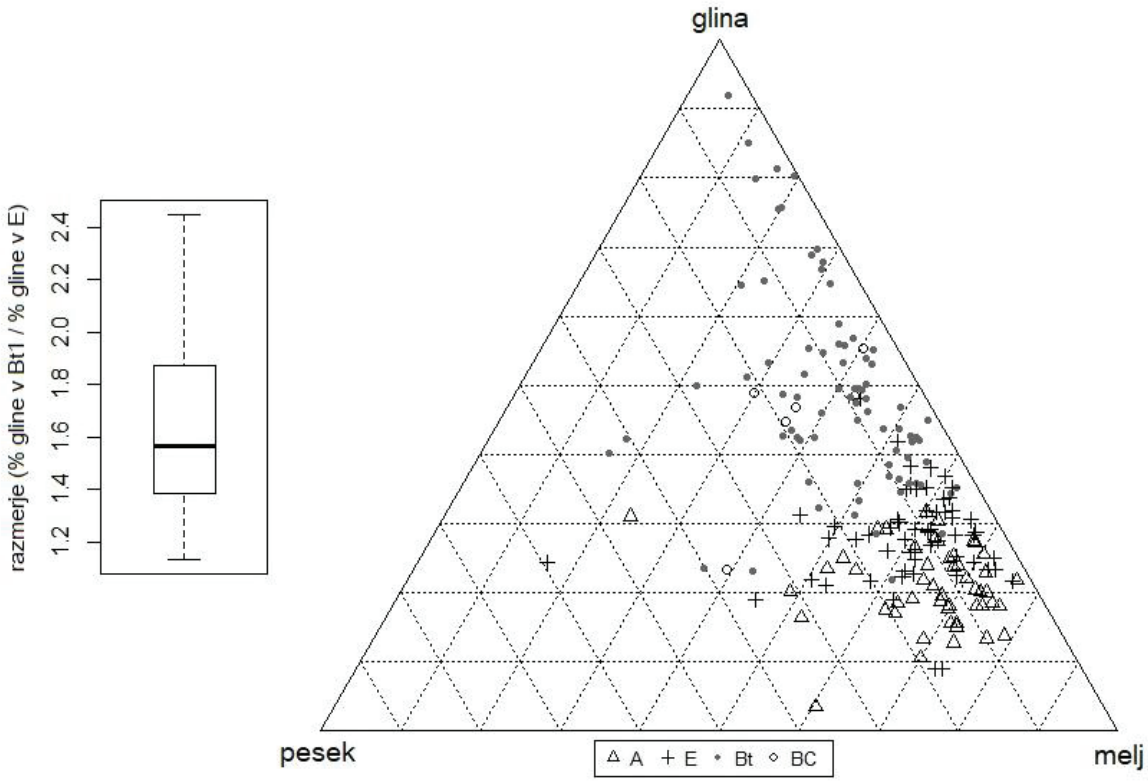
*povprečna barva – tehtano glede na pojavnost in skupno globino, izračunano po Beaudette (2015)

3.4 Kemične in fizikalne lastnosti izpranih tal

Glede na povprečno teksturo profila so izprana tla v večini opredeljena kot ilovnata (n = 27) ali glinasta (n = 18), v štirih primerih meljasta tla. Vsebnost peska v izpranih tleh variira med 1 in 59 %, melja med 5 in 79 % in gline med 9 in 92 %. V vseh profilih se kaže teksturna razlika. B_t in BC horizonti imajo značilno večjo vsebnost gline v primerjavi z E horizonti. Prevladujoči teksturni razredi po skupinah horizontov so prikazani v preglednici 5. Izprana tla so pretežno sestavljena iz gline (< 2 μm) in melja (2-50 μm). V E horizontih je mediana vsebnosti gline 28,4 % in vsebnosti melja 61,4 %, medtem ko je v B_t horizontih mediana vsebnosti gline 46,6 % in vsebnosti melja 42 % (Preglednica 5). Velika vsebnost gline v Bt horizontih je pričakovana, saj se večina profilov nahaja na matičnih podlagah apnenec ali dolomit, kar je značilno tudi za izprana tla na Hrvaškem (povprečje 48.5 %) (Bašić in sod. 2003), na italijanskem krasu (mediana 39 %) (Costantini in sod., 2013) in v Španiji (25–50 %) (Carballas in sod., 2016). Za izprana tla srednje in vzhodne Evrope, ki se pojavljajo na puhlici in drugih sedimentih je značilno več melja in manj gline (Kühn in sod., 2006; Blume in sod., 2016). Slika 5 jasno pokaže teksturno razliko v izpranih tleh. Peska je v tleh relativno malo (mediana < 12,1 %), razen v BC horizontih zaradi vpliva matične podlage. V E horizontu neposredno nad iluvialnim horizontom je povprečna vsebnost gline 28,5 %, v B_t1 46,1 % (Slika 5). B_t1 horizont, ki leži neposredno pod E horizontom, ima v povprečju za 1,63-krat več gline kot E horizont. 75 % tal ima količnik vsebnosti gline med B_t1 in E horizontom večji od 1,38. Najmanjši količnik v vsebnosti gline med B_t in E horizontom za tla, ki so v bazi pedoloških profilov opredeljena kot izprana, je 1,13.

Preglednica 5: Prevladujoči teksturni razredi in mediane vsebnosti peska, melja in gline po horizontih
Table 5: Prevailing textural classes and medians of content of sand, silt and clay by horizon

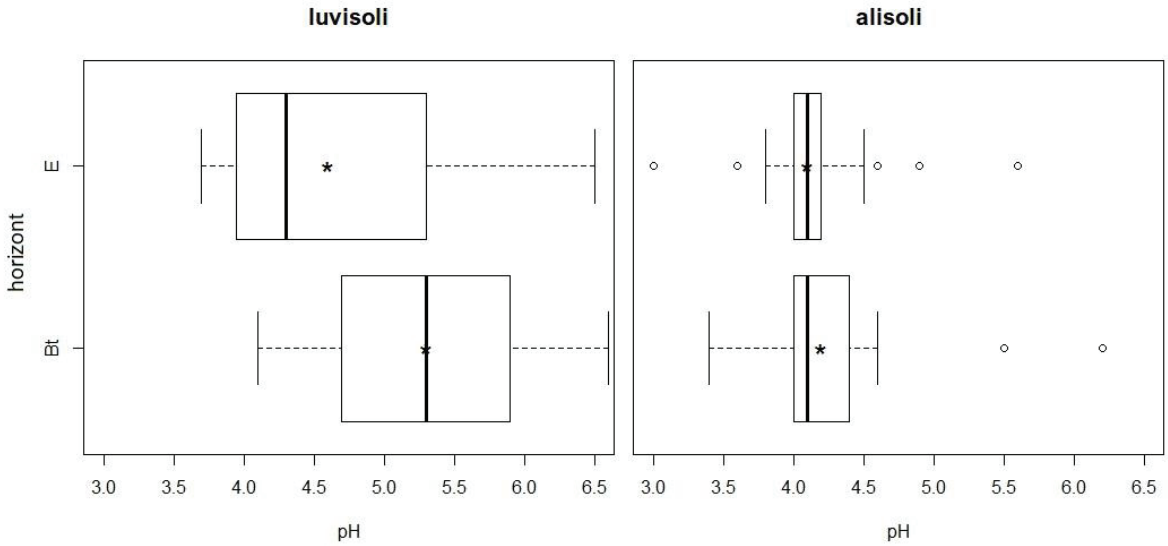
Horizont	Prevladujoči teksturni razredi	Mediana (min–max)		
		pesek (%)	melj (%)	glina (%)
A (n = 49)	MI, MGI	12,1 (1,7 – 45,6)	68,1 (23,4 – 78,9)	20,4 (10,6 – 31,7)
E (n = 58)	MI, MGI, GI	9,1 (2,3 – 59,3)	61,4 (16,3 – 76,0)	28,4 (8,90 – 48,1)
B_t (n = 78)	MGI, MG, G	7,8 (0,5 – 43,7)	42,0 (5,2 – 63,7)	46,6 (21,8 – 92,0)
BC (n = 6)	MG, G	18,2 (4,2 – 37,4)	37,8 (29,9 – 42,7)	47,7 (23,3 – 55,3)



Slika 5: Okvir z ročaji (odebeljena črta = mediana; okvir = kvartil 25 %, 75 %; sp. in zg. ročaj = min, max; krožec = osamelec) za prikaz razmerja vsebnosti gline med B_{t1} in E horizontom (n = 49, levo) in teksturni podatki horizontov izpranih tal, prikazani na ternarnem diagramu (pesek-glina-melj)

Figure 5: The box plot (black thickened line = median; rectangles = quartiles 25 %, 75 %; ranges = min, max; circles = outliers) of the ratio of clay content - B_{t1}/E (n = 49, left) and ternary diagram of textural composition of leached soil (all horizons, right)

pH vrednosti so v razponu od 2,9 do 6,8. Izprana tla imajo običajno nizek pH, pH vrednosti blizu nevtralnega območja zasledimo samo na dnu profila (spodnji B_t horizonti) in v stiku z matično podlago (BC horizonti), kot posledico akumulacije bazičnih kationov in vpliva karbonatne matične podlage; povprečna vrednost pH v BC horizontih je 5,7. V organskih in Ah horizontih je povprečna pH vrednost 4,4, deloma kot posledica surovega humusa, deloma zaradi izpiranja bazičnih kationov, kar je značilno za E horizonte, kjer je povprečna vrednost pH 4,4. V B_t horizontih je povprečna vrednost pH 4,6. Po WRB klasifikaciji (IUUS Working group WRB, 2015) so proučevani profili uvrščeni med luvisole in alisole. Slika 6 prikazuje razlike med B_t in E horizonti znotraj posamezne skupine tal. Za argične horizonte v luvisolih je značilna zasičenost z bazičnimi kationi nad 50 %, medtem ko je zasičenost pri alisolih manjša od 50 %, kar se kaže tudi v razlikah pH vrednosti B_t horizontov med obema skupinama. Očitne so razlike med B_t in E horizonti v luvisolih, kjer argični horizonti še niso tako močno izprani. Pri alisolih je slika drugačna, ker je argični horizont osiromašen bazičnih kationov in ima posledično tudi nižji pH, zato med B_t in E ni večjih sprememb v pH vrednosti (Slika 6).



Slika 6: Okvir z ročaji (odebeljena črta = mediana; okvir = kvartil 25 %, 75 %; sp. in zg. ročaj = min, max; krožec = osamelec) za pH vrednost v E in B_t horizontih pri luvisolih in alisolih, * - povprečna vrednost; luvisoli (n =22), alisoli (n =18)

Figure 6: Boxplots (black thickened line = median; rectangles = quartiles 25 %, 75 %; ranges = min, max; circles = outliers) of pH values in E and B_t horizon in Luvisols and Alisols, sign * present average values; Luvisols (n =22), Alisols (n =18)

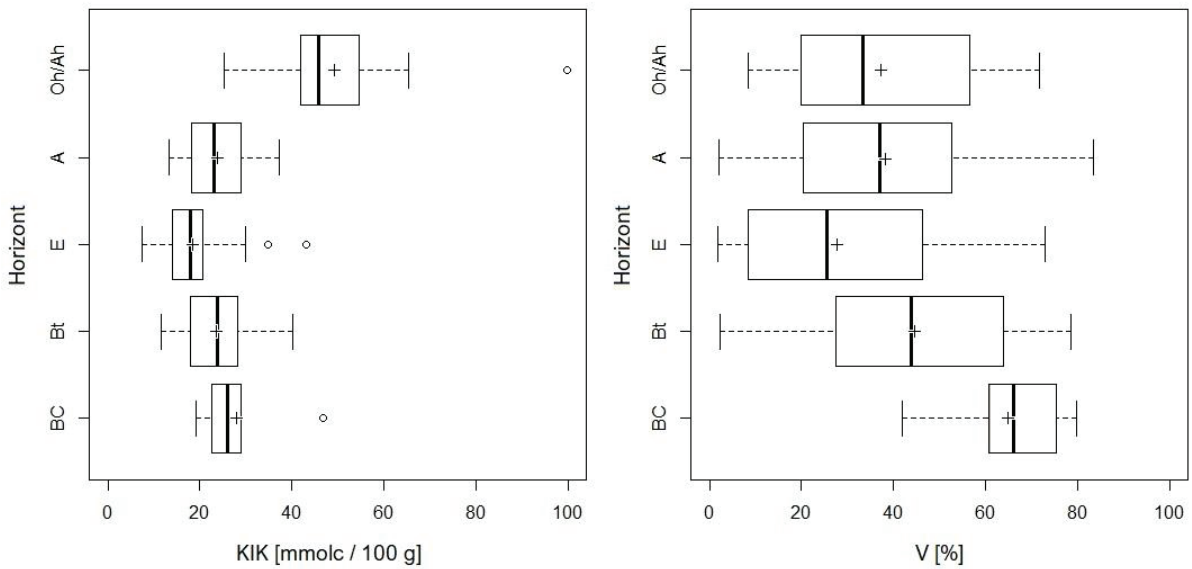
Med izmenljivimi bazičnimi kationi je v tleh največ Ca^{2+} (od 0,1 do 62,3 $mmol_c$ /100 g), sledijo Mg^{2+} (od 0,06 do 9,2 $mmol_c$ /100 g), K^+ (od 0,04 do 62,3 $mmol_c$ /100 g) in Na^+ (od 0,01 do 0,57 $mmol_c$ /100 g) (Preglednica 6). Njihova razporeditev po profilu je za vse bazične katione podobna in značilna za izprana tla (Preglednica 6). Največ bazičnih kationov na sorptivnem delu tal je v organskih in humusno akumulativnih horizontih, bogatih z organsko snovjo (Ćirić, 1984), kot posledica biološkega kroženja (premeščanja) in v BC horizontih, zaradi vpliva karbonatne matične podlage. Izpranost bazičnih kationov se ponekod kaže že v A horizontih, najbolj izrazita je v E horizontih, kjer je delež bazičnih kationov najmanjši (mediana = 4,2 $mmol_c$/100 g). Zaradi akumulacije v nižjih plasteh se vsebnost bazičnih kationov v B horizontih povečajo (B_t: 10,2 $mmol_c$ /100 g, BC: 17,1 $mmol_c$ /100 g).

Kationska izmenjalna kapaciteta v izpranih tleh je v razponu od 7,4 do 100 $mmol_c$/100 g (Slika 7). K sorptivni sposobnosti tal v največji meri prispeva organska snov, količina in vrste glinenih mineralov in kislost tal (Blume in sod., 2016). Horizonti z veliko organske snovi O_h/A_h imajo največjo kationsko izmenjalno kapaciteto. Izprani horizonti imajo zaradi najmanjšega deleža gline najmanjšo kationsko izmenjalno kapaciteto, k temu lahko prispeva tudi nizek pH, ki zmanjšuje količino negativnega naboja železovih in aluminijevih hidroksidov (Weil and Brady, 2017). V iluvialnih horizontih B_t se kationska izmenjalna kapaciteta zopet poveča, kar razlagamo s povečanjem vsebnosti glinene frakcije in/ali z dvigom pH (Preglednica 6, Slika 7). Horizonti v izpranih tleh so večinoma distrični. Najmanjša povprečna nasičenost z bazičnimi kationi je v E horizontih (28,1 %), kar je v skladu s pričakovanji. Povprečna nasičenost z bazičnimi kationi (V) v B_t horizontih je 44,7 %, v BC horizontih 65 %. Razmeroma visoke vrednosti v BC horizontih pripisujemo vplivu matične podlage. Razlike so med luvisoli in alisoli; argični horizonti v luvisolih so evtrični, mediana deleža bazičnih kationov je 64,5 %, medtem ko so pri alisolih distrični, z vrednostjo 32,2 %.

Preglednica 6: Mediane (min–max) vsebnosti izmenljivih bazičnih kationov, izmenljive kislosti in vsote bazičnih kationov po horizontih ($mmol_c$/100 g)

Table 6: Median values (min–max) of exchangeable cations and sum of exchangeable base cations by horizons ($mmol_c$/100 g)

Horizont	Ca^{2+} ($mmol_c$/100 g)	Mg^{2+} ($mmol_c$/100 g)	K^+ ($mmol_c$/100 g)	Na^+ ($mmol_c$/100 g)	H^+ ($mmol_c$/100 g)	Vsota bazičnih kationov
$O_h + A_h$ (n = 18)	14,25 (0,96 – 62,3)	1,94 (0,59 – 7,7)	0,54 (0,12 – 0,9)	0,13 (0,07 – 0,57)	28,90 (12,4 – 41,2)	18,30 (2,3 – 65,8)
A (n = 46)	6,16 (0,20 – 28,1)	0,88 (0,06 – 7,4)	0,16 (0,04 – 0,6)	0,07 (0,01 – 0,33)	12,93 (5,7 – 32,7)	8,05 (0,3 – 29,2)
E (n = 55)	3,42 (0,09 – 20,6)	0,56 (0,07 – 6,4)	0,10 (0,07 – 6,4)	0,05 (0,01 – 0,20)	12,65 (3,2 – 33,4)	4,20 (0,3 – 25,4)
B_t (n = 74)	6,97 (0,10 – 29,5)	1,04 (0,13 – 9,2)	0,17 (0,06 – 0,4)	0,09 (0,01 – 0,19)	12,17 (4,8 – 32,1)	10,20 (0,7 – 30,9)
BC (n = 6)	15,33 (4,76 – 33,0)	2,15 (0,78 – 3,9)	0,26 (0,18 – 0,3)	0,10 (0,08 – 0,17)	9,33 (5,6 – 11,4)	17,10 (8,1 – 37,3)



Slika 7: Okvirja z ročaji (odebeljena črta = mediana; okvir = kvartil 25 %, 75 %; sp. in zg. ročaj = min, max; krožec = osamelec) kationske izmenjalne kapacitete ($mmol_c$/100 g) (levo) ter deleža bazičnih kationov (desno) po horizontih; znak + prikazuje povprečno vrednost

Figure 7: Boxplots (black thickened line = median; rectangles = quartiles 25 %, 75 %; ranges = min, max; circles = outliers) of cation exchange capacity ($mmol_c$ /100 g) and base saturation by horizon; sign + present average values

Gozdna raba tal omogoča bolj ali manj nemoten razvoj tal v smeri procesov izpiranja, medtem ko kmetijska raba z oranjem, gnojenjem in apnenjem zavira in blaži posledice izpiranja. Po pričakovanjih se zato večina, 34 od 49 profilov, izpranih tal nahaja v gozdu. Vrhnji O_h/A_h horizonti imajo povprečno 15 % organskega ogljika, humusno akumulativni horizonti 3,2 %. Z globino se vsebnost organske snovi zmanjšuje. V eluvialnih in iluvialnih horizontih je običajno manj kot 1 % organskega ogljika. V nekaterih iluvialnih horizontih so vrednosti organskega ogljika tudi večje, ponekod nad 2 %, kar je lahko posledica načina vzorčenja; v vzorec se lahko zajame rove korenin ali deževnikov, ki so bogati z organsko snovjo. Podobno razporeditev po globini kaže tudi vsebnost skupnega dušika v tleh. Največje vsebnosti so v O_h/A_h horizontih (mediana 0,78 %) in v A horizontih (mediana 0,22 %), najmanjše v E, B_t in BC horizontih (mediana < 0,11 %). C/N razmerje v zgornjih O_h/A_h horizontih je v povprečju nad 20, kar je značilno za gozdna tla s prhninastim ali surovim humusom. Z globino se razmerje zmanjšuje in je v B_t horizontih povprečno 9,4.

Vsebnosti rastlinam dostopnega fosforja (P_2O_5) in kalija (K_2O) so majhne, kar je značilno za gozdna tla, ki jih ne gnojimo. Podobno kot pri bazičnih kationih na sorptivnem delu tal, tudi pri izmenljivem fosforju in kaliju opazimo obogatitev v O_h/A_h horizontih, ki je posledica biološke migracije in akumulacije. Z globino se vsebnost izmenljivega fosforja zmanjšuje, kar je značilno za vsa tla. Eluvialno-iluvialni procesi pri fosforju niso opazni, kar kaže, da je fosfor dobro vezan na sorptivnem delu tal, predvsem na aluminijevih oksidih in hidroksidih. Vsebnost izmenljivega kalija (K_2O) je večja kot vsebnost fosforja. Izvor izmenljivega kalija v tleh je preperevanje primarnih silikatov in glinenih mineralov, ki s površinskim in medplastovnim negativnim nabojem predstavljajo tudi mesto vezave za kalijeve ione. Razporeditev izmenljivega kalija z globino potrjuje eluvialno-iluvialne procese; najmanjše vsebnosti so v izpranem E horizontu (Preglednica 7).

Preglednica 7: Podatki o organskem ogljiku (%), dušiku (%), C/N razmerju in izmenljivem fosforju in kaliju (mg/100 g tal) po horizontih, mediana (min-max)

Table 7: Data for C_{org} (%), nitrogen (%), C/N ratio and readily available phosphorus and potassium (mg/100 g) by horizons, median (min–max)

Horizon	$C_{org.}$ (%)	N (%)	C/N	P_2O_5* (mg/100 g)	K_2O ** (mg/100 g)
$O_h + A_h$ (n = 17)	15,04 (8,9 – 40,99)	0,78 (0,15 – 1,68)	21,2 (15,5 – 100,2)	10,95 (1,3 – 44,5)	23,8 (8,6 – 71,1)
A (n = 44)	3,23 (0,75 – 8,22)	0,22 (0,07 – 0,6)	14,6 (3,8 – 24,6)	3,15 (0,3 – 31,8)	8,7 (1,4 – 34,2)
E (n = 46)	1,34 (0,29 – 5,7)	0,11 (0,03 – 0,6)	12,6 (0,58 – 28,3)	1,8 (0,1 – 4)	5,0 (1,2 – 12,9)
B_t (n = 48)	0,77 (0,12 – 3,1)	0,08 (0,04 – 0,22)	9,5 (1,93 – 19,3)	0,95 (0,83 – 1,5)	6,75 (4,5 – 9,0)
BC (n = 6)	1 (0,24 – 2,73)	0,09 (0,06 – 0,22)	9,45 (3,96 – 16,28)	0,9 (0,5 – 1,3)	10,45 (10,2 – 11,2)

* $O_h + A_h$ (n = 16), A (n = 32), E (n = 28), B_t (n = 8), BC (n = 2)
** $O_h + A_h$ (n = 16), A (n = 42), E (n = 33), B_t (n = 8), BC (n = 2)

V skladu s pričakovanji obstajajo razlike med izpranimi tlemi z gozdno in kmetijsko rabo. V zgornjih 30 cm izpranih tal s kmetijsko rabo so vrednosti pH in zasičenosti z bazičnimi kationi statistično značilno večje (n = 15, pH = 5, V = 43,3 %), kot pri tleh v gozdu (n = 34, pH = 4,3, V = 25,5 %). Večje vrednosti so lahko posledica gnojenja ali apnenja, v zgornjem delu »travniških tal« so lahko tudi posledica biološke migracije. Izprana tla s stališča kmetijstva veljajo za slabša tla (Stritar, 1984), kar pojasnjuje dejstvo, da večina profilov izpranih tal s kmetijsko rabo leži na travnikih, razen ponekod na Gorenjskem na prodnatih ledenodobnih terasah, kjer pridelujejo krompir (Vidic in sod., 2015). Kühn in sod. (2006) za izprana tla v njivski rabi poročajo o relativno visokih pH vrednostih v evtričnih A_p, E in B_t horizontih (mediana > 6), kar razlagajo s kmetijsko rabo.

4 SKLEPI

Izprana tla pokrivajo 2,3 % Slovenije. Najbolj pogosti matični podlagi, na katerih se pojavljajo v Sloveniji, sta apnenec in dolomit, sledijo sedimenti in konglomerat. V posameznih primerih jih najdemo na nekarbonatnih sedimentnih kamninah, na moreni in na flišnih kamninah. Večina izpranih tal se po WRB klasifikaciji uvršča med luvisole in alisole, kar je primerljivo z Italijo. V Evropi (Soil Atlas of Europe, 2005) se poleg luvisolov, retisolov in alisolov pojavljajo tudi akrisoli, ki pa jih pregled podatkov pedološke karte Slovenije ni potrdil, čeprav smo v dosedanjih študijah (Vidic in sod., 2015) izprana tla v Beli krajini opisovali kot akrična, na osnovi nizke pH vrednosti. V prihodnosti bo potrebno nameniti več pozornosti proučevanju akričnih tal ter upoštevati kriterije WRB klasifikacije glede kationske izmenjalne kapacitete. Eluvialno-iluvialni procesi se kažejo v teksturni razliki; razmerje v deležu gline med B_t in E horizontom je v povprečju 1,63. Eluvialni horizonti imajo v primerjavi z iluvialnimi horizonti nižjo pH vrednost, svetlejšo barvo, manjšo količino bazičnih kationov ter manjšo kationsko izmenjalno kapaciteto. Pri pregledu klasifikacijskih kriterijev za izprana tla smo ugotovili, da v Slovenski klasifikaciji tal (Prus in sod., 2015) ni dovolj natančno opredeljena teksturna razlika. Za diagnosticiranje iluvialnega horizonta bi bilo smiselneje upoštevati merila, ki jih ima

WRB klasifikacijski sistem (IUUS Working group WRB, 2015). Pri izpranih tleh na klastičnih sedimentnih kamninah lahko k teksturni diferenci med horizonti prispeva tudi razlika v granulometrijski sestavi posameznih plasti sedimentnih kamnin. V takih primerih bi procese premeščanja gline lahko nedvoumno dokazali s prisotnostjo glinenih prevlek, kar je bilo v preteklosti pri izkopu profila premalo natančno popisano. V prihodnjih raziskavah bi bilo potrebno več pozornosti usmeriti v mikromorfološki pregled tal.

5 ZAHVALA

Avtorja se zahvaljujeva pedologom in tehničnim sodelavcem vseh generacij, ki so delovali na Katedri za pedologijo in varstvo okolja Biotehniške fakultete in prispevali vzorce ter analitske podatke pedoloških profilov v bazo pedološke karte 1 : 25.000. Za konzultacije se še posebej zahvaljujeva višjemu predavatelju mag. Tomažu Prusu. Hvala tudi obema recenzentoma za kritičen pregled in koristne predloge.

6 VIRI

Arbeitkreis für Bodensystematik der Deutschen Bodenkundlichen Gesellschaft. (1998). *Systematik der Böden und der Bodenbildenden Substrate Deutschlands*. Göttingen: Deutschen Bodenkundlichen Gesellschaft.

Bašić, F. (2013). The soils of Croatia. Dordrecht: Springer. doi:10.1007/978-94-007-5815-5

Beaudette, D. E., Roudier, P., O'Geen, A.T. (2013). Algorithms for quantitative pedology: A toolkit for soil scientists. Computers & Geosciences, 52, 258–268. doi:10.1016/j.cageo.2012.10.020

Beaudette, D. E. (2015). Some ideas on summarizing soil color. R-Forge. Retrieved on https://r-forge.r-project.org/scm/viewvc.php/*checkout*/docs/sharpshootR/aggregate-soil- color.html?root=aqp

Blume, H. P., Brümmer, G. W., Fleige, H., Horn, R., Kandeler, E., Kögel-Knabner, I., Kretzschmar, R., Stahr, K., Wilke, BM. (2016). Scheffer/Schachtschabel soil science. Berlin: Springer. doi:10.1007/978-3-642-30942-7

Bockheim, J. G., Hartemink, A. E. (2013). Distribution and classification of soils with clay-enriched horizons in the USA. Geoderma, 209–210, 153–160. doi:10.1016/j.geoderma.2013.06.009

Camargo, M. N., Klamt, E., Kauffman, J. H. (1986). Soil classification as used in Brazilian soil surveys. In: Annual report 1986 (pp. 7–42). Wageningen: ISRIC.

Carballas, T., Rodríguez-Rastrero, M., Artieda, O., Gumuzzio, J., Díaz-Raviña, M., Martín, Á. (2016). Soils of the temperate humid zone. In J. F. Gallardo (Ed.), The soils of Spain (pp. 49–144). Switzerland, Cham: Springer. doi:10.1007/978-3-319-20541-0_3

Costantini, E. A. C., Barbetti, R., Fantappiè, M., L'Abate, G., Lorenzetti, R., Magini, S. (2013). Pedodiversity. In E. A. C. Costantini & C. Dazzi (Eds.). The soils of Italy (pp. 105–178). Dordrecht: Springer. doi:10.1007/978-94-007-5642-7_6

Ćirić, M. (1984). Pedologija. Sarajevo: Svjetlost.

Digitalni model višin (DMV 100). Ljubljana: Ministrstvo za okolje in prostor, Geodetska uprava RS.

ESRI - Environmental Systems Research Institute. (2014). ArcGIS 10.2.2 for Desktop (software)

Fedoroff, N. (1997). Clay illuviation in Red Mediterranean soils. Catena, 171, 171–189. doi:10.1016/S0341-8162(96)00036-7

IUUS Working group WRB. (2015). International soil classification system for naming soils and creating legends for soil maps. World soil resources reports No. 106. Rome: FAO.

Janousek, V., Farrow, C. M., Erban, V. (2006). Interpretation of whole-rock geochemical data in igneous geochemistry: introducing Geochemical Data Toolkit (GCDkit). Journal of Petrology, 47 (6), 1255–1259. doi:10.1093/petrology/egl013

Kobal, M. (2011). Vpliv sestojnih, talnih in mikrorastiščnih razmer na rast in razvoj jelke (Abies alba Mill.) na visokem krasu Snežnika. Doktorsko delo. Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo: 151 str.

Keys to soil taxonomy. (2014). Washington, United States Department of Agriculture, Natural Resources Conservation Service.

Kralj, T. (2008). Primerjava sistemov za razvrščanje tal na izbranih tleh v Sloveniji. Doktorsko delo. Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo: 151 str.

Kühn, P., Billwitz, K., Bauriegel, A., Kühn, D., Eckelmann, W. (2006). Distribution and genesis of

Fahlerden (Albeluvisols) in Germany. Journal of plant nutrition and soil science, 169, 420–433. doi:10.1002/jpln.200521963

Lavkulich, L. M., Arocena, J. M. (2011). Luvisolic soils of Canada: Genesis, distribution, and classification. Canadian journal of soil science, 91, 781–806. doi:10.4141/cjss2011-014

Nestroy, O., Aust, G., Blum, W.E.H., Englisch, M., Hager, H., Herzberger, E., Kilian, W., Nelhiebel, P., Ortner, G., Pecina, E., Pehamberger, A., Schneider, W., Wagner, J. (2011). Systematische Gliederung der Böden Österreichs. Österreichische Bodensystematik 2000 in der revidierten Fassung von 2011. Wien: Österreichische Bodenkundliche Gesellschaft.

Prus, T., Kralj, T., Vrščaj, B., Zupan, M., Grčman, H. (2015). Slovenska klasifikacija tal. Ljubljana: UL, Biotehniška fakulteta in Kmetijski inštitut Slovenije.

Quénard, L., Samouëlian, A., Laroche, B., Cornu, S. (2011). Lessivage as a major process of soil formation: A revisitation of existing data. Geoderma, 167-168, 135–147. doi:10.1016/j.geoderma.2011.07.031

R Development Core Team. (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved on http://www.R-project.org/

Shaetzl, R., Anderson, S. (2005). Soils - Genesis and geomorphology. Cambridge, Cambridge university press. doi:10.1017/CBO9780511815560

Soil Atlas of Europe. (2005). Luxembourg: European Soil Bureau Network, European Commission.

Soil Survey Staff. (1992). Soil survey laboratory methods manual. Ver. 2.0. USDA/NRCS, Soil Survey Investigations Report No. 42. U.S. Government. Printing Office, Washington, DC.

Stritar, A. (1972). Mesto vrištine u klasifikaciji zemljišta Jugoslavije – 2. dio. Zemljište i biljka, 21, 429–436.

Stritar, A. (1984). Pedologija: (kompendij). Ljubljana: Partizanska knjiga.

Stritar, A. (1990). Krajina, krajinski sistemi; Raba in varstvo tal v Sloveniji. Ljubljana: Partizanska knjiga.

Sušin, J. (1964). Doprinos k poznavanju terre rosse v Slovenskem primorju. Doktorsko delo. Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo: 106 p.

Škorič, A., Filipovski, G., Čirič, M. (1985). Klasifikacija zemljišta Jugoslavije. Sarajevo: Akademija nauka i umjetnosti Bosne i Hercegovine.

TIS/ICPVO – Infrastrukturni center za pedologijo in varstvo okolja, Biotehniška fakulteta, Univerza v Ljubljani, Ljubljana 1999–2017 (Talni informacijski system Slovenije).

Turniški, R. (2016). Pedološke, mineralne in geokemične lastnosti izpranih tal v Sloveniji. Magistrsko delo. Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo: 55 p.

Urbančič, M., Simončič, P., Prus, T., Kutnar, L. (2005). Atlas gozdnih tal Slovenije. Ljubljana: Gozdarski inštitut Slovenije.

Vidic, N. (1989). Geokemija tal na terasah reke Save. Magistrsko delo. Univerza v Ljubljani, Fakulteta za naravoslovje in tehnologijo, VTO Montanistika, Odsek za geologijo: 58 p.

Vidic, N. J., Lobnik, F. (1997). Rates of soil development of the chronosequence in the Ljubljana Basin, Slovenia. Geoderma, 76, 35–64. doi:10.1016/S0016-7061(96)00098-5

Vidic, N., Prus, T., Grčman, H., Zupan, M., Lisec, A., Kralj, T., Vrščaj, B., Rupreht, J., Šporar, M., Suhadolc, M., Mihelič, R., Lobnik, F. (2015). Tla Slovenije s pedološko karto v merilu 1:250.000. Luksemburg: Publications office of the European Union.

Vrščaj, B., Repe, B., Simončič, P. (2017). The soils of Slovenia. Dordrecht: Springer. doi:10.1007/978-94-017-8585-3

Weil, R. R., Brady, N. C. (2017). The nature and properties of soils, 15th edition. Harlow: Pearson Education.

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Potrditev prisotnosti plenilske hržice *Feltiella acarisuga* (Vallot, 1827) in plenilskega kratkokrilca *Oligota oviformis* Casey, 1893 na navadni pršici (*Tetranychus urticae* Koch, 1836) v Sloveniji

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IZVLEČEK

Navadna pršica, *Tetranychus urticae* C. L. Koch, 1836 je eden najpomembnejših škodljivcev zavarovanih prostorov pri nas in v svetu. Zaradi svoje polifagnosti in hitrega razmnoževanja so populacije pršic velike, zato ima tudi veliko naravnih sovražnikov, ki jih lahko uporabimo v biotičnem varstvu proti temu škodljivcu. Poleg številnih plenilskih pršic (*Phytoseiulus persimilis* Athias-Henriot, 1957, *Amblyseius swirskii* Athias-Henriot, 1962,…) je izredno uspešen plenilec navadne pršice tudi plenilska hržica *Feltiella acarisuga* (Vallot, 1827), ki smo jo v letu 2017 množično zasledili v rastlinjaku Biotehniške fakultete v Ljubljani na jajčevcu sorte 'Matrona'. Poleg plenilske hržice smo zaznali tudi veliko število plenilskih hroščev kratkokrilcev in njihovih ličink iz družine Staphylinidae, vrsta *Oligota oviformis* Casey, 1893 Vrsto *F. acarisuga* se lahko tudi uvrsti na seznam domorodnih vrst organizmov in se jo uporablja v biotičnem varstvu, saj je v tujini že vrsto let med vodilnimi organizmi za varstvo gojenih rastlin pred pršicami.

Ključne besede: biotično varstvo; *Feltiella acarisuga*; *Oligota oviformis; Tetranychus urticae;* zavarovani prostor

ABSTRACT

CONFIRMATION OF PRESENCE OF A PREDATORY GALL MIDGE, *Feltiella acarisuga,* (Vallot, 1827) AND STAPHYLINID PREDATOR *Oligota oviformis* Casey, 1893 OF A TWO SPOTTED SPIDER MITE (*Tetranychus urticae,* Koch, 1836) IN SLOVENIA

The two spotted spider mite, *Tetranychus urticae* C. L. Koch, 1836 is one of the most important pests of greenhouse crops worldwide. Due to its polyphagic range of hosts and rapid development it forms great populations and as such represents a suitable host/prey for lots of natural enemies usable in biological control. Most commonly used predators of Tetranychid mites are predatory mites (*Phytoseiulus persimilis* Athias-Henriot, 1957, *Amblyseius swirskii* Athias-Henriot, 1962 ,…), but among most voracious predators is the larva of a predatory gall midge, *Feltiella acarisuga* (Vallot, 1827) that was found also in greenhouses of the Biotechnical Faculty in Ljubljana on eggplant leaves in 2017. Besides the predatory gall midge also another predator, staphylinid *Oligota oviformis* Casey, 1893 beetles and larvae were found in great numbers. After positive identification of *F. acarisuga* found naturally in Slovenia, it can be added to the list of indigenous species of natural enemies and thus can be used in biological control programs in greenhouse crop protection against spider mites.

Key words: biological control; *Feltiella acarisuga*; greenhouse; *Oligota oviformis*; *Tetranychus urticae*

1 UVOD

Navadna (fižolova ali hmeljeva) pršica *Tetranychus urticae* Koch, 1836 (Acari, Tetranychidae) je eden najpomembnejših škodljivcev gojenih rastlin v zavarovanih prostorih in na prostem (Opit in sod., 2004). Tako odrasli osebki kot tudi nimfe se s sesanjem hranijo na rastlinah in povzročajo večjo škodo. Hiter razvoj, velik razmnoževalni potencial in pogosta raba akaricidov vodijo do hitrega razvoja odpornosti na

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fitofarmacevtska sredstva (Xiao in sod., 2013). Biotično varstvo je alternativna možnost omejevanja populacij škodljivih organizmov z uporabo naravnih sovražnikov. Poleg plenilskih pršic, ki lahko zmanjšajo populacije navadne pršice (Amano, 2001; Escudero in Ferragut, 2005), so izredno učinkovite tudi plenilske žuželke, še posebej se izpostavlja plenilsko hržico *Feltiella acarisuga* (Vallot, 1827), ki je tudi tržno dostopna (Gagné, 1995). Pogosto se omenja štiri rodove plenilskih žuželk, in sicer *Oligota* (Coleoptera: Staphylinidae), *Stethorus* (Coleoptera: Coccinellidae), *Scolothrips* (Thysanoptera, Thripidae) in *Feltiella* (Diptera; Cecidomyiidae), ki uspešno plenijo navadno pršico (Abe in sod., 2011; Shimoda in sod. 2015). Dva od njih, *Stethorus* in *Feltiella* sta tudi uvrščena na seznam tujerodnih vrst organizmov za biotično varstvo rastlin (UVHVVR, 2017).

V Sloveniji zakonodaja na področju varstva rastlin zahteva, da je za vnos in uporabo tujerodnih vrst organizmov na območju Republike Slovenije potrebno pridobiti dovoljenje Uprave za varno hrano, veterinarstvo in varstvo rastlin, ki se izda v soglasju z ministrstvom, pristojnim za ohranjanje narave (UVHVVR, 2017). Zato si prizadevamo najti in potrditi čim več organizmov iz seznama tujerodnih vrst, da so naravno prisotni na območju Slovenije in jih tako uvrstiti na Seznam domorodnih vrst, ki se jih lahko uporablja v okviru biotičnega varstva pri nas.

1.1 Plenilska hržica *Feltiella acarisuga* (Vallot, 1827)

Odrasla hržica meri do 2 mm v dolžino, ima svetlo rjavo do oranžno barvo telesa in dolge noge. Imago živi 12-14 dni in se hrani z nektarjem rastlin. Samica izleže okrog 33 drobnih prozornih jajčec (0.25 mm) iz katerih se razvijejo žerke, ki imajo 3 stopnje (se dvakrat levijo) in dosežejo dolžino do 3 mm ter so tipične rdečkasto-oranžne barve (Slika 1). Zabubijo se v svilen zapredek. Ličinke plenilske hržice so ene najpomembnejših naravnih sovražnikov navadne pršice in drugih pršic prelk iz družine Tetranychidae (Gagné, 1995; Osborne in sod., 2017).

Slika 1: Žerka plenilske hržice (vir: https://www.mindenpictures.com/search/preview/predatory-midge-larve-fetiella-acarisuga-larva-red-after-feeding-on-red/0_80113713.html, 1.12.2017)
Figure 1: The predatory gall midge larva (source: https://www.mindenpictures.com/search/preview/predatory-midge-larve-fetiella-acarisuga-larva-red-after-feeding-on-red/0_80113713.html, 1.12.2017)

1.2 Plenilski kratkokrilec *Oligota oviformis* (Casey, 1893)

Vrsta *O. oviformis* pripada rodu *Oligota*, ki je majhna skupina kratkokrilcev iz poddružine Aleocharinae in zajema okoli 300 vrst. Vrste iz tega rodu so majhni hroščki, ki merijo povprečno le 1.0 mm (Williams, 1976). Vrsta *O. oviformis* je zanimiva, saj tako odrasli osebki kot tudi ličinke plenijo navadno pršico in so tako koristni z agronomskega stališča (Moore in sod., 1975). Imagi vrste *O. oviformis* so majhni hroščki črne barve z značilno kratkimi pokrovkami. Običajno hodijo s pokonci dvignjenim zadkom. Njihova ličinka je podolgovata, meri okoli 2.1 mm v dolžino in ima črno liso na osmem členu zadka (Slika 2).

Slika 2: Ličinka vrste *O. oviformis* na listu jajčevca (foto F.A. Celar)
Figure 2: Predatory *O. oviforms* larva on eggplant leaf (photo F.A. Celar)

2 MATERIALI IN METODE

Konec meseca septembra 2017 smo v rastlinjakih Biotehniške fakultete v Ljubljani našli velike populacije navadne pršice na jajčevcu sorte 'Matrona' (*Solanum melongena* L.). Napadene liste jajčevca smo pobrali in v laboratoriju s pomočjo stereolupe iskali morebitne naravne sovražnike (Olympus SZ30). Ličinke in odrasle osebke plenilskih hržic smo poslali v identifikacijo dr. Raymondu J. Gagné (Taxonomic Services Unit, USDA-ARS-Systematic Entomology Laboratory, ZDA), vrsto *O. oviformis* pa smo potrdili s pomočjo identifikacijskih ključev in opisov (Moore in sod., 1975; Williams, 1976).

3 REZULTATI IN DISKUSIJA

Na listih jajčevca smo našli številne plenilske žuželke. Med njimi so bile tudi ličinke in odrasli osebki plenilskih stenic (*Orius* sp.), vendar pa je bilo največ ličink plenilske hržice, vrste *F. acarisuga*, katero nam je potrdil tudi strokovnjak za identifikacijo hržic iz družine Cecidomyiidae dr. R.J. Gagné, in ličink ter odraslih osebkov kratkokrilcev *O. oviformis*. Plenilska hržica *F. acarisuga* velja, poleg na pršice specializirane plenilske polonice iz rodu *Stethorus,* za najpomembnejšo plenilko pršic prelk. Odrasle hržice so tudi dobre letalke, imajo dobro sposobnost iskanja plena in velik potencial hranjenja na vseh razvojnih stadijih pršic. Razširjene so po večini kontinentov (kozmopoliti), plenijo pa lahko tudi druge pršice prelke. V laboratorijskih poskusih so Xiao in sod. (2013) dokazali da so ličinke hržice *F. acarisuga* uničile tudi do 50 jajčec pršic na dan, medtem ko so jih plenilske pršice uničile približno pol manj (25 jajčec samice *Phytoseiulus persimilis* (Anthias-Henriot, 1957) in 15 *Amblyseius swirskii* (Anthias-Henriot, 1962)). Ravno na jajčevcu je Sharaf (1984) opazil naravno prisotnost plenilske hržice, ki je zmanjšala populacijo pršic tudi preko 40 %. Vsaka žerka naj bi zaužila dnevno vsaj 15 odraslih pršic, 30 različnih razvojnih stadijev nimf ali po 80 jajčec. Razvoj hržice se odvija pri temperaturnem razponu od 15-25 °C. Jajčeca in ličinke so občutljive na visoke temperature (nad 30 °C) in suh zrak (relativna zračna vlaga pod 30 %). Optimalne razmere za njen razvoj so 20 °C in 90 % relativna zračna vlaga (Gillespie in sod. 1998).

V biotičnem varstvu je priporočljiva tudi kombinirana uporaba koristnih organizmov in pravočasen vnos. Ker je plenilska hržica leteča, lažje išče svoj plen na večje razdalje kot pa plenilske pršice (npr. *P. persimilis)*, vendar je priporočljivo uporabiti obe vrsti hkrati, če je plena dovolj. Če plena primanjkuje pa lahko plenilske pršice plenijo tudi jajčeca hržice in tako zmanjšajo učinkovitost kombiniranega biotičnega varstva (Gillespie in sod., 1998).

4 SKLEPI

Potrjujemo prisotnost dveh pomembnih plenilcev navadne pršice v Sloveniji, to sta plenilska hržica *F. acarisuga* in plenilski kratkokrilec *O. oviformis*. Obe vrsti sta bili močno zastopani in upamo, da nam bosta tudi v prihodnje pomagali pri omejevanju širjenja navadne pršice, oziroma pri zmanjševanju njenih populacij brez uporabe fitofarmacevtskih sredstev. Nepravilna in pogosta uporaba akaricidov lahko vodi do odpornosti pršic na tovrstna fitofarmacevtska sredstva. Naravni sovražniki nam lahko, ob pravočasni uporabi in v primernih razmerah, pomagajo, da na naraven način zatremo škodljivca in preprečimo razvoj odpornosti na akaricide. S pogosto uporabo insekticidov s širokim spektrom delovanja za zatiranje žuželk v rastlinjakih lahko močno zmanjšamo ali pa popolnoma uničimo favno občutljivih koristnih organizmov. Potrditev vrste *F. acarisuga* nam omogoča uvrstitev tega organizma na seznam domorodnih vrst koristnih organizmov v Sloveniji in njegovo uporabo, saj je v tujini že vrsto let tržno dostopen (Biobest, Koppert,…).

5 ZAHVALA

Najlepše se zahvaljujemo dr. Raymond J. Gagné (Taxonomic Services Unit, USDA-ARS-Systematic Entomology Laboratory, ZDA) za identifikacijo plenilske hržice.

6 VIRI

Abe, J., Ganaha-Kikumura, T., & Yukawa, J. (2011). Morphological features, distribution, prey mites, and life history traits of *Feltiella acarisuga* (Vallot) (Diptera: Cecidomyiidae) in Japan. *Applied Entomology and Zoology, 46*, 271-279. https://doi.org/10.1007/s13355-011-0038-x

Amano, H. (2001). Species structure and abundance of invertebrate natural enemies in sustainable agroecosystems. V: M. Shiyomi, H. Koizumi (Ur.), *Structure and Function in Agroecosystem Design and Management*, FL, USA (pp. 167-181) CRC Press LLC.

Escudero, L.A., & Ferragut, F. (2005). Life-history of predatory mites *Neoseiulus californicus* and *Phytoseiulus persimilis* (Acari: Phytoseiidae) on four spider mite species as prey, with special reference to *Tetranychus evansi* (Acari: Tetranychidae). *Biological Control, 32,* 378-384. https://doi.org/10.1016/j.biocontrol.2004.12.010

Gagné, R.J. (1995). Revision of Tetranychid (Acarina) mite predators of the genus *Feltiella* (Diptera: Cecidomyiidae). *Annals of the Entomological Society of America, 88*, 16-30. https://doi.org/10.1093/aesa/88.1.16

Gillespie, D.R., Roitberg, B., Basalyga, E., Johnstone, M., Opit, G., Rodgers, J., & Sawyer, N. (1998). Biology and application of *Feltiella acarisuga* (Vallot) (Diptera: Cecidomyiidae) for biological control of twospotted spider mites on greenhouse vegetable crops. *Pacific Agri-Food Research Centre (Agassiz) Technical Report, No. 145*. Agriculture and Agri-Food Canada.

Moore, I., Legner, E.F., & Badgley, M.E. (1975). Description of the developmental stages of the mite predator, *Oligota oviformis* Casey, with notes on the osmeterium and its glands (Coleoptera: Staphylinidae). *Psyche, 82,* 181-188. https://doi.org/10.1155/1975/67561

Opit, G.P., Roitberg, B., & Gillespie, D.R. (1997). The functional response and prey preference of *Feltiella acarisuga* (Vallot) (Diptera: Cecidomiidae) for two of its prey: male and female two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychiidae). *Canadian Entomologist*,*129*, 221–227. https://doi.org/10.4039/Ent129221-2

Opit, G.P., Nechols, J.R., & Margolies, D.C. (2004). Biological control of twospotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), using *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseidae) on ivy geranium: assessment of predator release ratios. *Biological Control, 29*, 445-452. https://doi.org/10.1016/j.biocontrol.2003.08.007

Osborne, R.S., Kumar, V., Leppla, N., & Osborne, L.S. (2016). Predatory gall midge (unofficial common name) scientific name: *Feltiella acarisuga* (Vallot) (Insecta: Diptera: Cecidomyiidae). http://entnemdept.ufl.edu/creatures/beneficial/f_acarisuga.htm

Sharaf, N.S. (1984). Studies on natural enemies of tetranychid mites infesting eggplant in the Jordan Valley. *Zeitschrift für Angewandte Entomologie, 98,* 527-533. https://doi.org/10.1111/j.1439-0418.1984.tb02745.x

Shimoda, T., Kobori, Y., Yara, K., & Hinomoto, N. (2015). A simple method of rearing insect natural enemies of spider mites. *Biological Control, 80*, 70-76. https://doi.org/10.1111/j.1439-0418.1984.tb02745.x

UVHVVR. 2017. Seznam tujerodnih vrst organizmov za biotično varstvo rastlin. http://www.uvhvvr.gov.si/fileadmin/uvhvvr.gov.si/pageuploads/DELOVNA_PODROCJA/Zdravje_rastlin/2013/Bioticno_varstvo_rastlin/tujerodni_organizmi/Seznam_tujerodnih_23_maj_2017_NOVO.pdf

Willims, S.A. (1976). The genus *Oligota* (Coleoptera: Staphylinidae) in New Zeland. *New Zeland Journal of Zoology, 3*(3), 247-255. https://doi.org/10.1080/03014223.1976.9517914

Xiao, Y., Osborne, L.S., Chen, J., & McKenzie, C.L. (2013). Functional Responses and Prey-Stage Preferences of a Predatory Gall Midge and Two Predacious Mites with Twospotted Spider Mites, *Tetranychus* Urticae, as Host. *Journal of Insect Science, 13*(8), 1-12. https://doi.org/10.1673/031.013.0801

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Interlaboratory comparison of fig (*Ficus carica* L.) microsatellite genotyping data and determination of reference alleles

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ABSTRACT

Microsatellites have been identified as the marker of choice in plant genotyping projects. However, due to length discrepancies obtained between different laboratories for the same allele, interlaboratory comparison of fingerprinting results is often a difficult task. The objectives of this study were to compare genotyping results of two laboratories, to evaluate genetic parameters of microsatellite markers and to determine reference allele sizes for fig cultivars from the Istrian peninsula.

Genotyping results of ninety fig (*Ficus carica* L.) accessions were comparable between the laboratories despite differences observed when comparing electropherograms of different capillary electrophoresis systems. Differences in lengths of the same alleles were detected due to different PCR methods and laboratory equipment, but the distances between alleles of the same locus were preserved. However, locus FSYC01 exhibited one allele dropout which led to misidentification of 28 heterozygotes as homozygote individuals suggesting this locus as unreliable. Allele dropout was assigned to the tail PCR technology or to a touchdown PCR protocol.

Genotypes of twenty-four reference cultivars from the Istrian peninsula were confirmed by both laboratories. These results will contribute to the usage of markers with greater reliability, discrimination power and consequently, to more reliable standardization with other fig genotyping projects.

Key words: microsatellite marker; reference genotype; interlaboratory comparison; *Ficus carica* L.

IZVLEČEK

MEDLABORATORIJSKA PRIMERJAVA REZULTATOV GENOTIPIZACIJE FIGE Z MIKROSATELITSKIMI MARKERJI (*Ficus carica* L.) IN DOLOČITEV REFERENČNIH ALELOV

Mikrosateliti so se izkazali kot zelo uporabni markerji pri genetskih raziskavah rastlin. Zaradi odstopanj dolžin enakih alelov v različnih laboratorijih, je primerjava rezultatov med laboratoriji pogosto težavna. Namen raziskave je bil primerjati rezultate genotipizacije dveh laboratorijev, ovrednotiti genetske parametre mikrosatelitskih markerjev in določiti dolžine referenčnih alelov za sorte fig istrskega polotoka.

Rezultati genotipizacije devetdesetih vzorcev fige (*Ficus carica* L.) so bili primerljivi med laboratorijema, kljub razlikam, ki smo jih opazili pri primerjavi elektroferogramov različnih sistemov kapilarnih elektroforez. Razlike med dolžinami enakih alelov med laboratorijema so bile odkrite zaradi različnih metod PCR in analitske opreme, vendar pa so bile razlike med aleli istega lokusa ohranjene. Pri lokusu FSYC01 smo ugotovili izpad alela, kar je privedlo do napačne identifikacije; namesto 28 heterozigotov smo posameznike določili kot homozigote. Ugotovljena lastnost nakazuje na nezanesljivost lokusa FSYC01. Izpad alela smo pripisali uporabi ekonomične metode PCR ali uporabi protokola PCR s postopnim nižanjem temperature prileganja začetnih oligonukleotidov.

Genotipi štiriindvajsetih referenčnih sort istrskega polotoka so bili potrjeni v obeh laboratorijih. Rezultati raziskave bodo prispevali k uporabi bolj zanesljivih mikrosatelitskih markerjev, z večjo močjo razlikovanja in posledično k zanesljivi standardizaciji rezultatov z drugimi genetskimi raziskavami fige.

Ključne besede: mikrosatelitski marker; referenčni genotip; medlaboratorijska primerjava; *Ficus carica* L.

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1 INTRODUCTION

The integration of DNA molecular marker technology into fingerprinting studies of agricultural plants is extremely widespread and has become a standard procedure. The techniques rely on independence from environmental factors and phenotype stage of the plant under investigation and are thus complementary to traditional approaches that often include laborious morphological evaluations.

Microsatellite markers combine several properties of an ideal molecular marker including high polymorphism in the number of tandem repeats, co-dominant inheritance, abundance in genome, excellent reproducibility and ease of use. They are also considered as a marker of choice in plant genetic research for many applications (e.g. diversity studies, paternity testing, mapping and fingerprinting studies) (Nybom et al., 2014). The employment of fluorescently labeled microsatellite markers in genotyping procedures significantly improves the throughput, automation and lowers the error rate (Wenz et al., 1998). However, the use of microsatellites can be costly due to the high price of fluorescent tags which must be carried by one of the primers in the primer pair. To overcome this problem, an inexpensive and flexible procedure was introduced with the three primer protocol incorporating the addition of modified locus specific primer and the universal fluorescent-labelled M13 (-21) primer (Schuelke, 2000). This method was used in multiple genotyping projects (Bandelj et al., 2004; Kyung-Ho et al., 2009; Mandel et al., 2011; Soriano et al., 2011) and it was recognized as a good economic alternative to conventional method.

Simple numerical output makes microsatellite technology very attractive for exchanging data among laboratories and for the establishment of global genotyping databases (De Valk et al., 2009), but several authors discuss the problem of consistency of microsatellite genotyping data in different laboratories and suggest standardization procedures for allele sizing (Cryer et al., 2006; De Valk et al., 2009; Deemer & Nelson, 2010; Jones et al., 2008; Vemireddy et al., 2007). Variation in results among laboratories could be due to human factors, differing methodologies, technological limitations, poor DNA quality or locus specific properties, since some microsatellite markers are more prone to errors and produce more stutters (Deemer & Nelson, 2010; Doveri et al., 2008; Ellis et al., 2011; Sutton et al., 2011; This et al., 2004). Genotyping errors are often neglected even though they affect the data and can markedly influence the biological conclusions (Pompanon et al., 2005).

Studies which compare genotyping results between laboratories have been performed on olive cultivars (*Olea europaea* L.) (Baldoni et al., 2009; Doveri et al., 2008) and grapevines cultivars (*Vitis vinifera* L.) (This et al., 2004), while on figs (*Ficus carica* L.) which are recognized as underutilized fruit species, no such study has been published yet. With development of fig microsatellite markers (Ahmed et al., 2007; Bandelj et al., 2007; Giraldo et al., 2005; Khadari et al., 2001), they have been successfully used for genotyping fig genetic resources in Spain (Balas et al., 2014; Giraldo et al., 2008), Turkey (Caliskan et al., 2012), Tunisia (Abdelkrim et al., 2015; Chatti et al., 2010), France (Khadari, 2012), Morocco (Achtak et al., 2010; Khadari et al., 2005), California (Aradhya et al., 2010), Japan (Ikegami et al., 2009), and Egypt (Abou-Ellail et al., 2014). According to Zohary & Spiegel-Roy (1975) the existing genetic diversity of figs reflects their domestication process, their complex pollination biology, exchange of cultivars between the growing regions, clonal propagation, and the coexistence of wild, feral, and cultivated forms in natural and agro ecosystems. A common germplasm database to support fig research should be available in order to solve the confusion in naming varieties (synonyms, homonyms), support management of fig genetic resources with genetic tools across all growing regions, and to facilitate the exchange of genotyping data among different laboratories. Several properties of figs as agricultural products support this need: (1) the economic potential of fig fruits (figs are widely cultivated in North African and Middle Eastern countries, where they represent a significant source of agricultural income); (2) the nutritional value and functional properties (high antioxidant content (Solomon et al., 2006), rich fibre content, vitamins, and minerals (Vinson et al., 2005)); and (3) special pollination biology (mutualism with fig wasps, different sexual systems, distinct flower formation, parthenocarpy, and the existence of all these forms in fig production).

In order to provide comparable results among fig genotyping projects and further evaluation of fig germplasms worldwide, our objectives in the present study are: (1) to compare the genotyping results of two laboratories with expertise in fig genotyping generated by different PCR methodologies employing either a conventionally labeled primer with fluorescent label in each primer pair or the economic method described by Schuelke (2000); (2) to publish reference allele sizes for local figs cultivars from the Istrian peninsula (the North-east Adriatic coast); and (3) to discuss diversity parameters of selected microsatellite loci for use in cultivar identification and fig genetic resources investigations.

2 MATERIAL AND METHODS

2.1 Plant material

Ninety fig accessions were analyzed in this study. Eighty-four accessions were provided by the Slovenian research group (University of Primorska, Koper - SI in continuation) including 60 accessions collected from the North and East Adriatic coast (hereafter referred to as feral or wild forms) and 24 cultivars from the Istrian Peninsula. Six accessions (four wild samples and two cultivars) from the Mediterranean ex situ collection in the Porquerolles Island (southern France) were provided by the French research group (INRA, Montpellier – FR in continuation).

2.2 DNA extraction

The SI research group extracted genomic DNA from leaves of 84 fig accessions by a modified cetyl trimethylammonium bromide (CTAB) method following the procedure described by Kump & Javornik (1996). The FR research group extracted genomic DNA from leaves of six accessions with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the supplier's instructions and minor modification described by Achtak et al. (2010). DNA concentration was determined using the Invitrogen Qubit® Fluorometer (Turner Biosystems, Sunnyvale, CA, USA) and the Qubit dsDNA BR Assay Kit (Molecular Probes, Thermo Fisher Scientific, Carlsbad, CA, USA) by SI researchers and spectrofluorometry (GENios Plus, TECAN, Grödig, Austria) by the FR research group. Dilutions of DNA with a concentration of 50 ng/µl were prepared and exchanged between research groups. Both research groups analysed the same DNA of 90 accessions as described in the following sections.

2.3 Microsatellite assay

Six primer pairs from different sets of the developed microsatellites have been selected for the genotyping procedure: MFC1, MFC2, MFC3 (Khadari et al., 2001), MFC9 (Khadari B., Hochu I., Santoni S., unpublished data), LMFC30 (Giraldo et al., 2005) and FSYC01 (Ahmed et al., 2007). According to each group's laboratory equipment and preferences for different chemicals various individual strategies for optimization and generalization of PCR conditions were employed. In general, the FR group used a conventional method with each primer pair labeled with the required dye, while the SI group used the economic three-primer method developed by Schuelke (2000). PCR and electrophoresis conditions for individual microsatellite locus are summarized in Table S1and Table S2. Primer sequences used for conventional and economic methods are listed in Table S3.

2.4 Data analysis

The software packages GeneMapper version 3.7 (FR) and 4.1 (SI) (Applied Biosystems, Foster City, CA, USA) were used for determination of allele sizes, peak intensities (in relative fluorescence units, rfu), banding patterns, and number of amplified alleles per primer pair. SPSS (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.) were used to illustrate differences of peak balance (also peak height ratio or heterozygote balance). Peak balance was calculated for each heterozygous combination (at least five individuals per allele combination) according to Method #2, developed by Leclair et al. (2004), and defined as the ratio of peak height of the longer allele over that of the shorter allele. For comparison of allele sizes standard deviation and range were calculated for each marker using Microsoft Excel (2010).

Genetic parameters were calculated for 90 samples over all six analyzed microsatellite loci. Expected heterozygosity (H_e), observed heterozygosity (H_o), probability of identity (PI), polymorphic information content (PIC) and test for deviation from Hardy-Weinberg equilibrium (HWE) across all loci (chi-square (χ^2) test, p-value was assessed using Bonferroni correction (Kalinowski et al., 2007)) using the CERVUS 3.0.7 and Identity 1.0 (Wagner & Sefc, 1999) programs. Frequency of null alleles (F_{null}) was calculated with FreeNA (Chapuis & Estoup, 2007) and N_e was computed using GenAlEx 6.5 (Peakall & Smouse, 2006, 2012). The mean error rate per locus ($e_l = m_l / nt$) was calculated as ratio between m_l, the number of single-locus genotypes including at least one allelic mismatch, and *nt*, the number of replicated single-locus genotypes (Pompanon et al., 2005).

The identification of the minimum number of markers required to distinguish all the observed multilocus genotypes was performed with the AMaCAID program written in the R language, using model one (Caroli et al., 2011).

3 RESULTS

3.1 Visual and morphological characterization of amplified alleles

In order to assess visual characteristics of alleles amplified in both laboratories caused by instrument resolution, peak signal strength and peak morphology were examined. Altogether 33 different alleles were identified over six microsatellite loci in both laboratories.

The shape of the peaks and number of stutter bands were nearly the same for all alleles regardless of the methods used in each laboratory. The exceptions were the alleles of the LMFC30 locus, produced by the economic method, which exhibit more stuttering and additional n+1 peak (where n indicates allele length), and at the MFC9 locus where higher stutter bands were observed. Alleles of the FSYC01 locus exhibited single stutter band in the FR laboratory, while the procedure in the SI lab yielded no stuttering but showed n+1 peak (Figure 1).

The peak signal strength for each locus resulting from two different amplification techniques showed noticeable differences with much lower intensity values recorded in the SI laboratory (Table 1).

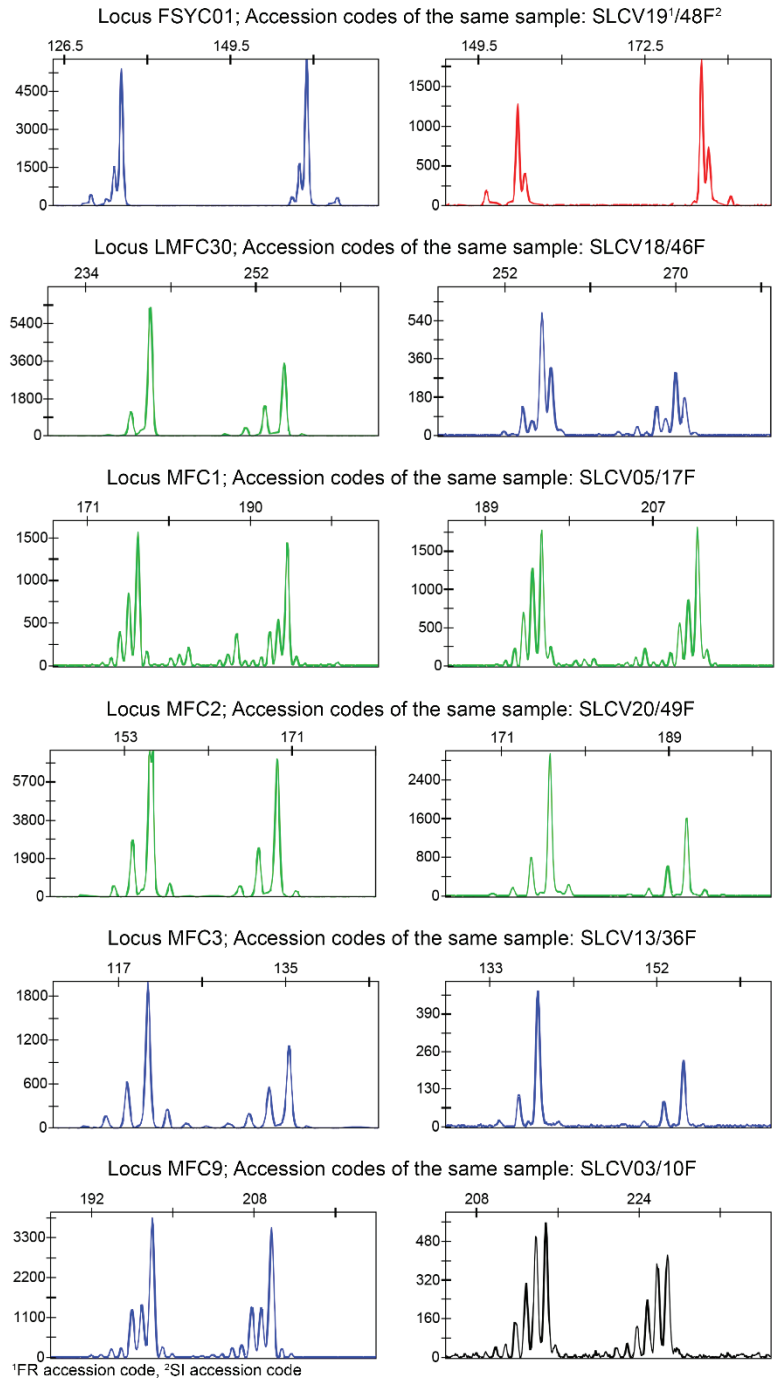

Figure 1: Allele patterns observed in the French research group (FR), on the left side, and in the Slovenian research group (SI), on the right side, at each locus. Heterozygous samples were chosen in order to present two alleles per sample.

Slika 1: Prikaz oblik alelov za vsak lokus, glede na elektroferograme francoske raziskovalne skupine (FR), prikazane na levi strani, in slovenske raziskovalne skupine (SI), prikazane na desni strani.

Table 1: Variability parameters of six microsatellite loci, studied on 90 fig (*Ficus carica* L.) accessions, and parameters linked to polymerase chain reaction conditions and electrophoresis systems. Parameters of microsatellite loci are identical for both laboratories, except for locus FSYC01, where discrepancy in homozygote and heterozygotes was found.

Preglednica 1: Parametri variabilnosti šestih mikrosatelitskih lokusov, testiranih na 90 vzorcih fige (*Ficus carica* L.) ter parametri, odvisni od verižne reakcije s polimerazo in sistemov kapilarnih elektroforez. Parametri mikrosatelitskih lokusov so enaki pri obeh laboratorijih, razen pri lokusu FSYC01, pri katerem smo odkrili neujemanje rezultatov pri določanju homozigotov in heterozigotov.

Locus	n	Ne	Ho	He	PIC	PI	F_{null}	HWE	Average intensity values (in rfu units)		Standard deviation of peak intensity values	
									FR group	SI group	FR group	SI group
FSYC01	5	2.17 / 1.73^{SI}	0.544 / 0.256^{SI}	0.539 / 0.423^{SI}	0.506 / 0.397^{SI}	0.311 / 0.409^{SI}	0.00000 / 0.13976^{SI}	0.4285 / 0.0002 *** ^{SI}	5186	2110	2087	737
LMFC30	8	5.25	0.833	0.810	0.782	0.118	0.00007	0.0603	5383	298	1605	176
MFC1	4	3.25	0.600	0.693	0.633	0.273	0.05099	0.0053*	2748	2215	1561	905
MFC2	5	2.76	0.667	0.683	0.571	0.331	0.00386	0.4867	6526	2068	1396	1022
MFC3	7	2.99	0.700	0.666	0.628	0.225	0.00000	0.4654	1912	269	813	128
MFC9	4	2.48	0.600	0.598	0.534	0.353	0.00003	0.4617	4569	605	1457	267
Combined PI for all loci						$2.66x10^{-4}$ / $3.50x10^{-4}$ ^{SI}						
Average	5.5	3.15 / 3.07^{SI}	0.657 / 0.609^{SI}	0.664 / 0.645^{SI}	0.609 / 0.590^{SI}							

n (number of alleles), Ne (effective number of alleles), Ho (observed heterozygosity), He (expected heterozygosity), PIC (polymorphic information content), PI (probability of identity), F_{null} (frequency of null alleles), HWE (Hardy-Weinberg equilibrium), ^{SI} (Calculated for Slovenian genotyping data), rfu (relative fluorescent values), * and ***: $p < 0.05$ and $p < 0.001$ (chi-square test, significance with Bonferroni correction)

The peak balance value was compared between laboratories to see the influence that distance between alleles in a heterozygote has on peak balance. In general, intensities were higher in shorter alleles for the majority of comparisons (peak balances lower than one). At two loci, LMFC30 and MFC3, the peak balance value was decreasing when the difference in allelic lengths was increasing (Figure 2). A similar pattern with similar peak balance values was observed in both the SI and FR laboratories.

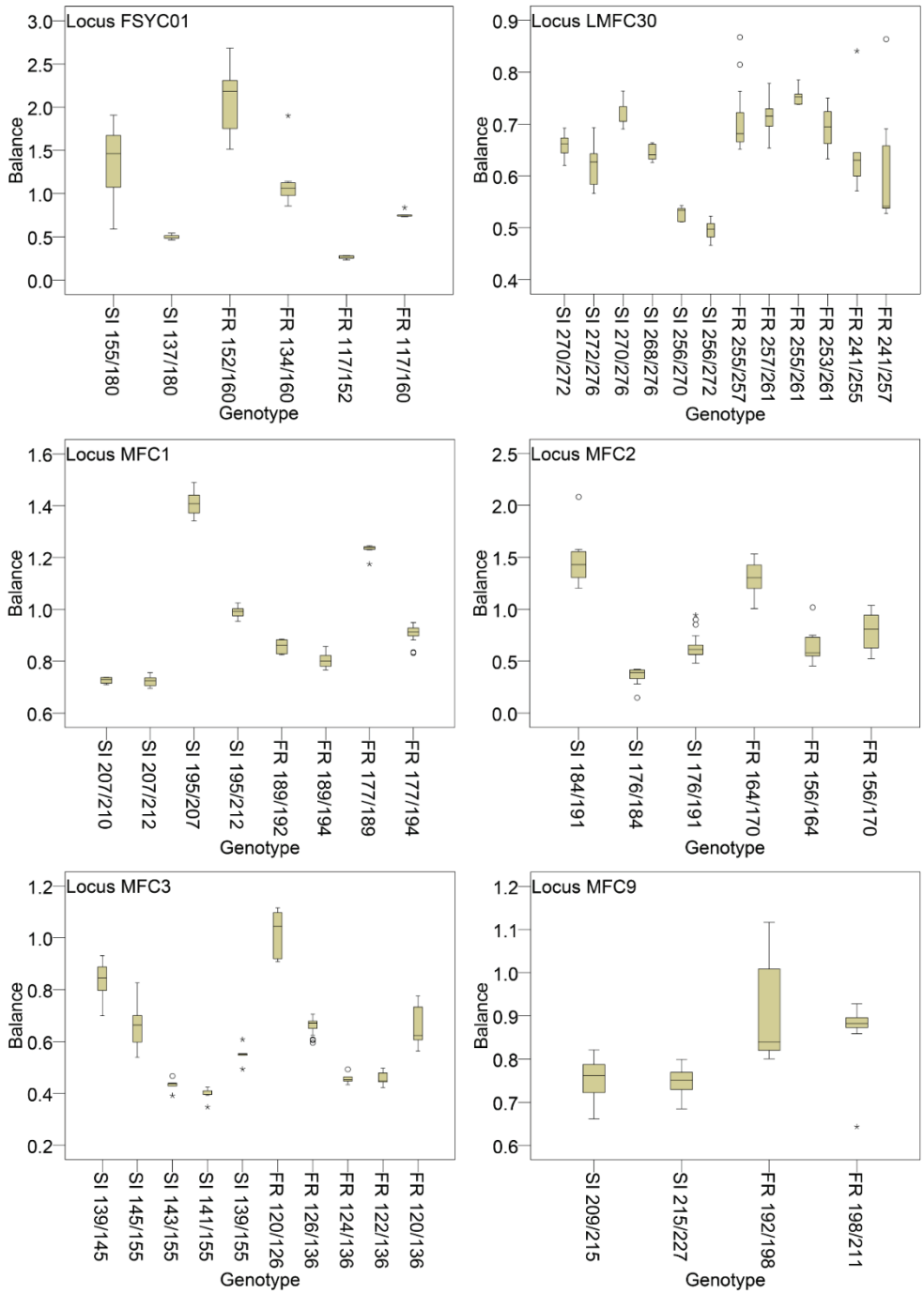

Figure 2: Box plots of peak balance values calculated for the Slovenian research group (SI) and French research group (SI) laboratories. Heterozygous samples with the same allele combinations were grouped together.

Slika 2: Prikaz vrednosti razmerij med intenziteto fluorescentnega signala daljšega in krajšega alela pri heterozigotih z okvirji z ročaji, na osnovi rezultatov slovenske (SI) in francoske (FR) raziskovalne skupine. Heterozigotni vzorci z enako kombinacijo alelov so uvrščeni v isto skupino.

3.2 Comparison of allele lengths among laboratories

Actual allele sizes determined by the GeneMapper software were sorted according to their length to determine the groups of alleles which differ by less than 1 bp. Alleles were also manually reviewed and final sizes were rounded to the nearest full number representing the final called allele length. For easier comparison of genotypes between laboratories letters were also assigned to alleles, as suggested by Doveri et al. (2008), where A represents the shortest allele of the locus (Table 2). As expected, sizes of alleles between the laboratories differ (from 14.65 bp to 21.44 bp for actual lengths and 15 bp to 21 bp for called allele lengths) due to the distinct PCR technology, dye analysis matrices and internal standards used in analyses. The differences were consistent between alleles of the same locus.

The range between the minimum and maximum allele lengths were calculated as the simplest measure of variability. The highest difference of 1.02 bp was observed at locus LMFC30 in FR data for allele 261 bp (H), while for SI data difference of 0.71 bp was encountered at locus MFC1 for allele 212 (D). The range between actual allele sizes within the allele were relatively low with an average of 0.33 bp and 0.23 bp for the FR and SI teams, respectively.

Further comparison showed that the standard deviations for actual sizes of individual alleles were relative low, but varied among teams. Standard deviations were between 0.021 to 0.405 for alleles genotyped by the FR team, while somewhat lower standard deviations have been calculated for alleles scored by the SI team and were between 0.005 and 0.151 (Table 2).

Table 2 List of alleles with average actual size, letter designation, total number of individual alleles used for calculation (n), standard deviation (SD), range, differences between average actual sizes obtained by Slovenian (SI) and French (FR) research group, differences between actual sizes after the removal of elongated primer sequence M13 (-21) and differences between allele sizes rounded to nearest integer with and without primer elongation sequences.

Preglednica 2: Seznam alelov s parametri: povprečna dejanska dolžina, črkovna oznaka, število alelov vključenih v izračun parametrov (n), standardni odklon (SD), variacijski razmik, razlika med povprečno dejansko dolžino določeno v slovenski (SI) in francoski (FR) raziskovalni skupini, razlika med dejansko dolžino po odstranjenem nukleotidnem zaporedju M13 (-21) začetnega oligonukleotida ter razlika med zaokroženimi vrednostmi dolžin na celo število z in brez podaljšanega zaporedja.

Locus	Allele	Average actual allele sizes	Letter designation	n	SD	Range	Differences in actual sizes	Differences in actual sizes after removal 18 or 17 bp	Differences in called allele sizes with and without primer elongation sequences
	FR group / SI group	FR group / SI group			FR group / SI group	FR group / SI group			
FSYC01	117 / 137	117.44 / 136.90	A	19	0.05 / 0.03	0.24 / 0.14	19.45	2.45	20 / 3
	126 / 146	126.04 / 146.02	B	7	0.03 / 0.06	0.10 / 0.13	19.98	2.98	20 / 3
	134 / 155	134.46 / 154.99	C	18	0.03 / 0.07	0.13 / 0.23	20.53	3.53	21 / 4
	152 / 172	151.78 / 172.51	D	2	0.07 / 0.00	0.30 / 0.00	20.72	3.72	20 / 3
	160 / 180	160.13 / 180.38	E	134	0.10 / 0.05	0.55 / 0.23	20.25	3.25	20 / 3
LMFC30	231 / 246	230.45 / 245.73	A	3	0.04 / 0.005	0.08 / 0.01	15.28	1.71	15 / -2
	239 / 254	238.80 / 253.60	B	4	0.09 / 0.06	0.20 / 0.15	14.80	2.20	15 / -2
	241 / 256	240.86 / 255.68	C	30	0.14 / 0.06	0.51 / 0.25	14.82	2.17	15 / -2
	247 / 262	246.76 / 261.74	D	3	0.40 / 0.07	0.75 / 0.14	14.98	2.02	15 / -2
	253 / 268	253.13 / 267.79	E	21	0.07 / 0.05	0.26 / 0.20	14.65	2.34	15 / -2
	255 / 270	255.07 / 269.77	F	35	0.11 / 0.05	0.52 / 0.21	14.70	2.29	15 / -2
	257 / 272	257.13 / 271.81	G	46	0.08 / 0.05	0.42 / 0.28	14.68	2.31	15 / -2
	261 / 276	261.24 / 275.91	H	38	0.15 / 0.04	1.02 / 0.22	14.66	2.33	15 / -2
MFC1	177 / 195	176.90 / 194.99	A	69	0.10 / 0.05	0.43 / 0.22	18.08	1.08	18 / 1
	189 / 207	189.49 / 207.00	B	44	0.08 / 0.05	0.29 / 0.21	17.51	0.51	18 / 1
	192 / 210	192.42 / 209.78	C	13	0.10 / 0.04	0.33 / 0.15	17.36	0.16	18 / 1
	194 / 212	194.37 / 211.64	D	54	0.17 / 0.12	0.99 / 0.71	17.27	0.24	18 / 1
MFC2	156 / 176	155.83 / 176.25	A	67	0.20 / 0.05	0.77 / 0.31	20.42	3.42	20 / 3
	158 / 178	157.76 / 178.31	B	7	0.17 / 0.09	0.46 / 0.24	20.55	3.55	20 / 3
	164 / 184	164.15 / 184.14	C	16	0.08 / 0.04	0.25 / 0.15	19.99	2.99	20 / 3
	166 / 186	166.24 / 186.15	D	7	0.07 / 0.04	0.21 / 0.12	19.91	2.91	20 / 3
	170 / 191	169.51 / 190.96	E	83	0.18 / 0.04	0.71 / 0.23	21.44	4.44	21 / 4
MFC3	104 / 123	104.30 / 123.33	A	1	0.00 / 0.00	0.00 / 0.00	19.03	1.03	19 / 1
	120 / 139	120.23 / 138.54	B	17	0.04 / 0.04	0.10 / 0.15	18.31	0.31	19 / 1
	122 / 141	122.42 / 140.94	C	16	0.05 / 0.05	0.17 / 0.19	18.51	0.51	19 / 1
	124 / 143	124.61 / 143.37	D	13	0.07 / 0.04	0.24 / 0.18	18.75	0.75	19 / 1
	126 / 145	126.43 / 145.43	E	38	0.04 / 0.08	0.18 / 0.49	18.99	0.99	19 / 1
	136 / 155	135.47 / 155.08	F	92	0.05 / 0.06	0.23 / 0.52	19.61	1.61	19 / 1
	142 / 162	141.87 / 161.60	G	2	0.02 / 0.00	0.03 / 0.00	19.72	1.72	20 / 2
MFC9	192 / 209	192.08 / 208.68	A	48	0.12 / 0.15	0.38 / 0.51	16.59	1.40	17 / -1
	198 / 215	197.97 / 214.75	B	99	0.05 / 0.04	0.28 / 0.25	16.77	1.22	17 / -1
	204 / 221	203.81 / 220.77	C	3	0.07 / 0.11	0.14 / 0.22	16.96	1.04	17 / -1
	211 / 227	209.70 / 226.65	D	30	0.05 / 0.08	0.24 / 0.45	16.94	1.05	16 / -1

3.3 Genotyping discrepancy

In total, twenty-eight discrepancies (2.6 %) were observed and contributed only by heterozygous/homozygous misreadings. All genotyping discrepancies were detected at locus FSYC01; in all cases heterozygous genotypes were determined in the FR laboratory and homozygotes in the SI laboratory. In eighteen samples short allele dropout cases were observed: heterozygotes were genotyped with profile DE (152:160) and homozygotes with profile EE (180:180). Long allele dropout and amplification of the short allele only was observed in ten samples from three different genotypes: 1) heterozygotes: AD (117:152), homozygotes: AA (137:137)); 2) heterozygotes CD (134:152), homozygotes: CC (155:155); 3) heterozygotes: BD (126:152); homozygotes: BB (146:146). Common to all cases, was the failure of amplification of the D allele (152 bp / 172 bp), although in the homozygous state, this allele was normally amplified in both laboratories. Due to the detected discrepancies at this locus, the calculated mean error rate for FSYC01 was 0.1556. On the other five loci, no allelic mismatches were discovered.

3.4 Discriminatory power of microsatellite loci

In order to estimate the discriminatory power of the loci used in the study, several variability parameters were calculated; the number of alleles, H_o, H_e, PI and PIC. All six microsatellite loci were polymorphic, revealing a total of 33 alleles with an average number of 5.5 alleles and an average of 3.1 effective alleles per locus (Table 1). The highest number of alleles (eight) was amplified on locus LMFC30, seven alleles were characteristic to locus MFC3, five alleles were found on loci MFC2 and FSYC01, and four alleles were characteristic to loci MFC9 and MFC1. Only one taxon specific allele A (104 bp (FR) / 123 bp (SI)) was found on locus MFC3 and was characteristic to the LBS16 fig genotype. In general, the number of effective alleles was relatively low, indicating that rare and frequent alleles are present in the examined population of samples. The highest number of effective alleles (5.25) was observed at locus LMFC30, where the frequencies of alleles were equally distributed.

At two loci, MFC3 and MFC9, three alleles were observed in the cultivar 'Belica' (SI / FR accesion code: 19F / SLCV06). At MFC3 the third allele length was 97 bp (FR) / 117 bp (SI). Since this allele was discovered only at this accession, it was discarded. At MFC9 all three alleles (SI allele lengths 209 / 215 / 227 bp; FR allele lengths 192 / 198 / 211 bp) were identified more than once, therefore we decided to exclude the longest allele from this analysis.

Expected heterozygosity varied between 0.539 (FSYC01) and 0.810 (LMFC30), with an average of 0.664. Similar values were obtained for observed heterozygosity, and were between 0.544 (FSYC01) and 0.833 (LMFC30).

The observed heterozygosity was higher than expected on four loci (MFC3, MFC9, LMFC30, and FSYC01 at the FR laboratory), showing an excess of heterozygotes, while excess of homozygotes was found on loci MFC1 and MFC2. An excess of homozygotes and a statistically significant deviation between expected and observed heterozygosity was also noted at locus FSYC01 ($\chi^2 = 14.35$ (using Yates correction), $p < 0.001$) calculated for the SI data set, where allele D (152 bp / 172 bp) was not amplified and thus influences the variability statistics of this locus. Statistically significant deviation from HWE was observed for MFC1 as well ($\chi^2 = 12.73$ (using Yates correction), $p < 0.05$). As expected, the frequencies of null alleles for FSYC01 SI data and for locus MFC1 were higher due to the deviation from HWE. Since the null allele frequencies of MFC1 and FSYC01 for the SI data were between 0.05 and 0.2, both loci were classified into the moderate class (Chapuis & Estoup, 2007), while the null allele frequency calculated for other loci were negligible ($F_{null} < 0.05$).

Calculated PIC values were in a range from 0.506 to 0.782 and classified all loci as informative markers (PIC > 0.5) and locus LMFC30 as suitable for mapping (PIC > 0.7). Regarding the probability of identity, the highest values were observed on loci MFC2, MFC9, and FSYC01. The minimum PI value (0.118) was calculated for loci LMFC30. The overall probability that the two samples in our study share the same genetic profile by chance was 2.66×10^{-4} (calculated for the FR data).

3.5 Fingerprinting and identification of reference cultivars

The genotyping data of twenty-four Istrian cultivars over five microsatellite loci are presented in Table S4, however due to the high error rate of FSYC01 (0.1556) it is excluded from the table. Altogether, 27 different alleles were amplified over five loci in a set of 24 cultivars. The molecular analysis demonstrated the existence of 17 different genotypes. Microsatellite loci used in this study allowed discrimination of 11 cultivars, the remaining 13 were indistinguishable due to identical DNA profiles observed between five pairs of cultivars and one triple: 'Bela Petrovka' / 'Črna Petrovka', 'Črnica' / 'Rovinj', 'Pinčica' / 'Zelenka', 'Termenjača' / 'Zuccherina', 'Vodenjača' / 'Bružetka bela' and 'Cikulina' / 'Kanora' / 'Grška črna'.

Analysis of discriminatory power of each combination of *k* markers among *n* available with AMaCAID program revealed that all 17 Istrian genotypes could be distinguished with only three loci (LMFC30, MFC1, and MFC9). With locus LMFC30 nine genotypes could be discriminated, while with loci LMFC30 and MFC1 and on the other hand with loci LMFC30, MFC1 and MFC9, 16 and 17 genotypes could be discriminated, respectively.

4 DISCUSSION

For successful evaluation of fig genetic resources and estimation of actual diversity of genotypes grown in specific geographical regions, the development and evaluation of proper genotyping protocols and construction of a database of the fig DNA profiles is necessary. This work requires collaboration between research groups and establishment of standardized protocols for the generation of easily comparable and interchangeable genotyping results.

In the present study we compared microsatellite genotyping data of fig trees generated in two different laboratories using their own protocols with the aim of comparing the data and the suitability of the used fig microsatellite loci for genotyping purposes. Ninety fig samples representing cultivars, feral, and wild figs were included in the analysis and genotyping was performed at six microsatellite loci proven to be suitable for discrimination of fig samples and genotyping cultivars (Ahmed et al., 2007; Giraldo et al., 2005; Khadari et al., 2001).

To introduce as much experimental variation as possible, each laboratory was allowed to optimize its own PCR condition and amplification protocols with their preferred supplier of chemicals (Table S1 and Table S2).

4.1 Comparison of genotyping results

Since instrument sensitivity is extremely important for interpretation, poor signal strength can result in poor morphology and potential for errors in sizing (Koumi et al., 2004). With the aim to assess similarity of electropherograms of the SI and FR groups, a comparison of peak morphology, signal strength, and peak balance were performed (Table 1, Figure 1, Figure 2). The lower signal intensity obtained by the SI group in comparison with the FR group may be due to the different PCR amplification protocols, different electrophoresis settings (e.g., injection time) and fluorescent dyes used for microsatellite labelling. Use of different fluorescent dyes has a strong impact on the results due to their different relative intensity values. Lower intensity dyes are also associated with the three-primer protocol (Culley et al., 2013), where part of the amplified fragments remains unlabelled.

Lower fluorescent values did not have influence on the proper allele calling step of the SI laboratory electropherograms. The differences observed at the electropherograms allelic patterns between the SI and the FR laboratory did not influence genotyping either, since results were comparable and the distances between alleles of the same locus were consistent.

Peak balances of different allele pairs per each locus was comparable between laboratories, despite the different fluorescence values. Comparable peak balances were also obtained by Koumi et al. (2004), where they analysed comparability of the results of STR multiplex AmpFLSTR™ SGMplus™ (Thermo Fisher Scientific) (multiplex assay for human identification applications) between three different electrophoresis instruments (ABI 377, ABI 3700, ABI 3100).

Peak balance can be used in genetic studies as a threshold for determining two heterozygous alleles as a possible genotype, where values of 50 % or 60 % are typically used (calculated by dividing the weaker intensity allele peak height by the stronger intensity allele peak height) (Butler, 2014). Debernardi et al. (2011) observed that a threshold at 60 % to be too stringent when analysing genotypes, obtained with AmpFLSTR™ Identifiler™ STR kit (Thermo Fisher Scientific), while in our study even a threshold at 50 % would be too stringent at loci FSYC01, LMFC30, MFC2 and MFC3. However, at loci MFC1 and MFC9 a threshold at 60 % could be applied.

Lower peak balance values indicate favourable amplification of the shortest allele. This phenomenon was most noticeable at loci LMFC30 and MFC3 with greater differences between short and long allelic combinations in heterozygous individuals. Such phenomenon can lead to a dropout effect of the longest allele (Tvedebrink et al., 2012), which is contributed by non-amplification of the allele. Analysis of peak balance in plant SSR genotyping studies is not the practice, but according to our opinion, it could improve the genotyping process because it helps to identify samples with larger deviations from the median and these should be checked once again with greater caution.

4.2 Comparison of allele length

Comparison of the allele lengths (after removal of 17 or 18 bp from the SI called allele lengths) showed differences between 2 bp and 4 bp which are in the range of previously reported investigations. This et al. (2004) have compared microsatellites of grape cultivars obtained from different laboratories, and mostly similar alleles were obtained, in some cases the raw data of identical alleles differed by as much as 5 bp. The differences are mainly contributed due to the use of different dyes, which contributes different molar weights to the final PCR products and due to the use of different molecular standards.

Standard deviation values of allele lengths were low, indicating that the sizing of identical alleles was very reproducible; differences among research groups could be assigned to different platform technologies used in the analysis. Differences in allele size are observed even if the same allele is repeatedly typed by the same CE machine (Pasqualotto et al., 2007). Very similar results have been obtained by Haberl & Tautz (1999) in comparative allele sizing of microsatellites of honey bees (0.05 - 0.17).

4.3 Genotyping discrepancies

Altogether twenty-eight discrepancies were observed, but all were a consequence of FSYC01 locus D allele dropout. We assume that this is an experimental problem, associated with the PCR protocol due to the tailed primers creating conditions that encourage competition between alleles and prevent amplification of some alleles. Different amplification temperature profiles, i.e. touchdown protocol used by SI group could also influence the amplification of the D allele, since increased specificity allowed by touchdown PCR protocol could cause allele dropout due to polymorphism in primer-binding sites (Mullins et al., 2007).

However, since long-allele dropout was observed in one sample, provided by FR group with DNA extracted with Dneasy Plant Mini Kit (Qiagen), we assume that different DNA extraction methods did not influence the D allele dropout, although it is known that different methods of DNA extraction can cause different results (Benjak et al., 2006).

According to Pompanon et al. (2005) error rates between 0.5 % and 1 % are common in many laboratories. In our study, this measure was calculated for locus FSYC01 only, where the allelic dropout was the main cause of error. Due to the high mean error rate (15.56 %) associated with locus FSYC01 it should be considered as error-prone and thus its use in identification studies is unreliable.

4.4 Diversity parameters of selected microsatellite loci

Six microsatellite loci used in this study were chosen based on their confirmed discriminatory power and ease of scoring as observed in previous studies. In the study of Achtak et al. (2009) selected loci demonstrated higher discriminating power, while a combination of loci LMFC30, MFC2, MFC3, FSYC01 and MFC9 was able to discriminate all 75 accessions (except one pair) from Moroccan fig collection. All six loci exhibited higher discriminating power in the analysis of 277 cultivated trees from Morocco (Achtak et al., 2010).

Selected microsatellite loci were tested on a genetically diverse plant material, which was confirmed with high He values (from 0.598 to 0.810). Similar values for the selected loci were obtained from the several fig collections including: the ELGO ‘Demeter’ collection in Greece (with accessions from different Mediterranean countries) (Ganopoulos et al., 2015), local Turkish fig accessions (Caliskan et al., 2012), and a Morrocan fig collection (Achtak et al., 2009). Lower values were obtained by Abdelkrim et al. (2015) when analysing wild and cultivated Tunisian figs.

According to the diversity parameters all microsatellite loci analyzed in this study (except FSYC01, which was identified as unreliable) are suitable for fig cultivar characterization. They exhibit high PIC values (from 0.534 to 0.782), which classified them as informative molecular markers. The total probability of identity calculated with five SSR loci (without locus FSYC01) was 8.47×10^{-4} and it is comparable with other fig fingerprinting studies. Giraldo et al. (2005) obtained a total PI of 6.8×10^{-4} when analyzing 209 fig accessions from Spain with 11 microsatellite loci. In a previously mentioned study of Achtak et al. (2009) calculated total PI for six loci, that were able to discriminate all 75 accession, was 2.3×10^{-4}. However, due to the high mean error rate observed at locus FSYC01, we suggest replacing it in future sets of microsatellite markers.

At two loci, MFC3 and MFC9, three alleles were observed in the cultivar ‘Belica’. Amplification of more than two alleles in diploid agricultural plants is rather common due to conservation of sequences through the eukaryotic genome and the presence of duplicated loci. Cipriani et al. (2002) have found 17 % out of 30 developed loci in olive cultivars to amplify two different loci. In fig trees, Giraldo et al. (2008) reported amplification of more than two markers in some genotypes on 45 % of tested loci. Such loci are usually discarded from analysis or they are marked as only partially suitable for comparison studies, but since in our study third allele was observed only in one sample

both loci were recognized as reliable for fig genotyping studies.

4.5 Reference allele sizes for Istrian cultivars

Genotyping results of 24 reference cultivars from the Istrian peninsula, confirmed by the SI and FR research groups, can serve as a reference for identification purposes, fig collection management, and for standardising fig genotyping projects from the Balkan and surrounding regions.

Using the selected set of five microsatellite loci eleven out of twenty-four cultivars were distinguishable. The possibility of synonyms for 'Cikulina', 'Kanora' and 'Grška črna' / 'Termenjača' and 'Zuccherina' / 'Vodenjača' and 'Bela Bružetka' was already observed with a different set of loci of FCUP series (Bandelj et al., 2007, 2008). Interestingly, using the selected microsatellite primers in this study we were not able to distinguish between 'Pinčica' and 'Zelenka', or 'Črnica' and 'Rovinj', which were already discriminated with loci FCUP44-6 and FCUP66-7 or FCUP62-2 and FCUP66-7, respectively (Bandelj et al., 2008). The varieties 'Bela Petrovka' and 'Črna Petrovka' were not discriminated on a DNA level, while it is known that they are different varieties, since 'Bela Petrovka' produces fruits with green skin and 'Črna Petrovka' produces fruits with brown green skin.

A minimum set of loci (LMFC30, MFC1, MFC9) was determined to be sufficient to identify all 17 genotypes among the 24 reference cultivars examined in this study. This set of loci can be used for preliminary screening of fig genetic resources, while for discrimination of all 24 reference cultivars additional microsatellite loci should be utilized.

5 CONCLUSIONS

The analysis performed in this study showed that the comparability of allele sizes between two laboratories was very good and deviations in allele sizes were in the expected range, although different PCR technology, chemicals, and laboratory machinery were used. Coding alleles with letters, after the results standardisation, simplified genotypes comparisons. The published allele sizes of 24 reference cultivars from the Istrian peninsula (north-east Adriatic coast) in this work will serve for standardisation of new genotyping projects. The defined minimum subset of markers represents a step toward efficient identification of fig genetic resources in other fig growing countries. Such studies are essential because they enable identification of error prone loci under different PCR technologies. The high error rate encountered with Locus FSYC01 (15.56 %) indicated it should be excluded from genotyping projects. All other loci were identified as reliable for fig genotyping studies.

A very important goal which can be achieved with standardized molecular identification tools is the identification of unique local genotypes. This would support management of fig collections and promote cultivation and breeding of new and interesting cultivars, either through exchanging and introducing different cultivars in new regions or to give importance to newly identified local unique cultivars. Traditional local products with protected designation of origin or with other quality schemes are in great demand and genetic analysis can help to identify cultivars which are characteristic for a specific geographical region.

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7 SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found online at the repository at University of Primorska.

8 REFERENCES

Abdelkrim, A. B., Baraket, G., Essalouh, L., Achtak, H., Khadari, B., & Salhi-Hannachi, A. (2015). Use of morphological traits and microsatellite markers to characterize the Tunisian cultivated and wild figs (*Ficus carica* L.). *Biochemical Systematics and Ecology, 59*, 209-219. doi:10.1016/j.bse.2015.01.026

Abou-Ellail, M., Mahfouze, S. A., El-Enany, M. A. M., & Mustafa, N. S. A. (2014). Using biochemical and simple sequence repeats (SSR) markers to characterize (*Ficus carica* L.) cultivars. *World Applied Sciences Journal, 29*(3), 313-321. https://doi:10.5829/idosi.wasj.2014.29.03.13835

Achtak, H., Oukabli, A., Ater, M., Santoni, S., Kjellberg, F., & Khadari, B. (2009). Microsatellite markers as reliable tools for fig cultivar identification. *Journal of the American Society for Horticultural Science, 134*(6), 624-631.

Achtak, H., Ater, M., Oukabli, A., Santoni, S., Kjellberg, F., & Khadari, B. (2010). Traditional agroecosystems as conservatories and incubators of cultivated plant varietal diversity: the case of fig (*Ficus carica* L.) in Morocco. *BMC Plant Biology, 10*(1), 28. doi:10.1186/1471-2229-10-28

Ahmed, S., Dawson, D. A., Compton, S. G., & Gilmartin, P. M. (2007). Characterization of microsatellite loci in the African fig *Ficus sycomorus* L. (Moraceae). *Molecular Ecology Notes, 7*(6), 1175-1177. doi:10.1111/j.1471-8286.2007.01822.x

Aradhya, M. K., Stover, E., Velasco, D., & Koehmstedt, A. (2010). Genetic structure and differentiation in cultivated fig (*Ficus carica* L.). *Genetica, 138*(6), 681-694. doi:10.1007/s10709-010-9442-3

Balas, F. C., Osuna, M. D., Domínguez, G., Pérez-Gragera, F., & López-Corrales, M. (2014). Ex situ conservation of underutilised fruit tree species: establishment of a core collection for *Ficus carica* L. using microsatellite markers (SSRs). *Tree Genetics & Genomes, 10*(3), 703-710. doi:10.1007/s11295-014-0715-3

Baldoni, L., Cultrera, N., Mariotti, R., Ricciolini, C., Arcioni, S., Vendramin, G., . . . Testolin, R. (2009). A consensus list of microsatellite markers for olive genotyping. *Molecular Breeding, 24*(3), 213-231. doi:10.1007/s11032-009-9285-8

Bandelj, D., Jakše, J., & Javornik, B. (2004). Amplification of fluorescent-labelled microsatellite markers in olives by a novel, economic method. *Acta agriculturae Slovenica, 83*(2), 323-329.

Bandelj, D., Javornik, B., & Jakše, J. (2007). Development of microsatellite markers in the common fig, *Ficus carica* L. *Molecular Ecology Notes, 7*(6), 1311-1314. doi:10.1111/j.1471-8286.2007.01866.x

Bandelj, D., Jakše, J., Javornik, B. (2008). Development of molecular markers for identification of fig varieties in Istria. In D. Bandelj, M. Bučar-Miklavčič & I. Vrhovnik (Eds.), *The common fig (Ficus carica L.) in Istria: morphological, molecular and some chemical characteristics* (pp. 84-89). Koper, SI: Annales.

Benjak, A., Konradi, J., Blaich, R., & Forneck, A. (2006). Different DNA extraction methods can cause different AFLP profiles in grapevine (*Vitis vinifera* L.). *Vitis, 45*(1), 15-21.

Butler, J. M. (2014). *Advanced Topics in Forensic DNA Typing: Interpretation* (1st ed.). San Diego, CA: Academic Press.

Caliskan, O., Polat, A. A., Celikkol, P., & Bakir, M. (2012). Molecular characterization of autochthonous Turkish fig accessions. *Spanish Journal of Agricultural Research, 10*(1), 130-140. doi:10.5424/sjar/2012101-094-11

Caroli, S., Santoni, S., & Ronfort, J. (2011). AMaCAID: a useful tool for accurate marker choice for accession identification and discrimination. *Molecular Ecology Resources, 11*(4), 733-738. doi:10.1111/j.1755-0998.2011.02993.x

Chapuis, M. P., & Estoup, A. (2007). Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution, 24*(3), 621-631. doi:10.1093/molbev/msl191

Chatti, K., Baraket, G., Abdelkrim, A. B., Saddoud, O., Mars, M., Trifi, M., & Salhi Hannachi, A. (2010). Development of molecular tools for characterization and genetic diversity analysis in Tunisian fig (*Ficus carica*) cultivars. *Biochemical Genetics, 48*(9-10), 789-806. doi:10.1007/s10528-010-9360-1

Cipriani, G., Marrazzo, M. T., Marconi, R., Cimato, A., & Testolin, R. (2002). Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. *TAG Theoretical and Applied Genetics, 104*(2), 223-228. doi:10.1007/s001220100685

Cryer, N., Fenn, M., Turnbull, C., & Wilkinson, M. (2006). Allelic size standards and reference genotypes to unify international cocoa (*Theobroma*

cacao L.) microsatellite data. *Genetic Resources and Crop Evolution, 53*(8), 1643-1652. doi:10.1007/s10722-005-1286-9

Culley, T. M., Stamper, T. I., Stokes, R. L., Brzyski, J. R., Hardiman, N. A., Klooster, M. R., & Merritt, B. J. (2013). An efficient technique for primer development and application that integrates fluorescent labeling and multiplex PCR. *Applications in Plant Sciences, 1*(10), 1300027. doi:10.3732/apps.1300027

De Valk, H. A., Meis, J. F. G. M., Bretagne, S., Costa, J. M., Lasker, B. A., Balajee, S. A.,. Klaassen, C. H. W. (2009). Interlaboratory reproducibility of a microsatellite-based typing assay for *Aspergillus fumigatus* through the use of allelic ladders: proof of concept. *Clinical Microbiology and Infection, 15*(2), 180-187. doi:10.1111/j.1469-0691.2008.02656.x

Debernardi, A., Suzanne, E., Formant, A., Pène, L., Dufour, A. B., & Lobry, J. R. (2011). One year variability of peak heights, heterozygous balance and inter-locus balance for the DNA positive control of AmpFLSTR Identifiler STR kit. *Forensic Science International: Genetics, 5*(1), 43-49. doi:10.1016/j.fsigen.2010.01.020

Deemer, D. L., & Nelson, D. C. (2010). Standardized SSR allele naming and binning among projects. *BioTechniques, 49*, 835-836. doi:10.2144/000113540

Doveri, S., Sabino Gil, F., Díaz, A., Reale, S., Busconi, M., da Câmara Machado, A., . . . Lee, D. (2008). Standardization of a set of microsatellite markers for use in cultivar identification studies in olive (*Olea europaea* L.). *Scientia Horticulturae, 116*(4), 367-373. doi:10.1016/j.scienta.2008.02.005

Ellis, J. S., Gilbey, J., Armstrong, A., Balstad, T., Cauwelier, E., Cherbonnel, C., . . . Stevens, J. R. (2011). Microsatellite standardization and evaluation of genotyping error in a large multi-partner research programme for conservation of Atlantic salmon (*Salmo salar* L.). *Genetica, 139*(3), 353-367. doi:10.1007/s10709-011-9554-4

Ganopoulos, I., Xanthopoulou, A., Molassiotis, A., Karagiannis, E., Moysiadis, T., Katsaris, P., . . . Madesis, P. (2015). Mediterranean basin *Ficus carica* L.: from genetic diversity and structure to authentication of a Protected Designation of Origin cultivar using microsatellite markers. *Trees, 29*(6), 1959-1971. doi:10.1007/s00468-015-1276-2

Giraldo, E., Viruel, M. A., López-Corrales, M., & Hormaza, J. I. (2005). Characterisation and cross-species transferability of microsatellites in the common fig (*Ficus carica* L.). *Journal of Horticultural Science and Biotechnology, 80*(2), 217-224. doi:10.1080/14620316.2005.11511920

Giraldo, E., Lopez-Corrales, M., & Hormaza, J. I. (2008). Optimization of the management of an ex-situ germplasm bank in common fig with SSRs. *Journal of the American Society for Horticultural Science, 133*(1), 69-77.

Haberl, M., & Tautz, D. (1999). Comparative allele sizing can produce inaccurate allele size differences for microsatellites. *Molecular Ecology, 8*(8), 1347-1349. doi:10.1046/j.1365-294X.1999.00692_1.x

Ikegami, H., Nogata, H., Hirashima, K., Awamura, M., & Nakahara, T. (2009). Analysis of genetic diversity among European and Asian fig varieties (*Ficus carica* L.) using ISSR, RAPD, and SSR markers. *Genetic Resources and Crop Evolution, 56*(2), 201-209. doi:10.1007/s10722-008-9355-5

Jones, H., Bernole, A., Jensen, L., Horsnell, R., Law, J., Cooke, R., & Norris, C. (2008). Minimising inter-laboratory variation when constructing a unified molecular database of plant varieties in an allogamous crop. *TAG Theoretical and Applied Genetics, 117*(8), 1335-1344. doi:10.1007/s00122-008-0867-3

Kalinowski, S. T., Taper, M. L., & Marshall, T. C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology, 16*(5), 1099-1106. doi:10.1111/j.1365-294X.2007.03089.x

Khadari, B., Hochu, I., Santoni, S., & Kjellberg, F. (2001). Identification and characterization of microsatellite loci in the common fig (*Ficus carica* L.) and representative species of the genus *Ficus*. *Molecular Ecology Notes, 1*(3), 191-193. doi:10.1046/j.1471-8278.2001.00072.x

Khadari, B., Oukabli, A., Ater, M., Mamouni, A., Roger, J. P., & Kjellberg, F. (2005). Molecular characterization of Moroccan fig germplasm using intersimple sequence repeat and simple sequence repeat markers to establish a reference collection. *HortScience, 40*(1), 29-32.

Khadari, B. (2012). Ex situ management of fig (*Ficus carica* L.) genetic resources: Towards the establishment of the Mediterranean reference collection. *Acta Horticulturae, 940*, 67-74. doi:10.17660/ActaHortic.2012.940.7

Koumi, P., Green, H. E., Hartley, S., Jordan, D., Lahec, S., Livett, R. J., . . . Ward, D. M. (2004). Evaluation and validation of the ABI 3700, ABI 3100, and the MegaBACE 1000 capillary array electrophoresis instruments for use with short tandem repeat microsatellite typing in a forensic environment.

Electrophoresis, 25(14), 2227-2241. doi:10.1002/elps.200305976

Kump, B., & Javornik, B. (1996). Evaluation of genetic variability among common buckwheat (*Fagopyrum esculentum* Moench) populations by RAPD markers. *Plant Science, 114*(2), 149-158. doi:10.1016/0168-9452(95)04321-7

Kyung-Ho, M., Nam-Soo, K., Gi-An, L., Sok-Young, L., Ju Kyong, L., Jung Yoon, Y., . . . Soon-Jae, K. (2009). Development of SSR markers for studies of diversity in the genus *Fagopyrum*. *Theoretical and Applied Genetics, 119*(7), 1247-1254. doi:10.1007/s00122-009-1129-8

Leclair, B., Fregeau, C., Bowen, K., & Fourney, R. (2004). Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. *Journal of Forensic Sciences, 49*(5), 968-980. doi:10.1520/JFS2003312

Mandel, J. R., Dechaine, J. M., Marek, L. F., & Burke, J. M. (2011). Genetic diversity and population structure in cultivated sunflower and a comparison to its wild progenitor, *Helianthus annuus* L. *Theoretical and Applied Genetics, 123*(5), 693-704. doi:10.1007/s00122-011-1619-3

Mullins, F. M., Dietz, L., Lay, M., Zehnder, J. L., Ford, J., Chun, N., & Schrijver, I. (2007). Identification of an intronic single nucleotide polymorphism leading to allele dropout during validation of a CDH1 sequencing assay: implications for designing polymerase chain reaction-based assays. *Genetics In Medicine, 9*, 752. doi:10.1097/GIM.0b013e318159a369

Nybom, H., Weising, K., & Rotter, B. (2014). DNA fingerprinting in botany: past, present, future. *Investigative Genetics, 5*(1), 1-35. doi:10.1186/2041-2223-5-1

Pasqualotto, A. C., Denning, D. W., & Anderson, M. J. (2007). A cautionary tale: Lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. *Journal of Clinical Microbiology, 45*(2), 522-528. doi:10.1128/JCM.02136-06

Peakall, R., & Smouse, P. E. (2006). GENEALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes, 6*(1), 288-295. doi:10.1111/j.1471-8286.2005.01155.x

Peakall, R., & Smouse, P. E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics, 28*(19), 2537-2539. doi:10.1093/bioinformatics/bts460

Pompanon, F., Bonin, A., Bellemain, E., & Taberlet, P. (2005). Genotyping errors: causes, consequences and solutions. *Nature Reviews Genetics, 6*(11), 847-859. doi:10.1038/nrg1707

Schuelke, M. (2000). An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology, 18*(2), 233-234. doi:10.1038/72708

Solomon, A., Golubowicz, S., Yablowicz, Z., Grossman, S., Bergman, M., Gottlieb, H. E., . . . Flaishman, M. A. (2006). Antioxidant activities and anthocyanin content of fresh fruits of common fig (*Ficus carica* L.). *Journal of Agricultural and Food Chemistry, 54*(20), 7717-7723. doi:10.1021/jf060497h

Soriano, J., Zuriaga, E., Rubio, P., Llácer, G., Infante, R., & Badenes, M. (2011). Development and characterization of microsatellite markers in pomegranate (*Punica granatum* L.). *Molecular Breeding, 27*(1), 119-128. doi:10.1007/s11032-010-9511-4

Sutton, J. T., Robertson, B. C., & Jamieson, I. G. (2011). Dye shift: a neglected source of genotyping error in molecular ecology. *Molecular Ecology Resources, 11*, 514-520. doi:10.1111/j.1755-0998.2011.02981.x

This, P., Jung, A., Boccacci, P., Borrego, J., Botta, R., Costantini, L., . . . Maul, E. (2004). Development of a standard set of microsatellite reference alleles for identification of grape cultivars. *TAG Theoretical and Applied Genetics, 109*, 1448-1458. doi:10.1007/s00122-004-1760-3

Tvedebrink, T., Eriksen, P. S., Mogensen, H. S., & Morling, N. (2012). Statistical model for degraded DNA samples and adjusted probabilities for allelic drop-out. *Forensic Science International: Genetics, 6*(1), 97-101. doi:10.1016/j.fsigen.2011.03.001

Vemireddy, L. R., Archak, S., & Nagaraju, J. (2007). Capillary electrophoresis is essential for microsatellite marker based detection and quantification of adulteration of Basmati rice (*Oryza sativa*). *Journal of Agricultural and Food Chemistry, 55*(20), 8112-8117. doi:10.1021/jf0714517

Vinson, J. A., Zubik, L., Bose, P., Samman, N., & Proch, J. (2005). Dried fruits: excellent in vitro and in vivo antioxidants. *Journal of the American College of Nutrition, 24*(1), 44-50. doi:10.1080/07315724.2005.10719442

Wagner, H. W., & Sefc, K. M. (1999). IDENTITY 1.0. Vienna, AT: University of Agricultural Sciences, Centre for Applied Genetics.

Wenz, H. M., Robertson, J. M., Menchen, S., Oaks, F., Demorest, D. M., Scheibler, D., . . . Efcavitch, J. W. (1998). High-precision genotyping by denaturing capillary electrophoresis. *Genome Research, 8*(1), 69-80. doi:10.1101/gr.8.1.69

Zohary, D., & Spiegel-Roy, P. (1975). Beginnings of fruit growing in the old world. *Science, 187*(4174), 319-327. doi:10.1126/science.187.4174.319

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Effectiveness of different control measures against western corn rootworm larvae *Diabrotica virgifera virgifera* LeConte, 1868

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ABSTRACT

The Western Corn Rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, 1868, [Coleoptera, Chrysomelidae], whose larvae cause damage to maize roots, is an important economic insect pest in America and Europe. Its larvae are usually controlled by granular soil insecticides or insecticide-treated seeds. Biological control options, such as entomopathogenic nematodes (EPN) have played an important role as an alternative for synthetic chemical insecticides. Therefore, for the WCR larvae control we compared the effectiveness of inundative biological control on the basis of EPN *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae); (commercial product Dianem®) and the conventional insecticides Force 1.5 g (active substance tefluthrin) from the group of synthetic pyrethriods and Sonido (active substance thiacloprid) from the group of neonicotinoids. Field experiments were carried out at geographically different locations under different population pressure of the insect pest in a), Bučečovci (Prlekija; Eastern Slovenia) and b), Šmartno (Gorenjska: northern Slovenia). The differences between the treatments were very similar at both locations; although the population of WCR in Gorenjska was approximately 5-fold lower than in Prlekija. The highest number of WCR beetles was caught in the negative control, followed by the product Sonido, Force and Dianem®, in decreasing order. Statistical analysis showed that only in the treatment where EPN were used, significantly less WCR was caught than in the control. The results of the WCR larvae control in maize using *Heterorhabditis bacteriophora* are comparable to published literature. However, the weather conditions in the 2016 trial were very favorable for the development and survival of EPN in the soil.

Key words: western corn rootworm; *Diabrotica virgifera virgifera;* inundative biological control; entomopathogenic nematodes; *Heterorhabditis bacteriophora;* field trial*; Zea mays*

IZVLEČEK

UČINKOVITOST RAZLIČNIH METOD ZATIRANJA LIČINK KORUZNEGA HROŠČA *Diabrotica virgifera virgifera* LeConte, 1868

Koruzni hrošč (WCR) (*Diabrotica virgifera virgifera* LeConte, 1868, [Coleoptera, Chrysomelidae], katerega ličinke povzročajo škodo z objedanjem korenin koruze, je pomemben gospodarski škodljivec v Ameriki in v Evropi. Ličinke navadno zatiramo z granularnimi talnimi insekticidi ali pa insekticidi, ki so naneseni na semena. Biotično varstvo je pomemben način nekemičnega varstva rastlin in med drugim predstavlja pomembno alternativo rabi sintetičnih kemičnih insekticidov. S tem namenom smo v letu 2016 primerjali učinkovitost preplavnega biotičnega varstva na osnovi entomopatogenih ogorčic (*Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae); v Sloveniji na voljo v obliki tržnega pripravka dianem®) in konvencionalnih insekticidov (teflutrin (Force 1,5 G) iz skupine sintetičnih piretriodov in tiakloprid (Sonido) iz skupine neonikotinoidov) za zatiranje ličink koruznega hrošča. Poljski poskus je potekal na geografsko različnih lokacijah z različnim populacijskim pritiskom škodljivca: a), v Bučečovcih v Prlekiji in b), v Šmartnem pri Cerkljah na Gorenjskem. Razlike med obravnavanji so bile zelo podobne na obeh lokacijah, čeprav je bila populacija hroščev na Gorenjskem približno 5-krat manjša kot v Prlekiji. Največ koruznih hroščev smo zabeležili v kontroli. Po padajočem številu ulovljenih hroščev so si sledili pripravki Sonido, Force in Dianem®. Statistična analiza je pokazala, da se je le v postopku, kjer smo uporabili entomopatogene ogorčice (EPN) ulovilo statistično manj koruznih hroščev kot v kontroli. Rezultati zatiranja ličink koruznega hrošča s pripravkom na osnovi vrste *H. bacteriophora* so primerljivi z objavljenimi rezultati študij iz tujine. Pri tem moramo upoštevati dejstvo, da so bile vremenske razmere v času poskusa ugodne za razvoj in preživetje EPN.

Ključne besede: koruzni hrošč; *Diabrotica virgifera virgifera;* preplavno biotično varstvo; entomopatogene ogorčice; *Heterorhabditis bacteriophora;* poljski poskus; koruza; *Zea mays*

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1 INTRODUCTION

LeConte wrote the first formal description of the species *Diabrotica v. virgifera* in 1868 from beetles collected on pumpkin flowers *Cucurbita foetidissima* Kunth in Humb near Fort Wallace, Kansas, USA (Smith in Lawrence, 1967). As an insect pest of corn it was referred for the first time in Kolorado in 1909 (Gillette, 1912). The WCR was first discovered in Europe in 1992 in Yugoslavia (Bača, 1994) and has become a threat to maize production areas in many European countries (Kiss et. al., 2005).

Official monitoring of the spreading of WCR in Slovenia has been organized since 1997**.** Its presence was first reported in 2003 in the eastern, northeastern (Prekmurje, Pomurje) and western part (Gorica) of Slovenia (Urek and Modic, 2004). In 2009 the entire territory of Slovenia was officially declared an infested area. Eight years after the occurrence of WCR the first damage caused by larval feeding was observed in Prekmurje (village Benica). In the next five years damage by larval feeding was observed in more Slovenian regions, mainly in the fields with continuous maize.

In an effort to eradicate or contain the species, legislation has been put into place (U.l. RS 21/04), which forces farmers to rotate their fields (thereby interrupting the life cycle of WCR) or to apply granular soil insecticides or to use insecticide-coated maize seeds (to target the root feeding larvae) or to spray insecticides against the adult (decrease the population density of larvae in the next year) (U. l. RS 21/04, 2006/565/ES). In the European Union the WCR is no longer considered a "quarantine pest" since 2014 (2014/63/EU), consequently crop rotation is no longer a legally required control measure. In Slovenia, in the measures of the Rural Development Program KOPOP (2014-2020), one of the mandatory requirements is a five-year crop rotation that limits the production of maize in monoculture.

Possible management options to control WCR in Europe include crop rotation, which is one of the oldest control measures (Roush et al., 1990), the development of maize hybrids that possess native resistance against WCR (Ivezić et al., 2009), or the use of conventional chemical insecticides (Levine and Oloumi-Sadeghi, 1991; Sutter et al., 1989). Biological control options have been recommended for WCR in south-eastern Europe in 1998 (Kuhlmann and Burgt, 1998). The use of entomopathogenic nematodes (EPN) against the larvae is one of the most promising alternative biological control options (Toepfer et al., 2012a), in particular species *Heterorhabditis bacteriophora* Poinar, 1976 which in the field trials reduced the population of the WCR larvae to 65 % and the lodging of plants to 60 %, which is comparable with soil insecticides (Toepfer et al., 2005; Kahrer et al., 2015; Toepfer et al., 2010). Importantly, the species *H. bacteriphora* was confirmed in Slovenia in 2009 (Laznik et al., 2009), thereby making it a potential biological agent for controlling larvae of WCR and important alternative to synthetic insecticides.

The aim of our study was to assess *H. bacteriphora*-based biological control and compare its efficacy to two commonly used synthetic insecticides for controlling WCR larvae.

2 MATERIALS AND METHODS

2.1 Study sites and experimental set-up

The field studies were carried out in geographically different maize growing areas in Slovenia (Table 1). Both field experiments had a natural pest population of WCR (field A since 2004, field B since 2008) and were four years under corn monoculture production regime.

Table 1: Characteristic of the two field experiments in Slovenia

Field	A	B
Location	Bučečovci	Šmartno pri Cerkljah
Coordinates	46°35'07"N 16°06'37"E	46°15'08.8"N 14°29'54.7"E
Date of sowing/nematode application	22th April 2016	11th May 2016
Cultivar of maize seed	Chapalu	LG 34.90
Field size	0.11 ha	0.11 ha
Soil texture	silty loam	sandy loam

In Bučečovci 'Chapalu' hybrid seeds (FAO 330) were sown by machine Monosem NC classic, at a inter row spacing of 70 cm and an intra row spacing of 16 - 17 cm, resulting in a theoretical stand of 85.000 plants per ha. The maize seeds of the hybrid LG 34.90 (FAO 430) were sown in Šmartno by machine Gaspardo by the same procedure. In total, on both locations, four treatments were conducted in 2016: 1. untreated control; 2. entomopathogenic nematodes (EPN); 3. synthetic pyrethroid insecticide Force 1,5 G (a.i. tefluthrin) and 4. seed coating with a neonicotinoid insecticide Sonido (a.i. thiacloprid) (Table 2). Each treatment was performed in 4 rows 70 m in length, giving a plot size of 0.028 ha; total size of whole experimental fields at both locations was 0.112 ha.

Table 2: WCR management of the two field experiments

Field sites	Date of applications	Treatment	Dose
A	22th April 2016	1. Untreated control	/
		2. *H. bacteriophora* (EPN)	2.000.000 nematodes/ha with 400 l water
		3. Force 1,5 G (a.i. tefluthrin)	12 kg/ha (1.5 % a.i.)
		4. Sonido (a.i. thiacloprid)	0.125 l / 50000 seeds (40 % a.i.)
B	11th May 2016	1. Untreated control	/
		2. *H. bacteriophora*	2.000.000 nematodes/ha with 400 l water
		3. Force 1,5 G (a.i.tefluthrin)	12 kg/ha (1.5 % a.i.)
		4. Sonido (a.i. thiacloprid)	0.125 l / 50000 seeds (40 % a.i.)

2.2 Source and handling of entomopathogenic nematode

The product Dianem®, based on entomopathogenic nematode *Heterorhabditis bacteriophora*, was produced and supplied by e-nema GmbH (Germany). Infective juveniles of *H. bacteriophora* were shipped by the producer directly to the Agricultural Institute of Slovenia. Upon arrival, they were stored in their shipping material at 7-9 °C in darkness until use. They were applied as liquid formulations with application rates 2×10^9 infective juveniles per ha in 400 litres water per ha as a liquid stream directly into seed furrows (Figure 1).

Figure 1: (A, B) Nematodes were applied to the field plots during sowing of maize as a fluid stream into the seed furrows, at a depth of 8-10 cm

2.3 Evaluation

Efficacy was assessed by comparing the number of emerging adult WCR beetles between treatments and untreated control. Twelve maize plants (1 m^2) were covered under gauze cages (1000 mm x 1000 mm x 2300 mm). A yellow sticky trap (Trécé) was placed 1.5 m above soil in the middle of the cage. Adult emergence on yellow sticky trap (Trécé) was recorded once a month.

2.4 Data analyses

The absolute number of WCR beetles caught on yellow sticky traps placed in the field cages was analysed by one way ANOVA and Dunnett's multiple comparison post-test in case of normal distribution, or with Kruskall-Wallis test and Dunn's post-test in case the distribution was not normal (Motulsky, 1995). The absolute number of beetles caught in the various treatments was normalized to negative control values, and then the data from both locations were pooled and Log-transformed. The transformed data was normally distributed and analysed by one way ANOVA and Dunnett's multiple comparison post-test. The analyses were carried out with the statistical software GraphPad Prism 5.00 (GraphPad Software, Inc., La Jolla, CA, USA). The number of replicates (n) is indicated in the figure.

3 RESULTS

3.1 Effect of treatments on the number of emerged WCR beetles

The highest number of WCR beetles was caught in negative control, followed by Sonido and Force, and the least in the EPN treatment. The treatments had a significant effect on the number of WCR beetles caught in the experiment in Šmartno (P = 0.042), but not in Bučečovci (P = 0.334). No individual treatment had a significant effect on the number of beetles caught at both experimental sites. The average number of WCR beetles caught in the EPN treatment was approximately three times smaller (one third) in Šmartno and two times smaller (half) in Bučečovci compared to negative control catches (Figure 2).

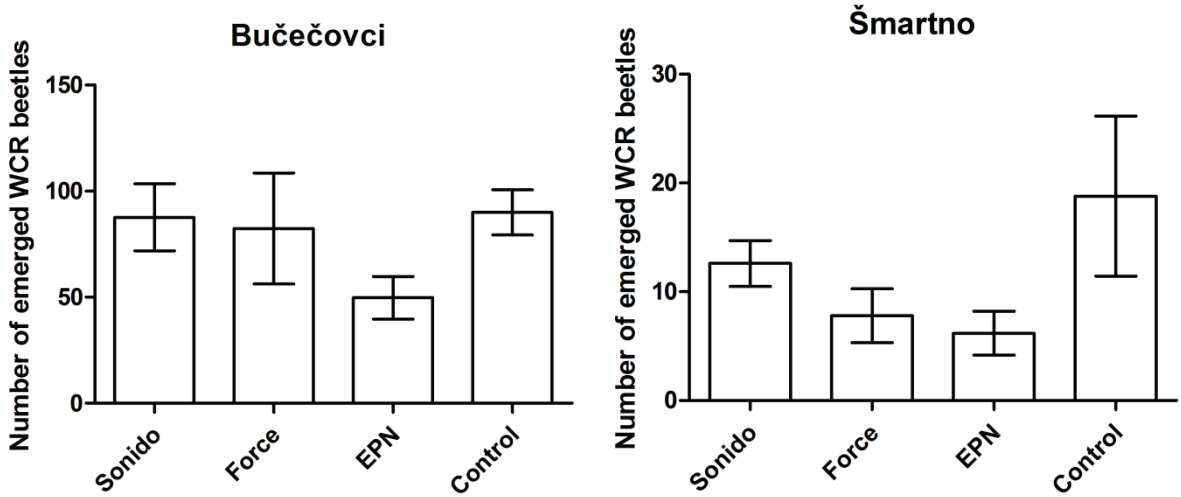

Figure 2: The absolute (cumulative whole season) number of WCR beetles caught on the yellow sticky traps in the field cages. Left: results from Bučečovci experiment; right: results from Šmartno experiment. Error bars were drawn from 5 replicates (n=5).

Differences between treatments, or trends, however, were very similar in Bučečovci and Šmartno. Additionally, markedly different pressure of the WCR population between the locations of the experiments was observed. Because of these two factors the results were normalized to each experiment's negative control and the data from both trials pooled. One-way ANOVA performed on pooled data showed a significant effect of treatments on the number of beetles caught on the yellow sticky traps (p = 0.017). The treatment with EPN resulted in significantly smaller beetle catches (Figure 3).

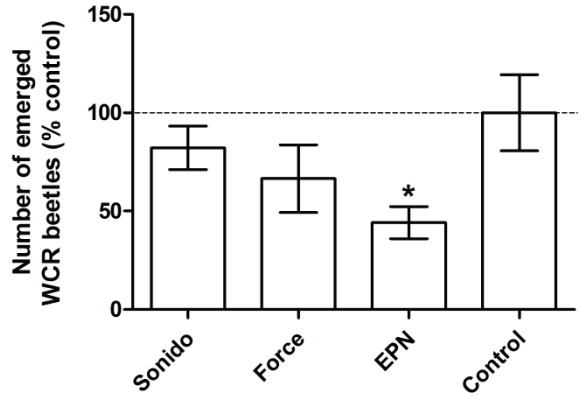

Figure 3: The relative (cumulative whole season) number of WCR beetles caught on the yellow sticky traps in the field cages. Data of experiments from both locations were normalized to their respective controls, and then pooled. Error bars were drawn from 10 replicates (n=10). Asterisk denotes significant difference from negative control ($P < 0.05$).

4 DISCUSION

Results of the present study provide a comparison between efficacy of a commercial product Dianem® on the basis of EPN *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) with the conventional insecticides Force 1.5 g (active substance tefluthrin) from the group of synthetic pyrethriods and with Sonido (active substance thiacloprid) from the group of neonicotinoids for the WCR larvae control. We found that the highest number of WCR beetles was caught in negative control, followed by product Sonido, Force and Dianem®, in decreasing order. The evaluation of emerging beetles showed a significant reduction of 55.8 % for Dianem (2.33 emerging beetles per plant or 28.0 m^{-2}) against the untreated control (4.53 emerging beetles per plant or 54.4 m^{-2}). This result is comparable to published study of Kahrer et al. (2015) where the authors reported a significant reduction in number of emerging beetles amounting 67.6 % for Dianem against the untreated control (81.4 emerging beetles per plant). The much higher emergence rate reported by Kahrer et al. (2015) is probably the result of artificial infestation of the maize plants with WCR eggs.

Crop rotation is the most effective way of controlling WCR populations as its three larval instars feed almost exclusively on maize roots (Moeser and Hibbard, 2005). However, a small percentage of WCR (< 5 %) also disperses into neighbouring fields other than maize, and less than 15 % of all eggs of a population may be laid into such non-maize crop habitats. Despite of this, any crop can be rotated with maize to offer potentially successful control of this pest in Europe (Toepfer et al., 2012b). However, in an Austrian several-season field study, it was reported that crop rotation is no longer a sufficient measure to control WCR, in case of rotation of maize and oil pumpkin (Fragner, 2017). The same study also reports that in 2016, the efficacy of EPN was comparable to certain soil insecticides (cypermethrin and lambda-cyhalothrin), and that EPN use reduced maize lodging on average by 10 % (Fragner, 2017).

Maize is one of the major crops in Slovenia covering about 40 % of all arable land. Sixty percent of maize is grown in north-east Slovenia (Čergan et al., 2008). However, there is the need to plant maize each season, because silage maize presents energetically and economically convenient source of feed for dairy cows and fattening bulls. The maize with its high dry matter yield per hectare fulfils the stakeholder`s demand after large quantities of feed for the increasing numbers of animals per farm in the last decade. Due to fragmented arable land (fields), we can expect the marginal effects of successive corn crops and the occurrence of damages on parts of fields where the surfaces are close together (Modic et al., 2016). Therefore, a system approach is needed to provide sustainable control of the WCR. Several control measures will need to be implemented at the same time: crop rotation, soli treatment, fertilization, corn variety, biological control agents and pheromone mating disruption control.

5 CONCLUSION

The results of WCR larvae control with entomopathogenic nematodes of the species *Heterorabditis bacteriophora* are promising and comparable to the results of studies from abroad (Kurtz et al., 2007; Toepfer et al., 2010; Pilz et al., 2014; Kahrer et al., 2015). However, we must take into account the fact that the weather conditions in 2016 (high precipitation in May) were favourable for the survival and persistence of the EPN in the soil (soil moisture). Such optimal conditions cannot be expected every year. Repetition of the experiment throughout several seasons is needed.

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7 REFERENCES

Bača, F. (1994). New member of the harmful entomofauna of Yugoslavia, *D. virgifera virgifera* LeConte (Coleoptera, Chrysomelidae). *Zaštita bilja*, *45*, 125-131.

Čergan, Z., Jejčič, V., Knapič, M., Modic, Š., Moljk, B., Poje, T., Simončič, A., Sušin, J., Urek, G., Verbič, J., Vrščaj, B., Žerjav, M. (2008) . *Koruza*. Založba kmečki glas: 314 str.

Fragner, H. (2017). Versuche und Erfahrungen aus der Steiermark: Maiswurzelbohrer 2016. *Der Pflanzenarzt*, *1-2*, 24-26.

Gillette, C.P. (1912). *Diabrotica virgifera* LeConte as a corn rootworm. *Journal of Economic Entomology*, *54*, 4, 364-366. doi:10.1093/jee/5.4.364a

Ivezić, M., Raspudić, E., Bremež, M., Majić, I., Brkić, I., Tollefson, J.J., Bohn, M., Hibbard, B., Šimić, D. (2009). A review of resistance breeding options targeting western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *Agricultural and forest Entomology*, *11*, 307-311. doi:10.1111/j.1461-9563.2009.00434.x

Kahrer, A., Pilz, C., Heimbach, U., Wechselberger, K., Grabenweger., G. (2015). Five years of experience in biocontrol of the western corn rootworm, *Diabrotica virgifera virgifera* by entomoparasitic nematodes. New Challenges for Biological Control: *15th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate Pests*": Programme and Abstract Book; June 7 - 11, 2015, Riga, Latvia.

Kuhlmann, U., Burgt, W.A.C.M. (1998). Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe. *Biocontrol News and Information*, *19*, 59–68.

Kurtz, B., Toepfer, S., Ehlers, R.-U., Kuhlmann, U. (2007). Assessment of establishment and persistence of entomopathogenic nematodes for biological control of western corn rootworm. *Journal of Applied Entomology, 131*(6), 420-425. doi:10.1111/j.1439-0418.2007.01202.x

Kiss, J, Edwards, C.R., Berger, H.K., Cate, P, Cean, M., Cheek, S., Derron, J., Festic, H., Furlan, L., Igrc-Barcic, J., Ivanova, I., Lammers, W., Omelyuta, V., Princzinger, G., Reynaud, P., Sivcev, I., Sivicek, P., Urek, G., Vahala, O. (2005). Monitoring of western corn rootworm (*Diabrotica v. virgifera* LeConte) in Europe 1992–2003. In S. Vidal, U. Kuhlmann, C. R. Edwards (Eds.), *Western corn rootworm: ecology and management* (pp. 29-39). Wallingford, UK: CABI Publishing. doi:10.1079/9780851998176.0029

Laznik, Ž., Tóth, T., Lakatos, T., Trdan, S. (2009). *Heterorhabditis bacteriophora* (Poinar) – the first member from Heterorhabditidae family in Slovenia. *Acta agriculturae Slovenica, 93* (2), 181–187. doi:10.2478/v10014-009-0013-1

Levine, E., & Oloumi-Sadeghi, H. (1991). Management of Diabroticite Rootworms in Corn. *Annual Review of Entomology, 36*, 229-255. doi:10.1146/annurev.en.36.010191.001305

Modic, Š., Urbančič Zemljič, M., Razinger, J., Kolmanič, A. (2016). *Priporočila za obvladovanje koruznega hrošča*. Kmetijski inštitut Slovenije. Retrieved from http://www.kis.si/f/docs/Entomologija/Priporocila_za_obvladovanje_koruznega_hrosca__2017.pdf.

Moeser, J., Hibbard, B.E. (2005). A synopsis of the nutritional ecology of larvae and adults of *Diabrotica virgifera virgifera* (LeConte) in the new and old world - nouvelle cuisine for the invasive maize pest *Diabrotica virgifera virgifera* in Europe? In S. Vidal, U. Kuhlmann, C.R. Edwards (Eds.), *Western corn rootworm: ecology and management* (pp. 41-65). Wallingford, UK: CABI Publishing. doi:10.1079/9780851998176.0041

Odločbe Komisije 2003/766/ES z dne 24. oktobra 2003 o nujnih ukrepih za preprečevanje širjenja koruznega hrošča *Diabrotica virgifera* Le Conte v Skupnosti. Uradni list EU, št. 275/49.

Pilz, C., Toepfer, S., Knuth, P., Strimitzer, T., Heimbach, U., Grabenweger, G. (2014). Persistence of the entomoparasitic nematode *Heterorhabditis bacteriophora* in maize fields. *Journal of Applied Entomology, 138,* 202-212. doi:10.1111/j.1439-0418.2012.01743.x

Pravilnik o fitosanitarnih ukrepih za preprečevanje širjenja koruznega hrošča (Uradni list RS, št. 21/2004, 106/2006 in 22/2009).

Priporočilo Komisije 2006/565/ES z dne 11. avgusta 2006 o zadrževalnih programih za omejitev nadaljnjega širjenja koruznega hrošča *Diabrotica virgifera* Le Conte na območjih v Skupnosti, kjer je njegova prisotnost potrjena. Uradni list Evropske unije, št. 225/28.

Priporočilo Komisije 2014/63/ES z dne 6. februarja 2014 glede ukrepov za obvladovanje koruznega hrošča *Diabrotica virgifera virgifera* Le Conte na območjih Unije, kjer je njegova navzočnost potrjena. Uradni list Evropske unije, št. 38/46.

Roush, R.T., Hoy, C.W., Ferro, D.N., Tingey, W.M. (1990). Insecticide resistance in the Colorado potato beetle (Coleoptera: Chrysomelidae): Influence of crop rotation and insecticide use. *Journal of Economic Entomology, 83*, 315-319. doi:10.1093/jee/83.2.315

Smith, R.F., Lawrence, J.F. (1967). Clarification of the type specimens of Diabroticites (Coleoptera, Chrysomelidae, Galerucinae). *University of California publications in entomology, 45*, 1-174.

Sutter, G.R., Branson, T.F., Fisher, J.R., Elliot, N.C., Jackson, J.J. (1989). Effect of insecticide treatments on root damage ratings of maize in controled infestations of western corn rootworms (*Coleoptera*: *Chrysomelidae*). *Journal of Economic Entomology, 82*, 1792-1798. doi:10.1093/jee/82.6.1792

Toepfer, S., Gueldenzoph, C., Ehlers, R.-U., Kuhlmann, U. (2005). Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, Diabrotica virgifera virgifera (Coleoptera: Chrysomelidae) in Europe. *Bulletin of Entomological Research, 95*, 473-482. doi:10.1079/BER2005379

Toepfer, S., Burger, R., Ehlers, R.-U., Peters, A., Kuhlmann, U. (2010). Controlling western corn rootworm larvae with entomopathogenic nematodes: effect of application techniques on plant-scale efficacy. *Journal of Applied Entomology, 134*, 467-480. doi:10.1111/j.1439-0418.2009.01469.x

Toepfer, S., Hatala-Zseller, I., Ehlers, R.-U., Peters, A., Kuhlmann, U. (2012a). The effect of application techniques on field-scale efficacy: can the use of entomopathogenic nematodes reduce damage by western corn rootworm larvae?. *Agricultural and Forest entomology, 12*, 389-402. doi:10.1111/j.1461-9563.2010.00487.x

Toepfer, S., Zellner, M., Haye, T., Kuhlmann, U. (2012b). Dispersal and oviposition behaviour of *Diabrotica virgifera virgifera* in non-maize crops to improve advice and guidelines for crop rotation. In H. Kehlenbeck, Heimbach U., Zellner M. (Eds.) *Proceedings International Conference on the German Diabrotica Research Program* (pp. 112-122). Berlin: Bundesforschungsinstitut für Kulturpflanzen, Julius Kühn-Institut.

Urek, G., Modic, Š. (2004). Occurrence of the Western Corn Rootworm (*Diabrotica virgifera virgifera* Le Conte) in Slovenia. *Acta agriculturae Slovenica, 83*, 5-13.

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Cadmium treatment effects on the growth and antioxidant system in barley plants under optimal and low temperatures

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ABSTRACT

The cadmium effect (100 μM) on the barley (*Hordeum vulgare* L.) growth, the content of *HvCu/ZnSOD*, *HvCAT2* and *HvPRX07* transcripts and the antioxidant enzymes activity (SOD, CAT and PRX) in roots and leaves of seedlings under optimal (22 °C) and low (4 °C) temperatures were studied. Exposure to cadmium at 22 °C did not inhibit the plants' growth. In this case, the rate of the oxidative processes in the cells remained at the control level. This was achieved by a corresponding increase of the gene transcripts and the antioxidant enzymes activity in roots and leaves. In contrast, exposure to cadmium at 4 °C inhibited the seedlings' growth despite of the lower metal content in the plants. Moreover the rate of lipid peroxidation in the roots and leaves increased significantly. It is assumed that this effect was connected with the accumulation of excess amounts of hydrogen peroxide due to a misbalance between its generation and neutralization. This assumption is confirmed by the obtained data, according to which the level of *HvCu/ZnSOD* expression and the total activity of SOD increased significantly under exposure to cadmium at 4 °C, although *HvCAT2* and *HvPRX07* transcripts and CAT and PXR activity did not rise.

Key words: *Hordeum vulgare* L.; cadmium; low temperature; growth; antioxidant enzymes; gene expression

IZVLEČEK

UČINKI OBRAVNAVE S KADMIJEM NA RAST IN ANTIOKSIDACIJSKI SISTEM JEČMENA PRI OPTIMALNIH IN NIZKIH TEMPERATURAH

Preučevan je bil učinek kadmija (100 μM) na rast ječmena (*Hordeum vulgare* L., vsebnost *HvCu/ZnSOD*, *HvCAT2* in *HvPRX07* transkriptov in na aktivnost antioksidacijskih encimov (SOD, CAT in PRX) v koreninah in listih sejank pri optimalni (22 °C) in nizki temperaturi (4 °C). Izpostavitev kadmiju pri 22 °C ni zavrla rasti rastlin. V teh razmerah so oksidacijski procesi v celicah ostali na ravni kontrole, kar je bilo doseženo s povečanjem ustreznih prepisov genov in aktivnostjo antioksidacijskih encimov v koreninah in listih. Nasprotno je izpostavitev kadmiju pri 4 °C zavrla rast sejank kljub manjši vsebnosti kovine v rastlini. Peroksidacija maščob v koreninah in listih se je značilno povečala. Domnevamo, da je bil ta učinek povezan s kopičenjem presežnih količin vodikovega peroksida zaradi neuravnoteženosti njegovega nastanka in nevtralizacije. To domnevo potrjujejo podatki, da sta se ekspresija *HvCu/ZnSOD* in celopkupna aktivnost SOD značilno povečali pri izpostavitvi kadmiju pri 4 °C, pri čemer vsebnosti *HvCAT2* in *HvPRX07* transkriptov in aktivnosti CAT ter PXR niso narasle.

Ključne besede: *Hordeum vulgare* L.; kadmij; nizke temperature; rast; antioksidacijski encimi; ekspresija genov

1 INTRODUCTION

One of the most harmful effects of adverse environmental factors on plants is the development of oxidative stress in cells, caused by the formation of an excessive amount of reactive oxygen species (ROS) (Foyer and Noctor, 2005; Gill and Tuteja, 2010). In small amounts, ROS are formed during metabolic processes and normally they are inactivated due to the components of antioxidant system. Under unfavorable conditions, the formation of ROS sharply increases, leading to oxidative stress, resulting in damage to proteins and nucleic acids, lipid oxidation and cell membrane damage, and eventually causing inhibition of plant growth and development, and sometimes their death (Avasthi et al., 2015). The same reaction is

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observed in plants exposed to cadmium. Cadmium cannot directly generate ROS, but it causes their accumulation by mediating of disorders in the structure and functions of chloroplasts and mitochondria (Sandalio et al., 2001) or a decrease in the antioxidant enzymes activity (Schützendübel and Polle, 2002). At the same time it was found that the ability of some species (varieties, genotypes) to grow at high cadmium concentrations is largely due to the high activity of antioxidant system (AOS) components, which allows preserving the structural integrity and functional activity of cell membranes (Wu et al., 2003; Khan et al., 2007).

In recent years, the attention of researchers has been aimed at studying the influence of cadmium on antioxidant gene expression (Smeets et al., 2009; Luo et al., 2011). However, as a rule, such studies are conducted at optimum temperatures. At the same time, in the wild, especially in northern regions, in addition to heavy metal contamination, low temperatures during the growing season affect the growth and development of plants. As a result, as shown so far only in few studies, their metal resistance decreases, presumably due to a reduction of AOS components activity (Sergeant et al., 2014; Venzhik et al., 2015). In this connection, the purpose of this study was to investigate the effect of cadmium on the growth, the antioxidant gene transcripts content and the activity of antioxidant enzymes in the roots and leaves of barley, as one of the important cereal crops in the world, under optimal and low temperatures.

2 MATERIALS AND METHODS

2.1 Plant growth conditions and treatments

Barley seedlings (*Hordeum vulgare* 'Zazersky 85') were grown (water culture) for 7 days on nutrition solution with micronutrients in a growth chamber at 22 °C and 60 – 70 % humidity under PAR 100 µmol $(m^2s)^{-1}$ with 14/10 h light/dark regime. The composition of the basic nutrition solution: NH_4NO_3 252 mg/l^{-1}, $MgSO_4 \cdot 7\ H_2O$ 187 mg/l^{-1}, KH_2PO_4 210 mg/l^{-1}, H_3BO_3 3 mg/l^{-1}, $CuSO_4 \cdot 5\ H_2O$ 1 mg/l^{-1}, $MnSO_4 \cdot 5\ H_2O$ 3 mg/l^{-1}, $ZnSO_4 \cdot 7\ H_2O$ 0.5 mg/l^{-1}, $(NH_4)_6Mo_7O_{24}$ 1 mg/l^{-1}, Fe-citrate 10 mg/l^{-1}. The solution pH = 6.2 was adjusted with $Ca(OH)_2$. After that, the seedlings were exposed to cadmium at a concentration of 100 µmol (as sulphate), which is sub-damaging for barley, at the optimum (22 °C) and low (4 °C) temperatures or at the 4 °C without cadmium. The control plants remained under the initial conditions. After 96 h, the increment of length of the best developed root, shoot height, the underground and aboveground biomass were measured. The content of cadmium in the roots and leaves was analyzed. Along with this, the rate of lipid peroxidation was estimated by the accumulation of malondialdehyde (MDA). The *HvCu/ZnSOD*, *HvCAT2* and *HvPRX07* transcripts content and the activity of superoxide dismutase (SOD), catalase (CAT) and peroxidase (PRX) were also determined.

2.2 Cadmium content assays

The concentration of cadmium was measured by the method of inversion voltammetry using the polarograph ABC-1.1 (Volta, Russia). The decomposition of plant samples was carried out in a 4:1 mixture of HNO_3 and H_2O_2 using the microwave system of sample preparation MS-6 (Volta, Russia).

2.3 Gene transcripts content

The transcripts content of the genes involved in antioxidant cell defense was determined by qRT- PCR. For RNA isolation, the roots or leaves of barley (50 mg) were ground in liquid nitrogen. Total RNA was isolated by ExtractRNA ("Eurogen"). The cDNA was synthesized using a reverse transcription kit with M-MLV reverse transcriptase and random hexaprimers ("Eurogen"). The quantity and quality of the isolated RNA and synthesized cDNA were measured spectrophotometrically (SmartSpecPlus, "Bio-Rad"). Amplification was carried out in the iCycler with an iQ5 optical pre-amplifier ("Bio-Rad") using amplification kits with an intercalating SYBR Green dye ("Eurogen"). The 25 µl PCR mixture contained 1 µl of cDNA (100 ng), 5 µl of the reaction mixture, 1 µl of forward and reverse primers (10 µmol) and 17 µl of nuclease-free deionized water. Gene transcripts content was detected using the primers shown in the Table 1. Primers for qRT-PCR were made by "Labtech" (Russia). Actin was used as the reference gene. PCR protocol: 5 min at 95 °C, then 45 cycles of 15 s at 95 °C, 50 s at 60 °C. The specificity of the amplification products was checked by melting the PCR fragments: 1 min at 95 °C, 1 min at 60 °C, 10 s at 56 °C (80 cycles, increasing the temperature by 0.5 °C in each cycle). The accumulation of gene transcripts was calculated from ΔΔ St (Livak and Schmittgen, 2001). cDNAs isolated from plants not exposed to stress factors were selected as the control samples.

Table 1: Primers for qRT-PCR

Gene	Primer	Nucleotide sequence 5´…3´	Accession number in NCBI GenBank
HvCu/ZnSOD	forward	CCTGCCCTTTCCACTCG	HM537232
	reverse	TGTCGTAGGACCGTCATCG	
HvCAT2	forward	GACAAGTCGTGCGGGATG	HVU20778
	reverse	CCTTATTGCTGGCTGGTT	
HvPRX07	forward	TCCACCCTCATCTCCTCCTT	X62438
	reverse	ACGGCTTGAACGGTCCTC	

2.4 Malondialdehyde content and antioxidant enzymes activity assays

The MDA content and antioxidant enzymes activity were determined on a spectrophotometer (Spectrum, Russia). To analyze the MDA content, a reaction medium containing 0.2 5 % solution of thiobarbituric acid (TBA) in 10 % trichloroacetic acid was used according to Heath and Packer (1968). The plant material was homogenized in the reaction medium. The homogenate was aged in a water bath at 95 °C for 30 minutes, quickly cooled in an ice vessel and centrifuged for 10 min at 10,000 g. The absorbance of the supernatant was measured at D = 532 and 600 nm. The concentration of TBA-reacting products was calculated using the formula $C_{MDA} = (D_{532}–D_{600}) / \varepsilon \bullet m$, where C_{MDA} is the concentration of MDA (μmol g^{-1} wet mass), D_{532} and D_{600} are the optical densities of the sample at the appropriate wavelengths, ε is the MDA extinction coefficient equal to 155 $mmol^{-1}$ cm^{-1} , m is the mass of the sample (g).

To determine the content of soluble proteins and the activity of antioxidant enzymes the plant material was homogenized in 0.1 M K / Na-phosphate buffer (pH = 7.8) at 2-4 °C. The homogenate was centrifuged for 20 minutes at 15,000 g and 4 °C. The supernatant was used for the analysis. The soluble protein content was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

The determination of the total activity of SOD (EC 1.15.1.1) was carried out according to Beauchamp and Fridovich (1971), based on the ability of SOD to inhibit the photochemical reduction of nitrogen tetrazolium to formazan. The amount of the enzyme capable of suppressing the reduction of nitrogen tetrazolium by 50 % was taken as the unit of SOD activity. The activity of CAT (EC 1.11.1.6) was measured by the amount of decomposed hydrogen peroxide per unit time at 240 nm (Aebi, 1984). The enzyme activity was calculated using the extinction coefficient of hydrogen peroxide (E = 36 mol^{-1} cm^{-1}). The PRX activity (EC 1.11.1.7) was measured by the increase in optical density at 470 nm as a result of guaiacol oxidation (E = 26.6 $mmol^{-1}$ cm^{-1}) in the presence of hydrogen peroxide (Maehly and Chance, 1954).

2.5 Statistical analysis

Experiment was conducted in complete randomized design with 3 replications. Biological replication within each variant for different measurements was from 3 to 10 plants, analytical replication was 3-4-fold. Statistical processing of the data was carried out using the Microsoft Excel software package. The data are presented as the mean ± standard error and were tested by paired Student's test at the 5 % probability level.

3 RESULTS

3.1 Plant growth

The results showed that at a temperature of 22 °C cadmium has a minor effect on the growth of 7-day barley seedlings. There was only a shoot height increment (for 96 h) decrease (by 35 % of the control) (Table 2). However, this did not affect the plant biomass storage. The low temperature leads to inhibition of seedling growth.

All the growth parameters were lowered (compared to the control). The strongest decline in growth parameters was observed in the presence of cadmium.

Table 2: Effect of cadmium (100 µmol) on the barley growth under the optimum (22 °C) and low (4 °C) temperatures

Parameter	Treatment			
	22 °C	Cd^{2+}, 22 °C	4 °C	Cd^{2+}, 4 °C
Root length increment, mm/96 h	0.29 ± 0.11a	0.17 ±.0.05a	0.23 ± 0.04a	0.05 ± 0.02b
Shoot height increment, mm/96 h	5.12 ± 0.39a	1.30 ± 0.16b	0.39 ± 0.04c	0.25 ± 0.03c
Root dry biomass increment, mg/96 h	2.06 ± 0.49a	1.65 ± 0.35a	0.74 ± 0.41ab	0.31 ± 0.12b
Shoot dry biomass increment, mg/96 h	7.45 ± 1.44a	7.35 ± 0.96a	3.71 ± 1.37ab	2.87 ± 0.62b

Values perform mean ± SE (n = 20) * Significant differences at $p < 0.05$ from control level.

3.2 Cadmium content

Usually, the inhibition of plant growth at high cadmium concentrations is associated with its direct effect on cell division and elongation. In our experiments, however, after a four-day exposure to 4 °C temperature, the metal content in the roots and leaves of the seedlings was 3-4 times lower than at the optimum temperature (Table 3).

Table 3: Cadmium content (µg/g FM) in the barley root and leaves after 96 h exposure to metal (100 µmol) at the optimum (22 °C) and low (4 °C) temperatures

Organ	Treatment			
	22 °C	Cd^{2+}, 22 °C	4 °C	Cd^{2+}, 4 °C
Root	0.02 ± 0.001a	43.21 ± 2.50c	0.02 ± 0.001a	16.61 ± 0.87b
Leaf	0.02 ± 0.001a	5.52 ± 0.52c	0.03 ± 0.001a	2.26 ± 0.013b

Values perform mean ± SE (n = 10) * Significant differences at $p < 0.05$ from control level.

3.3 Lipid peroxidation rate

Since one of the main reasons for the slowing down of growth processes in plants under unfavorable environmental conditions is the formation of an excessive amount of ROS, we investigated the effect of cadmium on the lipid peroxidation rate in relation to temperature. It was found that under the cadmium at optimum temperature the MDA content slightly increased in the leaves and did not change in the roots, whereas at 4 °C it increases significantly both in the roots (by 73 % of the control) and in the leaves (by 23 %) (Table 3). Interestingly, at low temperature without cadmium, the MDA content did not rise.

Table 4: Effect of cadmium (100 µmol) on the MDA content and antioxidant enzymes activity in the roots and leaves of barley under the optimum (22 °C) and low (4 °C) temperatures

Parameter	Organ	Treatment			
		22 °C	Cd^{2+}+22 °C	4 °C	Cd^{2+}+4 °C
MDA content, µmol g^{-1}FM	root	2.56 ± 0.14a	2.77 ± 0.11a	4.11 ± 0.22b	4.42 ± 0.16b
	leaf	4.27 ± 0.16a	5.57 ± 0.22b	4.39 ± 0.16a	5.26 ± 0.22b
Total SOD activity, units·mg^{-1} protein	root	5.33 ± 0.39a	8.85 ± 0.51b	13.69 ± 1.93c	13.73 ± 1.24c
	leaf	2.60 ± 0.24a	3.99 ± 0.22b	4.03 ± 0.13b	3.93 ± 0.30b
Total CAT activity, µmol H_2O_2 min^{-1} mg^{-1} protein	root	5.93 ± 0.81a	17.59 ± 2.27b	4.29 ± 1.08ac	2.78 ± 0.53c
	leaf	39.90 ± 5.73a	47.49 ± 5.57ab	22.93 ± 2.09d	33.95 ± 1.90ac
Total PRX activity, µmol guaiacol min^{-1} mg^{-1} protein	root	16.34 ± 2.34a	29.09 ± 1.84b	17.56 ± 1.70a	21.26 ± 2.24a
	leaf	2.34 ± 0.24a	8.72 ± 1.13b	0.97 ± 0.10c	0.86 ± 0.07c

Values perform mean ± SE (n = 10) * Significant differences at $p < 0.05$ from control level.

3.4 Gene transcript content

A low level of ROS in plants under stress is maintained owing to the efficient operation of AOS, including antioxidant enzymes. The most important role belongs to SOD, which catalyzes the dismutation of the superoxide anion radical reaction, as well as to CAT and PRX, which decompose the hydrogen peroxide (Foyer and Noctor, 2015). In our study, after 96 h of cadmium impact on barley seedlings at 22 °C in the roots the

content of gene transcripts of all antioxidant enzymes increased practically equally (approximately 2 times) (Fig.). In leaves, the *HvCu/ZnSOD* mRNA level did not change, while *HvCAT2* and *HvPRX07* also increased. In contrast, when seedlings exposed to cadmium at 4 °C the *HvCu/ZnSOD* transcripts content increased in both roots and leaves, while the *HvCAT2* and *HvPRX07* mRNA level did not change in the roots and even decreased in the leaves. Low temperature effect without cadmium led to an increase of the *HvCu/ZnSOD* and *HvPRX07* mRNA level.

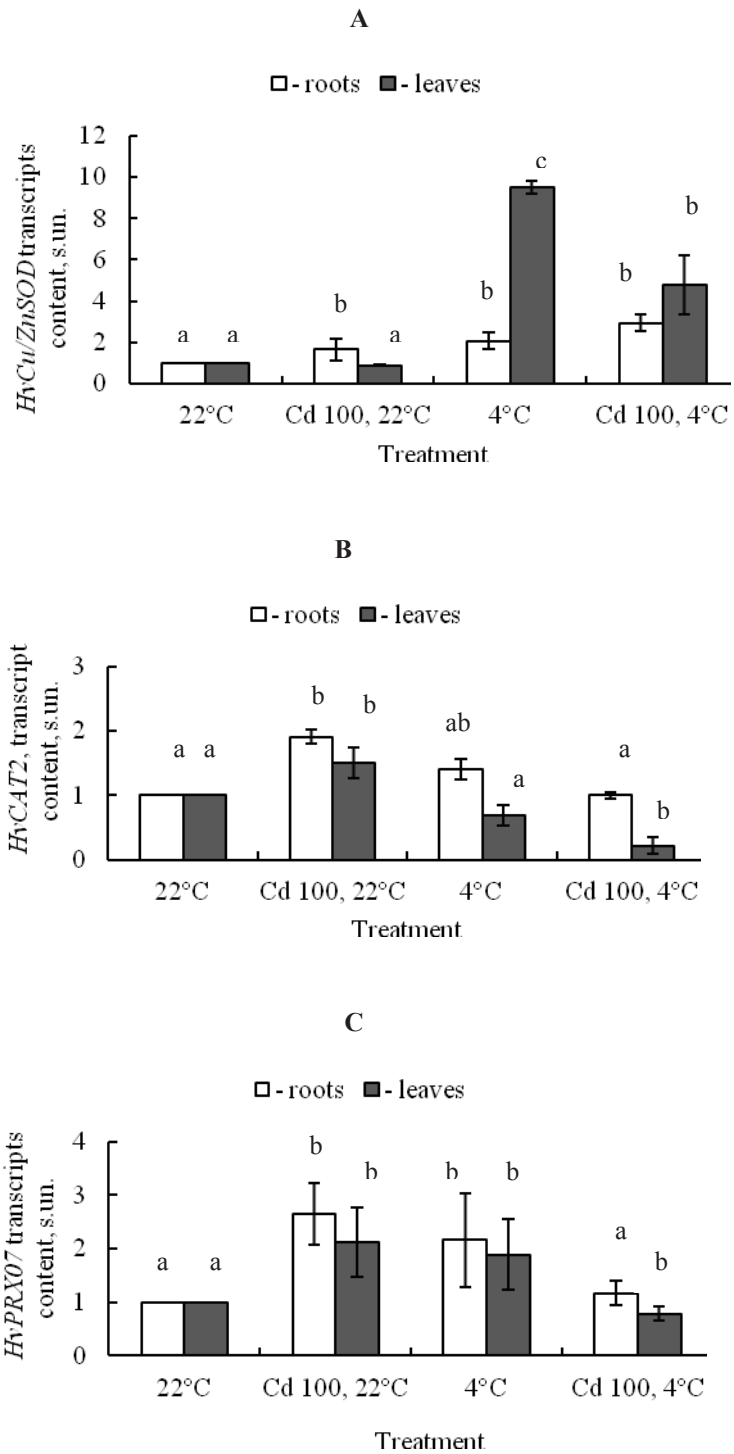

Figure 1: Effect of cadmium (100 µmol) on the *HvCu/ZnSOD* (A)*, HvCAT2* (B) and *HvPRX07* (C) transcripts level in the roots and leaves of barley seedlings under the optimum (22 °C) and low (4 °C) temperatures

3.5 Antioxidant enzymes activity

The change in the antioxidant enzymes activity in most cases corresponded to changes in the gene transcript content. For example, in the roots SOD and PRX activity in the presence of cadmium and under the optimum temperature increased almost two-fold compared to the control, and CAT activity increased three-fold (Table 3). At 4 °C, on the other hand, SOD activity was significantly increased (tripled compared to the control), while PRX activity did not change and CAT activity decreased. In the leaves at 22 °C antioxidant enzymes activity was higher than in the control, while at low temperatures SOD activity increased three-fold and CAT and PRX activity significantly decreased (two- and three-fold, respectively). At a low temperature without cadmium only SOD activity was increased also.

4 DISCUSSION

It is known that plants are able to grow normally at high cadmium concentrations in the environment. This is possible due to a number of adaptation mechanisms at different organizational levels. Among them, the antioxidant system plays an important role. In our experiments, cadmium at the optimum temperature had no adverse effect on the growth of barley seedlings. The high cadmium resistance of the plants was partly due to owing the consistent rise of the antioxidant gene transcripts content and the activity of antioxidant enzymes in roots and leaves. As a result, there was no increase in the lipid peroxidation rate in the cells, indicating the absence of oxidative stress. Similar data were obtained by other authors. For example, in the roots and leaves of *Arabidopsis thaliana* (L.) Heynh. exposed to this metal there was an increase in the gene expression level of such antioxidant enzymes as ascorbate peroxidase (APX) and CAT (Smeets et al., 2008), in rice roots – glutathione-S-transferase, APX and glutathione reductase (GR) (Lee et al., 2010), in the leaves of *Lolium perenne* L. – Cu/ZnSOD, APX and GR (Luo et al., 2011), which was accompanied by an increase in the enzymes activity and contributed to the adaptation of the plants to cadmium.

Under exposure to cadmium at 4° C, the growth of both roots and shoots of the seedlings was inhibited, despite the lower metal content in the plants. The cells experienced an acceleration of oxidative processes, as evidenced by an increased of lipid peroxidation rate. In this case, the *HvCu/ZnSOD* transcripts content and the total activity of SOD in the roots and leaves increased significantly. At the same time, there was no rise of the *HvCAT2* and *HvPRX07* mRNA level and the CAT and PRX activity. The fact that the activity of H_2O_2 decomposing enzymes was not enhanced under low temperatures has been mentioned in other studies. For example, APX activity in the leaves of a cold-resistant potato variety did not change at 5 °C (Sinkevich et al., 2011). In chilling-resistant varieties of tobacco (Gechev et al., 2003), barley, rice and wheat (Janda et al., 2003) exposed to 5 °C temperature a decrease in CAT activity was observed. In these cases the amount of hydrogen peroxide in the cells increased.

It is known that an agreed increase of the antioxidant enzymes activity is essential for maintaining the balance between the generation and neutralization of ROS (Gechev et al., 2003; Avasthi et al., 2015). Hence, the enhanced SOD activity alone, without an increase in the activity of CAT and/or PRX, as was observed in our experiments, leads to a build up of the hydrogen peroxide concentration in cells, and, accordingly, to an acceleration of oxidative processes. Probable development of oxidative stress in plants exposed to cadmium at low temperature was one of the reasons for the inhibition of their growth.

Attention must also be given to the high level of *HvPRX07* transcripts and a significant increase in PRX activity in the seedlings' roots and leaves under exposure to cadmium at 22° C. This effect may be associated with its various functions in the cell. For example, with its participation in the lignifications of cell walls, where they can provide additional protection of cells from the toxic ions and thereby contribute to an increase of metal resistance (Lukačová et al., 2013). Since at low temperature without cadmium all growth parameters changed to a lesser degree than in the presence of the metal, it can be assumed that with the combined action of low temperature and cadmium, their negative effects are summarized.

5 CONCLUSIONS

Thus, the effect of cadmium on the growth and antioxidant enzymes activity of barley seedlings is temperature-dependent. In the treatment with exposure to cadmium under optimal temperature the consistent increase of the *HvCu/ZnSOD, HvCAT2* and *HvPRX07* transcripts content and the activity of SOD, CAT and

PRX prevented the development of oxidative stress in the cells, enabling the plants to grow normally at a high concentration of cadmium in the medium and in the plants' organs. In contrast, exposure to cadmium coupled with low temperature inhibited plant growth, since the lipid peroxidation rate in the roots and leaves was accelerated. The activation of oxidative processes in this case is likely to be connected with the accumulation of excess amounts of hydrogen peroxide due to a misbalance between its generation and neutralization.

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7 REFERENCES

Aebi, H. E. (1984). Catalase *in vitro*. *Methods in Enzymology*, *105*, 121-126. doi:10.1016/S0076-6879(84)05016-3

Avasthi, R., Bhandari, K., Nayyar, H. (2015). Temperature stress and redox homeostasis in agricultural crops. *Frontiers in Environmental Science*, *3*, 1-24. doi:10.3389/fenvs.2015.00011

Beauchamp, C., Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical of Biochemistry*, *44*, 276-287. doi:10.1016/0003-2697(71)90370-8

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical of Biochemistry*, *72*, 248-254. doi:10.1016/0003-2697(76)90527-3

Foyer, C.H., Noctor, G. (2005). Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant, Cell and Environment*, *29*, 1056-1071. doi:10.1111/j.1365-3040.2005.01327.x

Foyer C. H., Noctor, G. (2015). Defining robust redox signalling within the context of the plant cell. *Plant, Cell and Environment*, *38*, 239-239. doi:10.1111/pce.12487

Gechev, T., Willekens, H., Van Montagu, M., Inze, D., Van Camp, W., Toneva, V., Minkov, I. (2003) Different responses of tobacco antioxidant enzymes to light and chilling stress. *Journal of Plant Physiology*, *160*, 509-515. doi:10.1078/0176-1617-00753

Gill, S. S, Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*. *48*, 909-930. doi:10.1016/j.plaphy.2010.08.016

Heath, R. L., Packer, L. (1968). Photoperoxidation in isolated cloroplasts. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, *125*, 189-198. doi:10.1016/0003-9861(68)90654-1

Janda, T., Szalai, G., Rios-Gonzaier, K., Veisz, O., Páldi, E. (2003). Comparative study of frost tolerance and antioxidant activity in cereals. *Plant Science*, *164*, 301-306. doi:10.1016/S0168-9452(02)00414-4

Khan, M. A., Samiullah, S., Singh, S., Nazar, R. (2007). Activities of antioxidative enzymes, sulphur assimilation, photosynthetic activity and growth of wheat (*Triticum aestivum*) cultivars differing in yield potential under cadmium stress. *Journal of Agronomy and Crop Science*, *193*, 435-444. doi:10.1111/j.1439-037X.2007.00272.x

Lee, K., Bae, D.W., Kim, S. H., Han, H. J., Liu, X., Park, N. C., Lim, C. O., Lee, C. Y., Chung, W. S. (2010). Comparative proteomic analyses of the short-term responses of rice roots and leaves to cadmium. *Journal of Plant Physiology*, *167*, 161-168. doi:10.1016/j.jplph.2009.09.006

Livak, K. J., Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, *25*, 402-408. doi:10.1006/meth.2001.1262

Lukačová, Z., Švubová, R., Kohanová, J., Lux, A. (2013). Silicon mitigates the Cd toxicity in maize in relation to cadmium translocation, cell distribution, antioxidant enzymes stimulation and enhanced endodermal apoplasmic barrier development. *Plant Growth Regulation*, *70*, 89-103. doi:10.1007/s10725-012-9781-4

Luo, H., Li, H., Zhang, X., Fu, J. (2011). Antioxidant responses and gene expression in perennial ryegrass (*Lolium perenne* L.) under cadmium stress.

Ecotoxicology, *20*, 770-778. doi:10.1007/s10646-011-0628-y

Maehly, A. C., Chance, B. (1954). The assay of catalase and peroxidase. *Methods of Biochemical Analysis*, *1*, 357-424.

Sandalio, L. M., Dalurzo, H. C., Gómez, M., Romero-Puertas, M. C., Del Rio, L. A. (2001). Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of Experimental Botany*, *52*, 2115-2126. doi:10.1093/jexbot/52.364.2115

Schützendübel, A., Polle, A. (2002). Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany*. *53*, 1351-1365. doi:10.1093/jexbot/53.372.1351

Sergeant, K., Kieffer, P., Dommes, J., Hausman, J.-F., Renaut, J. (2014). Proteomic changes in leaves of poplar exposed to both cadmium and low-temperature. *Environmental and Experimental Botany*, *106*, 112-123. doi:10.1016/j.envexpbot.2014.01.007

Sin'kevich, M. S., Naraikina, N. V., Trunova, T. I. (2011). Processes hindering activation of lipid peroxidation in cold-tolerant plants under hypothermia. *Russian Journal of Plant Physiology*, *58*, 1020-1026. doi:10.1134/S1021443711050232

Smeets, K., Opdenakker, K., Remans, T., Van Sanden, S., Van Belleghem, F., Semane, B. (2009). Oxidative stress-related responses at transcriptional and enzymatic levels after exposure to Cd or Cu in a multipollution context. *Journal of Plant Physiology*, *166*, 1982-1992. doi:10.1016/j.jplph.2009.06.014

Smeets, K., Ruytinx, J., Semane, B., Van Belleghem, F., Remans, T., Van Sanden, S., Vanginsveld. J., Cuypers, A. (2008). Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environmental and Experimental Botany*, *63*, 1-8. doi:10.1016/j.envexpbot.2007.10.028

Venzhik, Yu. V., Talanova, V. V., Titov, A. F., Kholoptseva, E. S. (2015). Similarities and differences in wheat plant responses to low temperature and cadmium. *Biology Bulletin*, *42*, 508-514. doi:10.1134/S1062359015060126

Wu, F., Zhang, G., Dominy, P. (2003). Four barley genotypes respond differently to cadmium: lipid peroxidation and activities of antioxidant capacity. *Environmental and Experimental Botany*, *50*, 67-78. doi:10.1016/S0098-8472(02)00113-2

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Pridelek, morfološki razvoj in hranilna vrednost zelinja lucerne med rastno sezono v osrednji Sloveniji: analiza časovnih potekov

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IZVLEČEK

Čas košnje je zelo pomemben dejavnik količine in kakovosti pridelka krme enovrstnih koševin. Zato smo želeli analizirati časovne poteke pridelka zelinja, morfološkega razvoja in parametrov kakovosti lucerne med rastno sezono ter jih povezati s časom košnje. Poljski poskus v split-plot zasnovi z dvema blokoma smo izvedli v Ljubljani leta 2016. Glavne parcele so predstavljale 4 rastne cikluse (C1-C4), podparcele pa 9 tedenskih terminov za merjenje pridelka in vzorčenje zelinja. Rast lucerne je bila v prvi polovici rastne sezone boljša kot v drugi. Pridelek zelinja je v zadnjih dveh ciklusih hitro dosegel maksimum in se nato zmanjševal. Statistična analiza je pokazala, da so linearni regresijski modeli primerni za opisovanje časovnih potekov naslednjih spremenljivk: morfološkega razvoja (PRF), surovih beljakovin (SB), vlaken, netopnih v nevtralnem detergentu (NDV) in neto energije za laktacijo (NEL). Morfološki razvoj lucerne je bil hitrejši poleti kot pomladi ali jeseni, vendar je po tej lastnosti med ciklusi izrazito odstopal samo prvi. Kakovost lucerne je bila predvsem zaradi počasnejšega razvoja v celoti najboljša v C1, kar se je bolj odrazilo pri NDV in NEL kot pri SB. C1 je bil po kakovosti lucerne enakovreden samo C4 na začetku rasti. Upoštevajoč pridelek in vsebnost NEL je bila optimalna starost lucerne ob košnji od 28 do 35 dni spomladi in poleti ter od 35 do 42 dni jeseni.

Ključne besede: lucerna; pridelek; morfološki razvoj; hranilna vrednost; časovni potek

ABSTRACT

HERBAGE YIELD, MORPHOLOGICAL DEVELOPMENT AND NUTRITIVE VALUE OF LUCERNE DURING GROWTH SEASON IN CENTRAL SLOVENIA: ANALYSIS OF TIME PATTERNS

Cutting time is a pivotal factor affecting herbage yield and nutritive value of forage crop monocultures. Therefore, our objectives in this lucerne study were to analyse temporal patterns of herbage yield, morphological development and quality parameters over growth season, and to relate these patterns to the time of cutting. A field experiment in split-plot design with two block replications was conducted in Ljubljana in 2016. Four growth cycles (C1-C4) were assigned to the main plots, and nine weekly intervals at which herbage yield was measured and herbage samples taken were assigned to the sub-plots. Dry-matter herbage yield accumulated faster during the first half of the season than during the second one. It peaked early in each of the last two growth cycles and after that started to decrease. Statistical analysis showed that linear regression models are acceptable to describe time patterns of morphological development stages (MSW) and contents of crude protein (CP), neutral detergent fibre (NDF) and net energy for lactation (NEL). MSW increased faster during the summer than during the spring or autumn, but only C1 was distinct in this pattern. Lucerne forage quality was generally the highest in C1 mainly due to slower morphological development. This high quality reflected more in NDF and NEL than in CP. In respect of the quality, only C4 at the beginning of the growth was equivalent to C1. Considering yield and content of NEL optimal age of lucerne at cutting was from 28 to 35 days in spring and summer and from 35 to 42 days in autumn.

Key words: lucerne; herbage yield; morphological development; nutritive value; time pattern

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1 UVOD

Lucerna (*Medicago sativa* L.) je v svetovnem merilu najpomembnejša gojena krmna metuljnica. Njena ocenjena pridelava obsega 32 mio ha (Russelle, 2001). Kot krmna rastlina je cenjena zaradi velikega rastnega potenciala, kakovostne krme in obstojnosti sestojev. Letni pridelek krme je običajno večji od 10 t sušine ha^{-1}, v zelo ugodnih razmerah za rast, ob namakanju in dolgi rastni sezoni lahko ta znaša od 20 t do 25 t sušine ha^{-1} (Lloveras in sod., 2012). Lucerna je predvsem beljakovinsko bogata voluminozna krma z vsebnostjo surovih beljakovin (SB) okoli 200 g kg^{-1} sušine. Pri zelo mladi krmi lahko vsebnost SB preseže 300 g kg^{-1} sušine (McGraw in Marten, 1986). Odlikuje se tudi po hitri prebavljivosti v vampu in s tem povezanim potencialom za povečano zauživanje krme (Marten in sod., 1988). Kot druge gojene metuljnice tudi lucerna na splošno vsebuje trikrat več kalcija in dvakrat več železa kot gojene trave (Whitehead, 1972). Lucerna ima tudi pridelovalne in prehranske pomanjkljivosti. Slabo prenaša prekomerno vlažnost tal, pomanjkanje glavnih mineralnih hranil in kisla tla (< pH 6). V primerjavi s črno deteljo se njena hranilna vrednost v smislu prebavljivosti in vsebnosti vlaknine znatno hitreje slabša s časom rasti in zorenjem, kar je posledica povečane lignifikacije stebel (Kühbauch in Pletl, 1981; Kühbauch in Voigtländer, 1981; Albrecht, 1983). Siliranje lucerne je v primerjavi s travami zahtevnejše zaradi njene večje puferske kapacitete in manjše vsebnosti vodotopnih ogljikovih hidratov (Rizk, 2004; Verbič in Verbič, 2017).

Morfološki razvoj lucerne, ki poteka skupaj z rastjo, je zelo kompleksen zaradi tvorbe stebelnih stranskih poganjkov, številnih socvetij in s tem povezanega dolgega obdobja cvetenja ter odganjanja naknadnih poganjkov iz prizemnih brstov med staranjem sestojev (po 7 tednu rasti). Pravimo tudi, da ima lucerna poudarjen nedeterminanten način rasti. Iz tega izhaja, da je morfološka sestava sestoja lucerne – razen na začetku rasti – zelo raznolika, in da ni mogoče vizualno zadovoljivo opredeliti njegove razvojne faze. Zato sta Kalu in Fick (1981) za potrebe posrednega ocenjevanja kakovosti krme razvila postopek, s katerim določimo tehtano povprečno morfološko razvojno fazo sestoja (PRF). Pri tem postopku poganjke v vzorcu lucerne najprej razvrstimo v razvojne faze od 0 do 9. Maso suhih poganjkov posameznih faz nato uporabimo za izračun tehtanega povprečja, kjer je potrebno upoštevati tudi številčno kodo razvojne faze. Postopek je možno poenostaviti tako, da izpustimo sušenje in stehtamo sveže poganjke, kar ne vpliva na rezultat analize (Božičković in sod., 2013).

Ker se kakovost krme med rastjo lucerne razmeroma hitro slabša, je za njeno uspešno vključevanje v krmne obroke zelo pomembna izbira ustreznega termina košnje. Ta izbira je vedno kompromis med količino in kakovostjo pridelka. Na splošno se pridelek krme pri lucerni linearno povečuje med zgodnjo rastjo do cvetenja. Kasneje se povečevanje pridelka krme upočasni in se med zorenjem lucerne celo zmanjša. Slednje je predvsem posledica odpadanja listov s starejših poganjkov, ki ga stresne razmere (npr. glivične okužbe, zasenčenje, suša) lahko še pospešijo. Dosegljive informacije o spreminjanju pridelka lucerne med rastjo so skope in omejene glede števila zajetih rastnih ciklusov (Kühbauch in Pletl,1981; McGraw in Marten, 1986; Bowley in sod., 1988; Brink in sod., 2010; Kruz in sod., 2016). Nasprotno od količine se kakovost lucerne med rastjo zmanjšuje, vendar na splošno manj med prvim ciklusom kot med naslednjimi. Objavljene raziskave se večinoma nanašajo na spreminjanje vsebnosti SB, vlaken, netopnih v nevtralnem (NDV) in kislem detergentu (KDV) in prebavljivost suhe snovi (npr. Kalu in Fick, 1983; McGraw in Marten, 1986; Hall in sod., 2000; Brink in sod. 2010). Za časovni potek vsebnosti neto energije za laktacijo (NEL) smo v literaturi našli le podatke za prvo košnjo (Žnidaršič in sod., 2015). V tej raziskavi so časovni poteki preučevanih parametrov kakovosti opisani z linearnimi ali kvadratnimi funkcijami.

Namen raziskave je bil ugotoviti časovne poteke povečevanja pridelka suhega zelinja, morfološkega razvoja sestoja in spreminjanja kakovosti krme med rastjo lucerne po ciklusih ter jih med seboj primerjati z uporabo statističnih modelov. Namen raziskave je bil tudi ugotoviti zgornjo mejo za pridelek SB in NEL pri njunih sprejemljivih vsebnostih za krmljene živali. Poskus je potekal na območju s predalpskim podnebjem, za katerega navedena problematika še ni bila preučevana.

2 MATERIALI IN METODE

2.1 Postavitev in opis poskusa

Raziskavo smo opravili na osnovi poljskega poskusa, ki smo ga postavili 10. aprila 2015 na poskusnem polju Biotehniške fakultete v Ljubljani (46° 03' S, 14° 28' V, 300 m n. m.). Poskus je bil v split-plot zasnovi z dvema ponovitvama. Na glavne parcele so bili razporejeni štirje rastni ciklusi (C1-C4), ki so zajeli celo rastno sezono. Rastni ciklus pomeni 70 dnevno rast lucerne od

temperaturnega praga spomladi (C1), oziroma od predhodne košnje, opravljene ob začetku cvetenja lucerne (C2-C3). Na podparcele je bilo razporejenih devet tedenskih terminov za meritve pridelka in vzorčenja lucerne. Velikost osnovne parcele je bila 1,32 m × 5,0 m. Izbrana je bila srbska sorta lucerne 'NS Mediana ZMS V', ki je nastala s križanjem lucerne in srpaste meteljke. Setev smo opravili s parcelno sejalnico Wintersteiger na medvrstno razdaljo 11 cm pri setvenem odmerku 25 kg ha^{-1}. Seme je bilo tik pred setvijo inokulirano z bakterijo *Rhizobium meliloti* (Dangeard, 1926) De Lajudie et al., 1994, pripravek NS-Nitragin. Ob setvi smo posevek pognojili z mineralnim gnojilom 400 kg ha^{-1} (NPK 7:20:30), naslednje leto, 23. marca 2016 pa z 900 kg ha^{-1} (PK 10:30). Rastni ciklusi v letu 2016, rezultate katerega predstavlja ta prispevek, so se po vrsti od prvega do četrtega začeli: 25. marca, 6. maja, 17. junija in 29. julija. Začetek C1 je bil določen s temperaturnim pragom (5 °C), ostali pa z datumi predhodnih košenj.

2.2 Tla in vremenske razmere

Na poskusnem polju se nahajajo rjava aluvialna tla na karbonatnem pesku in produ. V zgornji 30 cm plasti je meljasta ilovica, pod njo je do globine 110 cm zmerno oglejena meljasto glinasta ilovica. Na začetku poskusa so bila tla v zgornji 22 cm plasti nevtralna (pH 6,9 v $CaCl_2$), srednje do dobro preskrbljena s fosforjem (140 g P_2O_5 kg^{-1} tal, AL metoda) in srednje preskrbljena s kalijem (168 g K_2O kg^{-1} tal, AL metoda). Vsebnost organske snovi je bila srednja (3,8 % v suhih tleh). Na poskusnem polju je postavljena cevna drenaža, po kateri odteka padavinska voda, ki pronica skozi tla.

Povprečna temperatura zraka je bila v letu 2016 nižja za 0,2 °C od povprečnih 10,9 °C za obdobje 1981-2010 na meteorološki postaji Ljubljana-Bežigrad. Najbolj so odstopali naslednji meseci: februar s 3 °C višjo temperaturo od referenčnega povprečja (1,9 °C), maj s 1,8 °C, avgust s 1,2 °C, oktober s 1,7 °C in december s 2,5 °C nižjimi temperaturami od referenčnih povprečij (15,8 °C, 20,6 °C, 11,2 °C, 1,2 °C). Konec aprila se je ohladilo in padlo je 15 cm snega. Padavin je bilo v letu 2016 za 82 mm manj od povprečja za referenčno obdobje (1361 mm). Njihova razporeditev med rastno sezono je bila ugodna za uspevanje rastlin razen v avgustu in septembru, ko je bila suša. Vremenske razmere za rast lucerne so bile na splošno dobre, razen v zadnji dekadi aprila in v sredini maja, ko so minimalne temperature zraka padle pod 5 °C, 30. aprila celo na -1,2 °C. Od sredine septembra so bile ugodne razmere za razvoj lucernine rje (*Uromyces striatus* Schröter), ki je oslabila rast lucerne.

2.3 Meritev pridelka in morfološka analiza

Za ugotavljanje pridelka zelinja smo pokosili notranji del parcele v velikosti 1,2 m × 5,0 m. Rez kosilnice je bila nastavljena na višino 7 cm. Z vsake parcele smo od pokošene lucerne odvzeli približno kilogram vzorca, kateremu smo določili vsebnost sušine ter vsebnosti SB, NDV in NEL. Pridelek suhega zelinja smo izračunali iz pridelka svežega zelinja in vsebnosti sušine. Pridelek SB in NEL smo izračunali z množenjem pridelka suhega zelinja in deležem SB oziroma NEL v sušini.

Analizo povprečne morfološke razvojne faze (PRF) smo opravili po postopku, ki sta ga razvila Kalu in Fick (1981) z upoštevanjem poenostavitve pri tehtanju razvojnih frakcij poganjkov (Božičković in sod., 2013). Posamezni vzorci lucerne, namenjeni za to analizo, so bili odvzeti s kvadratne površine 0,1 m^2 na višini 3,5 cm. Poganjke smo postrigli z ročnimi škarjami. PRF smo izračunali po enačbi:

$$PRF = \sum_{i=0}^{9} (i \cdot m_i) / \sum_{i=0}^{9} m_i ,$$

kjer pomeni *PRF* povprečno razvojno fazo sestoja na podlagi tehtanja svežih poganjkov, *i* zaporedno številčno kodo razvojne faze posameznega poganjka (0-9), m_i maso poganjkov v fazi *i*. Meritve pridelka in analize PRF smo delali tedensko na dveh ponovitvah za vseh devet terminov znotraj posameznega ciklusa. Vsi štirje ciklusi so bili obdelani na enak način. Prva meritev in analiza sta se začeli 14 dni po začetku rasti pri C1, C3 in C4 ter 21 dni po začetku rasti pri C2.

2.4 Analiza hranil in neto energije

Vsebnosti SB in NDV smo določili po NIRS metodi (bližnja infrardeča refleksijska spektrometrija; Roberts in sod., 2004). Vsebnosti NEL smo izračunali z enačbami, ki jih je za lucerno in detelje predlagal GfE (2016). Pri tem smo uporabili podatke o sestavi krme [SB, surova vlaknina (SVl), surove maščobe (SM), pepel, KDV] in volumnu plina, ki nastane pri inkubaciji vzorcev z vampovim sokom. Vse navedene parametre, potrebne za izračun NEL, smo določili po NIRS metodi. Kalibracijske enačbe za NIRS metodo so bile razvite na podlagi 485 vzorcev trav in metuljnic, med njimi 88 vzorcev lucerne (od tega 11 iz našega poskusa), ki smo jim vsebnosti SB določili po Kjeldahlovi metodi (International organisation for standardisation, 2005), vsebnosti SVl po International organisation for standardisation (2000), vsebnosti SM brez predhodne hidrolize po Uredbi komisije (ES) 152/2009, vsebnosti pepela po International organisation for standardisation (2002), vsebnosti KDV in NDV po Van Soestu in sod. (1991), količino plina, ki nastane pri inkubaciji vzorcev z vampovim sokom pa s pomočjo Hohenheimskega

plinskega testa (Menke in sod., 1979), oziroma z modificirano različico, ki sta jo opisala Blümmel in Ørskov (1993). Med vzorci za NIRS umeritve je bilo tudi 11 vzorcev iz tega poskusa. NIRS meritve so potekale na zračno suhih in skozi 1 mm sito zmletih vzorcih zelinja.

2.5 Statistična analiza podatkov

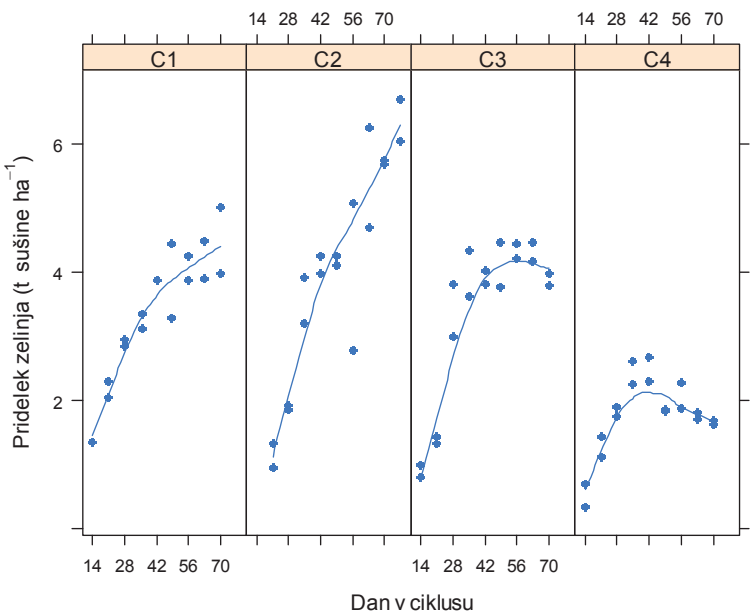
Za vsak preučevani parameter (pridelek, PRF, SB, NDV in NEL) posebej smo po ciklusih grafično prikazali njegovo odvisnost od časa rasti. Za to smo uporabili metodo lokalne regresije. Slike gladilnikov so pokazale, da je linearni regresijski model sprejemljiv za PRF, SB, NDV in NEL. V linearnem regresijskem modelu smo upoštevali tri napovedne spremenljivke: dan v ciklusu (začeli smo z vrednostjo 0, ki se nanaša na prvo meritev, opravljeno na 14. oziroma 21. dan), ciklus ter njuno interakcijo. S takim modelom dobimo ocene parametrov s standardno napako za vsak ciklus posebej ter koeficient determinacije za celoten model. Vrednost za presečišče *a* predstavlja napoved ob začetku meritev, vrednost za naklon premice *b* pa trend. Na osnovi navedenega linearnega regresijskega modela smo paroma primerjali presečišča in naklone po ciklusih. Za odvisnost pridelka zelinja od časa rasti nismo izdelali napovednih modelov. Opise časovnega spreminjanja pridelka smo naredili na podlagi gladilnikov.

Analiza je bila izvedena s programskim in grafičnim okoljem R s paketi lattice, nlme ter multcomp (R Core Team, 2016).

3 REZULTATI

3.1 Časovni potek pridelka zelinja

Pridelek suhega zelinja lucerne se je linearno povečeval v vseh ciklusih do 42. dneva rasti (Slika 1). Po tem dnevu se je priraščanje malo zmanjšalo v prvih dveh ciklusih in občutno v zadnjih dveh. Odstopa C2, kjer je bila rast v začetku zelo počasna zaradi nizkih temperatur zraka. Tako smo prvo meritev pridelka lahko opravili en teden kasneje kot v drugih treh ciklusih. Povprečni pridelek 21. dne v tem ciklusu (1,1 t sušine ha^{-1}) je bil podoben povprečnima pridelkoma v predhodnem in naslednjem ciklusu na 14. dan. Temu počasnemu začetku rasti je sledila najhitrejša rast. Zato je bil v C2 dosežen tudi maksimalni pridelek (6,4 t sušine ha^{-1} na 70. dan). V C3 in C4 se je pridelek začel zmanjševati od 63. oziroma 42. dne naprej.



Slika 1: Časovni potek pridelka zelinja lucerne po ciklusih (C1-C4) med rastno sezono 2016 v predalpskem podnebju Slovenije. Krivulje so gladilniki, dobljeni z lokalno regresijo.

Figure 1: Time pattern of herbage dry matter yield accumulation during four growth cycles (C1-C4) in the pre-Alpine climate of Slovenia in the season 2016. Smooth curves were obtained by local regression.

3.2 Modelirani časovni poteki PRF ter vsebnosti SB, NDV in NEL

Na osnovi grafičnih prikazov časovnih potekov PRF ter vsebnosti SB, NDV in NEL po ciklusih smo ugotovili, da je linearna zveza sprejemljiva (Slika 2). Vse premice se dobro prilegajo gladilnikom. Pojasnjena variabilnost (R^2) v regresijskem modelu za posamezne parametre je velika in znaša od 88,7 % za SB do 93,9 % za PRF (Preglednica 1).

Analiza časovnega poteka PRF je pokazala, da med presečišči premic po ciklusih ni statistično značilnih razlik, so pa statistično značilne razlike med nakloni. Največji naklon imata premici pri C2 in C3, med katerima ni statistično značilne razlike ($p > 0,05$). Srednji naklon ima premica pri C4, najmanjšega pa premica pri C1. Razlike v naklonih premic med ciklusi so razen navedene izjeme statistično značilne ($p < 0,01$). Najmanjši naklon pri C1 je 2,6 krat manjši kot pri najbližjem C4 (Preglednica 1).

Analiza časovnega poteka SB je pokazala, da ima največjo vrednost presečišče premice pri C4. Razlike med njim in drugimi tremi presečišči so statistično značilne ($p < 0,05$). Največja razlika v presečiščni vsebnosti SB (58,7 g kg^{-1} sušine) je bila ugotovljena med C3 in C4. Po naklonu so premice razdeljene v dve skupini in sicer v skupino z manjšim absolutnim naklonom (C1, C3) in v skupino z večjim absolutnim naklonom (C2, C4). Med skupinama so v naklonu premic statistično značilne razlike ($p < 0,001$), razen med C2 in C3, kjer je razlika mejno statistično značilna ($p = 0,06$). Primerjava dveh najbolj različnih naklonov pokaže, da se je vsebnost SB pri C1 dvakrat počasneje zmanjševala kot pri C4.

Preglednica 1: Ocene koeficientov (*a*, *b*) in njihove standardne napake za linearne regresije časovnih potekov PRF ter vsebnosti SB, NDV in NEL v zelinju lucerne po ciklusih (C1-C4). Koeficienti determinacije (R^2) so podani za vse štiri cikluse skupaj. Presečišča znotraj posameznega parametra kakovosti, označena z isto črko, niso statistično značilno različna ($p > 0,05$). Enako velja za naklone.

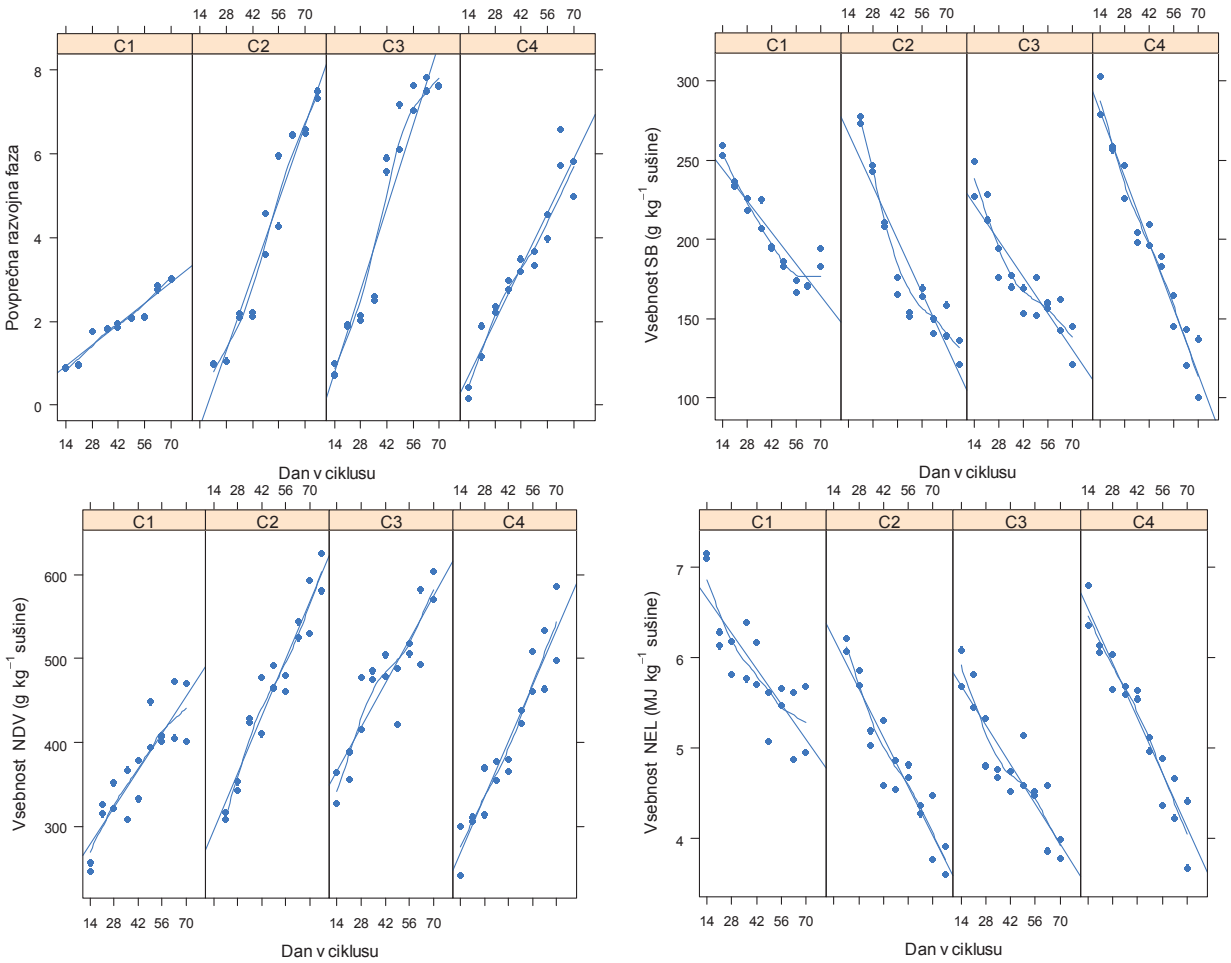
Table 1: Estimated linear regression coefficients (*a, b*) and their standard errors for predicting time patterns of MSW, and contents of CP, NDF and NEL in herbage dry matter of lucerne analysed separately for cycles (C1-C4). Coefficients of determination (R^2) are given for all four cycles together. Intercepts denoted with the same letter are not significantly different within particular parameter of quality ($p > 0.05$). The same holds for slopes.

Parameter kakovosti	Ciklus	Presečišče *a*	St. napaka (*a*)	Naklon *b*	St. napaka (*b*)
PRF (R^2 = 93,9 %)	C1	0,96a	0,269	0,036 c	0,008
	C2	0,44a	0,268	0,128a	0,008
	C3	0,81a	0,268	0,140a	0,008
	C4	0,73a	0,268	0,093 b	0,008
SB (R^2 = 88,7 %)	C1	244 bc	7,114	-1,43a	0,204
	C2	250 b	6,792	-2,40 bc	0,204
	C3	222 c	6,792	-1,64ab	0,204
	C4	281a	6,792	-2,96 c	0,204
NDV (R^2 = 90,4 %)	C1	279 bc	14,35	3,12 b	0,398
	C2	328ab	14,29	4,87a	0,397
	C3	366a	14,29	3,72ab	0,397
	C4	268 c	14,29	4,77a	0,397
NEL (R^2 = 89,5 %)	C1	6,65a	0,145	-0,028a	0,004
	C2	5,93 b	0,145	-0,039ab	0,004
	C3	5,70 b	0,145	-0,031ab	0,004
	C4	6,53a	0,145	-0,043 b	0,004

Opomba: Vrednosti za presečišča pri C1, C3 in C4 se nanašajo na 14. dan rasti, pri C2 pa na 21. dan rasti.
Note: *a* = intercept, *b* = slope, MSW = PRF, CP = SB, NDF = NDV

Regresijske premice časovnih potekov NDV so glede presečišč razdeljene na skupino z manjšima vrednostma (C1, C4) in na skupino z večjima vrednostma (C2, C3). Med skupinama so v vrednosti presečišč po parih statistično značilne razlike ($p < 0,05$), razen med C1 in C2, kjer je razlika mejno statistična značilna ($p = 0,07$). Največja razlika v presečiščni vsebnosti NDV (97 g kg^{-1} sušine) je bila ugotovljena med C3 in C4. V naklonih premic se ciklusi manj razlikujejo kot po presečiščih. Statistično značilni razliki v naklonu premic sta med C1 in C2 oziroma C4 ($p < 0,05$).

Odnosi med regresijskimi premicami časovnih potekov NEL so podobni kot pri NDV. Presečišča delijo premice na skupino z večjima vrednostma (C1, C4) in na skupino z manjšima vrednostma (C2, C3). Med skupinama so v vrednostih presečišč po parih močno statistično značilne razlike ($p < 0{,}001$). Povprečna razlika v vsebnosti NEL med skupinama znaša 0,8 MJ kg^{-1} sušine. V naklonih premic je bila ugotovljena edina statistično značilna razlika med C1 in C4 ($p < 0{,}05$). Pri C4 se je vsebnost NEL s časom rasti 1,5 krat hitreje zmanjševala kot pri C1.

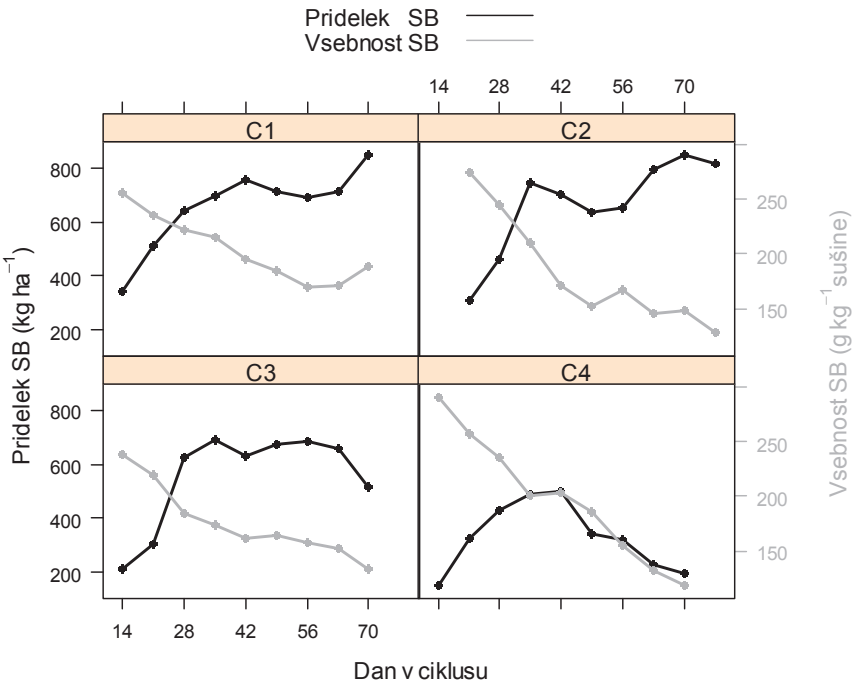


Slika 2: Časovni potek PRF ter vsebnosti SB, NDV in NEL v zelinju lucerne po ciklusih (C1-C4) med rastno sezono 2016 v predalpskem podnebju Slovenije. Na slikah so regresijske premice in gladilniki.

Figure 2: Time pattern of MSW and contents of CP, NDF and NEL in herbage dry matter of lucerne during four growth cycles (C1-C4) in the pre-Alpine climate of Slovenia in the season 2016. Both linear regression curves and smooth curves are presented.

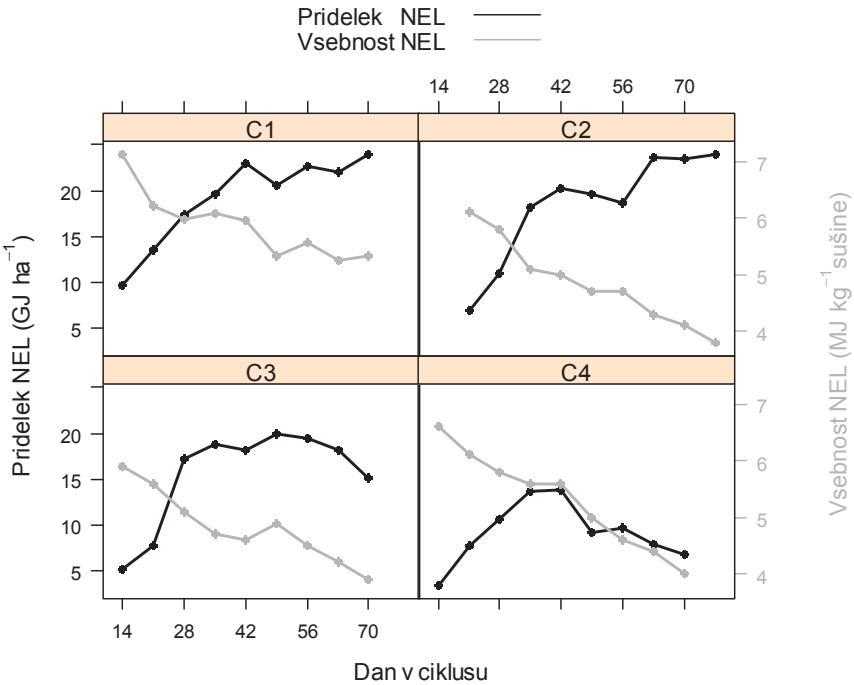
3.3 Primerjava časovnih potekov pridelkov in vsebnosti SB in NEL

Sliki 3 in 4 prikazujeta časovne poteke povprečnega pridelka in vsebnosti SB oziroma NEL v zelinju lucerne po posameznih ciklusih, narejenih na podlagi meritev. Namen slik je predvsem prikazati odnose med poteki navedenih parametrov. Pridelka SB in NEL sta na splošno potekala podobno med sezonsko rastjo lucerne – v prvih dveh ciklusih sta oba naraščala do konca, v zadnjih dveh pa se od sredine naprej nista povečevala oziroma sta padala. Vsebnosti SB in NEL so se s časom rasti pri vseh ciklusih zmanjševale v mejah od 291 g do 119 g SB kg^{-1} sušine in od 7,1 MJ do 3,8 MJ NEL kg^{-1} sušine. Pri C1 je vsebnost SB tudi pri zelo poznih vzorčenjih presegala 150 g kg^{-1} sušine, pri ostalih ciklusih pa se je zmanjšala na približno 120 g kg^{-1} sušine. Vsebnosti NEL so se pri C1 proti koncu vzorčenja približale vrednosti 5 MJ kg^{-1} sušine, pri C2, C3 in C4 pa so se zmanjšale pod 4 MJ kg^{-1} sušine.



Slika 3: Časovni potek pridelka in vsebnosti SB v zelinju lucerne po ciklusih (C1-C4) v rastni sezoni 2016 v predalpskem podnebju Slovenije. Posamezne točke so povprečja dveh meritev.

Figure 3: Time pattern of yield and content of CP in lucerne herbage during four growth cycles (C1-C4) in the pre-Alpine climate of Slovenia in the season 2016. Data points are averages of two independent measurements.



Slika 4: Časovni potek pridelka in vsebnosti NEL v zelinju lucerne po ciklusih (C1-C4) v rastni sezoni 2016 v predalpskem podnebju Slovenije. Posamezne točke so povprečja dveh meritev.

Figure 4: Time pattern of yield and content of NEL in lucerne herbage during four growth cycles (C1-C4) in the pre-Alpine climate of Slovenia in the season 2016. Data points are averages of two independent measurements.

4 RAZPRAVA

4.1 Časovni potek pridelka

Časovni potek povečevanja pridelka zelinja smo modelirali z metodo lokalne regresije, ker se je izkazala za zadostno pri primerjavi le-teh med ciklusi. Izračunani gladilniki tudi kažejo obliko poteka pridelka zelinja in prelomna mesta. Po podobnosti potekov lahko cikluse razdelimo na spomladanski in zgodnje poletni par (C1 in C2) ter na poletni do jesenski par (C3 in C4). Prva dva ciklusa imata bolj linearni potek pridelka. Lucerna naj bi v teh dveh ciklusih tudi dosegla večja končna pridelka zelinja kot v drugih dveh zaradi ugodnejših, sezonsko povezanih, vremenskih razmer. To se je zgodilo v drugem ciklusu, ne pa v prvem, kjer je bila rast lucerne v drugi polovici ciklusa zelo ovirana zaradi poleglosti lucerne, ki so jo povzročile snežne padavine 27. aprila. Snežna odeja, debeline približno 15 cm, je obstala na poskusu dva dni. Ker se polegla lucerna potem ni več dvignila, je to zmanjšalo njeno rast in povečalo razlike med ponovitvama. Bolj ugodne razmere za rast lucerne v prvi polovici sezone so v osrednji Sloveniji zato, ker v tem času ni izrazitih suš in ker so temperature zraka podobne optimalnim (24 do 25 °C podnevi in 18 do 19 °C ponoči; Stock, 1971). Pri drugih dveh ciklusih je potek pridelka podoben krivulji kvadratne funkcije. Priraščanje lucerne se je hitro začelo zmanjševati, v prvem primeru predvsem zaradi suše, v drugem pa zaradi suše in bližanja koncu rastne sezone. Negativni vpliv suše se je odrazil na masi in velikosti stebel, kar je dobro poznano (Fick in sod., 1988). Zasenčenost sestoja lucerne povzroči staranje in odpadanje listov na spodnjih delih poganjkov (Albrecht, 1983; Fick in sod., 1988). Pri lucerni v našem poskusu se je to odpadanje v večji meri začelo po 42. dnevu rasti. Obenem se je odmiranje in odpadanje listov povečalo v C4 zaradi okuženosti z lucernino rjo. Ker je bila rast proti koncu C3 in C4 manjša od mase odpadlih listov, se je pridelek zmanjševal. Vsi poteki povečevanja pridelka izkazujejo prelomno območje med 35. in 42. dnevom – prva dva ciklusa manj kot druga dva – po katerem se je rast lucerne zmanjšala. V tem času je lucerna začela cveteti, razen v C1, ko je cvetenje nastopilo šele proti koncu ciklusa. Hitrejša rast lucerne pred cvetenjem kot po njem je dobro poznana in se odraža tudi v največjem letnem pridelku pri košnji, ko ima od 10 do 50 % poganjkov cvetove (Albrecht, 1983). Primerjava časovnih potekov povečevanja pridelka zelinja v tej raziskavi z drugimi je težavna zaradi metodoloških razlik pri izvajanju poskusov in številu vključenih ciklusov. Vendar lahko ugotovimo, da je večina teh potekov iz drugih raziskav imelo tudi linearne ali v vrhnjem delu ukrivljene krivulje (npr. Kühbauch in Voigtländer, 1981; McGraw in Marten, 1986; Brink in sod., 2010).

4.2 Modelirani časovni poteki PRF ter vsebnosti SB, NDV in NEL

Morfološki razvoj lucerne, določen kot PRF, je bil najhitrejši v poletnih mesecih, to je v C2 in C3, kar je bila predvsem posledica višjih temperatur v tem času kot spomladi ali jeseni. Manj na to vpliva tudi večja dolžina dnevnega sončnega sevanja (fotoperioda). Navedena vpliva sta bila dokazana v več raziskavah (npr. Kalu in Fick, 1981). Najpočasneje se je lucerna razvijala v spomladanskem času, kar se je v veliki meri pozitivno odrazilo tudi na kakovosti krme. Morfološki razvoj je namreč zelo pomemben dejavnik kakovosti, čeprav ni edini, kot so dokazali Marvin in sod. (2000). Kultivarji lucerne, ki so izkazovali večjo kakovost krme med rastjo, se namreč niso razlikovali po razvojni fazi od tistih z manjšo kakovostjo. Linearna oblika časovnega poteka PRF, ugotovljena v tej raziskavi, se sklada z nekaterimi drugimi (Ohlsson, 1991; Božičković in sod., 2013) oziroma od njih odstopa (Kalu in Fick, 1981; Ohlsson, 1991). V teh zadnjih primerih so poteki podobni krivuljam kvadratne funkcije.

Kljub ugotovljenim razlikam v potekih vsebnosti SB med ciklusi so si ti z dvema izjemama na splošno podobni. Prvič, zmanjševanje vsebnosti SB v zadnjem delu C1 se je praktično ustavilo, kar je bilo povezano tako s počasnejšim morfološkim razvojem kot z nižjimi temperaturami zraka v maju. Verjetno so slednje povečale razmerje med listi in stebli in tudi upočasnile razvoj, kar je v skladu z navedbami Ficka in sod. (1988). Oboje se je potem poznalo na vsebnosti SB. Drugič, vsebnost SB je bila na začetku C4 zelo velika (291 g kg^{-1} sušine), na koncu pa najmanjša (119 g kg^{-1} sušine). Prvo bi lahko povezali s sušo v tem času, saj ta povečuje razmerje med listi in stebli s tem, da bolj zavira rast slednjih (Fick in sod. 1988). Drugo bi lahko povezali s povečanim odpadanjem zasenčenih in z rjo okuženih listov. Ta negativni vpliv na kakovost je bil očitno močnejši od nasprotnega vpliva suše, ki je trajala do konca tega ciklusa.

V obdobju do 56. dne, ki je lahko še pomembno za pridelovanje krme, so bile vse vsebnosti SB nad 150 g kg^{-1} sušine. Podobno velike vsebnosti SB v zelinju ostarele lucerne so ugotovili McGraw in Marten (1986) ter Hall in sod. (2000). V teh dveh virih je tudi oblika časovnega poteka vsebnosti SB enaka naši.

Časovni poteki vsebnosti NDV in NEL v lucerni med rastno sezono imajo nekaj skupnih značilnosti, kar kaže na precejšnjo povezanost teh dveh kazalcev kakovosti. Lucerna je bila po obeh kazalcih izrazito boljše kakovosti v C1 kot v drugih. To pomeni prehransko ugodnejšo najmanjšo vsebnost NDV in največjo

vsebnost NEL skozi cel prvi ciklus. S kakovostjo lucerne se je C1 približal C4, a samo na začetku rasti. Razlogi za navedene razlike v potekih vsebnosti NDV in NEL so enaki kot pri SB: prvič, večja olistanost in počasnejši razvoj lucerne zaradi nižjih temperatur spomladi, drugič, večja olistanost mlade lucerne zaradi sušnih razmer v drugi polovici poletja in tretjič, povečano odpadanje listov lucerne zaradi zasenčevanja in okuženosti z rjo proti koncu rastne sezone.

Časovni poteki NDV v literaturi so, enako kot poteki v tej raziskavi, linearni oziroma so rahlo ukrivljeni (Kühbauch in Pletl, 1981; Ohlsson, 1991; Hall in sod., 2000). Sulc in sod. (1999) navajajo 400 g NDV kg^{-1} sušine kot optimalno vsebnost v lucerni. Temu kriteriju je v našem poskusu lucerna ustrezala do približno 45. dne rasti v spomladanskem in pozno poletnem času, v osrednjem delu rastne sezone pa le do 30. dne. Objav o primerjavi rastnih ciklusov glede na potek NDV nismo našli. Prav tako nismo našli nobene objave o primerjavi rastnih ciklusov v poteku vsebnosti NEL. Žnidaršič in sod. (2015) so poročali le o zmanjševanju vsebnosti NEL med prvim ciklusom. Spremembe so bile hitrejše (-0,041 MJ NEL kg^{-1} sušine na dan) kot v C1 tega poskusa (-0,028 MJ NEL kg^{-1} sušine na dan), a podobne z vrednostmi za C2 in C4 (-0,039 MJ in -0,043 MJ NEL kg^{-1} sušine na dan; Preglednica 1).

Krave molznice morajo dobiti v določenih fazah laktacije obroke z večjo vsebnostjo energije, kot jo vsebuje najkakovostnejša voluminozna krma (> 7 MJ NEL kg^{-1} sušine). Pri oblikovanju priporočil za optimalen čas košnje upoštevamo načelo, da naj bo le ta opravljena v času, ko dovolj velik pridelek krme omogoča gospodarno spravilo. Za sejane trave velja, da jih je smiselno kositi najkasneje pri pridelku 3,5 t sušine ha^{-1}, ko je pridelek že dovolj velik, energijska vrednost pa tudi še sprejemljiva (Van Middelaar in sod., 2014). V tem poskusu je bil pri prvih treh ciklusih omenjeni pridelek dosežen med 28. in 35. dnem po začetku rasti. V tej fazi rasti je lucerna C1 dosegla približno 6,0 MJ NEL, lucerna C2 in C3 pa približno 5,0 MJ NEL kg^{-1} sušine. V C4 priporočen pridelek ni bil dosežen. Pokositi bi jo bilo smiselno med 35. in 42. dnem po začetku rasti, ko je že dosegla največji pridelek (približno 2,5 t sušine ha^{-1}), vsebnost NEL pa je bila približno 5,6 MJ kg^{-1} sušine. Rezultati kažejo, da z lucerno ni mogoče pridelati krme, ki bi zadostila kakovostnemu kriteriju za vsebnost energije v odlični travniški krmi (> 6,1 MJ NEL kg^{-1} sušine; Verbič in sod., 2011). Ta kriterij je sicer bil dosežen ali presežen pri mladi lucerni v prvih 14. ali 21. dneh rasti (C1, C4), vendar je takrat pridelek premajhen za košnjo. Ob tem je treba upoštevati, da molznice slabo energijsko vrednost lucerninega sena ali silaže deloma ali v celoti kompenzirajo s približno 10 % večjo količino zaužite krme in ob tem dajo enako količino mleka kot z zelo kakovostno travniško krmo (Hoffman in sod., 1998; Bulang in sod., 2006).

4.3 Primerjava časovnih potekov pridelkov in vsebnosti SB in NEL

Dobra kazalnika uspešnosti pridelovanja voluminozne krme sta pridelka SB in NEL, ki morata biti dosežena ob sprejemljivi vsebnosti NEL. Ob zgoraj predlaganih časih košnje (med 28. in 35. dnem po začetku rasti pri C1, C2 in C3 in med 35. in 42. dnem po predhodni košnji pri C4), so se pri prvih treh ciklusih pridelki NEL gibali med približno 17 GJ in 20 GJ ha^{-1}, pridelki SB pa med 600 kg in 700 kg ha^{-1}. Pri C4 sta bila pridelka manjša (približno 14 GJ NEL ha^{-1} in 500 kg SB ha^{-1}). Zaradi razlik v dinamiki rasti (Slika 1) je bil v tem času pri C3 in C4 potencial za pridelek NEL že dosežen, pri C1 in C2 pa je bilo doseženo približno 80 % največjega potenciala (Slika 4). Priraščanje pridelka SB se je ob predlaganih časih košnje pri vseh ciklusih v glavnem že zaključilo (Slika 3).

5 ZAKLJUČKI

Rast lucerne je bila v prvi polovici rastne sezone boljša kot v drugi, kar je posledica sezonsko pogojenih ugodnejših vremenskih razmer v tem času in običajno manjših okužb z glivami. Pridelek zelinja je v zadnjih dveh ciklusih hitro dosegel vrh in se nato začel zmanjševati zaradi povečanega odpadanja listov.

Morfološki razvoj lucerne je bil najhitrejši v poletnem času, med C2 in C3. Vendar je med vsemi ciklusi prvi izrazito odstopal s počasnim razvojem, kar je pozitivno vplivalo na kakovost krme.

Kakovost zelinja lucerne, vrednotena z vsebnostmi SB, NDV in NEL, je bila v celoti najboljša med C1. To bolj velja za NDV in NEL kot SB. Lucerna v C4 je bila na začetku enako kakovostna kot v C1, a se je nato njena kakovost hitreje zmanjševala.

Rezultati raziskave so pokazali, da je optimalna starost lucerne ob košnji, glede na pridelek in vsebnost NEL, od 28 do 35 dni spomladi in poleti ter od 35 do 42 dni jeseni. Ob takšnem času rabe vsebuje lucerna C1 približno 6,0 MJ NEL, lucerna C2 in C3 približno 5,0 MJ NEL, lucerna C4 pa približno 5,6 MJ NEL kg^{-1} sušine.

Raziskava je bila opravljena v okviru Ciljnega raziskovalnega programa »Zagotovimo.si hrano za

jutri«, projekt Tehnološke rešitve za boljše izkoriščanje lucerne v prehrani prežvekovalcev (V4-1414). Projekt sta sofinancirala Ministrstvo za kmetijstvo, gozdarstvo in prehrano ter Javna agencija za raziskovalno dejavnost Republike Slovenije iz državnega proračuna.

6 VIRI

Albrecht, K. A. (1983). Studies on nitrogen accumulation, fiber chemistry, and in vitro digestibility of alfalfa. *Retrospective Theses and Dissertations. 8448*. URL http://lib.dr.iastate.edu/rtd/8448

Blümmel, M., & Ørskov, E. R. (1993). Comparison of in vitro gas production and nylon bag degradability of roughages in predicting feed intake in cattle. *Animal Feed Science and Technology, 40*, 109-119. doi:10.1016/0377-8401(93)90150-I

Bowley, S. R., Dougherty, C. T., Taylors, N. L., & Cornelius, P. L. (1988). Comparison of yield components of red clover and alfalfa. *Canadian Journal of Plant Science, 68*, 103-114. doi:10.4141/cjps88-011

Božičković, A., Grubić, G., Verbič, J., Žnidaršič, T., Djordjević, N., & Stojanović, B. (2013). A modified method for assessment of the morphological stage of development as a predictor of alfalfa herbage chemical composition and nutritive value. *Journal of Agricultural Science, 151*, 590-598. doi:10.1017/S0021859613000129

Brink, G., Hall, M., Shewmaker, G., Undersander, D., Martin, N., & Walgenbach, R. (2010). Changes in alfalfa yield and nutritive value within individual harvest periods. *Agronomy Journal, 102*, 1274-1282. doi:10.2134/agronj2010.0080

Brown, H. E., Moot, D. J., & Pollock, K. M. (2005). Herbage production, persistence, nutritive characteristics and water use of perennial forages grown over 6 years on a Wakanui silt loam. *New Zealand Journal of Agricultural Research, 48*, 423-439. doi:10.1080/00288233.2005.9513677

Bulang, M., Kluth, H., Engelhard, T., Spike, J., & Rodehutscord, M. (2006). Zum Einzatz von Luzernesilage bei Kühen mit hoher Milchleistung. *Journal of Animal Physiology and Animal Nutrition, 90*, 89-102. doi:10.1111/j.1439-0396.2005.00568.x

Fick, G. W., Holt, D. A., & Lugg, D. G. (1988). Environmental physiology and crop growth. In A. A. Hanson, D. K. Barnes & Hill R. R. (Eds.), *Alfalfa and alfalfa improvement* (pp. 163-194). Madison, Wisconsin, USA: ASA, CSSA, SSSA.

GfE [Gesellschaft für Ernährungsphysiologie]. (2016). Equations for predicting metabolisable energy and digestibility of organic matter in forage legumes for ruminants. *Communications of the Committee for Requirement Standards of the Society of Nutrition Physiology*. URL http://www.gfe-frankfurt.de/download/Equations_in_forage_legumes_ruminants.pdf

Hall, M. H., Scott Smiles, W., & Dickerson, A. (2000). Morphological development of alfalfa cultivars selected for higher quality. *Agronomy Journal, 92*, 1077-1080. doi:10.2134/agronj2000.9261077x

Hoffman, P. C., Combs, D. K., & Casler, M. D. (1998). Performance of lactating dairy cows fed alfalfa silage or perennial ryegrass silage. *Journal of Dairy Science, 81*, 162-168. doi:10.3168/jds.S0022-0302(98)75563-8

International organisation for standardisation. (2000). Animal feeding stuffs. Determination of crude fibre content – *Method with intermediate filtration (ISO Standard 6865).*

International organisation for standardisation. (2002). Animal feeding stuffs. *Determination of crude ash (ISO Standard 5984).*

International organisation for standardisation. (2005). Animal feeding stuffs. Determination of nitrogen content and calculation of crude protein content. *Part 1: Block digestion/steam distillation method (ISO Standard 5983-2).*

Kalu, A. B., & Fick, W. G. (1981). Quantifying morphological development of alfalfa for studies of herbage quality. *Crop Science, 21*, 267-271. doi:10.2135/cropsci1981.0011183X002100020016x

Kalu, B. A., & Fick, G. W. (1983). Morphological stage of development as a predictor of alfalfa herbage quality. *Crop Science, 23*, 1167-1172. doi:10.2135/cropsci1983.0011183X002300060033x

Kühbauch, W., & Pletl, L. (1981). Berechnung der Futterqualität bei Weißklee, Rotklee und Luzerne nach morphologischen Kriterien und/oder aus Pflanzeninhaltsstoffen. II. Mitteilung: Berechnung der Stengel- und Blätterverdaulichkeit bei Rotklee und Luzerne aus Komponenten von Zellwand und Zellinhalt. *Zeitschrift für Acker- und Pflanzenbau, 150*, 281-290.

Kühbauch, W., & Voigtländer, G. (1981). Berechnung der Futterqualität bei Weißklee, Rotklee und Luzerne mit morphologischen Kriterien und/oder aus Pflanzeninhaltsstoffen. III. Mitteilung: Ertragsbildung und Futterqualtät bei Rotklee und Luzerne unter besonderer berücksichtigung der morphologischen Differenzierung. *Zeitschrift für Acker- und Pflanzenbau, 150*, 339-348.

Lloveras, J., Chocarro, C., Torres, L., Viladrich, D., Costafreda, R., & Santiveri, F. (2012). Alfalfa yield components and soil potassium depletion as affected by potassium fertilization. *Agronomy Journal, 104*, 729-734. doi:10.2134/agronj2011.0293

Marten, G. C., Buxton, D. R., & Barnes R. F. (1988). Feeding value (Forage quality). In: A. A. Hanson, D. K. Barnes & Hill R. R. (Eds.), *Alfalfa and alfalfa improvement* (pp. 463-491). Madison, Wisconsin, USA: ASA, CSSA, SSSA.

McGraw, R. L., & Marten, G. C. (1986). Analysis of primary spring growth of four pasture legume species. *Agronomy Journal, 78*, 704-710. doi:10.2134/agronj1986.00021962007800040029x

Menke, K. H., Raab, L., Salewski, A., Steingass, H., Fritz, D., & Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they were incubated with rumen liquor in vitro. *Journal of Agricultural Science, 93*, 217-222. doi:10.1017/S0021859600086305

Ohlsson, C. (1991). Growth, development, and composition of temperate forage legumes and grasses in varying environments. *Retrospective Theses and Dissertations. 9563*. URL http://lib.dr.iastate.edu/rtd/9563

R Core Team (2016). R: A language and environment for statistical computing. *R Foundation for Statistical Computing,* Vienna, Austria. URL https://www.R-project.org/.

Rizk, C. (2004). Effects of inoculation on alfalfa silage quality and its feeding on the performance of dairy cattle. *Master of Science Thesis* (98 p.), Montreal, Quebec: McGill University.

Roberts, C. A., Stuth, J., & Finn, P. C. (2004). NIRS applications in forages and feedstuffs. In: C. A. Roberts, J. Workman & Reeves J. B. (Eds.), *Near-Infrared Spectroscopy in Agriculture* (pp. 231-268). Agronomy Monograph 44. Madison, Wisconsin, ASA, CSSA, SSSA.

Russelle, M. P. (2001). Alfalfa: After an 8,000-year journey, the "Queen of Forages" stands poised to enjoy renewed popularity. *American Scientist, 89*, 252-261. doi:10.1511/2001.3.252

Stock, H. G. (1971). Die Wirkung von Temperatur, Bodenfeuchte und Windgeschwindigkeit auf das Wachstum von Rotklee und Luzerne. *Archiv für Acker- und Pflanzenbau und Bodenkunde, 15*, 951-962.

Sulc, R. M., Albrecht, K. A., Owens, V. N., & Cherney, J. H. (1999). Update on predicting harvest time for alfalfa. In M. L. Eastridge (Ed.), *Proceedings Tri-State Dairy Nutrition Conference* (pp. 167-177). Fort Wayne, Indiana: The Ohio State University.

Uredba Komisije (ES) 152/2009. (2009). Uredba Komisije (ES) št. 152/2009 z dne 27. januarja 2009 o določitvi metod vzorčenja in analitskih metod za uradni nadzor krme. URL http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:054:0001:0130:SL:PDF

Van Middelaar, C. E., Dijkstra, J., Berentsen, P. B. M., & De Boer, I. J. M. (2014). Cost-effectiveness of feeding strategies to reduce greenhouse gas emissions from dairy farming. *Journal of Dairy Science, 97*, 2427–2439. doi:10.3168/jds.2013-7648

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science, 74*, 3583-3597. doi:10.3168/jds.S0022-0302(91)78551-2

Verbič, J., Čeh, T., Gradišer, T., Janžekovič, S., Lavrenčič, A., Levart, A., Perpar, T., Velikonja Bolta, Š., & Žnidaršič, T. (2011) Kakovost voluminozne krme in prireja mleka v Sloveniji. V: T. Čeh (ur.), S. Kapun (ur.), *Zbornik predavanj 20. mednarodno znanstveno posvetovanje o prehrani domačih živali "Zadravčevi-Erjavčevi dnevi"* (str. 97-110). Murska Sobota, Kmetijsko gozdarska zbornica Slovenije, Kmetijsko gozdarski zavod.

Verbič, J., & Verbič, J. (2017). Lastnosti lucerne za siliranje. *Naše travinje, 11*, 18-19.

Whitehead, D. C. (1972). Chemical composition. In C. R. W. Spedding & E. C. Diekmahns (Eds.), *Grasses and legumes in British agriculture* (pp. 326-342). Farnham Royal, Bucks, England: CAB.

Žnidaršič, T., Verbič, J., Verbič, J., & Kopač, P. (2015). Kemična sestava in energijska vrednost posameznih vrst trav in metuljnic prve košnje v povezavi s časom košnje. *Naše travinje, 9*, 17-20.

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Temperaturna odvisnost razgradnje opada v tleh travnikov v zaraščanju

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IZVLEČEK

Namen raziskave je bil ugotoviti, ali lahko dvig temperature, ki je predviden v projekcijah podnebnih sprememb, pospeši razgradnjo opada v tleh travnikov v zaraščanju. Poskus smo izvedli v naravnih razmerah na lokacijah Bohinj-Polje in Uskovnica s podobnimi okoljskimi razmerami (padavine, matična podlaga in razvoj tal, rastlinske združbe), ter razliko v temperaturah zraka. Povprečne mesečne temperature med poskusom so bile v Bohinju za 4,4 °C (± 1,5 °C) višje kot na Uskovnici. Jeseni 2007 smo na obeh lokacijah v Of horizont talnega profila vstavili mrežaste najlonske vrečke, v katerih je bila mešanica rastlinskega opada z obeh lokacij. Vrečke z opadom smo vzorčili zaporedno v 4 terminih do maja 2009 v 5 ponovitvah. Razgradnja opada, izražena z izgubo mase, je bila v celotnem obdobju raziskave 57,1 ±1,2 % (0 - 526 dni) v Bohinju oz. 57,3 ±2,6 % (0 - 555 dni) na Uskovnici. Med lokacijama nismo ugotovili statistično značilnih razlik v hitrosti razgradnje opada in sezonskem vzorcu zmanjševanja mase. Dinamika skupne vsebnosti celuloze in lignina, Corg in N ter njunih topnih oblik (DOC in DON) je bila med lokacijama prav tako podobna. Vsebnost lignina v rastlinskem opadu se v času našega poskusa ni statistično značilno spreminjala. Rezultati poskusa niso potrdili vpliva razlike v povprečni mesečni temperaturi zraka med lokacijama na hitrost razgradnje opada.

Ključne besede: podnebne spremembe; organska snov tal; kroženje ogljika; hitrost razgradnje; lignin

ABSTRACT

LITTER DECOMPOSITION IN SOILS OF OVERGROWN GRASSLANDS IN DEPENDANCE OF TEMPERATURE

The aim of the study was to examine whether the effect of projected temperature rises due to the global climate change could accelerate plant litter decomposition in soils of overgrown grasslands. The experiment was carried out under natural conditions at the locations of Bohinj-Polje and Uskovnica with similar environmental conditions (precipitation, parent material and soil development, plant communities) and the difference in air temperatures. The average difference in monthly air temperatures during our study were higher in Bohinj for 4.4 °C (± 1.5 °C) than in Uskovnica. Nylon mesh bags with mixed plant litter from both locations were placed into the Of horizon of the soil profiles at both locations in autumn 2007. The litter bags were sampled successively at 4 sampling times until May 2009 in 5 replicates. The litter degradation, expressed as mass loss, was throughout our study 57.1 ± 1.2 % (0 - 526 days) in Bohinj, 57.3 ± 2.6 % (0 - 555 days) at Uskovnica. No statistically significant differences in litter decomposition rate and seasonal pattern of mass loss was found between the sites. The dynamics of the total content of cellulose and lignin, Corg and N and their soluble forms (DOC and DON) were similar between the sites as well. The lignin content in the plant material did not statistically significantly change during the experiment. The results of our experiment did not confirm the effect of the difference in average air temperature on decomposition rate decreases. The results did not confirm any effect from the difference in the average monthly air temperature between the sites on the plant litter decomposition in our study.

Key words: climate change; soil organic matter; carbon cycling; decomposition rate; lignin

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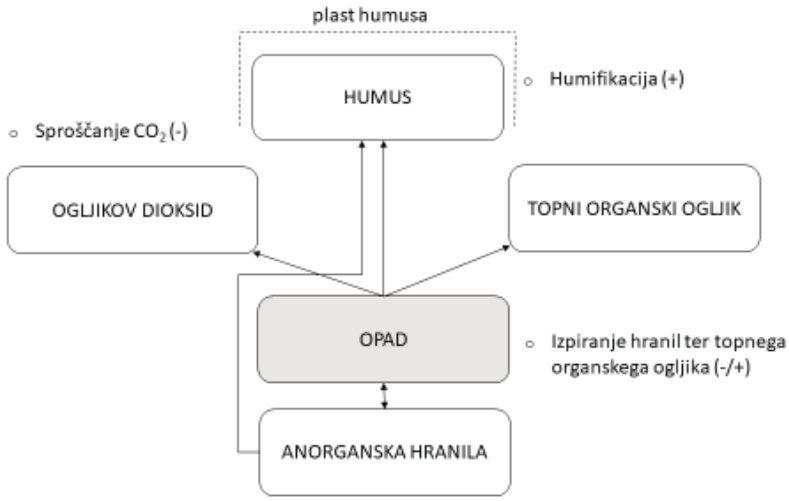
1 UVOD

Slovenija se nahaja v geografskih širinah, kjer lahko glede na modele globalnega kroženja ozračja pričakujemo hitrejše in izrazitejše ogrevanje kot v svetovnem povprečju (Kajfež Bogataj in sod., 2010). Območje Alp se segreva še nekoliko hitreje od ostale Evrope (Okolje se …, 2010). Rezultati IPCC poročila (IPCC, 2014) kažejo, da lahko do konca 21. stoletja pričakujemo zaradi dosedanjih in prihodnjih izpustov toplogrednih plinov globalno ogrevanje med 1,1 in 4,8 °C glede na povprečne razmere v obdobju 1986-2005, odvisno od tega, kateri izmed scenarijev izpustov toplogrednih plinov in delcev se bo v prihodnje uresničil. V Sloveniji se je v obdobju 1951-2000 temperatura zraka zvišala za 1,1 °C, v zadnjih 30 letih pa je ogrevanje preseglo mejo 1,5 °C (Kajfež Bogataj in sod., 2010). V skladu s predvidenim segrevanjem po celotni Evropi tudi scenariji značilnega poteka vsebnosti toplogrednih plinov v Sloveniji do leta 2100 predvidevajo naraščanje temperatur, s srednjim razponom od 1 do 4 °C in sicer RCP2.6 za 1 °C, RCP4.5 za približno 2 °C in RCP8.5 za 4 °C (Ocena podnebnih …, 2017). Z globalnim dvigom temperature zraka in spremembami ostalih podnebnih dejavnikov, npr. trajanjem in debelino snežne odeje, se zvišujejo tudi temperature tal (Houle in sod., 2012), kar vpliva na hitrost razgradnje organskih ostankov (Dar, 2010). Pogačar in sod. (2018) so ugotovili, da se tla v zadnjih letih ogrevajo tudi v Sloveniji, statistično značilen trend naraščanja letne temperature tal znaša med 0,62 in 0,76 °C/10 let, trendi naraščanja pa so večji v toplejšem delu leta. Med možnimi odzivi kopenskih ekosistemov na povišanje globalne temperature je poleg sprememb v rastlinski sestavi tudi povečana hitrost mineralizacije organske snovi v tleh, kar lahko vodi do degradacije tal, posebno zmanjšanja vsebnosti organske snovi, kar zmanjšuje obstojnost strukturnih agregatov in posledično povečuje občutljivost tal za erozijo.

Razgradnja organskih ostankov (na primer, rastlinskega opada) je sestavni in nujni del procesov globalnega kroženja ogljika (C) in hranil, ki vodi tako do sproščanja ogljikovega dioksida (CO_2) v ozračje in lahko-topnih C spojin v talno raztopino, kot tudi do vzporednega preoblikovanja organskih ostankov v bolj stabilne oblike in povezovanja z mineralnim delom tal humifikacija. Humus izboljšuje rodovitnost tal in pomeni dolgoročno zalogo C v tleh (skladiščenje ogljika) (Slika 1). Hitrost razgradnje lahko opišemo z izgubo mase opada, ki ga v naravnih ekosistemih dobro opisuje negativni eksponentni model, še posebno v prvi fazi razgradnje, ko so na voljo lahko razgradljive C spojine (sladkorji in aminokisline), sledi razgradnja srednje labilnih in najbolj zastopanih spojin (celuloze in hemiceluloz), najobstojnejši strukturni material je lignin. Izgubo mase opada in nastajanje humusa uravnava set kompleksnih in medsebojno povezanih dejavnikov, med katerimi lahko izpostavimo kemijsko sestavo opada, podnebje, dostopnost hranil, sestavo mikrobnih združb, ter specifične dejavnike mikrolokacije (pregled v Berg in McClaugherty, 2014).

Z raziskavo smo želeli ugotoviti, ali lahko dvig temperature zaradi globalnih podnebnih sprememb, pospeši razgradnjo opada v tleh travnikov v zaraščanju, ter posledično vpliva na kroženje C v kopenskih ekosistemih. Poskus smo izvedli v naravnih razmerah na lokacijah Bohinj in Uskovnica, ki imata podobne okoljske razmere za razgradnjo opada (padavine, matična podlaga in razvoj tal, rastlinske združbe), ter razliko v temperaturah zraka okrog 4 °C, kakršne so tudi projekcije povečanja letnih temperatur zraka v primeru uresničitve najbolj pesimističnega scenarija za Slovenijo do leta 2060.



Slika 1: Razgradnja opada in pretvorbe C spojin (modificirano po Berg in McClaugherty, 2014)
Figure 1: Litter decomposition and conversion of C compounds (modified after Berg and McClaugherty, 2014)

2 MATERIALI IN METODE

2.1 Lokaciji poskusa

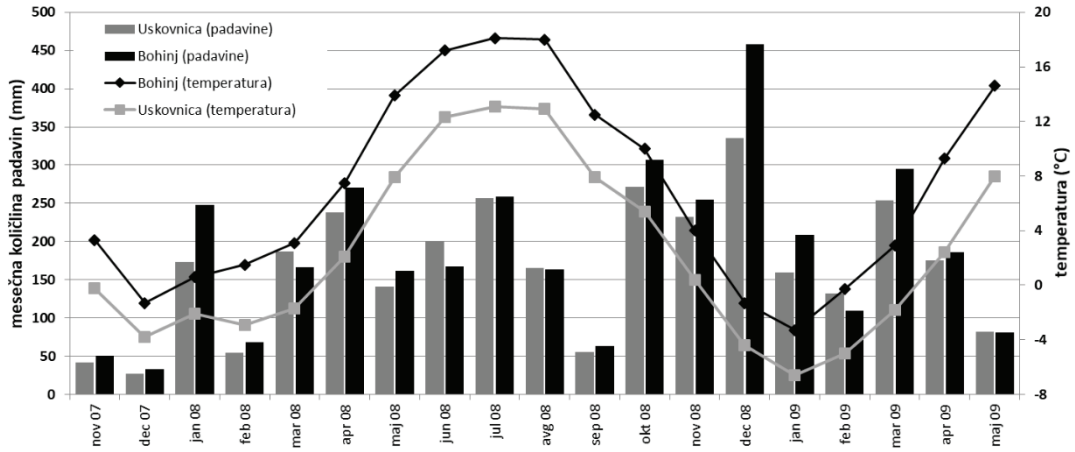
Bohinj-Polje in Uskovnica. se zaradi različne nadmorske višine (599 in 1138 m n.m.v.) razlikujeta v povprečnih dolgoletnih temperaturah, po količini in razporedu padavin pa sta si podobni (ARSO, 2017). Mikrolokaciji poskusa sta si podobni tudi po rabi tal in rastlinski sestavi, topografiji in matični podlagi. V obeh primerih gre za zaraščen, grbinasti travnik oziroma opuščeni pašnik. Matična podlaga na obeh lokacijah je karbonatna morena, na kateri se je razvila rendzina. Površinski Ah horizont je v obeh primerih zelo močno humozen, z meljasto ilovnato teksturo, določeno s sedimentacijsko pipetno metodo (ISO 11277, 2009), ter s podobnim pH (5,4 v Bohinju in 5,1 na Uskovnici), določenim po ekstrakciji tal z 0,01 mol l^{-1} $CaCl_2$ (SIST ISO 10390, 2005), ki se z globino povečuje. Na zakisanost oz. izpranost bazičnih kationov v zgornjem horizontu tal nakazuje tudi popis rastlin. Rastlinska združba na lokaciji v Bohinju je bila uvrščena v *Mesobrometum erecti* Koch 1926 (zveza: *Bromion erecti*, razred: *Festuco-Brometea*), na Uskovnici pa je združba nekoliko bolj prehodna in sicer med zvezami *Bromion erecti* in *Seslerio-Mesobromion* (Eler, osebna komunikacija).

2.2 Zasnova poskusa

Jeseni 2007 smo na obeh mikrolokacijah nabrali rastlinski opad (trave in zeli tekoče rastne sezone), ga posušili na 35 °C, narezali na 0,5-1 cm dolžine in premešali v razmerju 1:1. V najlonske mrežaste vrečke z odprtinami 0,04 x 0,04 mm smo dali mešanico opada z okvirno težo 10 g, ter vsako natančno stehtali. Vrečke z opadom smo 29.10.2007 na obeh lokacijah vgradili v Of horizont (2-3 cm od vrha tal). Skupaj smo pripravili 40 vrečk (2 lokaciji x 4 vzorčenja x 5 ponovitev).

2.3 Temperatura in padavine v času poskusa

V času trajanja poskusa je bilo povprečje mesečnih povprečnih temperatur zraka 6,9 °C v Bohinju in 2,3 °C na Uskovnici (Slika 2). Najtoplejša meseca sta bila na obeh lokacijah julij in avgust 2008, s povprečno temperaturo zraka v Bohinju 18,0 °C, na Uskovnici pa 13,0 °C. Najhladnejši mesec je bil januar 2009 s povprečno mesečno T v Bohinju -3,3 °C, na Uskovnici je dosegla -6,6 °C. V obdobju 1981-2010 je bila povprečna letna vsota padavin na izbranih lokacijah podobna, na padavinski postaji Bohinjska Bistrica (Bohinj) 2065 mm, v Gorjušah (Uskovnica) pa 1870 mm (ARSO, 2017). V času trajanja poskusa je bila skupna količina padavin v Bohinju 3548,7 mm, oz. povprečno mesečno 186,8 mm, na Uskovnici 3181,4 mm, oz. povprečno mesečno 167,4 mm. Leto 2008 je bilo na obeh lokacijah med bolj mokrimi leti zadnjega obdobja (2584 in 2309,8 mm). Največ padavin je bilo decembra 2008, medtem ko je bil december 2007 najbolj sušen mesec (32,5 in 27,4 mm) (Slika 2). Na lokaciji v Bohinju je padlo več padavin kot na Uskovnici, predvsem v bolj mokrih mesecih. Dinamika padavin je med lokacijama podobna.



Slika 2: Povprečne mesečne temperature zraka (°C)/padavine (mm) od novembra 2007 do maja 2009 (ARSO, 2017)
Figure 2: Average monthly air temperatures (°C)/precipitation (mm) from November 2007 to May 2009 (ARSO, 2017)

2.4 Spremljanje razgradnje opada

Vrečke smo pobirali sukcesivno: 2 tedna po vgradnji, 6 mesecev po vgradnji (oz. 2-3 tedne po snegu), 8 mesecev po vgradnji (10 tednov po snegu), ter 17. oz. 18 mesecev po vgradnji (2-3 tedne po snegu). Poskus je potekal od oktobra 2007 do maja 2009. Vrečke z opadom smo po vzorčenju sušili 96 ur pri 70 °C, nato ohladili v eksikatorju ter določili maso, ter jo izrazili v % začetne teže ob vgradnji. Rastlinski material smo zmleli in shranili pri sobni temperaturi za nadaljnje analize. Vsebnost suhe snovi v rastlinskem materialu smo določili s sušenjem na 105 °C.

Vsebnost celuloze in lignina v rastlinskem materialu je bila določena po metodi za določanje vlaken netopnih v kislem detergentu (KDV) in metodi za določanje v kislem detergentu netopnega lignina (KDL) na Oddelku za Zootehniko Biotehniške fakultete (Lavrenčič, 2003). Rezultat KDL nam poda oceno vsebnosti lignina v materialu, razlika med KDV (g kg^{-1}) in KDL (g kg^{-1}) pa nam poda oceno vsebnosti celuloze.
Skupno vsebnost ogljika (%) in skupno vsebnost dušika (%) smo v rastlinskem materialu (0,3 g) določili po sežigu pri 900 °C na elementnem CNS analizatorju (VarioMAX, Elementar). Topne oblike C in N (DOC in DON) smo določili z ekstrakcijo rastlinskih vzorcev (0,5 g) z 0,01 M $CaCl_2$ (10 ml) (Houba, 1986). Ekstrakcija je potekala s stresanjem 20 minut, nato smo vzorce 30 min centrifugirali pri 4000 obratih min^{-1}. Ekstrakt smo vakuumsko filtrirali skozi filter premera 0,4 µm (Whatman Nr. 111207 PC MB 50 mm). Ekstrakte smo shranili v zamrzovalnik do določitve C in N. Nadaljnje analize so bile izvedene na Tehnični Univerzi v Münchenu, na katedri za talno ekologijo. Skupni C v ekstraktu rastlinskega opada (DOC, g C kg^{-1} SS opada) smo določili s TC analizatorjem (DIMATEC, Germany). Amonijski, nitratni in skupni dušik (g N kg^{-1} SS opada) smo določili fotometrično na analizatorju s kontinuiranim pretokom (Skalar Analytical, The Netherlands). Topni organski dušik (DON, g N kg^{-1} SS opada) smo izračunali iz razlike med skupnim N in mineralnimi oblikami N.

2.5 Obdelava podatkov

Rezultate poskusov smo analizirali z analizo kovariance (ANOVA) z uporabo SPSS paketa, trendne črte (modelirane vrednosti) smo prikazali z eksponentnim modelom.

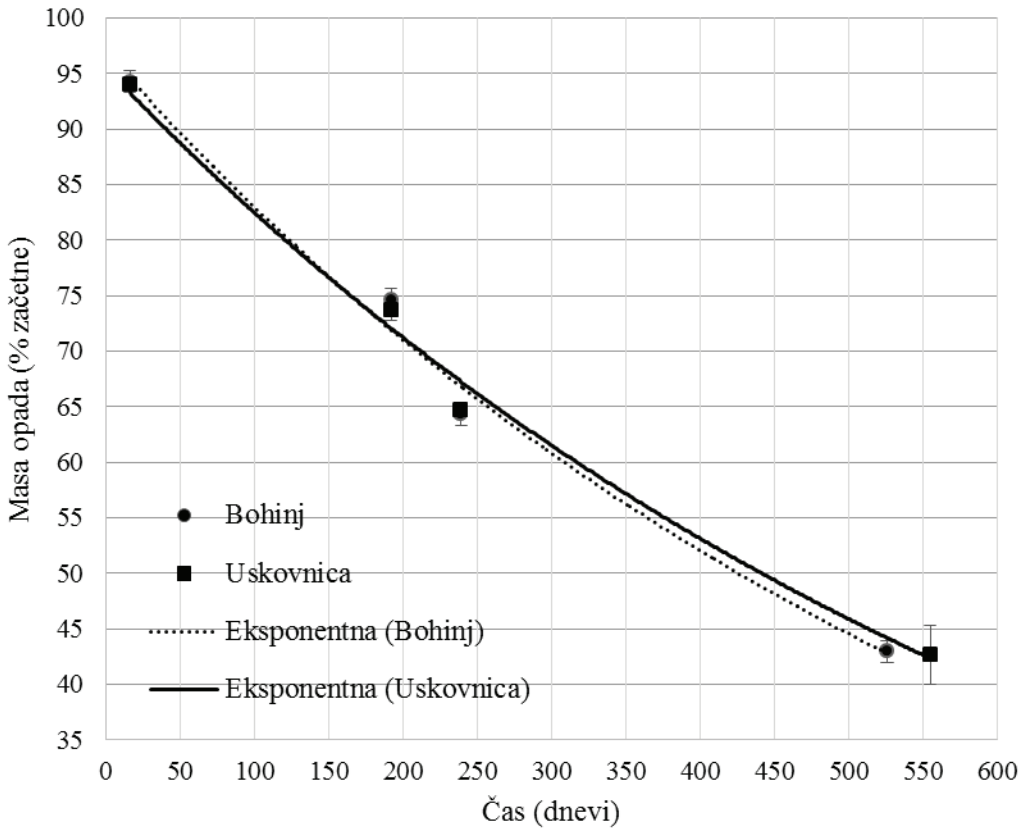
3 REZULTATI IN DISKUSIJA

Razgradnja opada je kompleksen proces, v katerem se prepletajo fizikalni, kemijski in biološki procesi, izguba mase opada, ki smo jo spremljali v naši študiji, odraža skupni, agregirani rezultat (Welsch in Yavitt, 2003).

3.1 Izguba mase opada

V času naše študije, od vgradnje vrečk z rastlinskim opadom 29.10.2007 do zadnjega vzorčenja 6.5.2009, statistično značilnih razlik med lokacijama v hitrosti in poteku razgradnje nismo ugotovili (Slika 3). Podobno kot v drugih študijah je bila izguba mase opada največja v prvih tednih po vgradnji (pregled v Berg in McClaugherty, 2014; Santonja in sod., 2015; Duan in sod., 2018), v Bohinju je znašala 5,7 ± 0,3 odstotnih točk in na Uskovnici 6,1 ± 0,6 odstotnih točk. V prvih šestih mesecih smo ugotovili zmanjšanje mase za 25,4

±1,1 odstotnih točk v Bohinju oz. 26,3 ± 0,9 odstotnih točk na Uskovnici. Zanimivo je, da razlik v razgradnji opada med lokacijama ni bilo, čeprav so bile razlike v temperaturi zraka med lokacijama precejšnje (Slika 2). Na Uskovnici so bile na primer temperature zraka pod ledišcem vse do začetnih dni v aprilu, v Bohinju pa le do konca februarja. Posledično je bila tudi pokritost s snežno odejo daljša na Uskovnici, spomladi 2008 za teden dni dlje, spomladi 2009 pa celo tri tedne dlje v primerjavi z Bohinjem. Sneg ima sicer tudi izolativno vlogo ter je na ta način lahko regulator procesov v tleh. Preprečuje zamrznitev talne vode in na ta način omogoča mikrobno pogojeno razgradnjo organskih ostankov tudi pod snežno odejo. V pozni pomladi smo pričakovano zaznali povečano razgradnjo zaradi višjih temperatur zraka, ki so ugodno vplivale na mikrobno aktivnost. Dolgoletna povprečna majska temperatura zraka za Bohinj znaša 12,7 °C, maja 2008 je znašala 13,9 °C, maja 2009 pa kar 14,6 °C. Masa opada se je, na primer, med drugim in tretjim vzorčenjem zmanjšala za 10,3 odstotnih točk v Bohinju in za 9,0 odstotnih točk na Uskovnici, vendarle pa je razlika v izgubi mase opada med lokacijama le 1 odstotna točka, čeprav so bile povprečne temperature zraka v Bohinju aprila 2008 za 5,5 °C in maja 2008 za 6 °C višje kot na Uskovnici. Razgradnja opada se je nato pričakovano upočasnila zaradi spremenjene kemijske sestave preostalega rastlinskega materiala (Sliki 4, 5). Do konca naše raziskave se je razgradilo 57,1 ± 1,2 % (0 - 526 dni) v Bohinju oz. 57,3 ± 2,6 % (0 - 555 dni) na Uskovnici začetne (vgrajene) mase opada (Slika 3).



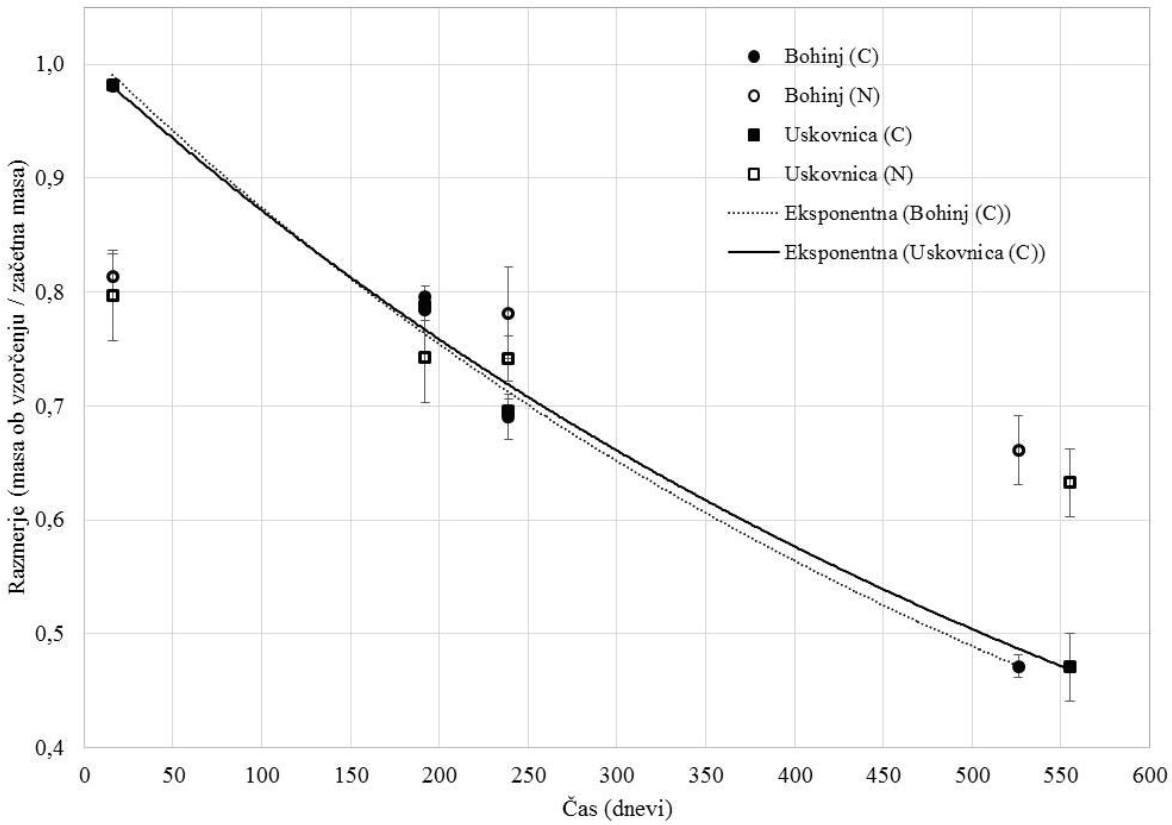
Slika 3: Izguba mase opada od novembra 2007 do maja 2009 na lokacijah Bohinj in Uskovnica. Prikazana so povprečja in standardni odkloni 5 ponovitev (preostale) mase izražene v % vgrajenega rastlinskega materiala

Figure 3: Litter mass loss from November 2007 to May 2009 at sites Bohinj and Uskovnica. Average and standard deviation of 5 replicates of remaining mass, expressed as % of incorporated plant material, are shown

3.2 Kemijska sestava opada

Med razgradnjo se spreminja tudi kemijska sestava razgrajajočega se opada. Te spremembe niso nujno linearno povezane z izgubo mase. Prav tako so te spremembe v kemijski sestavi razgrajajočega opada lahko različne v različnih okoljskih razmerah, četudi gre za podobno sestavo izvornega rastlinskega materiala (Coleman in sod., 2004). V naši raziskavi se je delež skupnega organskega ogljika (Corg) v rastlinskem opadu zmanjševal linearno z zmanjševanjem mase opada, medtem ko je bilo zmanjševanje deleža skupnega dušika počasnejše (Slika 4). Posledično se je ožilo C/N razmerje preostalega opada, od C/N 47:1 ob vgradnji do

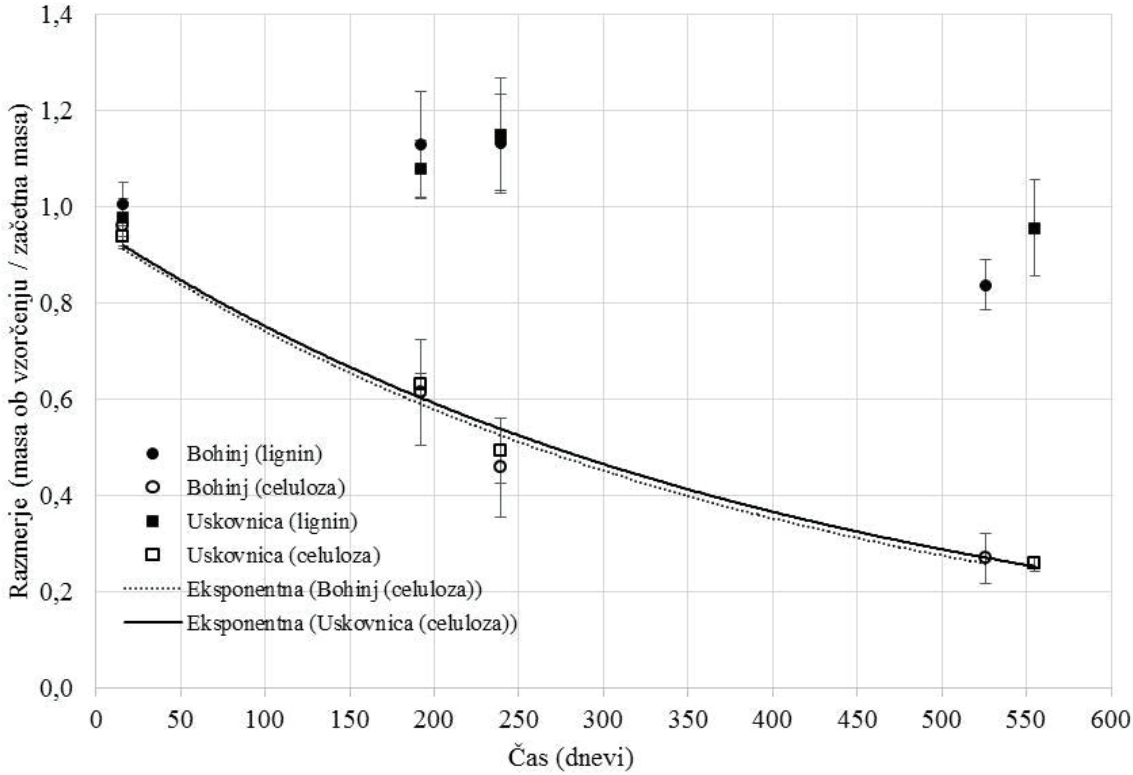
25:1 v Bohinju, oz. 26:1 na Uskovnici ob koncu poskusa. Nekateri avtorji poročajo celo o akumulaciji N med razgradnjo, kar pojasnjujejo z imobilizacijo sproščenega N med razgradnjo v mikrobno biomaso in/ali prenosom N iz tal na opad (Coleman in sod., 2004). Vsebnost topnega organskega ogljika (DOC) v rastlinskem materialu se je prav tako zmanjševala tekom poskusa, vendar brez značilnih razlik med lokacijama (podatki niso prikazani). Do največjega zmanjšanja DOC je pričakovano prišlo ob prvem vzorčenju in sicer iz 23,1 ± 2,3 g kg^{-1} ob vgradnji na 12,6 ± 1,1 g kg^{-1} v Bohinju in 11,2 ± 0,9 g kg^{-1} na Uskovnici. Ob koncu poskusa je bila vsebnost DOC 5,2 ± 0,5 g kg^{-1} opada na Uskovnici in 6,2 ± 0,2 g kg^{-1} opada v Bohinju.



Slika 4: Razmerje med začetno vsebnostjo ob vgradnji in vsebnostjo ob vzorčenju za parametra skupni Corg in skupni N v času od vgradnje (dan 0, 29.10.2007) do zadnjega vzorčenja maja 2009 na lokacijah Bohinj in Uskovnica. Prikazana so povprečja in standardni odkloni 5 ponovitev

Figure 4: Ratio between initial and sampling time contents of total Corg and of total N from the litter bag incorporation (day 0, 29th of October 2007) till last sampling in May 2009 at sites Bohinj and Uskovnica. Average and standard deviation of 5 replicates are shown

Sestava rastlinskega materiala glede na vsebnost celuloze in lignina v času naše študije prav tako potrjuje enak potek razgradnje opada na obeh lokacijah (Slika 5). Vsebnost celuloze se je značilno zmanjševala ves čas poskusa, medtem ko se vsebnost lignina ni značilno spreminjala. Šele ob koncu poskusa, po letu in pol razgrajevanja, smo zaznali določeno zmanjšanje vsebnosti lignina, ki pa ni bilo statistično značilno. Rezultat nakazuje na večjo razgradnjo lignina v Bohinju v primerjavi z Uskovnico. Lignin je kompleksnejši substrat v primerjavi s celulozo, zato ima večjo aktivacijsko energijo, kar bi lahko pojasnilo večjo občutljivost na temperaturne spremembe (Davidson in Janssens, 2006), vendarle pa so si rezultati različnih študij o temperaturni občutljivosti organske snovi glede na razgradljivost substrata nasprotujoči. Ker je v proces razgradnje vključenih več mehanizmov, ima lahko vsak od njih svojo specifično občutljivost na spremembe v temperaturi (Davidson in Janssens, 2006; Lützow in Kögel-Knabner, 2009).



Slika 5: Razmerje med začetno vsebnostjo ob vgradnji in vsebnostjo ob vzorčenju za parametra celuloza in lignin v času od vgradnje opada (dan 0, 29.10.2007) do zadnjega vzorčenja maja 2009 na lokacijah Bohinj in Uskovnica. Prikazana so povprečja in standardni odkloni 5 ponovitev

Figure 5: Ratio between initial and sampling time contents of cellulose and lignin from the litter bag incorporation (day 0, 29th of October 2007) till last sampling time in May 2009 at sites Bohinj and Uskovnica. Average and standard deviation of 5 replicates are shown

Naše hipoteze, da bo razgradnja rastlinskega opada na lokaciji Bohinj hitrejša zaradi višjih povprečnih temperatur zraka, rezultati poskusa niso potrdili. Rezultati drugih študij kažejo na nasprotujoče si ocene temperaturne občutljivosti razgradnje organske snovi tal (Davidson in Janssens, 2006; Lützow in Kögel-Knabner, 2009; Kirschbaum, 2010; Ding in sod., 2016). Odziv razgradnje organske snovi na temperaturo je odvisen od več dejavnikov: (i) kemijske sestave (obstojnosti) organske snovi, (ii) razpoložljivosti substrata, ki jo določa ravnotežje med vnosom opada, stabilizacijo in mineralizacijo, (iii) fiziologije mikrobioma tal ter njegove učinkovitosti pri izkoriščanju substrata in temperaturnega optimuma ter (iv) fizikalno-kemijskih parametrov, ki vplivajo na razgradnjo, kot so na primer pH in vsebnost vode v tleh, oskrba s kisikom in hranili. V naši raziskavi smo vgradili enak rastlinski material na obeh lokacijah, ki sta si bili po pedoloških lastnostih podobni. Učinek temperature bi lahko zmanjšala lega mikrolokacij, saj relief in ekspozicija lahko vplivata tako na temperaturo tal kot na vodno-zračni režim. V bodoče bi bilo potrebno izvesti tudi meritve temperature na globini tal, kjer je potekala razgradnja opada, saj lahko časovni zamik temperature tal v primerjavi s temperaturo zraka do določene mere vpliva na rezultate poskusa. Poudariti moramo tudi, da so bile povprečne dnevne temperature zraka na obeh lokacijah v celotnem času trajanja poskusa relativno nizke z vidika optimalnih temperatur za aktivnost mikroorganizmov: na lokaciji Bohinj 6,8 °C in na lokaciji Uskovnica 2,5 °C, zato bi bilo smiselno raziskavo razširiti v toplejšem območju.

4 SKLEPI

Razlika 4,4 °C (± 1,5 °C) v povprečnih mesečnih temperaturah zraka ni vplivala na hitrost in potek razgradnje rastlinskega opada v travniških tleh v zaraščanju v leto in pol dolgem obdobju po vgradnji. Rezultate bi lahko pojasnili naslednji razlogi: (i) razgradnja opada je na spremembe v temperaturi manj občutljiva od napovedi, (ii) dejanske razlike v temperaturi tal so bile lahko manjše od razlik v spremljani temperaturi zraka, (iii) čas študije je bil prekratek, saj do razgradnje lignina v tem času še ni prišlo, (iv) povprečne dnevne temperature zraka so bile na izbranih lokacijah prenizke z vidika optimalnih temperatur za aktivnost mikroorganizmov, ki so glavni akterji v razgradnji.

Razgradnja rastlinskega opada je pomemben del globalnega kroženja ogljika. Skupaj s primarno produkcijo določa zaloge ogljika v tleh in s tem ravnotežja ogljika v kopenskih ekosistemih. Glede na napovedi spreminjanja globalnih temperatur zraka ter povezanosti s temperaturami tal, so potrebne nadaljnje raziskave v spremenjenih razmerah.

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6 VIRI

ARSO. (2017). Agencija Republike Slovenije za okolje (ARSO), izpis meteoroloških podatkov iz baze podatkov za leta 2007-2009. http://meteo.arso.gov.si/met/sl/climate/

Berg, B., McClaugherty, C. (2014). *Plant litter: decomposition, humus formation, carbon sequestration*, Springer, 3rd edition, 315 str.

Coleman, C. D., Crossley, A. D., Hendrix, F. A. (2004). *Fundamentals of soil ecology*. 2nd edition. New York, Elsevier. 287 str.

Dar, G.H. (2010). Soil microbiology and biochemistry. New Delhi, New India publishing Agency, 512 str.

Davidson, E. A., Janssens, I. A. (2006). Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. *Nature*, *440*, 9, https://doi.org/10.1038/nature04514

Ding, J., Chen, L., Zhang, B., Liu, L., Yang, G., Fang, K., Chen, Y., Li, F., Kou, D., Ji, C., Luo, Y., Yanget, Y. (2016). Linking temperature sensitivity of soil CO_2 release to substrate, environmental, and microbial properties across alpine ecosystems, *Global Biogeochem. Cycles*, *30*, 1310–1323. https://doi.org/10.1002/2015GB005333

Duan, H., Wang, L., Zhang, Y., Fu, X., Tsang, Y., Wu, J., Le, Y. (2018). Variable decomposition of two plant litters and their effects on the carbon sequestration ability of wetland soil in the Yangtze River estuary. *Geoderma*, v tisku, https://doi.org/10.1016/j.geoderma.2017.10.050.

Houba, V. J. G. (1986). Comparision of soil extractions by 0.01 M CaCl2, by EUF and by some conventional extraction procedures. *Plant and Soil, 96*, 433-437. https://doi.org/10.1007/BF02375149

Houle, D., Bouffard, A., Duchesne, L., Logan, T., Harvey, R. (2012). Projections of future soil temperature and water content for three southern Quebec Forested Sites. *Journal of Climate*, *25*, 7690-7701. https://doi.org/10.1175/JCLI-D-11-00440.1

IPCC. (2014). Climate Change 2014: Synthesis Report. *Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. R.K. Pachauri R.K., Meyer L.A. (ur.), IPCC, Geneva, Switzerland, 151 str.

ISO 10390. (2005). Soil quality - determination of pH. *International Organization for Standardization*, Genève, Switzerland

ISO 11277. (2009). Determination of particle size distribution in mineral soil material-method by sieving and sedimentation. *International Organization for Standardization*, Genève, Switzerland

Kajfež Bogataj, L., Pogačar, T., Ceglar A., Črepinšek, Z. (2010). Spremembe agroklimatskih spremenljivk v Sloveniji v zadnjih desetletjih. *Acta agriculturae Slovenica, 95*, 1, 97-109.

Kirschbaum, M.U.F. (2010). The temperature dependence of organic matter decomposition: seasonal temperature variations turn a sharp short-term temperature response into a more moderate annually averaged response. *Global Change Biology, 16*, 2117–2129. https://doi.org/10.1111/j.1365-2486.2009.02093.x

Lavrenčič, A. (2003). *Vaje pri predmetu Prehrana domačih živali*. Ljubljana, Veterinarska fakulteta, 115 str.

Lützow, M., Kögel-Knabner, I. (2009). Temperature sensitivity of soil organic matter decomposition—what do we know? *Biology and Fertility of Soils, 46*, 1. https://doi.org/10.1007/s00374-009-0413-8

Okolje se spreminja: podnebna spremenljivost Slovenije in njen vpliv na vodno okolje. (2010). Ljubljana, Ministrstvo za okolje in prostor RS: 162 str.

Pogačar, T., Zupanc, V., Kajfež Bogataj, L., Črepinšek, Z. (2018). Soil temperature analysis for various locations in Slovenia. *Italian Journal of Agrometeo*rology (v tisku).

Santonja, M., Fernandez, C., Gauquelin, T., Baldy, V. (2015). Climate change effects on litter decomposition: intensive drought leads to a strong decrease of litter mixture interactions. *Plant and Soil, 393*, 69-82. https://doi.org/10.1007/s11104-015-2471-z

Welsch, M., Yavitt, J.B. (2003). Early stages of decay of *Lythrum salicaria* L. and *Typha latifolia* L. in a standing-dead position. *Aquatic Botany*, *75*, 45-57.

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Evaluation of turmeric-mung bean intercrop productivity through competition functions

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ABSTRACT

An intercropping experiment was conducted with varying combinations of turmeric and mung bean to find out the efficacy of productivity and economic return through competition functions. Treatments were evaluated on the basis of several competition functions, such as land equivalent ratio (LER), aggressiveness, competitive ratio (CR), monetary advantage index (MAI) and system productivity index (SPI). Results showed that rhizome yields of turmeric were higher in intercropping system than in mono crop. It indicated that intercropping of mung bean did not affect the rhizome yield of turmeric. However, turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system exhibited maximum yield of both the crops as well as turmeric equivalent yield, LER, competitive indices values, SPI and MAI (Tk. 2,44,734.46 ha^{-1}) compared to the other intercropping combinations and the mono crops. Aggressiveness of intercrop indicated dominance of turmeric over mung bean in all the combinations except turmeric (100 %) + 1 row mung bean (33 %). Competition functions of intercroping suggested beneficial association of turmeric and mung bean crops. The study revealed that mung bean could be introduced as intercrop with turmeric without hampering rhizome yield with higher benefit additionally increasing mung bean production area.

Key words: competition functions; economics; intercropping; turmeric; mung bean

IZVLEČEK

OVREDNOTENJE PRIDELKA KOMBINIRANEGA GOJENJA KURKUME IN ZLATE VINJE GLEDE NA MEDSEBOJNO KOMPETICIJIO

Povečanje učinkovitosti izrabe kmetijskih površin za večji pridelek je pomembno za prehranjevanje naraščajoče človeške populacije. Medsadnja kurkume v posevke zlate vinje (zelenega mungo fižola, *Vigna radiata* (L.) R. Wilczek) v optimalnih gostotah lahko poveča učinkovitost izrabe površin. Ekvivalent zemljišča (LER) je bil večji od 1 v vseh sistemih mešanega gojenja. Poskus z mešanim gojenjem omenjenih poljščin je bil izveden v sezonah 2014 in 2015 z različnimi kombinacijami kurkume in zlate vinje na Regional Agricultural Research Station, Bangladesh Agricultural Research Institute, Ishwardi, Pabna, Bangladesh. Namen raziskave je bil ugotoviti učinkovitost in gospodarnost takšne pridelave v povezavi s tekmovalnostjo med obema poljščinama. Poskus je temeljil na naključnem bločnem razporedu s tremi ponovitvami. Obravnavanja so bila ovrednotena na osnovi različnih kompeticijskih funkcij kot so ekvivalent zemljišča (LER), agresivnost, kompeticijsko razmerje (CR), denarni indeks (MAI) in sistemski produktivnostni indeks (SPI). Rezultati so pokazali, da je bil pridelek korenik kurkume večji v vseh sistemih z medposevki kot v čisti sadnji. Pokazali so tudi, da kombinirano gojenje z zlato vinjo ni povzročilo zmanjšanja pridelka korenik kurkume. Površine z vrstami čiste kurkume (100 %), kombiniranimi s trovrstnimi pasovi zlate vinje (100 %) so imele večji pridelek obeh poljščin kot tudi največje vrednosti za ekvivalent pridelka, LER, kompeticijski indeks, SPI in MAI (Tk. 2,44,734.46 ha^{-1}) za kurkumo v primerjavi z drugimi sistemi mešanega gojenja in čistimi kulturami. Analiza agresivnosti pri različnih kombinacijah mešanega gojenja je pokazala prevlado kurkume nad zlato vinjo v vseh kombinacijah, razen v sistemu, kjer je bil nasad kurkume (100 %), kombiniran s po eno vrsto zlate vinje (33 %). Kompeticijske funkcije mešanih načinov gojenja so pokazale, da je glede na pozitiven učinek smiselno kombinirati omenjeni poljščini. Raziskava je pokazala, da bi se zlata vinja lahko uvedla kot medkultura kurkume, ne da bi zmanjašala pridelek njenih korenik, hkrati pa bi to predstavljalo dodatno možnost povečanja površin za pridelavo zlate vinje.

Ključne besede: kompeticijske funkcije; gospodarnost; medposevki; kurkuma; zlata vinja

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1 INTRODUCTION

Turmeric (*Curcuma longa* L.) belongs to the family Zingiberaceae is one of the important tropical and subtropical rhizomatous species widely cultivated in Asia including Bangladesh. About 8307 hectares of land remain under turmeric cultivation in Pabna, Rajshahi, Faridpur, Jessore, Kushtia districts which were 37 % of the total turmeric cultivation in Bangladesh (BBS, 2011). It is a long duration crop remains under field about 270-300 days. However, adoption of long duration turmeric varieties by cultivators poses a threat to some popular and time demanding traditionally grown pulse crop like mung bean in this region.

In Bangladesh, mung bean (*Vigna radiata* (L.) R. Wilczek) is a significant seed legume among pulses. It is widely cultivated in the worldwide for its' high content of protein in seeds (Hasan et al., 2017). Moreover, being a leguminous crop mung bean improves soil fertility through fixation of atmospheric nitrogen and provides additional yield advantages to the companion crop, which may contribute to gross return. It also performs well in a low-input intercropping system with non-legume and provides nitrogen, consequently the companion crop can grow faster and therefore improve yield (Esmaeilia et al., 2011).

The efficient use of natural and biological cycles such as nitrogen fixation by legumes may stimulate yield of the non-legume crops in an intercropped system (Hauggaard-Nielsen et al., 2001). In addition, mung bean supply 56, 61 and 67 kg N under low, moderate and high nutrient level, respectively (Mian, 2008). Ability to tolerate shading is one additional advantage of turmeric in intercropping systems. It is reported that higher fresh turmeric yield was obtained in intercropping system than mono cropping in open sunlight due to shady condition in India (Joyachandran et al., 1991). Furthermore, after planting of turmeric (rhizome), it takes 60 to 70 days to 100 % emergence. During this period, farmers can easily grow short duration mung bean (65-75 days) crop in association with turmeric for higher benefit. In Bangladesh, majority of the farmers in farming community are small holders having 0.02-1.01 hectares of cultivated lands which also shrinking progressively (MOA, 2014).

In such background, intercropping offers the higher potentials of yield enhancement relative to mono cropping through yield stability and improved yield in tropical and sub-tropical areas (Nazir et al., 2002; Malik et al., 2002; Bhatti, 2005). Therefore, the way out is to grow the mung bean as an intercrop without losing turmeric production. However, studies on mung bean-turmeric intercropping are not much available. The competition functions viz land equivalent ratio (LER), relative crowding coefficient (K), competitive ratio (CR), aggressiveness, monetary advantage index (MAI) and system productivity index (SPI) have been developed to describe the competition and possible economic advantages of intercropping systems (Banik et al., 2000 ; Ghosh, 2004; Yilmaz et al., 2008; Midya et al., 2005; Oseni et al., 2010).

The extreme increase in population in Bangladesh needs to maximize the total production of legume crops for overcome the deficiency of protein through cultivation in the newly lands (Rahman et al., 2017). The importance of pulses is very much pertinent for food and improving the farm-family income in order to ensure food security, nutritional security and economic security (Islam et al., 2017). Hence, this study was undertaken to find out the efficacy of productivity and economic return of intercropping mung bean with turmeric through different competition functions.

2 MATERIALS AND METHODS

2.1 Experimental Site

The experiment was conducted at the Regional Agricultural Research Station (RARS), BARI, Ishwardi, Pabna, Bangladesh during 2014 and 2015 to find out the efficiency of productivity and economic return from intercropping mung bean with turmeric through competition functions.

2.2 Data sources and treatments

The treatments *viz.* T_1 = Turmeric (100 %) + 1 row mung bean (33 %) in between turmeric lines; T_2 = Turmeric (100 %) + 2 row mung bean (67 %) in between turmeric lines; T_3 = Turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines; T_4 = Turmeric (100 %) + mung bean broadcast (100 %) in between turmeric lines; T_5 = only turmeric as a mono crop, and T_6 = only mung bean as a mono crop were used. The experiment was laid out in a randomized complete block design with three replications. The unit plot size was 4.5m × 4m. Turmeric was established as main crop and mung bean was used as the intercrop in the study. Mung bean was intercropped in between turmeric row at 33, 67 and 100 % population densities. Turmeric ('BARI Halud-4') and mung bean ('BARI mung-6') were planted/sown on 22 March 2014 and

2015, respectively. Turmeric 'BARI Halud-4' was harvested on 31 and 28 December 2014 and 2015, respectively. Mung bean was harvested on 20-30 May in both years, respectively. Except broadcasting, mung bean seeds were sown keeping row spacing 30 cm following continuous seeding. The mono crop of turmeric and intercrops was fertilized with 140-54-117 kg ha^{-1} of N-P-K with 5 t ha^{-1} cow dung. In case of intercropping mung bean with turmeric full amount of P, $^1/_4$ N and $^1/_4$ of K with 5 t ha^{-1} cow dung were applied during final land preparation. Rest N and K were applied three equal components at 70, 100 and 120 days after planting/sowing. For mung bean mono crop treatment, fertilizer was applied at 20-20-20 kg ha^{-1} of N-P-K. All fertilizers were applied as basal at final land preparation. Weeding and other intercultural operations were done as per requirement of the crops. After emergence, mung bean was thinned out for keeping plant to plant distance of 5 cm. Earthing up of turmeric was done after harvesting mung bean (100 days after planting).

2.3 Measurements and Data analysis

Data on yield and yield contributing characteristics were recorded and statistically analysed. The mean values were adjudged by LSD (0.05). Turmeric equivalent yield (TEY) was converted by converting yield of intercrops on the basis of presenting market price of individual crop following the formula:

$$\text{TEY} = \text{Yield of intercrop turmeric} + \frac{\text{Yi} \times \text{Pi}}{\text{Price of mungbean}}$$

Where, Yi = Yield of intercrop, and Pi =Price of intercrop.

The important tool that agricultural researchers commonly use to assess the relative advantage of intercropping compared to sole crops is the land equivalent ratio (LER) (Mead and Willey, 1980). If the value of LER shows >1, the intercropping favors the growth and yield of the species. When LER demonstrate <1, the intercropping negatively effects the growth and yield of crops grown in mixtures (Caballero et al. 1995). It was calculated for each proportion on a plot basis using the total land equivalent ratio (LER):

$$\text{LER} = \text{RY}_t + \text{RY}_i = \frac{\text{T}_{IY}}{\text{T}_{SY}} + \frac{\text{M}_{IY}}{\text{M}_{SY}}$$

Where, RY_t= Relative yield of turmeric (main crop),
RY_i= Relative yield of intercrops (mung bean),
T_{IY} = Intercrop yield of turmeric,
T_{SY} = Sole crop yield of turmeric,
M_{IY} = Intercrop yield of mung bean, and
M_{SY}= Sole crop yield of mung bean

Replacement value of intercropping (RVI) is a slightly more complex tool that used to measure for economic feasibility of intercropping or mixed cropping (Moseley, 1994) which computed as:

$$\text{RVI} = \frac{aP_1 + bP_2}{aM_1 - C}$$

Where, P_1 & P_2 are the yield of intercrops and a and b are the respective prices of these crops. M_1 is the yield and C is the input cost of the primary (main) crop in sole stand.

The entire the competition indices MAI give an indication of the economic advantage of the intercropping system. The higher the MAI value the more profitable is the cropping system (Ghosh 2004). MAI was calculated as described by Ali and Mishra (1993) as follows:

MAI = Value of combined intercrop yield × (LER-1)/LER

Where, MAI = Monetary advantage index, LER = Land equivalent ratio

Competitive ratio (CR) gives better measure of competitive ability of the crops as well as evaluation whether the association of the two component crops is beneficial or not (Mahapatra, 2011). It measures the ratio of individual LERs of the two component crops and the proportion in which they were sown in the mixture. The competitive ratio (CR) among different combinations was calculated using the following formula (Willey, 1990):

$$\text{CR} = \frac{\text{LER of crop (a)}}{\text{LER of crop (b)}}$$

Agressivity (A) indicates the relative yield increase in "a" crop is greater than of "b" crop in an intercropping system (McGilchrist 1965). Aggressiveness was determined according to Willey and Rao (1980) using mean grain yield values of treatments averaged across years and replications as:

$$\text{Aggressiveness of turmeric (Aab)} = \frac{Y_{ab}}{Y_{aa} \times Z_{ab}} - \frac{Y_{ba}}{Y_{bb} \times Z_{ba}}$$

$$\text{Aggressiveness of mung bean (Aba)} = \frac{Y_{ba}}{Y_{bb} \times Z_{ba}} - \frac{Y_{ab}}{Y_{aa} \times Z_{ab}}$$

Where,
Y_{ab}= Intercropped yield of turmeric,
Y_{ba} = Intercropped yield of mung bean,
Y_{aa} = Mono crop yield of turmeric,

Y_{bb} = Mono crop yield of mung bean,
Z_{ab} = Sown proportion of turmeric, and
Z_{ba} = Sown proportion of mung bean.

The system productivity index (SPI) was calculated based on (Odo, 1991):

$$\text{System productivity index (SPI)} = \frac{S_a}{S_b} Y_b + Y_a$$

Where,
S_a = Mean yield of turmeric in Mono culture,
S_b = Mean yield of mung bean in Mono culture,
Y_a = Mean yield of turmeric in mixed culture,
Y_b= Mean yield of mung bean in mixed culture.

3 RESULTS AND DISCUSSION

3.1 Yield and yield attributes of turmeric

Rhizome yield and yield attributes of turmeric were significantly varied among the intercropping treatments (Table 1). It was evident that the entire yield and yield attributes in the intercropping treatments increased with the increasing of mung bean population. This might be due to the N fixation ability of the legume which lead an improvement of turmeric (rhizome) yield as well as yield attributes. Values of yield contributing characters were maximum under turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system compared to other intercropping systems and mono cropping. Our data clearly showed that turmeric showed yield ranged of 17.52-20.01 t ha^{-1} in intercropping systems, which was higher than that of mono culture (17.43 t ha^{-1}). However, the maximum value was found under turmeric (100 %) + 3 row mung bean (100 %) in turmeric lines intercropping system. The results further revealed that intercropping mung bean with turmeric did not hamper the normal growth of turmeric but it significantly enhanced the growth and development, which lead the highest rhizome yield in mung bean-turmeric intercropping system compared to cultivation of turmeric alone. These results are in agreement with the findings of (Joyachandran et al., 1991) who reported that higher fresh turmeric yield was obtained in intercropping systems than mono crop (in open sunlight) due to shady condition. The rhizome yield increased up to 15 % in intercropping systems than mono cropping of turmeric (Table 1).

Table 1: Yield and yield contributing characters of turmeric (pooled average of 2014 and 2015)

Treatments	Plant height (cm)	Number of mother rhizomes/ plant	Number of fingers/ plant	Mass of mother rhizome/plant (g)	Mass of fingers/plant (g)	Rhizome yield (t ha^{-1})	Rhizome yield (%) increased over sole turmeric
T_1	119.47	6.06	15.78	180.18	363.16	17.52	0.52
T_2	120.13	7.18	16.49	190.19	371.09	18.16	4.19
T_3	124.69	7.40	17.49	241.59	409.85	20.01	14.80
T_4	123.96	7.51	17.55	238.29	411.13	19.74	13.25
T_5	119.16	5.39	15.16	163.34	349.75	17.43	-
LSD (0.05)	4.48	0.90	0.90	15.10	17.14	1.18	-
CV (%)	3.01	10.94	4.46	6.09	3.68	5.19	-

3.2 Yield and yield attributes of mung bean

Yield and yield attributes of mung bean were significantly influenced by different intercropping system (Table 2). The longest plants (52.42 cm) was recorded from turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system (T_3). The shortest mung bean plants were observed in the treatment of one row mung bean between two turmeric lines (T_1). The maximum number of pods per plant (15.16) was recorded in the turmeric (100 %) + 1 row mung bean (100 %) in between turmeric lines intercropping system. Reduction in number of pods per plant was found with increased plant population of mung bean. Similar results were found in case of pod length, seeds/pod and 1000-seed mass. Mung bean produced the maximum seed yield in mono culture (1.08 t ha^{-1}). Higher mung bean seeds were harvested from the higher percentage of mung bean populations in the intercrops resulted the highest seed yield of mung bean (1.05 t ha^{-1}) in the turmeric (100 %) + 3 row mung

bean (100 %) in between turmeric lines intercropping system (T_3) than other combinations. The lowest seed yield (0.51 t ha^{-1}) was recorded in turmeric (100 %) + 1 row mung bean (100 %) in between turmeric lines intercropping system (T_1), probably due to the lowest plant population of mung bean per unit area. Mung bean showed 3 % to 53 % higher yield in mono cropping systems as compared to their corresponding intercropping systems.

Table 2: Yield contributing characters and yield of mung bean (pooled average of 2014 and 2015)

Treatments	Plant height (cm)	Pods/ plant (no.)	Pod length (cm)	Seeds/ pod (no.)	1000-seed mass (g)	Yield (t ha^{-1})	Yield decreased (%) over sole sesame
T_1	45.90	15.16	9.52	11.65	51.11	0.51	52.78
T_2	48.88	13.61	8.68	9.98	50.68	0.72	33.33
T_3	52.42	13.36	8.32	9.79	50.62	1.05	2.78
T_4	50.53	12.10	8.15	9.59	46.99	1.00	7.41
T_5							
T_6	47.16	12.35	8.33	9.76	50.45	1.08	-
$LSD_{(0.05)}$	3.73	1.28	0.914	1.14	2.00	0.04	-
CV (%)	6.23	7.84	8.68	9.19	3.27	4.12	-

T_1 = Turmeric 100 % + 1 line mung bean (33 %) in between two turmeric lines; T_2 = Turmeric 100 % + 2 lines mung bean (67 %) in between two turmeric lines; T_3 = Turmeric 100 % + 3 lines mung bean (100 %) in between two turmeric lines; T_4 = Turmeric 100 % + mung bean broadcast (100 %) in between two turmeric lines; T_5 = Sole Turmeric and T_6 = Sole mung bean

3.3 Turmeric equivalent yield (TEY)

TEY was referred to total productivity and it ranged from 19.05 to 23.16 t ha^{-1} in intercropping system, which was higher compared to mono cropping treatments (Table 3) indicating higher biomass production and efficient land use and recourse availability under intercropping than mono cropping. However, the highest TEY was recorded with turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system (T_3). The total productivity increase of 9 % to 33 % over mono cropping turmeric where turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping combination increase the highest total productivity (33 %).

Figure 3: Economics of intercropping mung bean with turmeric (average of two years)

Treatments	TEY (t ha^{-1})	Gross return (Tk ha^{-1})	Total cost (Tk ha^{-1})	Gross margin (Tk ha^{-1})	BCR
T_1	19.05	381000	149014	231986	2.56
T_2	20.32	406400	151714	254686	2.68
T_3	23.16	463200	154414	308786	3.00
T_4	22.74	454800	154564	300236	2.94
T_5	17.43	348600	137064	211536	2.54
T_6	3.24	64800	30260	34540	2.14

Market price: Turmeric: TK 20 kg^{-1} and Mung bean: Tk 60 kg^{-1}

3.4 Economics

In the present study, all the intercrop combinations showed higher monetary return than mono crops (Table 3). The maximum gross return (Tk. 4, 63,200 ha^{-1}) was found to be in turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system (T_3). Mono crop of mung bean showed the lowest gross return (Tk.64,800 ha^{-1}). The highest cost of cultivation was observed under all intercropping systems while

maximum was observed in turmeric 100 % + mung bean broadcast (100 %) in between two turmeric lines intercropping system. It was mainly due to more cost in extra labour required for sowing, harvesting, and other agronomic operations of two crops. The highest benefit cost ratio (BCR) was obtained (3.00) in turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system compared to all other combinations.

3.5 LER, RVI, MAI, SPI and Aggressiveness of turmeric-mung bean intercrop

The outcome of different intercropping systems on LER, RVI, MVI, SPI and aggressiveness are presented in Table 4. The LER is the relative area of mono crop required to produce the yield achieved in intercropping (Khan, 1988). The LER values were >1.0 for all the intercropping systems showed the efficacy of all intercropping systems. The increased value of LER over 1 (unity) indicated more land utilization facility in intercropping over actual mono cropping land (Mian, 2008). It also indicated yield advantage of intercropping over mono cropping with regard to the use of environmental resources for plant growth. The LER of different intercrop combinations ranged from 1.48 to 2.12 indicating 48-112 % yield increase by intercropping. The maximum LER value (2.12) was found in turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system. The LER value was increased proportionately in both crops in the different intercropping system. The result revealed that LER>1.00 in intercropping rendered better productivity than their mono crops.

RVI is a way to determine the economic advantage of intercropping. The maximum RVI (2.19) was observed in turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system (Table 4) implying that the farmers who practice intercropping of turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines will be making 119 % profit more than the farmers who are practicing mono-cropping of these crops. Therefore, the reason to popular intercropping systems among farmers is well-understood.

Table 4: Land equivalent ratio (LER), replacement value of intercropping (RVI), monetary advantage index (MAI), system productivity index (SPI) and aggressivity of mung bean-turmeric intercropping system (average of two years)

Treatments	LER	RVI	MAI (Tk. ha^{-1})	SPI	Aggressivity	
					Turmeric	Mung bean
T_1	1.48	1.80	123112.04	25.75	-0.43	0.43
T_2	1.71	1.92	168537.27	29.78	0.05	-0.05
T_3	2.12	2.19	244734.46	36.96	0.18	-0.18
T_4	2.06	2.15	233857.71	35.88	0.21	-0.21
T_5	1.00	1.65	-	-	-	-
T_6	1.00	0.31	-	-	-	-

MAI values were positive in all the intercropping systems. The result showed positive yield and economic advantages of the intercropping system over their mono cropping. The highest MAI (Tk. 2, 44,734.46 ha^{-1})) was obtained in the turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system, which implied that the combination was highly economical and advantageous (Table 4).

SPI standardized the yield of the secondary crop (mung bean) in terms of the primary crop (turmeric) and identified the combinations that utilized the growth resources effectively. The highest SPI (36.96) was found in turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system over the other combinations and monoculture. Contrary, the lowest SPI (25.75) was observed in turmeric (100 %) + 1 row mung bean (100 %) in between turmeric lines intercropping system (Table 4). The results also revealed that mung bean in high densities (100 %), as in the intercropping with turmeric utilized resources more effectively over other combinations and thus had a higher SPI.

Aggressiveness is an important tool that measures the competitive ability of a crop when grown in association with another crop (Dhima et al., 2007). An aggressiveness value of zero indicates that the component crops are equally competitive. But the data regarding the aggressiveness values of turmeric and

mung bean revealed that the component crops did not compete equally (Table 4). Negative sign of aggressiveness values for mung bean indicates the dominance of turmeric in all the intercropping systems except in turmeric (100 %) + 1 row mung bean (100 %) in between turmeric lines intercropping system, in which mung bean dominated the turmeric.

Table 5: Competitive ratio (CR) of turmeric and mung bean

Treatments	CR of turmeric	CR of mung bean	Difference
T_1	2.13	0.47	1.66
T_2	1.56	0.64	0.92
T_3	1.18	0.85	0.33
T_4	1.22	0.82	0.41
T_5	-	-	-
T_6	-	-	-

T_1 = Turmeric 100 % + 1 line mung bean (33 %) in between two turmeric lines; T_2 = Turmeric 100 % + 2 lines mung bean (67 %) in between two turmeric lines; T_3 = Turmeric 100 % + 3 lines mung bean (100 %) in between two turmeric lines; T_4 = Turmeric 100 % + mung bean broadcast (100 %) in between two turmeric lines; T_5 = Sole Turmeric and T_6 = Sole mung bean.

3.6 Competitive ratio (CR)

CR is an important way to measure the degree of competitiveness in which one crop compete with the others. The results of CR were higher in turmeric (1.18-2.13) than mung bean (0.47-0.85) indicating that turmeric was more competitive than mung bean in all intercropping systems. The highest CR value of turmeric was recorded in turmeric (100 %) + 1 row mung bean (100 %) in between turmeric lines intercropping system showing a decreasing trend with the mung bean proportion increases. This was due to more intra-species competition at higher population of mung bean. Similarly, the highest CR value of mung bean (0.85) was found in turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system exhibiting a decreasing trend with the increase of CR values of turmeric. Lower difference of CR values indicated better utilization of growth resources. However, turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system produced higher productivity in terms of TEY (23.16 t ha^{-1}) with minimum CR difference of 0.33 (Table 5). The CR over 1 (unity) indicates the species as good competitor while less than 1 (unity) indicates the species as poor competitor when grown in intercropping (Jedel et al., 1998).

4 CONCLUSIONS

Our results confirmed that potential benefits of intercropping mung bean with turmeric especially for increasing cropping intensity, total productivity and economic return per unit land enhancing national food security against gradual declining cultivable land. Further, the results showed correlation on improving soil fertility by mung bean and sustaining crop productivity under intensive cropping systems. Moreover, the results encourage the farmers to grow long duration turmeric crop for getting higher economic return. The outcome of the results furthermore indicated that rhizome yield of turmeric was higher in intercropping system than in mono crop. However, turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines intercropping system gave maximum yield of both the crops as well as TEY, better land use efficiency, BCR and MAI. Therefore, turmeric (100 %) + 3 row mung bean (100 %) in between turmeric lines could be a better intercropping system.

5 REFERENCES

Ali, M., Mishra, J. S. (1993). Intercropping of blackgram (*Phaseolus mungo*) and greengram (*P. radiatus*) with spring sunflower (*Helianthus annuus*). *Indian Journal of Agricultural Sciences*, *63*(8), 493-495.

Banik, P., Sasmal, T., Ghosal, P. K., Bagchi, D. K. (2000). Evaluation of mustard (*Brassica compestris* var. *toria*) and legume intercropping under 1:1 and 2:1 row-replacement series systems. *Journal of Agronomy and Crop Science*, *185*, 9-14. doi.org/10.1046/j.1439-037X.2000.00388.x

BBS (Bangladesh Bureau of Statistics). (2011). *Statistical Year Book of Bangladesh*. Statistics Division, Ministry of Planning, Government of the Peoples Republic of Bangladesh, Dhaka.

Bhatti, I. H. (2005). *Agro-physiological studies on sesame-legume intercropping system in different geometric arrangements*. Ph. D. thesis, Department of Agronomy, University of Agriculture, Faisalabad, Pakistan.

Caballero, R., Goicoechea, E.L., Hernaiz, P. J. (1995). Forage yield and quality of common vetch and oat sown at varying seed ratios and seeding rates of common vetch. *Field Crops Research*, *41*, 135-140. doi.org/10.1016/0378-4290 (94)00114-R

Dhima, K. V., Lithourgidis, A. S., Vasilakoglou, I. B., Dordas, C. A. (2007). Competition indices of common vetch and cereal intercrops in two seeding ratio. *Field Crop Research*, *100*, 249–256. doi.org/10.1016/j.fcr.2006.07.008

Esmaeilia, A., Sadeghpourb, A., Hosseinia, S. M. B., Jahanzada, E., Chaichia, M. R., Hashem, M. (2011). Evaluation of seed yield and competition indices for intercropped barley (*Hordeum vulgare*) and annual medic (*Medicago scutellata*). *International Journal of Plant Production*, *5*(4), 395-404. doi: 10.22069/ijpp.2012.749

Ghosh, P. K. (2004). Growth, yield, competition and economics of groundnut/cereal fodder intercropping systems in the semi-arid tropics of India. *Field Crops Research*, *88*, 227-237. doi.org/10.1016/j.fcr.2004.01.015

Hasan, MD. K., Sabagh, A. EL., Sikdar, MD. S. I., Alam, MD. J., Ratnasekera, D., Barutçular, C., Islam, M.S. (2017). Comparative adaptable agronomic traits of black gram and mung bean for saline lands. *Plant Archives*, *17*(1), 589-593.

Hauggaard-Nielsen, H., Ambus, P., Jensen, E. S. (2001). Interspecific competition, N use and interference with weeds in pea-barley intercropping. *Field Crops Research*, *70*, 101-109. doi.org/10.1016/S0378-4290 (01)00126-5

Islam, M. S., EL Sabagh, A., Hasana, K., Akhter, M., Hasan Barutçulard, C. (2017). Growth and yield response of mung bean (*Vigna radiata* L.) as influenced by sulphur and boron application. *Scientific Journal of Crop Science* , *6*(1), 153-160. doi: 10.14196/sjcs.v6i1.2383

Jedel, P. E., Helm, J. H., Burnett, P. A. (1998). Yield, quality and stress tolerance of barley mixture in central Alberta. *Canadian Journal of Plant Science*, *78*, 429-436. doi.org/10.4141/P97-137

Joyachandran, B. K., Bari, M. M., Salam, M. A., Mannan, M. K., Mathew, K. P. (1991). Performance of turmeric/ginger under shade and open condition. *Indian Coca, Arecanut and Spices Journal*, *15*(2), 40-42.

Khan, Z., Ahmed, S., Nazir, M. S. (1988). Land equivalent ratios, relative yields and relative yield totals of intercropped maize and soybean. *Pakistan Journal of Agricultural Sciences, 9*, 453-457.

Malik, M. A., Saleem, M. F., Sana, M., Aziz, A. (2002). Agro economic expression of different relay crops after rice harvest under conventional and zero tillage. *International Journal of Agriculture and Biology*, *4*, 277-278.

Mahapatra, S. C. (2011). Study of Grass-Legume Intercropping System in Terms of Competition Indices and Monetary Advantage Index under Acid Lateritic Soil of India. *American Journal of Experimental Agriculture*, *1*(1), 1-6. doi:10.5281/zenodo.8092

McGilchrist, C.A. (1965). Analysis of competition experiments. *Biometrics*, *21*, 975-985. doi.org/10.2307/2528258

Mead, R., Willey, R.W. (1980). The concept of land equivalent ratio and advantages in yields for intercropping. *Experimental Agriculture, 16*, 217-228. doi.org/10.1017/S0014479700010978

Mian, M. A. K. (2008). *Performance of maize oriented cropping patterns under different nutrient management*. Ph. D. Dissertation, Dept. Agron., Bangladesh Agril. Univ., Mymensingh. pp. 31-137.

Midya, A., Bhattacharjee, K., Ghose, S. S., Banik, P. (2005). Deferred seeding of blackgram (*Phaseolus mungo* L.) in rice (*Oryza sativa* L.) field on yield advantages and smothering of weeds. *Journal of Agronomy and Crop Science*, *191*, 195-201. doi.org/10.1111/j.1439-037X.2005.00157.x

MOA (Ministry of Agriculture). (2014). *Hand Book of Agricultural Statistics*, December 2007. Government of the Peoples Republic of Bangladesh. http://www.moa.gov.bd/statistics/statistics.htm

Mosely, W. G. (1994). An equation for the replacement value of agroforestry. *Agroforestry System*, *26*, 47-52. doi**:** 10.1007/BF00705151

Nazir, M. S., Jabbar, A., Ahmad, I., Nawaz, S., Bhatti, I. H. (2002). Production potential and economics of intercropping in autumn-planted sugarcane. *International Journal of Agriculture and Biology*, *4*, 140-142.

Odo, P. E. (1991). Evaluating short and tall sorghum varieties in mixtures with cowpea in Sudan Savanna of Nigeria: LER, grain yield and system productivity index. *Experimental Agriculture, 27*(4), 435- 441. doi.org/10.1017/S0014479700019426

Oseni, T. O. (2010). Evaluation of sorghum-cowpea intercrop productivity in savanna agro-ecology using competition indexes. *Journal of Agricultural Science, 2*(3), 229-234. doi.org/10.5539/jas.v2n3p229

Rahman, Md. M., Zahan, F., Sikdar, Md. SI., EL Sabagh, A., Barutçular, C., Islam, M. S., Ratnasekera, D. (2017). Evaluation of salt tolerance mung bean genotypes and mitigation of salt stress through potassium nitrate fertilization. *Fresenius Environmental Bulletin*, *26*(12), 7218-7226.

Willey, R. W. (1990). Resource use in intercropping system. *Agricultural Water Management*, *17*, 215-231. doi.org/10.1016/0378-3774(90)90069-B

Willey, R. W., Rao, R. M. (1980). A competitive ratio for quantifying competition between intercropping. *Experimental-Agriculture, l6*(1), 117-125. doi : 10.1017/S0014479700010802

Yilmaz, S., Atak, M., Erayman, M. (2008). Identification of advantage of maize-legume intercropping over solitary cropping through competition indices in the east Mediterranian region. *Turkish Journal of Agriculture and Forestry*, *3q`2*, 111-119.

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Preservation of sweet chestnut genetic resources (*Castanea sativa* Mill.) against attack by chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu, 1951)

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ABSTRACT

European sweet chestnut (*Castanea sativa* Mill.) is one of the most important wood species due to its environmental and economic role in many agro-forestry systems. Chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu, 1951) is currently the most dangerous pest of sweet chestnut, including in Slovenia. Attack on vegetative buds (in which the eggs are deposited and on which galls are subsequently formed) disturbs the growth of shoots and reduces the yield. In the event of a strong attack, the tree can weaken and decay, which is already noticeable on the ground in Slovenia, especially in terms of the monitored genetic resources of the chestnut tree. Following Japanese experience, European countries are increasingly choosing biological control of chestnut gall wasp with the torymid wasp (*Torymus sinensis* Kamijo, 1982). Micropropagation is a way of ensuring effective preservation and reproduction while optimizing all phases of work. In the micropropagation of Slovenian sweet chestnut genetic resources, problems arise in the rooting phase.

Key words: sweet chestnut; *Castanea sativa;* chestnut gall wasp; *Dryocosmus kuriphilus;* torymid; *Torymus sinensis;* micropropagation; breeding

IZVLEČEK

OHRANITEV GENSKIH VIROV EVROPSKEGA PRAVEGA KOSTANJA (*Castanea sativa* Mill.) PRED NAPADOM KOSTANJEVE ŠIŠKARICE (*Dryocosmus kuriphilus* Yasumatsu, 1951)

Evropski pravi kostanj (*Castanea sativa* Mill.) je ena izmed najpomembnejših lesnatih vrst zaradi svoje okoljske in gospodarske vloge v mnogih kmetijsko-gozdnih sistemih. Trenutno je kostanjeva šiškarica (*Dryocosmus kuriphilus* Yasumatsu, 1951) tudi v Sloveniji najpomembnejši škodljivec pravega kostanja. Z napadom vegetativnih brstov, kamor odlaga jajčeca in posledično povzroča tvorbo šišk, moti rast poganjkov in zmanjšuje pridelek. Ob močnem napadu lahko drevo zelo oslabi in propade, kar je na terenu v Sloveniji že opazen pojav, predvsem pri spremljanih genskih virih kostanja. Po vzoru japonskih izkušenj se tudi evropske države vse pogosteje odločajo za biotično varstvo s parazitoidno oso *Torymus sinensis* Kamijo, 1982. Mikropropagacija je način, ki zagotavlja učinkovito ohranjanje in razmnoževanje ob optimizaciji vseh faz dela. Pri mikropropagaciji slovenskih genskih virov se pojavljajo težave v fazi koreninjenja.

Ključne besede: evropski pravi kostanj; *Castanea sativa;* kostanjeva šiškrica; *Dryocosmus kuriphilus;* parazitoid; *Torymus sinensis;* mikropropagacija; žlahtnjenje

1 INTRODUCTION

Sweet chestnut is a woody species that plays an important role in the world because of its wide functional value and because of its economic and environmental importance. Asian species, the Chinese chestnut, *Castanea mollissima* Blume, the Japanese chestnut, *C. crenata* Sieb. & Zucc. and another Chines species, *C. henryi* (Skan.) Rehd. & Wils., as well as the European chestnut , sweet chestnut, *C. sativa* (Mill.), have been a basic food for survival of the population for centuries in many parts of Asia, Southern Europe and most of the countries bordering the Mediterranean (Bounous, 2005).

The Romans spread European sweet chestnut (*C. sativa* Mill.) throughout the European continent mainly to produce wooden barrels for storing wine. During this period, chestnut as a source of food was not the main reason for its spread across Europe. Growing sweet chestnut as food for sustenance was mainly developed after the Roman period, in connection with the socio-

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economic system of the Middle Ages. The ancient Greeks were important growers of sweet chestnut for wood and fruit, although they never grew it on a large scale (Metaxas, 2013). In Europe, the Greeks were among the first to use the fruit and introduce the sweet chestnut to other cultures, from Asia Minor to Southern Europe and North Africa. Chestnut species and cultivars are traditionally grown today in China, Korea, Japan and the Mediterranean (Litz, 2005).

Except for timber extraction and tannin production, sweet chestnut fruit and honey are the only market-relevant crops within the family of Fagaceae in the temperate zone. Despite increasing demand, global chestnut production has been gradually decreasing over the last century, in particular due to fungal diseases and pests that have not only destroyed the chestnut population throughout its distribution but have also limited the creation of new growing chestnut areas (Litz, 2005; Metaxas, 2013).

There are three main areas of chestnut plantations around the world: (1) in Asia the most important area is in China, where the species *C. mollissima* and *C. henryi* grow in natural conditions, as well as in plantations, and in Japan, where the species *Castanea crenata* is widespread; (2) Europe is the second main area, in which the species *C. sativa* is predominant; (3) in North America, the American chestnut, *C. dentata* (Marsh.) Borkh. was widespread in nature, but is nowadays replaced by hybrids that are resistant to chestnut blight and ink disease (Pereira-Lorenzo and Ramos-Cabrer, 2007).

European sweet chestnut (*C. sativa*) is widespread in forests and artificial plantations across all Mediterranean countries and elsewhere. It extends from Spain and Portugal to the Caucasus, across Turkey, Greece, the Balkans, the former Soviet Union and the southern part of Great Britain. In North Africa, it is found in small areas of Morocco, Algeria and Tunisia. The main producers in Europe are Italy, Turkey, Portugal, Spain, France and Greece. Chestnuts are no longer a matter of survival in Europe but they still play an important role in diet, the timber industry and landscape strategies in many agri-economic systems. Over the past thirty years, the chestnut ecosystem has increased its ecological and landscape significance mainly through the planting of varieties resistant to fungal diseases and it has thus become a fundamental resource for the sustainable development of mountain areas (Bounous, 2005; Bounous, 2014).

Due to the rapid expansion of the chestnut gall wasp with planting material from the original areas of China to almost all of the world's growing areas, including Europe, and the rapid collapse of trees, new plant breeding programs have begun to appear, in order to obtain genotypes resistant to this very dangerous pest.

2 CHESTNUT GALL WASP: DESCRIPTION AND ITS SUPERVISION

The great dispersal of and economic damage by the chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu, 1951) is a consequence of trade in or transfer of attacked planting material. Mathematical models have assessed the natural spread of the chestnut gall wasp to be at a speed of 8 km per year, with a variation ranging from 3 to 12 km year^{-1}, which reflects the rate of natural spread of this species to other parts of Europe (EFSA, 2010; Gibbs et al., 2011).

By attacking the vegetative buds, through the development of the wasps the chestnut gall wasp disrupts the growth of shoots, the flow of assimilates and reduces the crop. In both commercial plantations and natural stands, the crop can be reduced from 50 – 70 %, depending on the severity of the attack. Severe damage can cause complete crop decline and the death of the tree. Chestnut gall wasp is still the worst insect pest of chestnut trees globally (*Dryocosmus kuriphilus*, 2005). Some researchers argue that severe and repeated injuries over several years gradually lead to a decline in vitality and often lead to the destruction of trees (Bosio et al., 2010). This was confirmed by our field visits carried out from 2003 for obtaining material for the design of *in vitro* cultures. At that time, we did not notice any damage from the chestnut gall wasp (Osterc et al., 2005). We sampled the material again in 2015 and 2016, and found that almost all the genetic resources that had been previously marked around Slovenia had been severely attacked by chestnut gall wasp and were rapidly decaying. In 2015, complete collapse was observed in the genotype from the surroundings of Pedrovo (Primorska), which was also supported by the age of the trees.

2.1 Physical and phytosanitary measures to suppress the chestnut gall wasp

Damage in small chestnut stands can be reduced by pruning and destroying damaged shoots but commercial growers do not make use of this due to the cost and uncontrollability of work. Insecticides can be effective against adult female and young larvae but with negative side effects on the environment (*Dryocosmus kuriphilus*, 2005; Knapič et al., 2010). Most insecticides

do not provide good control over the chestnut gall wasp. Systemic insecticides have an advantage over contact ones, since most of the developmental phases (eggs, larvae, bugs and part of the adult wasps) are protected in the galls. Several experiments have been carried out in the past in which very toxic insecticides were used. In China, good efficacy was achieved by injecting methanmidophos and ometoate into tree trunks and by spraying with dichlorvos, methylparation and methamidophos (Bosio et al., 2010).

In Italy, the Piedmont Region Plant Health Service, in collaboration with local research centres for fruits and vegetables, studied the effectiveness of some insecticides permitted in other fruit species but not chestnuts. Treatments were carried out in various phenological phases of young plants and development stages of the pests, in both nurseries and plantations. Treatments that were carried out at the time of the appearance of adult gall wasps leaving the kaolin galls, with a mineral powder that acts as a physical barrier or with lambda-cyhalothrin, alpha-cypermethrin and ethylchlorpyrofos with the addition of mineral oil, contributed to minor injuries to the plant tissue but increased the mortality of adult females and reduced the appearance of eggs in the buds. These results were obtained from young plants grown in pots. However, it took five to six treatments to protect plants during the flying season of the gall wasps. This means that it would be very expensive on larger trees, and an unacceptable technique for the environment. In addition, the risk of killing pollinators and toxic residues in honey need to be considered, since the first treatments may also overlap with the time of chestnut flowering. It is thus obvious that chemical control would pose more risk than benefit. In the Piedmont region, therefore, they decided to follow the Japanese example and selected biological control (Bosio et al., 2010).

Strict monitoring of the transport of infected plant material can significantly reduce the proliferation of gall wasps over a long-distance and to new areas across Europe. There are currently very limited options for managing existing pest populations and reducing the extent of their impact. Since the larvae and pupas of the pest are protected within galls, conventional chemical protection is very ineffective. The development of resistant varieties of *Castanea* sp. is a solution for new chestnut plantations but does not solve the spread of pests from existing attacked areas.

After the Second World War, Japanese fruit growers chose varieties of chestnut with some resistance, but the pest developed a new strain to overcome this resistance (*Dryocosmus kuriphilus*, 2005).

In some parts of China, where the pest is naturally present, populations of the chestnut gall wasp survive in small numbers but do not cause economic damage, probably due to natural enemies, but there has been little publicity and there is little knowledge of alternative sources of pest mortality in these areas (Gibbs et al., 2011).

2.2 A biological measure of suppression of the chestnut gall wasp

In its natural area of distribution in China, the chestnut gall wasp is controlled by natural enemies, especially indigenous Hymenoptera parasitoids. Many new parasitoids parasitizing the chestnut gall wasp have recently been described in China, Japan and Korea; e.g., *Torymus sinensis* Kamijo, 1982, *Torymus beneficus* Yasumatsu & Kamijo, 1979, *Megastigmus maculipennis* Yasumatsu & Kamijo, 1979, *Megastigmus nipponicus* Yasumatsu & Kamijo, 1979 (Chalcidoidea, Torymidae), *Ormyrus flavitibialis* Yasumatsu & Kamijo, 1979 (Ormyridae) and others. Some of these parasites have proved to be very effective (Yasumatsu and Kamijo, 1979; *Dryocosmus kuriphilus*, 2005; Kos and Trdan, 2010).

The parasitoid wasp *Torymus sinensis* (Kamijo) has been released as a biological control agent for controlling gall wasp populations in North America, Japan and Europe. The parasitoid seeks galls of chestnut gall wasp in early spring using visual and olfactory organs and hatches its eggs in a barnacle containing developing larvae of the pest. The parasitoid larvae feed on the larvae of the chestnut gall wasp and remain inside the galls until they are fully grown and leave the barnacle next spring (Graziosi and Rieske, 2015).

T. sinensis is a natural species in China and Moriya et al. reported in 2003 that this is the only Chinese chestnut gall wasp parasitoid species that was previously known and is also host specific and phenologically well coincides with chestnut gall wasp.

In 1979 and 1981, 260 females of *T. sinensis* (from about 5000 galls of chestnut gall wasps imported from China) were released as a biocontrol method on Japanese chestnut trees at a research station in the province of Ibaraki. By 1989, the population of *T. sinensis* had increased 25-fold and it had become the most common group of local chestnut gall wasp parasitoids. Following this increase, the parasitoid *T. sinensis* successfully expanded into areas with a population of Japanese chestnut gall wasp and achieved effective biological control. The parasitoid wasp *T. sinensis* was also released in the US state of Georgia in the late 1970s, in response to widespread chestnut gall wasp infestation in North America The number of

damaged shoots decreased, ensuring effective biological control (Gibbs et al., 2011).

However, some sources claim that the imported natural parasitoids in Japan and the United States, and most probably in Europe, will not ensure good control of the chestnut gall wasp, since they are not specific and do not coincide with the life cycle of the pest, as well as there being various environmental effects (*Dryocosmus kuriphilus*, 2005).

Preliminary studies on the spread of the *T. sinensis* parasitic wasp were firstly carried out in Europe in 2003 and 2004 in Italy, using imported parasitoid Japanese galls of *D. kuriphilus.* In these initial studies, the phenological discrepancy between the emergence of adult parasitoid *T. sinensis* and the development of the local chestnut gall wasp *D. kuriphilus* (due to temperature changes that had occurred during the transfer) was shown. Based on these results the parasitoid wasps could not be released into the open field. Behavioural experiments were also used, and they helped to improve later efforts in controlling adult females. In 2005, several chestnut galls of *D. kuriphilus* were imported from Japan. Their development was slowed down by artificial cooling. This enabled artificial adjustment of the appearance of imported adult parasitoid *T. sinensis* with populations of the chestnut gall wasps *D. kuriphilus,* and females of the parasitoid wasp *T. sinensis* were then released at three sites in Italy attacked by the chestnut gall wasp *D. kuriphilus*. Following successful adjustment of the *T. sinensis* parasitoid wasp at all three sites, a further cultivation program was set up to stimulate the release of the parasitoid *T. sinensis* to additional attacked areas in Italy (Gibbs et al., 2011).

However, it is still too early to assess the long-term effectiveness of biological control by the parasitoid wasp *T. sinensis* against Italian chestnut gall wasp populations of *D. kuriphilus*. Successful control would mean reducing chestnut damage to less than 30 % (Gibbs et al., 2011).

Further studies and confirmation of the effectiveness of the parasitoid wasp (*T. sinensis*) as a viable option for biological control of the chestnut gall wasp (*D. kuriphilus*) in Central Europe is indispensable. It has been suggested that more attention should be paid to determining: (i) the conditions under which the parasitoid wasp (*T. sinensis*) can attack the host chestnut gall wasp and (ii) the probability of the two genera crossing. Both issues are central to predicting the spread of the released parasitoid wasp (*T. sinensis*) and assessing the environmental risks associated with a more widespread release of this species into Europe (Gibbs et al., 2011).

2.2.1 Environmental risk assessment

A comprehensive assessment of environmental risk is based on the identification and evaluation of potential risks associated with the release, or planned dissemination, of a natural enemy and the development of a risk reduction plan. The last step before the introduction of a planned dissemination is to identify, evaluate and consider all negative and positive effects in terms of benefits, risks and costs.

The first question in the assessment of environmental risk relates to the origin and purpose of use of the biological controlling agent. It is necessary to evaluate the extent of the appearance of the parasitoid wasp (*T. sinensis*) and decide whether it can attack non-target species. Earlier studies have shown that wasps that cause galls on oak tree are attacked by similar parasitoids, so there is a general risk that the parasitoid wasp (*T. sinensis*) will pass over to autochthonous wasps that are related to chestnut gall wasps (*D. kuriphilus*), including an autochthonous species of the genus *Dryocosmus* that causes galls on oak trees. Information on cultivation and monitoring in China shows that the parasitoid wasp (*T. sinensis*) is very specific to chestnut gall wasp (*D. kuriphilus*). It should be noted that such an obvious monophagia is exceptional among many parasitoids that attack galls (Gibbs et al., 2011).

Two recent assessments of the parasitoid wasp (*T. sinensis*) as a candidate for biological control have shown many deficiencies in knowledge of the biology of this species. The key issue in relation to attacking non-target hosts is the latter's seasonal phenology and thus the potential for the parasitoid wasp (*T. sinensis*) to attack other hosts, in addition to chestnut gall wasp (*D. kuriphilus*). If *T. sinensis* could be confirmed as a specific parasitoid that does not attack non-target species, then it could be considered as a candidate for biological control of the chestnut gall wasp (*D. kuriphilus*) beyond the present scale. Conversely, if the parasitoid wasp (*T. sinensis*) has a wider host circle, it would be regarded as too risky to release, and unsuitable for biological control in other parts of Europe (Gibbs et al., 2011).

The possible crossing of a biological control organism with indigenous species is considered to be an environmental risk for non-target species and is, in general, a threat to autochthonous biodiversity. Theoretically, insects introduced as a biological control can cross with indigenous species. It is worth mentioning that the only such example so far reported involves *T. sinensis* and the Japanese autochthonous species *Torymus beneficus* Yasumatsu and Kamijo. It was suggested that there is a possibility of

interbreeding, which was confirmed by the successful crossing of *T. sinensis* and *T. beneficus* under laboratory conditions and by gaining fertile hybrid females. Hybrids were also found in the field and molecular markers confirmed their hybrid origin (Gibbs et al., 2011).

A thoroughly designed risk assessment would confirm or deny the risk of expanding the introduction of *T. sinensis* that would outweigh the risks associated with the use of other control options (chemical control). More attention should be given to determining: (i) the conditions under which *T. sinensis* could attack alternative hosts and (ii) the probability of crossing with indigenous species of the genus *Torymus*. It is necessary to consider factors such as: the type of host, host behaviour in the given area, the location of the galls on host plant and phenology, since these factors can influence the outcome and reliability of host specificity tests (Gibbs et al., 2011). In general, current data suggest that the release of *T. sinensis* could have a wide range of potential impacts and it is therefore important to consider all possible impacts before further release of *T. sinensis* across Europe. The only alternative to the mentioned measures is breeding tolerant/resistant genotypes to reduce and weaken the pest population, which does not solve the current situation on the ground.

3 BREEDING OBJECTIVES AND LACK OF TOLERANT/RESISTANT VARIETIES

Most phytosanitary measures for suppression have not been shown in experimental studies effectively to eradicate or reduce the presence of chestnut gall wasp. There are also concerns about biological control, so an alternative is identifying resistance genes and the targeted breeding of resistant varieties or hybrids to replace damaged trees.

In Europe and Asia, the main breeding objectives have been focused on the selection of economically interesting genotypes from natural sites and obtaining hybrids with added genes for tolerance or resistance to severe diseases and pests, in order to improve and obtain resistant varieties (Bounous, 2014). In America, Australia and New Zealand, efforts are directed towards acquiring new varieties with the desired tolerance/resistance properties, or a selection of elite genotypes among the best European and Asian varieties, with wide adaptation to local pedoclimatic conditions. Other breeding goals to improve the properties of wood and fruit have recently been of secondary importance (Van Fleet, 2014). For the rapid replacement of decayed trees and cultivation in plantations, many studies have focused on successful vegetative propagation of resistant varieties with the best compatible properties of the rootstock and the graft, which has a significant impact on the survival and quality of the seedling. It is well established that *C. crenata* and *C. dentata* are more easily propagated than *C. sativa* (Galic et al., 2014). *C. crenata* is therefore often included as one of the parents in interbreeding with *C. sativa* to improve rooting. However, there is a problem of compatibility between the rootstock and the graft. It has been found that there is better compatibility between grafted scions that are hybrids of the same parents as the rootstock (Bounous, 2005).

Breeders have recently used molecular markers to improve approaches to detecting resistant genes against major fungal pathogens, such as chestnut blight (*Cryphonectria parasitica* (Murr.) Barr.) and ink disease caused by *Phytophthora cambivora* (Petri) Buis. and *P. cinnamomi* (Rands.). Genes for resistance to ink disease have been found in *C. crenata* and *C. mollissima.* Japanese-European hybrids *C. crenata* x *C. sativa* obtained in France: 'Marsol', 'Maraval', 'Ferosacre', 'Marigoule', 'Marlhac' and 'Bouche de Betizac', have good resistance to *Phytophthora* fungal infections and are suitable for reproduction with layering and cuttings. Resistance to insect pests have been slightly less investigated, except for the gall wasp (*Dryocosmus kuriphilus*), in which the focus has been on studying the growth of shoots, crown density and the morphology of buds (Bounous, 2005). The cultivar 'Bouche de Betizac' shows tolerance here, if not resistance to this pest. In 2003, a project was started in Piedmont of establishing biological control using the parasitoid wasp (*T. sinensis*) and selection of resistant genotypes. The 'Bouche de Betizac' cultivar was found to be completely resistant to the gall wasp. The resistance mechanism of the 'Bouche de Betizac' cultivar is unknown but it has been found that dormant winter buds can contain eggs or larvae, although the galls are not formed in the spring after the development of buds (Dini et al., 2012). In this case, careful control and integration of trees with natural resistance into plant breeding programs is very promising but progress is very slow.

Anagnostakis et al. (2011) found in the US state of Georgia, that "chinquapins" (chestnuts with a single nut/fruit instead of three, in a thorny wrap) rarely showed symptoms of chestnut gall wasp attack, so they crossed the American chestnut with Ozark chinquapin (*Castanea ozarkensis* Ashe), which was crossed with Chinese chestnut. The authors found that American chestnut and Chinese chestnut are very susceptible to chestnut gall wasp attack and that American chestnut and Ozark chinquapin are susceptible to chestnut blight.

The female parents of the crosses they planted were four different trees of American chestnut, which were half-sisters/half-brothers. The male parents were two distinct trees of *C. ozarkensis* x *C. mollissima* (Ozark chinquapin crossed with Chinese chestnut; the parents of Ozark chinquapin and Chinese chestnut were not the same). Both parents had good resistance to chestnut blight. The descendants of this crossing had some resistance to chestnut blight, which enabled them to survive longer than sensitive genotypes. In addition, it was expected that if resilience to the attack of the gall wasp was inherited easily, some descendants might express some degree of resistance. In 1995, 93 trees were planted in North Carolina, in an area in which the gall wasp was already endemic. In 2009, 36 of these trees had survived, and 31 of them did not have galls (Anagnostakis et al., 2011).

The results obtained in the experiment encouraged the authors, Anagnostakis et al. (2011), to further cross the "chinquapin" chestnut with chestnut species, by selecting trees that were most resistant to the attack of the gall wasp. Resistant trees of Ozark chinquapins, which rarely had galls, began to be used in crosses with the aim of introducing resistance to the gall wasp into commercial chestnut cultivars and interesting hybrid species (Anagnostakis, 2014).

In Japan, selection and breeding began of genotypes that were resistant to chestnut gall wasp within the genetic resources of *Castanea crenata* Siebold & Zucc. and they obtained four resistant varieties. Two of these varieties, 'Tzukuba' and 'Tanzawa', together with the resistant 'Ginyose' variety, are still the most widespread varieties in Japan. Similar studies are under way in the US, where researchers want to introduce new resistance factors to the American chestnut *Castanea dentata* (Marsh.) Borkh. (Dini et al., 2012), using alternative sources of genetic material such as *Castanea mollissima* Blume, *Castanea pumila* (L.) Mill. and *Castanea henry* (Skan)Rehd. & Wils..

Resistance to chestnut gall wasp has also been reported in other species of the genus *Castanea* (*C. mollissima, C. pumila*), but not in the variety *C. sativa*. Based on the study of resistance, researchers have indicated that several mechanisms may be responsible for resistance or tolerance in various chestnut genotypes (Dini et al., 2012), which makes breeding work difficult and prolongs the time for obtaining tolerant/resistant varieties.

4 PRESERVATION AND REPRODUCTION OF EUROPEAN SWEET CHESTNUT (*Castanea sativa* Mill.)WITH MICROPROPAGATION

Chestnut is a hard-to-root tree species and grafting is the most common propagation method (Bounous, 2005). Failure to find an effective method of mass reproduction of selected genotypes has suggested micropropagation as a suitable method. The effectiveness of the micropropagation of forest trees depends on the responsiveness of the tissue in *in vitro* conditions, the effectiveness of vegetative propagation of selected varieties, as well as individual trees or genotypes. There are several forest varieties for which the successful establishment of *in vitro* cultures from adult/mature trees has not yet been achieved. Juvenile trees are generally easily reproduced by conventional techniques (Ballester et al., 1990; Ballester et al., 1992; Ballester et al., 1999; Litz, 2005).

Micropropagation, if there is optimization of all phases of work, provides a useful tool for preservation and reproduction of the genetic resources of chestnut. According to the literature data and our experience with Slovenian genotypes, optimization of the rooting phase is very difficult. *In vitro* culture with nodial cuttings was established from young axillary and lateral shoots, mainly from mature though partly also juvenile material. A key factor limiting clonal reproduction of woody varieties is the maturation and age associated non-responsiveness to the formation of roots. Mature and young - juvenile tissues of the same plant source may even have different responses to the added auxin (Pijut et al., 2011). These results can be explained by epigenetic changes during the maturation process. It had been repeatedly reported also for European chestnut, that the success in rooting strongly depends on maturation levels of the stock plant material (Sánchez and Vieitez, 1991; Gonçalves et al., 1998; Corredoira et al., 2017). Moreover, chestnut cuttings (*Castanea sativa*) from mature trees have an elevated level of methylation of DNA compared to cuttings from juvenile trees (Hasbun et al., 2007). After several subcultures obtained by *in vitro* reproduction, some micro-cuttings can root as a consequence of gaining a certain phase of rejuvenation (Pereira-Lorenzo and Ramos Cabrer, 2007).

Studies have shown that the formation of roots is a hereditary quantitative feature, controlled by several endogenous and environmental factors. The most important of these are the juvenility of the stock plant material, hormones, especially auxins, light, temperature and mineral nutrition (Pop et al., 2011; Pacurar et al., 2014).

Physiologically, the process of root development consists of three consecutive but interdependent phases: (1) induction, (2) initiation and (3) expression. The three stages of rooting differ from each other and have different hormonal requirements. Root buds are formed from the cells between the vascular contacts, which accumulate starch for the first 24 hours and the cells become capable of forming root buds between 72 - 96 hours (Pop et al., 2011).

European sweet chestnut (*Castanea sativa*) had been repeated categorised as especially difficult-to-root species via micropropagation. Other chestnut species, including interspecific hybrids (*C. crenata* × *C. sativa*) are much easier to root (Gonçalves et al., 1998; Tetsumura in Yamashita, 2004; Corredoira et al., 2017). Different methods *in vitro* had been tested in the past to improve rooting process in *Castanea* microshoots, including micrografting (Šiftar, 1992). Generally, all methods can be divided into two groups: methods where micro shoots are rooted on medium supplemented with auxin and methods where auxin had been added to micro shoots by dipping their basis into the auxin solution before transferring the shoots into the medium. Gresshoff and Doy (1972) medium is mostly used as a basal medium, some experiments used for rooting of sweet chestnut micro shoots also MS-medium. The majority of the references also reported that the basal medium should contain ½ or ⅓ of the full concentration of macro- and micronutrients when it is used for rooting. Tetsumura in Yamashita (2004) successfully rooted micro shoots of the Japanese chestnut by culturing the shoots on (½) MS-medium supplemented with 15 μM IBA in the dark. Gonçalves et al. (1998) used a combination of Gresshoff and Doy (macronutrients) and MS-medium (micronutrients) supplemented with 3 mg/l IBA for successful rooting of hybrid chestnut (*C. sativa* × *C. crenata*) microshoots. Sanchez and Vieitez (1991) and Vielba et al. (2011) succeeded to root European chestnut by quick-dipping (0.5 – 3 min. or 1 min.) micro shoots in aqueous solution of IBA before transferring the shoots to IBA-free medium. Nevertheless, the micropropagation is still remaining a very doubtful method for economic propagation of European sweet chestnut plants (rooting and acclimatisation problems), but it is a useful method for preservation interesting genotypes (Capuana and Di Lonardo, 2013).

5 CONCLUSIONS

Some interesting Slovenian chestnut genotypes have been recorded in the field but they are recently at risk of decay from chestnut gall wasp, so we decided to preserve them in tissue culture through micropropagation. We have managed to optimize most of the stages of work, except for rooting and acclimatization, which corresponds to the experience of other authors, who have reported that the phase of rooting *in vivo* and *in vitro* of European sweet chestnut (*Castanea sativa* Mill.) is difficult. It also affects plant breeding programs, which are additionally faced with problems in selecting the best parents from a variety of available genetic resources and with incorporating resistant genes into European genotypes. Limiting factors also include high heterozygosity, a long juvenile period and a lack of markers for the early selection of tolerant/resistant offspring. All of this causes a lack of elite planting material for the establishment of new chestnut crops and the replacement of damaged trees in forest stands.

6 REFERENCES

Anagnostakis S. L., Clark S., McNabb H. (2011). Resistance of chestnut trees to infestation by Asian gall wasp. *Annual Report of the Northern Nut Growers Association*, *101*, 15-17.

Anagnostakis S. L. (2014). *Asian chestnut gall wasp on Connecticut chestnut trees*. http://www.ct.gov/caes/lib/caes/documents/publications/fact_sheets/plant_pathology_and_ecology/asian_chestnut_gall_wasp_on_connecticut_chestnut_trees.pdf

Ballester A., Sanchez M. C., San-Jose M. C., Viéitez F. J., Viéitez A. M. (1990). Development of rejuvenation methods for *in vitro* establishment, multiplication and rooting of mature trees. *Plant Aging: Basic and Applied Approaches, 186*, 43-49. https://doi.org/10.1007/978-1-4684-5760-5_6

Ballester A., Sanchez M. C., Viéitez A. M. (1992). New strategies for *in vitro* propagation of chestnut. *Proceedings of the World Chestnut Industry Conference*, Morgantown, USA, 32-40.

Ballester A., San-Jose M C., Vidal N., Fernandez-Lorenzo J. L., Viéitez A. M. (1999). Anatomical and biochemical events during *in vitro* rooting of microcuttings from juvenile and mature phases of chestnut. *Annals of Botany, 83*, 619-629. https://doi.org/10.1006/anbo.1999.0865

Bosio G., Gerbaudo C., Piazza E. (2010). *Dryocosmus kuriphilus* Yasumatsu: an outline seven years after

the first report in Piedmont (Italy). *Acta Horticulturae, 866,* 341-348. https://doi.org/10.17660/ActaHortic.2010.866.43

Bounous G. (2005). The chestnut: a multipurpose resource for the new millennium. *Acta Horticulturae, 693*, 33-40. https://doi.org/10.17660/ActaHortic.2005.693.1

Bounous G. (2014). Perspectives and future of the chestnut industry in Europe and all over the world. In: Proceedings of the second European congress on chestnut. *Acta Horticulturae, 1043,* 19-22. https://doi.org/10.17660/ActaHortic.2014.1043.1

Capuana M., Di Lonardo S. (2013). In vitro conservation of chestnut (*Castanea sativa*) by slow growth. *In vitro cell and developmental biology-Plant, 49,* 605-610. https://doi.org/10.1007/s11627-013-9536-6

Corredoira E., Martinez M. T., Cernadas J. M., San José M. C. (2017). Application of biotechnology in the conservation of the genus *Castanea*. *Forests, 8,* 394-408. https://doi.org/10.3390/f8100394

Dini F., Sartor C., Botta R. (2012). Detection of a hypersensitive reaction in the chestnut hybrid 'Bouche de Betizac' infested by *Dryocosmus kuriphilus* Yasumatsu. *Plant Physiology and Biochemistry, 60*, 87-73. https://doi.org/10.1016/j.plaphy.2012.07.023

Dryocosmus kuriphilus. (2005). *Bulletin OEPP/EPPO Bulletin*, *35*, 3, 422-424.

Galic D., Dale A., Alward M. (2014). Vegetative propagation of American chestnut. *Acta Horticulturae*, *1019,* 99-103. https://doi.org/10.17660/ActaHortic.2014.1019.15

Gibbs M., Schönrogge K., Alma A., Melika G., Quacchia A., Stone G. N., Aebi A. (2011). *Torymus sinensis*: a viable management option for the biological control of *Dryocosmus kuriphilus* in Europe? *BioControl*, *56,* 4, 527-538. https://doi.org/10.1007/s10526-011-9364-8

Gonçalves J. C., Diogo G., Amãncio S. (1998). In vitro propagation of chestnut (*Castanea sativa* × *C. crenata*): Effects of rooting treatments on plant survival, peroxidase activity and anatomical changes during adventitious root formation. *Scientia horticulturae, 71,* 265-275. https://doi.org/10.1016/S0304-4238(97)00136-2

Graziosi I., Rieske L. K. (2015). A plant pathogen causes extensive mortality in an invasive insect herbivore. *Agricultural and Forest Entmology*, *17,* 366-374. https://doi.org/10.1111/afe.12116

Gresshoff, P.M. and C.H. Doy. (1972). Development and differentiation of haploid Lycopersicon esculentum. *Planta, 107,* 161-170. https://doi.org/10.1007/BF00387721

EFSA (2010). Risk assessment of the oriental chestnut gall wasp, *Dryocosmus kuriphilus* for the EU territory and identifi cation and evaluation of risk management options. *EFSA Journal, 8*, 6, 1-114.

Hasbun R., Valledor L., Santamaria E., Canal M. J., Rodriguez R. (2007). Dynamics of DNA methylation in chestnut trees development. *Acta Horticulturae*, *760,* 563-566. https://doi.org/10.17660/ActaHortic.2007.760.80

Knapič V., Seljak G., M. Kolšek M. (2010). Experience with *Dryocosmus kuriphilus* Yatsumatsu eradication measures in Slovenia. *OEPP/EPPO Bulletin*, *40,* 169-175. https://doi.org/10.1111/j.1365-2338.2010.02371.x

Kos K., Trdan S. (2010). Biotično zatiranje kostanjeve šiškarice (*Dryocosmus kuriphilus* Yasumatsu, Hymenoptera, Cynipidae). *Acta agriculturae Slovenica*, *95,* 89-96.

Litz R. E. (2005). Biotechnology of fruit and nut crops. *Biotechnology in agriculture series*. Wallingford, CABI: 723 pp. https://doi.org/10.1079/9780851996622.0000

Metaxas A. M. (2013). Chestnut (*Castanea* spp.) cultivar evaluation for commercial chestnut production in Hamilton county, Tennessee. Chattanooga, University of Tennessee: *Thesis. M. S. Environmental Science*: 124 pp.

Moriya S., Shiga M., Adachi I. (2003). Classical biological control of the chestnut gall wasp in Japan. V: Proceedings 1st International Symposium on Biological Control of Arthropodes. Honolulu. Hawai. *USDA-Forestry Service,* 407-415.

Osterc G., Zavrl Fras M., Vodenik T., Luthar Z. (2005). The propagation of chestnut (*Castanea sativa* Mill.) nodal explants. *Acta agriculturae Slovenica*, *85*(2), 411-418.

Pacurar D. I., Pernone I., Bellini C. (2014). Auxin is a central player in the hormone cross-talks that control adventitious rooting. *Physiologia Plantarum*, *151*, 83-96. https://doi.org/10.1111/ppl.12171

Pereira-Lorenzo S., Ramos-Cabrer A. M. (2007). Chestnut, an ancient crop with future. *Production Practices and Quality Assessment of Food Crops*, *1*, 105-161.

Pijut P. M., Woeste K. E., Michler C. H. (2011). Promotion of adventitious root formation of

difficult-to-root hardwood tree species. *Horticultural Reviews*, *38*, 213-251.

Pop T. I., Pamfil D., Bellini C. (2011). Auxin control in the formation of adventitious roots. *Notulae Botanicae Horti Agrobotanici Cluj*, *39*, 1, 307-316.

Sánchez M. C., Vieitez A. M. (1991). *In vitro* morphogenetic competence of basal sprouts and crown branches of mature chestnut. *Tree physiology, 8*, 59-70. https://doi.org/10.1093/treephys/8.1.59

Šiftar A. (1992). In vitro grow rejuvenilisated shots from plants taken wth grafting n the germinated seeds of chestnut. *Acta horticulturae*, *300*; 141-143.

Tetsumura T., Yamashita K. (2004). Micropropagation of japaneese chestnut (*Castanea crenata* Sieb. et Zucc.) seedlings. *Hortscience*, *39*, 1684-1687.

Van Fleet W. (1914). Chestnut breeding experience. *Journal of Heredity*, *5*, 19-25. https://doi.org/10.1093/jhered/5.1.19

Vielba J. M., Diaz-Sala C., Ferro E., Rico S., Lamprecht M., Abarca D., Ballester A. Sanchez C. (2011). CsSCL1 is differentially regulated upon maturation in chestnut microshoots and is specifically expressed n rooting-competent cells. *Tree physiology, 31*. 1152-1160. https://doi.org/10.1093/treephys/tpr086

Yasumatsu K., Kamijo K. (1979). Chalcidoid parasites of *Dryocosmus kuriphilus*. Japan, with descriptions of five new species (Hymenoptera). *Esakia*, *14*, 93-11.

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O vlogi trehaloze v rastlinah

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IZVLEČEK

Trehaloza je disaharid, pomemben pri obrambi pred stresom pri mnogih organizmih, vključno z zelenimi algami in nižjimi rastlinami. Dolgo je veljalo prepričanje, da je vloga trehaloze v višjih rastlinah marginalna in da je tu njene funkcije v evoluciji prevzela saharoza. Pred nekaj leti so odkrili, da ima trehaloza pomembne fiziološke funkcije tudi v presnovi višjih rastlin. Je pomemben signalni metabolit, ki uravnava raven in razpoložljivost saharoze, sintezo in razgradnjo škroba ter sintezo organskih kislin. Povezava trehaloze in saharoze je dokazano pomembna pri interakciji rastline s patogenimi organizmi in rastlinojedimi insekti. Trehaloza je udeležena tudi pri obrambi rastline pred abiotskimi stresorji kot so suša, mraz, slanost in hipoksija. V interakciji z abscizinsko kislino sodeluje pri regulaciji stomatalne prevodnosti. Glede na potrjene funkcije je trehaloza primarni metabolit, ki močno pripomore k rasti in razvoju rastline. Takšna primera sta njen vpliv na indukcijo cvetenja in stimulacijo fotosinteze.

Ključne besede: trehaloza; presnova sladkorjev; saharoza; škrob; stres

ABSTRACT

ABOUT THE ROLE OF TREHALOSE IN PLANTS

Trehalose is an important disaccharide which takes a major role of a stress protector in many organisms, including green algae and lower plants. It has long been thought that trehalose functions in higher plants are marginal and that they have been overtaken by sucrose. In the last years it has been discovered that trehalose takes on a lot of important physiological roles in vascular plants metabolism. It is an important signal metabolite of sucrose availability and maintains sucrose concentrations within an appropriate range. It also contributes to starch synthesis and degradation and to synthesis of organic acids. Trehalose-sucrose nexus was found to be very important in plant interactions with pathogenic organisms and herbivorous insects. Furthermore, trehalose is involved in response of plant to abiotic stressors such as drought, cold, salinity and hypoxia. It contributes in regulation of stomatal conductivity where it interacts with abscisic acid. All this makes trehalose an important primary metabolite which significantly influences plant growth and development such as induction of flowering and stimulation of photosynthesis.

Key words: trehalose; sugar metabolism; sucrose; starch; stress

Okrajšave:
TPS - trehaloze-fosfat sintaza
TPP – trehaloze-fosfat fosfataza
TS – trehaloze sintaza
T6P – trehaloze-6-fosfat
ROS – reaktivne kisikove zvrsti
ABA – abscizinska kislina
SnRK1 - nefermentirajoča s saharozo povezana kinaza 1
AtTPPG – gen za trehaloze-fosfat fosfatazo v navadnem repnjakovcu (*Arabidopsis thaliana* (L.) Heynh.)
AtTPS1 – gen za trehaloze-fosfat sintazo v vrsti *Arabidopsis thaliana*
AtTRE1 – gen za trehalaze v vrsti *Arabidopsis thaliana*
TPS – kompleks genov za sintezo trehaloze
Tps1 – sintaza (del TPS kompleksa)
Tps2 – trehaloze-P-fosfataza (del TPS kompleksa)
Tps3 in Tps4 – proteina z regulatorno in stabilizacijsko funkcijo (del TPS kompleksa)

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1 UVOD

Kljub obširnemu znanju o sladkorjih v rastlinah, še vedno spoznavamo nova dejstva o njih. Čeprav je od prvega članka, ki beleži odkritje trehaloze v rastlinah minilo že več kot 100 let (Anselmino in Gilg, 1913), je bilo o njeni vlogi v rastlinah do pred približno 20 leti znano bolj malo. Trehalozo so dobro poznali že pred tem odkritjem, vendar le v nižje razvitih organizmih (arhejah, mikobakterijah, kvasovkah in drugih glivah) ter v živalih (npr. nematodah, morskih rakih...) (Elbein in sod., 2003). Na začetku raziskav so na osnovi majhnih količin trehaloze v rastlinah sklepali, da je ta trehaloza glivnega ali mikrobnega izvora, njena presnova v rastlinah pa nepomembna oziroma celo odsotna. Te domneve so bile ovržene z odkritjem genov, ki v navadnem repnjakovcu (*Arabidopsis thaliana* (L.) Heynh.) kodirajo dva encima, pomembna za metabolizem trehaloze, trehaloze fosfat sintazo (TPS) in trehaloze fosfat fosfatazo (TPP) (Blázquez in sod., 1998; Vogel in sod., 1998; Leyman et al., 2001). To je spodbudilo nadaljnje raziskave in vodilo v mnoga zanimiva odkritja. Ugotovili so, da trehaloza v rastlinah sodeluje pri regulaciji več procesov, kot so uravnavanje količine saharoze in primarnega škroba (Yadav in sod., 2014) ter uravnavanje prevodnosti listnih rež (Gomez in sod., 2014). Je signalni metabolit pri interakciji rastline s patogenimi in simbiotskimi organizmi; npr. pri vzpostavitvi mikorize pripomore tudi k bolj učinkoviti vezavi dušika (Garg in Singla, 2016),. Vključena pa je tudi pri odzivu na napad rastlinojedcev (Tayeh in sod., 2014) in abiotske stresne dejavnike (Farrant in sod., 2015; Tapia in sod., 2015; Farooq in sod. 2017; Mostofa in sod., 2015; Krasensky in sod.; 2014, Shahbaz in sod, 2017; Garg in sod., 2016; Kretyschmar in sod., 2015; Chen in Hoehenwarter, 2015).

2 TREHALOZA – OPIS MOLEKULE IN SINTEZA

Trehaloza je disaharid, sestavljen iz dveh molekul glukoze, ki sta povezani z 1,1-glikozidno vezjo. Trehaloza je dobro topna v vodi, a zaradi svoje nereducirajoče narave kemijsko nereaktivna, zato se lahko v celici nahaja tudi v velikih koncentracijah (Lunn in sod., 2014). Obstajajo vsaj tri različne poti biološke sinteze trehaloze. Najbolj poznana in v rastlinah edina prisotna pot sinteze poteka preko intermediata trehaloze-6-P (T6P) in vsebuje dve encimatski stopnji. Trehaloze-fosfat sintaza (TPS) katalizira prenos glukoze iz UDP-glukoze na glukozo-6-P, s čimer nastane T6P. V naslednjem koraku encim trehaloze-fosfat fosfataza (TPP) pretvori trehalozo-fosfat v trehalozo. To reakcijo so prvič opisali na primeru kvasovk in je dokazano prisotna tudi v rastlinah in drugih organizmih (Elbein in sod., 2003). Odkrili so, da so za sintezo trehaloze zaslužni štirje homologni geni, katerih produkti tvorijo kompleks (TPS) sestavljen iz štirih podenot – sintaze (Tps1), trehaloze-P fosfataze (Tps2), ter dveh podenot, ki imata regulatorno in stabilizacijsko funkcijo (Tps3 in Tsl1) (Svanström in sod., 2014).

3 TREHALOZA V RASTLINAH

Za razkrivanje vloge trehaloze v rastlinah je bil ključen razvoj zadosti občutljivih analitskih metod (Lunn in sod., 2014), saj rastlinska tkiva vsebujejo približno stokrat manj trehaloze-6-P kot celice kvasovk, za katere so bile metode razvite. Različne študije so pokazale, da 1) je trehaloza vključena v regulacijo koncentracije saharoze in primarnega škroba v rastlinskih tkivih, 2) trehaloza sodeluje pri odzivu rastlin na abiotski stres, 3) je trehaloza signalni metabolit pri okužbi rastline ali napadu rastlinojedcev ter 4) regulira stomatalno prevodnost.

3.1 Regulacija koncentracije saharoze in primarnega škroba

S fotosintezo asimilirani ogljik se tekom dneva pretvarja v saharozo in transportira v ponore, presežek ogljika pa se akumulira v kloroplastih v obliki primarnega škroba. Ta se porablja v obdobjih, ko rastlina ne more vršiti zadostne fotosinteze ali pa ta sploh ne poteka, npr. ponoči. Sinteza saharoze in primarnega škroba sta soodvisna procesa (Vodnik, 2012), v regulaciji katerih je udeležena tudi trehaloza. Lunn in sod. (2014) so ugotovili, da je vsebnost T6P v rastlinah navadnega repnjakovca (*A. thaliana)*, ki jim primanjkuje ogljika, zelo majhna, z dodajanjem saharoze pa se hitro povečuje. Močno povezavo med T6P in saharozo so odkrili tudi v rozetah, semenih in brstih repnjakovca, v krompirjevih gomoljih in pšeničnih zrnih. Raziskava, v kateri so rastline navadnega repnjakovca oskrbovali s širokim spektrom rastlinskih sladkorjev, njihovih analogov ter z mineralnimi hranili (KNO_3, NH_4Cl, K_2SO_4 in KH_2PO_4),je pokazala, da ima največji vpliv na vsebnost T6P prav saharoza. Ostali sladkorji ter mineralna hranila imajo manjši vpliv na T6P ali pa nanjo delujejo posredno preko saharoze (Yadav in sod.,

2014). V poskusih s transgenimi rastlinami z zmanjšano ali povečano vsebnostjo T6P so ugotovili, da se ob genetsko inducirani spremembi v vsebnosti T6P spremeni tudi koncentracija saharoze. T6P kontrolira nivo saharoze v rastlinskih celicah in preprečuje, da bi se preveč zvišal ali znižal - podobno kot inzulin nadzoruje vsebnost glukoze v krvi živali. T6P naj bi bil tudi del regulativne mreže, ki ureja tok saharoze iz virov in porabo saharoze v ponorih (Figueroa in Lunn, 2016). Mehanizem delovanja T6P lahko vključuje encim SnRK1 (nefermentirajoča s saharozo povezana kinaza 1). SnRK1 vpliva na ekspresijo genov, ki se izrazijo ob stresu ter vplivajo na transport saharoze po rastlini, katabolne procese, zavirajo rast rastline in energijo usmerjajo v obrambo. T6P inhibira delovanje SnRK1, kar povzroči skladiščenje ogljikovih hidratov, pospeši anabolne procese, rast in razmnoževanje (Griffiths in sod., 2016). Transgene rastline s povečano vsebnostjo T6P so imele v poskusih znatno zmanjšano vsebnost saharoze, manjšopost-translacijsko aktivacijo nitrat reduktaze in fosfoenolpiruvat karboksilaze in povečano vsebnost organskih kislin in aminokislin, kar kaže na to, da je metabolizem trehaloze povezan tudi s sintezo spojin, ki vsebujejo dušik (Figueroa in sod., 2016). V eni nedavnih raziskav so proučevali vpliv T6P na tvorbo lateksa v brazilskem kavčukovcu (*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.). Tvorbo lateksa omogoča sprejem saharoze in njen metabolizem v celicah, ki proizvajajo lateks, zato predpostavljajo, da vpliv T6P na nivo saharoze posredno vpliva tudi na tvorbo lateksa (Zhou in sod., 2017). Trehaloza pa ne vpliva samo na vsebnost saharoze, ampak sodeluje tudi pri regulaciji sinteze in razgradnje primarnega škroba. Ni še do konca potrjena možnost, da naj bi v tem primeru T6P deloval preko redoks regulacije aktivnosti encima ADP-glukoze pirofosfataza (Kolbe in sod., 2005; Martins in sod., 2013). Regulacija razgradnje škroba s T6P je povezana s cirkadiano ritmiko (Martins in sod., 2013). Notranja ura rastline uravnava največjo možno porabo škroba preko noči, spremenljiva količina T6P pa regulira njegovo razgradnjo glede na potrebe po saharozi. Če so te velike, se vsebnost saharoze zmanjša, zaradi česar se zmanjšatudi koncentracija T6P. Zmanjšanje T6P omogoča pospešeno razgradnjo škroba, vendar le do stopnje, ki jo glede na zaloge dovoljuje notranja ura. Če je zalog več kot je potreb po saharozi, se nivo T6P in saharoze poveča, kar upočasni razgradnjo škroba (Martins in sod., 2013). Mehanizem, s katerim T6P ureja razgradnjo škroba, še ni znan, predvidevajo pa, da ni omejen na kloroplast (Lunn in sod., 2014).

3.2 Pomen trehaloze pri odzivu rastline na abiotični stres

V zadnjih letih vedno več raziskav poroča o pomenu trehaloze pri odzivu rastlin na abiotske stresne dejavnike npr. na sušo (Farrant in sod., 2015; Tapia in sod., 2015), nizke temperature (Farooq in sod. 2017), slanost (Mostofa in sod., 2015; Krasensky in sod., 2014; Shahbaz in sod., 2017; Garg in sod., 2016), hipoksijo (Kretzschmar in sod.., 2015) in oksidativni stres (Chen in Hoehenwarter, 2015).

Izpostavitev ekstremnim temperaturam vodi v kompleksne fiziološke in biokemične odzive v rastlinah, vključno s spremembami v koncentraciji presnovkov (sladkorjev, maščob in sekundarnih presnovkov). Pri rastlinah, ki so bile izpostavljene mrazu ali vročini, so odkrili spremembe v ekspresiji genov za TPS in TPP. Neposredni vpliv trehaloze se kaže v interakciji TPS v navadnem repnjakovcu z multiproteinskim vezavnim faktorjem 1c, ki je glavni regulator tolerance rastlin na neugodne temperature (Suzuki in sod., 2008; Lunn in sod., 2014). Ob večjih spremembah temperature se v rastlini spreminja tudi koncentracija saharoze, zato moramo razlikovati med neposrednim in posrednim vplivom trehaloze na termotoleranco rastlin (Lunn in sod., 2014). Pri nizkih temperaturah pa se v rižu, vinski trti in navadnem repnjakovcu, skladno s količino saharoze poveča vsebnost TPP (Premanik in Imai, 2005; Fernandez in sod., 2012; Iordachesu in Imai, 2008; Lunn in sod., 2014).

Koncentracija T6P se poveča tudi ob pomanjkanju kisika. Ni znano ali se spremembe T6P v navadnem repnjakovcu zgodijo zaradi porasta koncentracije saharoze ob hipoksiji, ali pa T6P dejansko igra kakšno vlogo pri prilagajanju presnove in rasti ter s tem preprečuje, da bi rastlina ostala popolnoma brez kisika (Thiel in sod., 2011).

Trehaloza prav tako pripomore k več procesom, ki omogočajo preživetje rastlinam v času slanostnega stresa. Majhne koncentracije eksogene trehaloze zmanjšujejo kopičenje Na^+ ionov v rastlini in prispevajo k ohranjanju absorpcijske vloge korenin, velike koncentracije pa preprečujejo razgradnjo klorofila v listih zaradi delovanja natrijevega klorida (Garcia in sod., 1997). Povečanje vsebnosti trehaloze ob slanostnem stresu avtorji povezujejo s spremembo v aktivnosti encimov sinteze in razgradnje trehaloze. Trehaloza naj bi se akumulirala zaradi povečane aktivnosti TPS (El-Bashiti in sod., 2005).Druga možnost je, da se trehaloza kopiči zaradi počasnejše razgradnje. V trnati meteljki (*Medicago truncatula* Gaertn.), izpostavljeni slanosti, so namreč zaznali manjše izražanje encima trehalaza. Nakopičena trehaloza bi lahko kot osmotik prispevala k večji toleranci rastline na slanostni stres (Muller in sod., 2001; Aeschbacher in sod, 1999).

Nekatere resurekcijske rastline, kot npr. *Selaginella lepidophylla* (Hook. in Grev.) Spring., ob izsuševanju akumulirajo velike količine trehaloze, ki v času

dormance – ta lahko traja tudi več let – skupaj s saharozo stabilizira celične membrane, proteine in druge celične komponente (Iturriaga in sod., 2006). Nivo trehaloze je znatno nižji v kulturnih rastlinah. Rahel dvig koncentracije trehaloze pa je bil zaznan v pšenici in bombažu, ki sta tolerantna na sušo. Koncentracija trehaloze se je najverjetneje povečala zaradi večje ekspresije TPS (El-Bashiti in sod., 2005), medtem ko lahko pri nekaterih sortah pšenice povečanje njene koncentracije pripišemo zmanjšanju vsebnostitrehalaze. Še vedno je neznano, ali so te manjše spremembe in majhna absolutna vsebnost trehaloze sploh pomembne pri obrambi pred sušnim stresom. Pri transgenem krompirju, odpornem na sušo, z vstavljenim genom za TPS, se je namreč v primerjavi z divjim tipom krompirja povečala le vsebnost prolina, inozitola in rafinoze (Kondrák in sod., 2012).

Trehaloza deluje tudi kot zaščita pred reaktivnimi kisikovimi zvrstmi (ROS), ki se pojavijo kot sekundarni stresorji zaradi delovanja drugih stresnih dejavnikov. ROS imajo pozitivno in negativno vlogo v rastlini. Delujejo kot signalne molekule ob napadu patogenov in sodelujejo pri programirani celični smrti (Grant in Loake, 2000; Dangl in Jones, 2001). Problem nastane, ker se ROS nakopičijo do toksičnih koncentracij, zato jih je potrebno odstraniti iz celice, saj lahko povzročijo oksidativne poškodbe. Za ta namen so rastline opremljene z različnimi obrambnimi mehanizmi, eden takšnih je tudi akumulacija sladkorjev. Obstajajo *in vivo* in *in vitro* dokazi, da trehaloza ščiti pred hidroksilnimi radikali (Couee in sod., 2006). Prekomerno izražanje TPS v tobaku in paradižniku je povečalo toleranco na oksidativni stres povzročen z metil-viologenom. Milimolarne koncentracije trehaloze namreč prispevajo k zaščiti encimov, ki odstranjujejo proste radikale, pred toplotno deaktivacijo. Na ta način trehaloza ohranja aktivnost askorbat katalaze in askorbat peroksidaze, le v manjši meri pa tudi delovanje encima superoksid dismutaza (Luo in sod., 2008). Lunn in sod. (2014) navajajo, da naj bi trehaloza - po sedaj še nepojasnjenem mehanizmu – proste radikale iz celic odstranjevala tudi direktno.

3.3 Trehaloza kot signalni metabolit pri biotičnem stresu

Ob napadu patogenih organizmov se rastlina brani tudi s tvorbo različnih kemičnih snovi, med katere spadajo tudi sladkorji. Da je med njimi tudi trehaloza, kažejo povečanja njene koncentracije in spremembe ekspresije genov za TPS in TPP ob okužbi (Golem in Culver, 2003; Shing in sod., 2011; Zhang in sod., 2016) ter odzivnost obrambnih mehanizmov na dodatke eksogene trehaloze (Tayeh in sod., 2014). Brodmann in sod. (2002) so vlogo trehaloze v obrambi proučevali pri navadnem repnjakovcu, okuženim z glivo *Plasmodiophora brassicae* Woronin, 1877. 38 dni po infekciji so imele okužene rastline v primerjavi z zdravimi močno povečano vsebnost trehaloze, ki je bila eden od dominantnih sladkorjev v koreninah in hipokotilu. Trehaloze ni bilo zaznati 14 - 23 dni po okužbi, kar pomeni, da se sintetizira v poznejših fazah razvoja bolezni. V koreninah in hipokotilu okuženih rastlin se je za 14- do 45-krat povečala tudi koncentracija škroba, za kar je prav tako zaslužna trehaloza. Ker tudi mikroorganizmi lahko sintetizirajo trehalozo, pa bi lahko k veliki koncentraciji trehaloze prispevala tudi gliva *P. brassicae* (Brodmann in sod., 2002). V raziskavi o primarnem vplivu trehaloze na odziv rastlin ob okužbi z žitno pepelovko (*Blumeria graminis* (DC.) E.O. Speer *f. sp. tritici* Em. Marchal.) so odkrili, da je okužba s trehalozo tretirane pšenice (*Triticum aestivum* L.) manjša kot pri netretirani kontroli. Trehaloza je inducirala aktivnost hitinaz in lipooksigenaz, širjenje okužbe pa se je v večini s trehalozo tretiranih rastlin ustavilo v stadiju razvoja apresorija. To nakazuje, da trehaloza sodeluje pri omejevanju infekcije patogena (Tayeh in sod., 2014). Raziskave ekspresije genov prav tako dokazujejo vlogo trehaloze kot signalnega metabolita pri odzivu na biotični stres. Rastline navadnega repnjakovca okužene s tobakovim mozaičnim virusom kažejo povečano izražanje gena za TPS (Golem in Culver, 2003), Zhang in sod. (2016) pa poročajo o spremenjeni ekspresiji genov za TPS in TPP po okužbi paradižnika z glivo *Botrytis cinerea* Pers. oz. bakterijo *Pseudomonas syringae* Van Hall, 1904. Tudi raziskava odziva navadnega repnjakovca ob napadu sive breskove uši (*Myzus persicae* (Sulzer, 1776)) je pokazala, da so obrambni mehanizmi povezani z geni, ki uravnavajo raven trehaloze (Singh in sod., 2011).

3.4 Regulacija stomatalne prevodnosti

Presnova trehaloze igra pomembno vlogo pri uravnavanju prevodnosti listnih rež in učinkovitosti izrabe vode. Raziskave so pokazale, da je ekspresija AtTPPG, AtTPS1 in AtTRE1 bolj izrazita v celicah zapiralkah v listih, kakor v drugih celicah in tkivih, kar nakazuje na vlogo trehaloze v regulaciji stomatalne prevodnosti. Listne reže na listih mutantov navadnega repnjakovca z zmanjšano aktivnostjo TPS so imele manjšo prevodnost listnih rež kot divji tipi (Gomez in sod., 2010).

Med sušnim stresom rastline priprejo oz. zaprejo listne reže, da zmanjšajo izgubo vode. Ta odziv med drugim sproža abscizinska kislina (ABA). Gensko spremenjene rastline navadnega repnjakovca z zmanjšano ekspresijo trehalaze, in s tem večjo vsebnostjo trehaloze, niso mogle zapreti listnih rež, ko jim je bila dodana ABA. Na drugi strani pa so bile rastline s povečano vsebnostjo trehalaze preveč občutljive na dodatek ABA in imele

tudi zmanjšano stomatalno prevodnost v primerjavi z divjimi tipi v nesušnih razmerah. To kaže na to, da je delovanje ABA močno povezano s trehalazo. Povezava med ABA in presnovo trehaloze je bila dokazana z odkritjem, da ABA inducira ekspresijo trehalaze (Van Houtte in sod., 2013). Čeprav signalni mehanizmi še niso definirani, obstajajo dokazi za več interakcij: ABA deluje na ekspresijo trehalaze, TPP in trehalaza pa delujeta na ABA pri zapiranju listnih rež (Figueroa in Lunn, 2016).

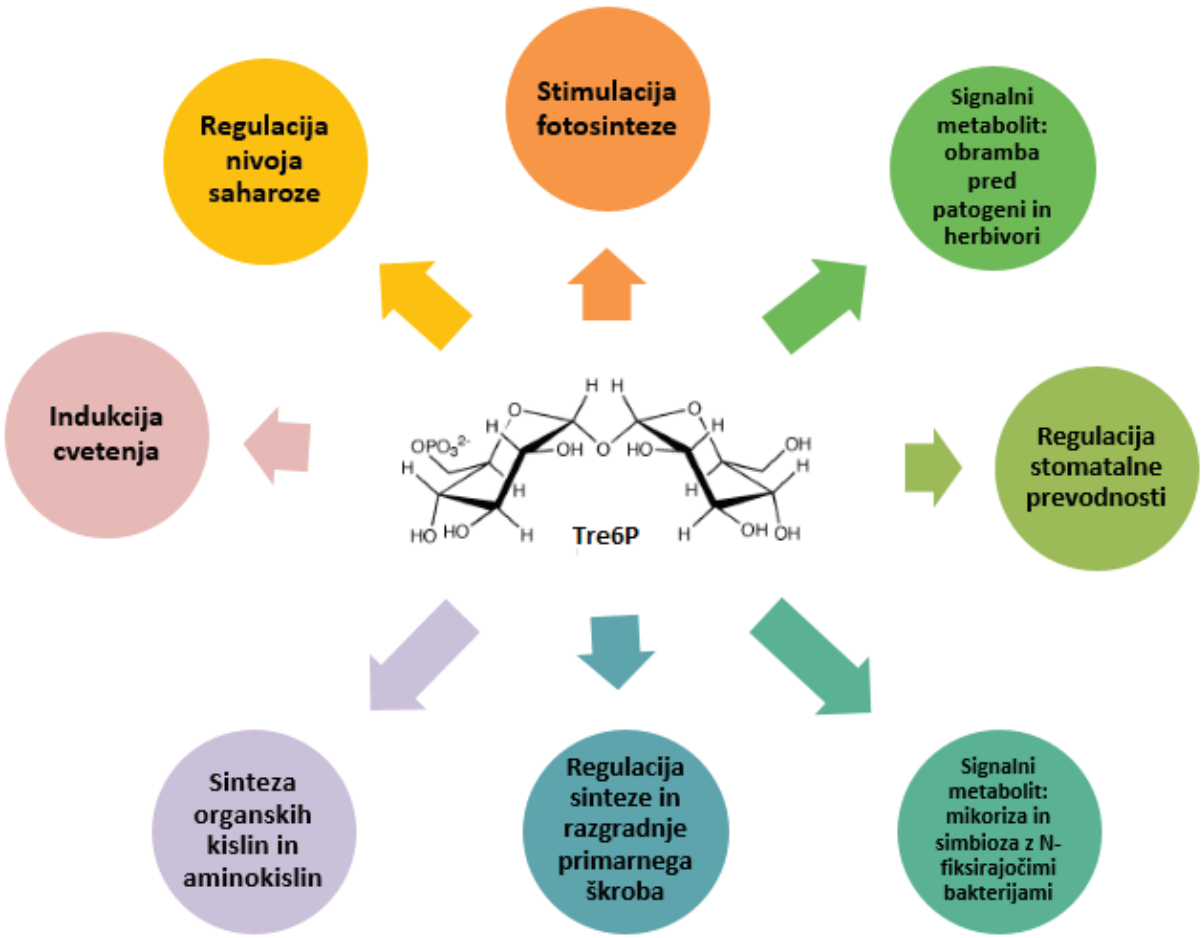
3.5 Nadzor rasti in razvoja rastline s trehalozo-6-fosfat

Rast heterotrofnih tkiv, kot so meristemska tkiva, korenine, cvetovi in razvijajoča se semena, je odvisna od zaloge fotoasimilatov ali remobilizacije škroba in drugih energetskih rezerv. Ogljik se najpogosteje po rastlini prenaša po floemu, povečini v obliki saharoze. Ko prispe do ponorov, izstopi iz floema in se s pomočjo encimov različnih celičnih organelov razcepi. Nastale heksoze nato vstopajo v primarno presnovo in se porabijo za izgradnjo celičnih komponent. Rast in razvoj rastlinskih tkiv sta zato zelo odvisna od razpoložljivosti saharoze (Fugueroa in Lunn, 2016). Kot smo že omenili, pa je vsebnost saharoze močno odvisna od vsebnosti T6P. V družini rožnic (Rosaceae), kjer je glavni transportni sladkor sorbitol, so dokazali tudi njuno soodvisnost. V rožnicah pa so poleg sorbitola zastopani tudi drugi sladkorji, med drugim tudi saharoza. Zanimivo je, da so pri poskusu na sorti jablane (*Malus domestica* 'Gala') odkrili, da ima T6P večji vpliv na sorbitol kakor na saharozo, kar še dodatno nakazuje na povezanost T6P z nadzorom rasti in razvoja rastline (Zhang in sod., 2017). Ugotovili so, da so imeli mutanti navadnega repnjakovca z večjo vsebnostjo T6P med rastjo večjo količino antocianov in škroba ter manj saharoze, glukoze-1-P in glukoze-6-P kakor divji tipi. Rastline imajo manjše liste, kar je večinoma posledica spremenjene zgradbe listov (npr. večja debelina lista), manjše listne površine in manjše vsebnosti suhe snovi (Yadav in sod., 2014; Lunn in sod., 2014). Obstaja več dokazov, da T6P uravnava razvojne programe rastline. Pri navadnem repnjakovcu povečanje T6P vodi v hitrejše cvetenje, medtem ko ob zmanjšanju koncentracije T6P dosežemo nasproten učinek. Po indukciji cvetenja rastline navadnega repnjakovca z dodatkom T6P kažejo povečano razrast, tvorijo pa manj semen. Pri rastlinah navadnega repnjakovca z manjšo vsebnostjo T6P pa poročajo o zapozneli senescenci. V rastlinah krompirja T6P zakasni brstenje, kar bi lahko bilo povezano tudi z zmanjšanim katabolizmom ABA. Zapoznelo cvetenje pa lahko povežemo tudi z vplivom trehaloze na razgradnjo škroba, kjer bi trehaloza lahko igrala vlogo signalne molekule, ki preprečuje začetek cvetenja ob premajhni vsebnosti škroba v rastlini, v transgenih rastlinah pa so v različnih raziskavah zaznali tudi stimulacijo fotosinteze. Povečanje fotosinteze naj bi bila posledica spremenjene morfologije listov, predvsem njihove večje debeline, s čimer se fotosinteza na površino lista poveča (Lunn in sod., 2014).

4 ZAKLJUČEK

Trehaloza ima v rastlini mnogo več kot le marginalno funkcijo. S filogenetsko analizo so ugotovili, da je povezava med saharozo in trehalozo zelo starodavna, da obstaja od samega začetka nastanka zelenih rastlin. V prvih rastlinah sta imela najverjetneje oba sladkorja funkcijo osmotika, energetske rezerve in obrambe proti stresu. Te funkcije si pri nižjih rastlinah delita še danes. Med evolucijo je zaradi neznanih razlogov saharoza prevzela vlogo glavnega transportnega sladkorja v višjih rastlinah. Kljub temu trehaloza še zmeraj opravlja nekaj pomembnih funkcij. Trehaloza preprečuje, da bi se vsebnost saharoze v določenih situacijah preveč povečala ali zmanjšala, uravnava pa tudi tok saharoze iz virov in porabo v ponorih. S tem je trehaloza posredno vključena v mnoge pomembne presnovne procese v rastlini; uravnava stomatalno prevodnost ter sintezo in razgradnjo škroba, vpliva tudi na rast in razmnoževanje rastlin. Vzajemno delovanje saharoze in trehaloze je pomembno tudi v obrambi rastlin pred abiotičnim stresom. Mnogi obrambni mehanizmi so namreč povezani s povečano vsebnostjo sladkorjev, katero regulira ravno trehaloza. Podoben mehanizem poznamo tudi pri obrambi rastlin pred patogeni. Trehaloza pa ne vpliva le na vsebnostsladkorjev, temveč tudi na vsebnost abscizinske kisline. Njen vpliv ni pomemben le pri stomatalni prevodnosti, temveč se lahko ob zmanjšanju njene vsebnosti ABA odraža tudi z zakasnelim brstenjem.

Nova dognanja o pomenu trehaloze v rastlinah so potencialno uporabna za področje agronomije. Rastline s spremenjeno vsebnostjo trehaloze bi lahko bile bolj strpne na stresne dejavnike in s tem tudi bolj produktivne. Vendar transgene rastline, v katere so vstavili bakterijske gene, ki kodirajo encime za sintezo trehaloze, v poskusih niso dale pričakovanih rezultatov v povezavi z odpornostjo na različne stresorje. To kaže, da posegi v kompleksno regulirane presnovne poti primarnega metabolizma niso enostavni.



Slika 1: Tre6P je fosforiliziran intermediat biosinteze trehaloze. Je pokazatelj koncentracije saharoze v rastlinah in vpliva na mnoge metabolne in razvojne procese, prikazane v barvnih krogih. Poleg Tre6P pri stomatalni prevodnosti sodeluje še sama trehaloza.

Figure 1: Tre6P is the phosphorylated intermediate of trehalose biosynthesis. It shows the concentration of sucrose in plants and influences many metabolic and developmental processes which are shown in the coloured circles. Both Tre6P and trehalose are involved in regulation of stomatal conductance.

5 VIRI

Aeschbacher R.A., Müller J., Boller T., Wiemken A. (1999). Purification of the trehalase GMTRE 1 from soybean nodules and cloning of its cDNA: GMTRE 1 is expressed at a low level in multiple tissues. *Plant Physiology*, *119*, 489–496. doi:10.1104/pp.119.2.489

Anselmino O., Gilg E. (2013). Űber das Vorkommen von Trehalose in *Selaginella lepidophylla*. *Berichte der Deutschen Chemischen Gesellschaft*, *23*, 309-320.

Blázquez M.A., Santos E., Flores C., Martinez-Zapater J.M., Salinas J., Gancedo C. (1998). Isolation and molecular characterization of the *Arabidopsis* TPS1 gene, encoding trehalose-6-phosphate synthase. *Plant Journal, 13*, 685-689. doi:10.1046/j.1365-313X.1998.00063.x

Brodmann D., Schuller A., Ludwig-Műller J., Aeschbacher R., Wiemken A., Boller T., Wingler A. (2002). Induction of trehalase in *Arabidopsis* plants infected with the trehalose-producing pathogen *Plasmodiophora brassicae*. *The American Phytopathological Society*, *15*, 693-700.

Chen Y., Hoehenwarter W. (2015). Changes in the phosphoproteome and metabolome link early signaling events to rearrangement of photosynthesis and central metabolism in salinity and oxidative stress response in *Arabidopsis*. *Plant Physiology*, *169*, 3021-3033. doi:10.1104/pp.15.01486

Couee I., Sulmon C., Gouesbet G., El-Amrani A. (2006). Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany*, *57*, 449-459. doi:10.1093/jxb/erj027

Dangl J.L., Jones J.D.G. (2001). Plant pathogens and integrated defence responses to infection. *Nature*, *411*, 826-833. doi:10.1038/35081161

De Smet K., Weston A., Brown I., Young D., Robertson D. (2000). Three pathways for trehalose byosinthesis in mycobacteria. *Microbiology*, *146*, 199-208. doi:10.1099/00221287-146-1-199

El-bashiti T., Hamamci H., Oktem H.A., Yucel M. (2005). Biochemical analysis of trehalose and its metabolizing anzymes in wheat under abiotic stress conditions. *Plant Science*, *169*, 47-54. doi:10.1016/j.plantsci.2005.02.024

Elbein A.D., Pan Y.T., Pastuszak I., Carroll D. (2003). New insights on trehalose: a multifunctional molecule. *Glycobiology*, *13*, 17R-27R. doi:10.1093/glycob/cwg047

Farooq M., Hussain M., Nawaz A., Lee D.J., Alghamdi S.S., Siddique K.H.M. (2017). Seed priming improves chilling tolerance in chickpea by modulating germination metabolism, trehalose accumulation and carbon assimilation. *Plant Physiology and Biochemistry*, *111*, 274-283. doi:10.1016/j.plaphy.2016.12.012

Farrant J.M., Cooper K., Hilgart A., Abdalla K.O., Bentley J., Thomson J.A., Dace H.J., Penton N., Mundree S.G., Rafudeen M.S. (2015). A molecular physiological review of vegetative desiccation tolerance in the resurrection plant *Xerophyta viscosa* (Baker). *Planta*, *242*, 407-426. doi:10.1007/s00425-015-2320-6

Fernandez J., Wilson R.A. (2012). Why no feeding frenzy? Mechanisms of nutrient acquisition and utilization during infection by the rice blast fungus *Magnoporthe oryzae*. *Molecular Plant-microbe Interactions*, *25*, 1286-1293. doi:10.1094/MPMI-12-11-0326

Figueroa C.M., Feil R., Ishihara H., Wanatabe M., Kolling K., Krause U., Hohne M., Encke B., Plaxton W.C., Zeeman S.C., Li Z., Schulze W.X., Hoefgen R., Stitt M., Lunn J.E. (2016). Trehalose 6-phosphate coordinates organic and amino acid metabolism with carbon availability. *Plant Journal*, *85*, 410-423. doi:10.1111/tpj.13114

Figueroa C.M., Lunn J.E. (2016). A tale of two sugars: trehalose 6-phosphate and sucrose. *Plant Physiology*, *172*, 7-27. doi:10.1104/pp.16.00417

Garg R., Shankar R., Thakkar B., Kudapa H., Krishnamurthy L., Varshney R.K., Bhatia S., Jain M. (2016). Transcritome analyses reveal genotype- and developmental stage-specific molecular responses to drought and salinity stress in chickpea. *Scientific Reports*, *6*: 19228. doi:10.1038/srep19228

Golem S., Culver J.N. (2003). *Tobacco mosaic virus* induces alterations in the gene expression profile of *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions*, *16*, 681-688. doi:10.1094/MPMI.2003.16.8.681

Goméz L.D., Gildaz A., Fell R., Lunn, J.E., Graham I.A. (2010). AtTPS mediated trehalose-6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. *Plant Journal*, *64*, 1-13.

Grant J.J., Loake G.J. (2000). Role of reactive oxygen intermediates and cognate redox signalling in disease resistance. *Plant Physiology*, *124*, 21-29. doi:10.1104/pp.124.1.21

Griffiths C.A., Paul M.J., Foyer C.H. (2016). Metabolite transport and associated sugar signalling systems underpinning source/sink interactions. *Biochimica et Biophysica Acta*, *1857*, 1715-1725. doi:10.1016/j.bbabio.2016.07.007

Iordachescu M., Imai R. (2008). Trehalose biosynthesis in response to abiotic stresses. *Journal of Integrative Plant Biology*, *50*, 1223-1229. doi:10.1111/j.1744-7909.2008.00736.x

Iturriaga G., Cushman M.A.F., Cushman J.C. (2006). An EST catalogue from the resurrection plant *Selaginella lepidophylla* reveals abiotic stress-adaptive genes. *Plant Science*, *170*, 1173-1184. doi:10.1016/j.plantsci.2006.02.004

Kolbe A., Tiessen A., Schluepmann H., Paul M., Ulrich S., Geigenberger P. (2005). Trehalose-6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. *Proceedings of the National Academy of Sciences*, *102*, 1118-11123. doi:10.1073/pnas.0503410102

Kondrák M., Marincs F., Antal F., Juhász Z., Bánfalvi Z. (2012). Effects of yeast trehalose-6-phosphate synthase 1 on gene expression and carbohydrate contents of potato leaves under drought stress conditions. *BMC Plant Biology*, 12: e74, doi: 10.1186/1471-2229-12-74: 12 str.

Krasensky J., Broyart C., Rabanal F.A., Jonak C. (2014). The redox-sensitive chloroplast trehalose-6-phosphate phosphatase AtTPPD regulates salt stress tolerance. *Antioxid Redox Signal*, *21*, 1289-1304. doi:10.1089/ars.2013.5693

Kretzschmar T., Pelayo M.A.F., Trijatmiko K.R., Gabunada L.F.M., Alam R., Jimenez R., Mendorio M.S., Slamet-Loedin I.H., Sreenivaslu N., Bailey-Serres J., Ismail A.M., Mackill D.J., Septiningsih E.M. (2015). A trehalose-6-phosphate phosphatase enhances anaerobic germination tolerance in rice. *Nature Plants*, *1,* 15124. doi:10.1038/nplants.2015.124

Leyman B., van Dijck P., Thevelein J.M. (2001). An unexpected plethora of trehalose biosynthesis genes in Arabidopsis thaliana. *Trends in Plant Science*, *6*, 510-513. doi:10.1016/S1360-1385(01)02125-2

Lunn J.E., Delorge I., Figueroa M.C., van Dijck P., Stitt M. (2014). Trehalose metabolism in plants. *The Plant Journal*, *79*, 544-567. doi:10.1111/tpj.12509

Luo Y., Li W.M., Wang W. (2008). Trehalose: protector of antioxidant enzymes or reactive oxygen species scavenger under heat stress? *Environmental and Experimental Botany*, *63*, 378-384. doi:10.1016/j.envexpbot.2007.11.016

Martins M.C.M, Hejazi M., Fettke J. (2013). Feedback inhibition of starch degradation in Arabidopsis leaves mediated by trehalose-6-phosphate. *Plant Physiology*, *163*, 1142-1163. doi:10.1104/pp.113.226787

Mostofa M.G., Hossain M.A., Fujita M. (2015). Trehalose pretreatment induces salt tolerance in rice (*Oryza sativa L.*) seedlings: oxidative damage and co-induction of antioxidant defense and glyoxalase systems. *Protoplasma, 252*, 461-475. doi:10.1007/s00709-014-0691-3

Müller J., Aeschbacher R.A., Wingler A., Boller T., Wiemken A. (2001). Trehalose and trehalase in *Arabidopsis*. *Plant Physiology*, *125*, 1086–1093. doi:10.1104/pp.125.2.1086

Pramanik M.H.R. in Imai R. (2005). Functional identification of a trehalose-6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. *Plant Molecular Biology*, 58,751-762. doi:10.1007/s11103-005-7404-4

Shahbaz M., Abid A., Masood A., Waraich E.A. (2017). Foliar-applied trehalose modulates growth, mineral nutrition, photosynthetic ability, and oxidative defense system of rice (*Oryza sativa* L.) under saline stress. *Journal of Plant Nutrition*, *40*, 584-599. doi:10.1080/01904167.2016.1263319

Singh V., Louis J., Ayre B.G., Reese J.C., Shah J. (2011). Trehalose phosphate synthase11-dependent trehalose metabolism promotes *Arabidopsis thaliana* defense against the phloem-feeding insect *Myzus persicae*. *Plant Journal, 67*, 94-104. doi:10.1111/j.1365-313X.2011.04583.x

Suzuki N., Bajad S., Shuman J., Shulaev V., Mitter R. (2008). The transcriptional co-activator MBF1c is a key regulator of thermotolerance in Arabidopsis thaliana. *The Journal of Biological Chemistry*, *283*, 9269-9275. doi:10.1074/jbc.M709187200

Svanström A., van Leeuwen M.R., Dijksterhuis J., Melin P. (2014). Trehalose synthesis in *Aspergillus niger*: characterization of six homologous genes, all with conserved orthologs in related species. *BMC Mikrobiology*, *14*, 90-106. doi:10.1186/1471-2180-14-90

Tapia H., Young L., Fox D., Bertozzi C.R., Koshland D. (2015). Increasing intracellular trehalose is sufficient to confer desiccation tolerance to *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*, *112*, 6122-6127. doi:10.1073/pnas.1506415112

Tayeh C., Randoux B., Vincent D., Bourdon N., Reignault P., (2014). Exogenous trehalose induces defenses in wheat before and during a biotic stress caused by powdery mildew. *Phytopathology, 104*, 293-305. doi:10.1094/PHYTO-07-13-0191-R

Thiel J., Rolletschek H., Friedel S., Lunn J.E., Nguyen T.H., Feil R., Tschiersch H., Műller M., Borisjuk L. (2011). Seed-specific elevation of non-symbiotic hemoglobin AtHb1: beneficial effects and underlying molecular networks in *Arabidopsis thaliana*. *BMC plant biology*, *15*, 11, 48. doi:10.1186/1471-2229-11-48

Van Houtte H., Vandesteene L., López-Galvis L. (2013). Overespression of the trehalase gene *AtTRE1* leads to increased drought stress tolerance in *Arabidopsis* and is involved in abscisic acid-induced stomatal closure. *Plant Physiology*, *161*, 1158-1171. doi:10.1104/pp.112.211391

Vodnik D. 2012. Osnove fiziologije rastlin. Oddelek za agronomijo, Biotehniška fakulteta, Univerza v Ljubljani: 141 str.

Vogel G., Aeschbacher R.A., Műller J., Boller T., Wiemken A. (1998). Trehalose-6-phosphate phosphatases from *Arabidopsis thaliana*: identification by functional complementation of the yeast tps2 mutant. *Plant Journal*, *13*, 673-683. doi:10.1046/j.1365-313X.1998.00064.x

Yadav P.U., Ivakov A., Feil R., Duan G., Walter D., Giavalisco P., Piques M., Carillo P., Hubberton M., Stitt M., Lunn J.E. (2014). The sucrose-trehalose-6-fosfat (Tre6P) nexus: specificity and mechanisms of sucrose signalling by Tre6P. *Journal of*

Experimental Botany, *65*, 1051-1068. doi:10.1093/jxb/ert457

Zhang H.,Hong Y., Huang L., Liu S., Tian L.,Dai Y.,Cao Z.,Huang L.,Li D., Song F.(2016). Virus-induced gene silencing-based functional analyses revealed the involvement of several putative trehalose-6-phosphate synthase/phosphatase genes in disease resistance against *Botrytis cinerea* and *Pseudomonas syringae* pv. tomato DC3000 in tomato. *Frontiers in Plant Science*, *7*, 1176. doi:10.3389/fpls.2016.01176

Zhang W., Lunn J.E., Feil R., Wang Y.F., Zhao J.J., Tao H.X., Guo Y.P., Zhao Z.Y. (2017). Trehalose 6-phosphate signal is closely related to sorbitol in apple (*Malus domestica* Borkh. cv. Gala). *Biology Open*, *6*, 260-268. doi:10.1242/bio.022301

Zhou B., Fang Y., Fan Y., Wang Y., Qi J., Tang C. (2017). Expressional characterization of two class I trehalose-6-phosphate synthase genes in *Hevea brasiliensis* (para rubber tree) suggests a role in rubber production. *New Forests*, *48*, 513-526. doi:10.1007/s11056-017-9578-4

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An overview of molecular identification of insect fauna with special emphasis on chalcid wasps (Hymenoptera: Chalcidoidea) of India

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ABSTRACT

Identifying organisms has grown in importance as we monitor the biological effects of global climate change and attempt to preserve species diversity in the face of accelerating habitat destruction. Classical taxonomy falls short in this race to catalogue biological diversity before it disappears. Differentiating subtle anatomical differences between closely related species requires the subjective judgment of highly trained specialists – and few are being trained in institutes today. DNA barcodes allow non-experts to objectively identify species – from small, damaged, or even industrially processed material. The aim of DNA barcoding is to establish a shared community resource of DNA sequences commonly used for identification, discrimination or taxonomic classification of organisms. It is a method that uses a short genetic marker in an organism's DNA to identify and distinguish its belonging from particular species, varieties or inter varieties. This simple technique has attracted attention from taxonomists, ecologists, conservation biologists, agriculturists, plant-quarantine officers and studies using the DNA barcode has rapidly increased. The extreme diversity of insects and their economical, epidemiological and agricultural importance have made them a major target of DNA barcoding. In this review, we present an overview of DNA barcoding of insects with emphasis on Chalcid wasps of India.

Key words: biological diversity; catalogue; chalcid wasps; classical taxonomy, DNA barcode; DNA sequence, genetic marker

IZVLEČEK

PREGLED MOLEKULARNEGA DOLOČANJA ŽUŽELK V INDIJI S POUDARKOM NA OSICAH NAJEZDNICAH (Hymenoptera: Chalcidoidea)

Določanje organizmov pridobiva na pomenu pri spremljanju globalnih podnebnih sprememb in pri poskusih ohranjanja biodiverzitete v procesu hitrega uničevanja habitatov. Klasična taksonomija v teh procesih ne uspe določiti vse biodiverzitete pred njenim propadom. Prepoznavanje majhnih anatomskih razlik med ozko sorodnimi vrstami zahteva presojo visoko usposobljenih specialistov, ki jih je danes vedno manj. Vrednotenje DNK zaporedij omogoča tudi nestrokovnjakom objektivno prepoznavanje vrst kot tudi njihovih malih ali poškodovanih ostankov ali celo industrijsko predelanih materialov. Namen te metode je ustvariti nabor DNK zaporedij za vzajemno rabo pri določanju in taksonomskem razvrščanju organizmov poznano tudi pod imenom DNK črtne kode. Pri tej metodi omogoča kratek genetski marker v DNK organizma njegovo določitev in razlikovanje od drugih vrst, različic. Ta preprosta tehnika je pritegnila pozornost taksonomov, ekologov, konzervatorskih biologov, agronomov, fitokarantenskih uradnikov in preučevanje na osnovi sekvenciranja DNK je hitro poraslo. Izjemna raznolikost žuželk in njihov ekonomski, epidemiološki in kmetijski pomen so jih naredile za tarčno skupino preučevanj na osnovi DNK črtnih kod. V tem sestavku predstavljamo pregled analiz z DNK črtnimi kodami žuželk s poudarkom na osicah najezdnicah iz Indije.

Ključne besede: biodiverziteta; seznam; osice najezdnice; klasična taksonomija; genetska koda; DNK zaporedje; genetski marker

1 INTRODUCTION

Chalcid wasps are one of the most diverse groups of insects numerically, structurally, and biologically belonging to the superfamily Chalcidoidea and order Hymenoptera. With about 150,000 described species, the Hymenoptera is the fourth largest insect order after Coleoptera, Lepidoptera, and Diptera (Grimaldi & Engel, 2005; Beutel & Pohl, 2006). With an estimated total diversity of some 22,500 known species and more than 500,000 morphologically distinct species (Munro et al., 2011) and an even larger number of cryptic

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species possible, the Chalcidoidea superfamily is likely the most diverse group of insects in order Hymenoptera. Most Chalcid wasps are parasitoids attacking immature and adult stages of virtually all insect orders, mostly Hemiptera and Holometabola and hence are used as biological control agents of agricultural and ornamental pests thus having tremendous importance in both natural and managed ecosystems both economically and ecologically (Preethi et al., 2016).

Species identification is a fundamental part of recognizing and describing biodiversity in an ecosystem. Traditionally, identification has been based on morphological diagnoses provided by taxonomic studies. Only experts such as taxonomists and trained technicians can identify taxa accurately, because it requires special skills acquired through extensive experience. As interest in biodiversity has increased in the fields of ecology, evolutionary biology, agriculture and economics, among others, it has become increasingly important to precisely identify species. However, the number of taxonomists and other identification experts has drastically decreased. The characterization based on morphometric characters is not well suited for phylogeographical studies because both phenotypic plasticity and genetic variability in the characters employed for species recognition can lead to incorrect identifications (Pires & Marinoni, 2010). It overlooks morphologically cryptic taxa, which are common in many groups (Jarman & Elliott, 2000) and the use of keys often demands such a high level of expertise that misdiagnoses are common. Faunal and floral studies are besieged by specimens in immature stages that lack the characters necessary for identification, or sexes that cannot be matched, especially if they are dimorphic such as some insects in which the sexes vary dramatically in size or colour (Pinzón-Navarro et al., 2010).Consequently, alternative and accurate identification methods that non-experts can use are required.

One of the most promising approaches to revitalize traditional taxonomy and help it rise above the taxonomic crisis is the use of molecular data for identifying taxa, which has long been a fundamental idea of many biologists (Busse et al., 1996; Blaxter, 2004). This method has received increased acceptance because it is simple and affordable (Padial & De La Riva, 2007). DNA barcoding promises the ability to automate the identification of specimens by determining the sequence of the barcode region, avoiding the complexities inherent in morphological identifications, and prompting advocates arguing for the establishment of a system that ultimately might be applied to all life (Tautz et al., 2003; Blaxter, 2004; Savolainen et al., 2005). Advances in DNA-sequencing technologies have enabled researchers studying biodiversity to conduct simple, cost-effective and rapid DNA analyses. This progress in biotechnology, and the taxonomy crisis itself, played a large role in the creation of DNA barcoding. DNA barcoding, in particular, was formally introduced more than a decade ago as an alternative way to assign species names to specimens, addressing concerns and limitations with traditional morphological identifications (Hebert et al., 2003). The use of DNA sequences to gain information about the taxonomic affinities of an unknown specimen saw its earliest adoption in the least morphologically amenable groups such as viruses and bacteria (Theron & Cloete, 2000). More recently, it has been applied to plants (Chase et al., 2005), to simple metazoan animals such as nematode worms (Floyd et al., 2002) and even to fascinating mega fauna such as birds, fish, and mammals (Ward et al., 2005; Clare et al., 2007; Kerr et al., 2007). This approach relies on the use of algorithms enabling DNA-sequence comparison, such as Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), in conjunction with DNA databases such as GenBank.

1.1 DNA barcoding and taxonomy

India is one of the mega biodiversity rich countries, home to hotspots like the Western Ghats and the Himalayas (ENVIS, 2011). In spite of this rich biodiversity heritage, well documented in the Fauna of British India volumes and having many endemics in all groups, much still remains to be understood about it. Many species are difficult to identify and are poorly known. Insects are the most abundant of all life forms on earth. India with about 2 % of the global land area is among the top 20 mega biodiversity nations in the world accounting for 7.10 % of the world insect fauna. It is estimated that over 900,000 species of insects are known across the globe with over 60,000 species described from India with nearly as many species yet to be named. However, the number of barcodes generated from India is 4.6 % of known species, while the corresponding global scenario is about 16 % of described species, and hence requires a lot of emphasis to catch up with the world scenario (Jalali et al., 2015).The first initiative in DNA barcoding was led by the Department of Biotechnology (DBT), India, to barcode species of butterflies and amphibians from the Western Ghats of India (Gaikward, 2014). To speed up taxonomic identification, DNA barcoding is now being considered as an alternative tool for insect biodiversity identification in India and the world.

Chalcid wasp species in India have been described and illustrated mostly at morphological level. Keeping in view various drawbacks of morphological taxonomy like lack of taxonomic experts, overlooking cryptic taxa, difficulty in using keys and due to phenotypic plasticity

and genetic variability with changing environmental conditions as has been found in other animal species, alternative and complementary approach (molecular taxonomy) has been used in identification of specimens. Molecular approach to Chalcid identification provides a grim scenario from India with very little work done so far at molecular level. Recently Kumar et al., (2009), Jalali et al., (2015) and Venkatesan et al., (2016) have carried some research work at molecular level in Chalcid fauna and came out with some interesting results. The rDNA internal transcribed spacers region 2 (ITS-2) (Kumar et al., 2009), cytochrome c oxidase subunit 1 (COI), NADH dehydrogenase subunit 1 (nadh1), and cytochrome b (cytb) markers used in recent molecular analysis have significantly increased our understanding of the phylogenetic relationships between insect species. Kumar et al. (2009) used Internal transcribed spacer-2 restriction fragment length polymorphism (ITS-2-RFLP) tool to differentiate some exotic and indigenous Trichogrammatid egg parasitoids from India whereas Venkatesan et al. (2011) studied characterization and identification of *Acerophagus papayae* Noyes & Schauff, 2003 (Hymenoptera: Encyrtidae), an introduced parasitoid of papaya mealybug, *Paracoccus marginatus* Williams & Granara de Willink, 1992 through DNA barcoding. The study was undertaken for the DNA barcoding of *A. papayae*, using CO1 region in order to boost and confirm that the introduced and native populations in Pune belonged to the same species. In addition DNA Barcoding for Identification of Agriculturally Important Insects of India was recently carried out by Jalali et al., (2015). Different parasitoids, predators and other insects were collected from various cities of India and were used for DNA barcoding studies. The specimens, thus collected and morphologically identified, were used for COI barcoding at the National Bureau of Agriculturally Important Insects (NBAII) Bangalore, India. Venkatesan et al. (2016) carried out study to unravel the discrimination success in the two molecular marker loci cytochrome oxidase I (COI) and internal transcribed spacer-2 (ITS-2) region of Trichogrammatids.

1.2 DNA barcoding of insect fauna

DNA barcoding, a taxonomic method that uses a short, standardized DNA sequence to identify species, has gained increased attention and acceptance from members of the scientific community interested in documenting the Earths' biodiversity (Hebert et al., 2003; Savolainen et al., 2005; Hajibabaei et al., 2007; Borisenko et al., 2009; Ivanova et al., 2009). One of the advantages of DNA barcoding with respect to traditional taxonomy is the speed and low costs involved in assemblage and analyzing data (Borisenko et al., 2009; Strutzenberger et al., 2010). The creation of the CBOL's online database (The Barcode of Life Data System – BOLD: www.barcodinglife.org) has provided an impetus for numerous researchers to join the barcode initiative. It is easy to access and provides free storage and retrieval of molecular, morphological and geographical data, besides a built-in, integrated analysis tools such as tree reconstructions on the basis of genetic similarity (Ratnasingham & Hebert, 2007; Frézal & Leblois, 2008). DNA barcoding relies on the premises that the genetic variation among species is greater than the variation within species (Hajibabaei et al., 2007). Mitochondrial genes as universal markers were mostly driven by the fact that the mitochondria is maternally inherited, avoiding problems with recombination. Also, the mitochondrial genome has a high mutation rate when compared with the nuclear genome, which results in high degrees of intra-specific polymorphism and divergence, important in evolutionary studies (Williams & Knowlton, 2001; Wheat & Watt, 2008; Hlaing et al., 2009). Taxonomy and systematics of insects using DNA barcoding has been enriched with several contributions from various authors. Molecular studies in the order Hemiptera were carried out by Foottit et al. (2009), Lee et al. (2010) and Shufran & Puterka (2011), whereas Smith et al. (2006), Ekrem et al. (2007) and Rivera & Currie (2009) barcoded Diptera. Hymenoptera was enriched by contributions of Smith et al. (2005), Sheffield et al. (2009) and Smith et al. (2009) while Yoshitake et al. (2008), Raupach et al. (2010) and Greenstone et al. (2011) carried out studies in Coleoptera. Molecular studies in Trichoptera were performed by Salokannel et al. (2010), Geraci et al. (2011) and Zhou et al. (2011). Characteristics intrinsic to insects, such as their diversity, biological control and the economic and epidemiological relevance of some groups, have made them the main target of DNA barcoding studies. This standard database can be used in studies on the taxonomy, phylogeny, ecology, agriculture and conservation of various groups of organisms (Jinbo et al., 2011).Several contributions focusing on identification using the mitochondrial COI have proved useful in the detection of cryptic insect species. Some of those cryptic species which were initially almost impossible to separate using morphological characters alone, have had their identities corroborated by other characters in their natural history and even characters in their morphology (Hebert et al., 2004; Smith et al., 2006; Pfenninger et al., 2007; Decaëns & Rougerie, 2008; Vaglia et al., 2008; Wheat & Watt, 2008; Dasmahapatra et al., 2010; Hausmann et al., 2011. Morphological differences, cases of sexual dimorphism, different castes, or different stages of development have made barcode sequences applicative (Miller et al., 2005; Geraci et al., 2011); Jinbo et al., 2011). Other applications include : identification of host plants by sequencing the stomach contents or plant tissues left on the outside of an insect's body (Jurado-Rivera et al., 2009); identification of the stomach contents of predators in biological control studies

(Greenstone et al., 2005); Greenstone (2006); additional data uncovering trophic relationships (Clare et al., 2009; Hrcek et al., 2011); and finally, population genetics, community ecology and biodiversity inventories (Hajibabaei et al., 2006; Lukhtanov et al., 2009; Craft et al., 2010).

1.3 Limitations of DNA barcoding

DNA barcoding has its pitfalls too. Its success is dependent on the strength of the pretension that interspecific variation exceeds intraspecific variation by one order of magnitude, thus establishing a "barcoding gap", or on the reciprocal monophyly of species (Wiemers & Fiedler (2007). The presence of multiple mitochondrial gene haplotypes, such as nuclear pseudogenes of the mitochondria genome (NUMT) or heteroplasmy also reduces the validity of DNA barcoding. This problem has been reported for many insects (Gellissen & Michaelis, 1987; Zhang & Hewitt, 1996; Bensasson et al., 2000; Brower, 2006; Rubinoff et al., 2006) and can also affect the barcoding results (Song et al., 2008).

1.4 Summary from barcode of life data system

Barcode of Life Data Systems (commonly known as BOLD) is a sequence database specifically devoted to DNA barcoding. It provides an online platform for analyzing DNA sequences. BOLD is populated with nearly 163617 insect species barcodes out of which India has only 3694 barcodes. There are about 5448764 records of specimens of insects in BOLD statistics with 4404476 specimens with sequences and 4092095 specimens with Barcodes. It represents 218968 species in which 170452 have been barcoded (Fig. 1). As far as hymenoptera are concerned there are 907902 specimen records with 666323 specimens with sequences. 563353 specimens are with barcodes representing 35907 species with 26017 species barcoded (BOLD v4) (Fig. 1).

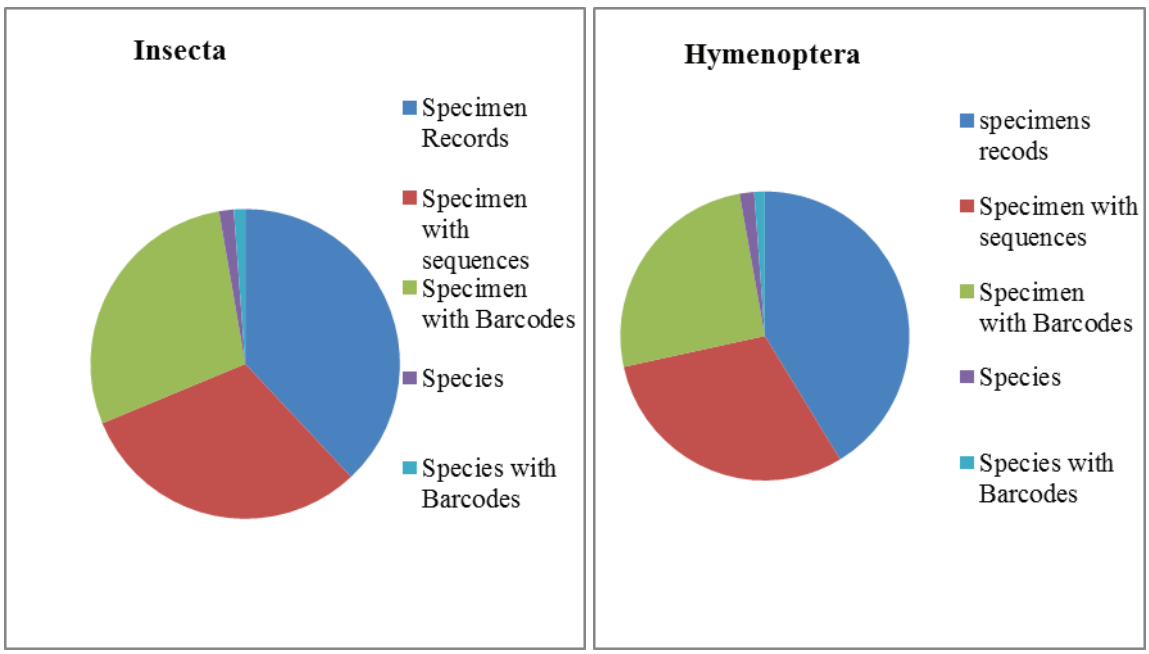

Figure 1: Barcoding status of Insecta (left) and Hymenoptera (right) in BOLD**. (**Data accessed on 30 July 2017)

1.5 DNA barcoding status of Chalcidoidea in India

An estimated 150000 Hymenopteran species of insects are reported worldwide of which 25169 species have been barcoded (Axel et al., 2013) (Fig. 2). In India, little work has been done so far at molecular level. With an estimated 10000 species, only 167 species of Hymenoptera have been subjected to barcoding in the Insect Barcode Informatica (IBIn): a platform to assist and manage acquisition, storage, analysis and to explore DNA barcode records for species identification and genetic analysis of status data of Indian insects (Fig. 2). Out of 167 hymenopteran species barcoded, 58 belong to superfamily Chalcidoidea including 44 Trichogrammatidae, 5 Eulophidae, 2 Torymidae and 7 Encyrtidae species (Fig. 3).

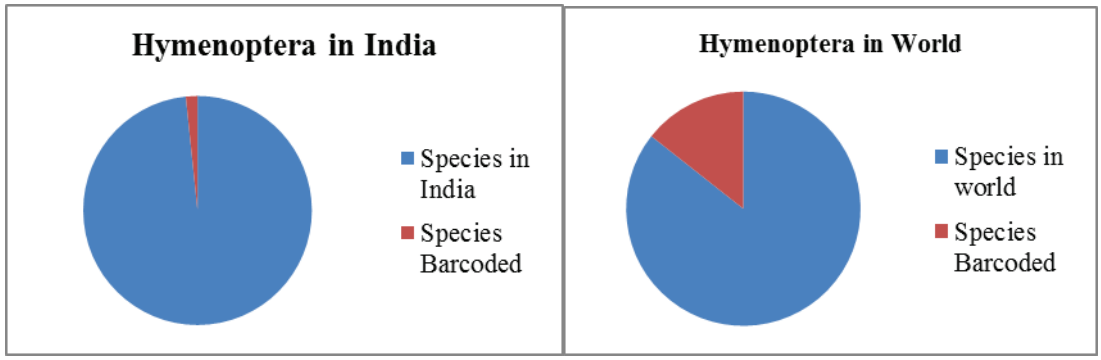

Figure 2: Hymenoptera: species and barcodes in the India (left) and in World (right) (Data accessed on 30 July 2017)

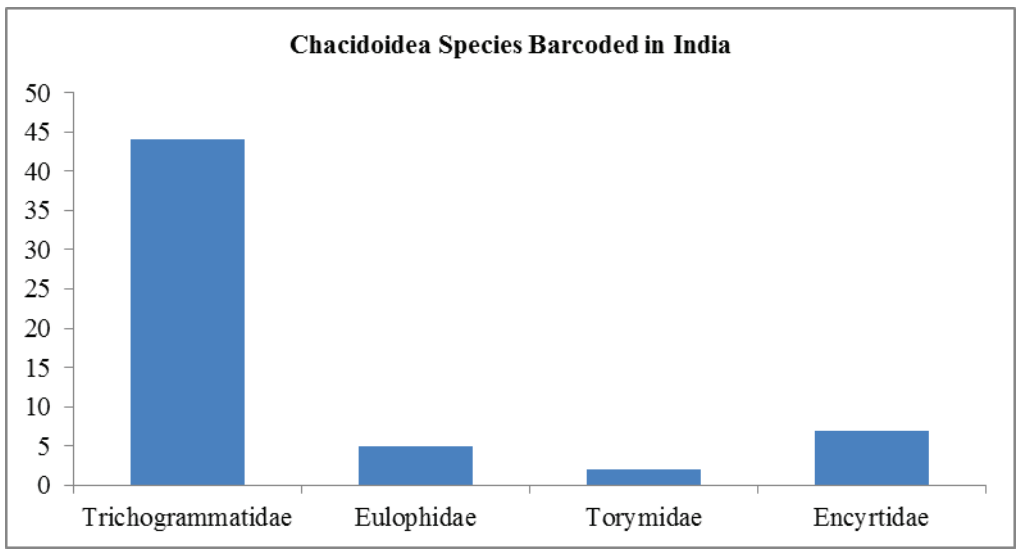

Figure 3: Number of chalcid wasps barcoded in India (Data accessed on 30 July 2017)

At global level an estimated 25169 Hymenopteran species have been barcoded so far among 150000 described species (Axel et al., 2013). Species of Chalcidoidea are richly represented among the species barcoded so far in the order Hymenoptera. Out of 38287 Specimens of Chalcid wasps with barcodes, only 3.6 % (1382) of species with barcodes are represented in Barcode of Life Data system (BOLD) with Eulophidae most represented (357) and Mymarommatidae and Rotoitidae least represented with just one species. Records of individual families of superfamily Chalcidoidea in BOLD are shown in Table 1.

Table 1: Current summary of DNA barcoding library of Chalcidoidea in the BOLD system

Sr. No.	Family	Specimen records	Specimen with sequences	Specimen With Barcodes	Species	Species with Barcodes
1	Agonidae	2410	2324	1569	372	271
2	Aphelinidae	7892	6803s	2875	56	47
3	Chalcididae	2493	1163	611	175	88
4	Encyrtidae	4622	4139	1799	92	66
5	Eulophidae	21321	18444	11574	642	357
6	Eucharitidae	241	147	81	44	29
7	Eupelmidae	1749	1190	700	126	58
8	Eurytomidae	3029	2474	1287	90	45
9	Leucospidae	80	34	09	14	06
10	Mymaridae	20379	18629	6637	61	27
11	Mymarommatidae	56	07	04	01	01
12	Ormyridae	278	223	159	22	12
13	Perilampidae	1211	784	543	87	58
14	Pteromalidae	13114	10946	6830	547	227
15	Rotoitidae	01	01	01	01	01
16	Signiphoridae	168	163	85	01	00
17	Tanaostigmatidae	36	15	05	07	04
18	Tetracampidae	21	17	14	07	05
19	Torymidae	2420	1999	1056	97	53
20	Trichogrammatidae	5707	5356	2448	31	27

(Data Accessed on 30 July 2017)

2 CONCLUSIONS

Species identification is a fundamental part of recognizing and describing biodiversity. Traditionally, identification has been based on morphological diagnoses provided by taxonomic studies. The classical use of morphological trait for species identification has several limitations and requires a high level of expertise for correct identification of species. The DNA barcoding approach might correctly present the best solution for identifying species when their morphology is of limited use (Hebert et al., 2003). DNA barcoding has recently picked up pace in India and helped in the unambiguous identification of insect species of India including Chalcid wasps. This latest method of species identification through DNA barcoding of mitochondrial cytochrome oxidase gene I (COI) (Hebert et al., 2003) clearly gives support to improve classifications and examine the precision of morphological traits commonly used in taxonomy critically.

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4 REFERENCES

Altschul, S.F., Gish, W., Miller, W.E., Myers, W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Axel, J., Seraina, K., Lars, V., Heraty, J.M., Sharkey, M., & Fredrik, R. (2013). "The Hymenopteran Tree of Life: Evidence from Protein-Coding Genes and Objectively Aligned Ribosomal Data". *PLoS ONE, 8*(8), e69344. https://doi.org/10.1371/journal.pone.0069344

Bensasson, D., Zhang, D. X., & Hewitt, G. M. (2000). Frequent assimilation of mitochondrial DNA by grasshopper nuclear genomes. *Molecular Biology and Evolution*, *17*(3), 406-415. https://doi.org/10.1093/oxfordjournals.molbev.a026320

Beutel, R.G., & Pohl, H. (2006). Endopterygote systematic- where do we stand and what is the goal(Hexapoda: Arthropoda). *Systematic Entomology*, *31*, 202–219. https://doi.org/10.1111/j.1365-3113.2006.00341.x

Blaxter, M.L. (2004). The promise of DNA taxonomy. *Philosophical Transactions of the Royal Society London B, 359*, 669-679. https://doi.org/10.1098/rstb.2003.1447

Brower, A. V. (2006). Problems with DNA barcodes for species delimitation:'ten species' of Astraptes fulgerator reassessed (Lepidoptera: Hesperiidae). *Systematics and Biodiversity*, *4*(2), 127-132. https://doi.org/10.1017/S147720000500191X

Busse, H.J., Denner, E.B.M., & Lubitz, W. (1996). "Classification and identification of bacteria: Current approaches to an old problem. Overview of methods used in bacterial systematic." *Journal of Biotechnology*, *47*, 3-38. https://doi.org/10.1016/0168-1656(96)01379-X

Chase, M.W., Salamin, N., Wilkinson, M., Dunwell, J.M., Kesanakurthi, R.P., Haidar, N., & Savolainen, V. (2005). Land plants and DNA barcodes: short-term and long-term goals. *Philosophical Transactions of the Royal Society B. Biological Sciences*, *360*, 1889–1895. https://doi.org/10.1098/rstb.2005.1720

Clare, E.L., Lim, B.K., Engstrom, M.D., Eger, J.L., & Hebert, P.D.N. (2007). DNA barcoding of Neotropical bats: species identification and discovery within Guyana. *Molecular Ecology Notes*, *7*, 184–190. https://doi.org/10.1111/j.1471-8286.2006.01657.x

Clare, E.L., Fraser, E.E., Braid, H.E., Fenton, M.B., & Hebert, P.D.N. (2009). Species on the menu of a generalist predator, the eastern red bat (*Lasiurus borealis*) - using a molecular approach to detect arthropod prey. *Molecular Ecology*, *18*, 2532-2542. https://doi.org/10.1111/j.1365-294X.2009.04184.x

Craft, K.J., Pauls, S.U., Darrow, K., Miller, S.E., Hebert, P.D.N., Helgen, L. E., Novotny, V., & Weiblen, G.D. (2010). Population genetics of ecological communities with DNA barcodes: an example from New Guinea Lepidoptera. *Proceedings of the National Academy of Science,* 11, 5041-5046. https://doi.org/10.1073/pnas.0913084107

Dasmahapatra, K.K., Elias, M., Hill, R.I., Hoffman, J.I., & Mallet, J. (2010) Mitochondrial DNA barcoding detects some species that are real, and some that are not. *Molecular Ecology Resources*, *10*, 264-273. https://doi.org/10.1111/j.1755-0998.2009.02763.x

Decaëns, T., & Rougerie, R. (2008). Descriptions of two new species of Hemileucinae (Lepidoptera: Saturniidae) from the region of Muzo in Colombia - evidence from morphology and DNA barcodes. *Zootaxa, 1944*, 34-52.

Ekrem, T., Willassen, E., & Stur, E. (2007). A comprehensive DNA sequence library is essential for identification with DNA barcodes. *Molecular Phylogenetics and Evolution*, *43*, 530-542. https://doi.org/10.1016/j.ympev.2006.11.021

ENVIS (2011) - *Centre on Floral Diversity, Ministry of Environment, Forest & Climate Change*, Governement of India, (accessed on 30 July 2017).

Floyd, R., Abebe, E., Papert, A., & Blaxter, M. (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology*, *11*, 839–850. https://doi.org/10.1046/j.1365-294X.2002.01485.x

Foottit, R.G., Maw, H.E.L., Havill, N.P., Ahern, R.G., & Montgomery, E. (2009). DNA barcodes to identify species and explore diversity in the Adelgidae (Insecta: Hemiptera: Aphidoidea). *Molecular Ecology Resource, 9*, 188-195. https://doi.org/10.1111/j.1755-0998.2009.02644.x

Frézal, L., & Leblois, R. (2009). Four years of DNA barcoding: Current advances and prospects. *Infection, Genetics and Evolution*, *8*, 727-736. https://doi.org/10.1016/j.meegid.2008.05.005

Gaikward, S. (2014). DNA Barcoding: The Indian Scenario. *Barcode Bulletin, International Barcode of life, 5*(4). Available from http://ibol.org/

Gellissen, G., & Michaelis, G. (1987). Gene Transfer. *Annals of the New York Academy of Sciences*, 503(1), 391-401. https://doi.org/10.1111/j.1749-6632.1987.tb40625.x

Geraci, C.J., Mohammed, A.A., & Zhou, X. (2011). DNA barcoding facilitates description of unknown faunas: a case study on Trichoptera in the headwaters of the Tigris River, Iraq. *Journal of the North American Benthological Society, 30*, 163-173. https://doi.org/10.1899/10-011.1

Greenstone, M.H. (2006). Molecular methods for assessing insect parasitism. *Bulletin of entomological research, 96*, 1-13. Available from https://doi.org/10.1079/BER2005402

Greenstone, M.H., Rowley, D.L., Heimbach, U., Lundgren, G., Pfannenstiel, R.S., & Rehner, S.A. (2005). Barcoding generalist predators by polymerase chain reaction: carabids and spiders. *Molecular Ecology, 14*, 3247-3266. https://doi.org/10.1111/j.1365-294X.2005.02628.x

Greenstone, M.H., Vandenberg, N.J., & Hu, J.H. (2011). Barcode haplotype variation in north American agroecosystem lady beetles (Coleoptera: Coccinellidae). *Molecular Ecological Resources*, *11*, 629-637. https://doi.org/10.1111/j.1755-0998.2011.03007.x

Grimaldi, D.A., & Engel M.S. (2005). *Evolution of the Insects.* Cambridge University Press, Cambridge.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W., & Hebert, P.D.N. (2006). DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences, USA, 103*, 968-971. https://doi.org/10.1073/pnas.0510466103

Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N., & Hickey, D.A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *TRENDS in Genetics*, *23*, 167-172. https://doi.org/10.1016/j.tig.2007.02.001

Hausmann, A., Haszprunar, G., & Hebert, P.D.N. (2011). DNA Barcoding the Geometrid Fauna of Bavaria (Lepidoptera): Successes, Surprises, and Questions. *PLoS ONE*, *6*, e17134. https://doi.org/10.1371/journal.pone.0017134

Hebert, P.D.N., Cywinska, A., Ball, S.L., & DeWaard, J.R. (2003). Biological Identification through DNABarcodes. *Proceedings of the Royal Society of London B: Biological Sciences, 270*, 313-321. https://doi.org/10.1098/rspb.2002.2218

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences, USA,* 101, 14812-14817. https://doi.org/10.1073/pnas.0406166101

Hlaing, T., Tun-lin, W., Somboon, P., Socheat, D., Setha, T., Min, S., Chang, M.S., & Walton, C. (2009). Mitochondrial pseudogenes in the nuclear genome of *Aedes aegypti* mosquitoes: implications for past and future population genetic studies. *BMC Genetics, 10*, 11. https://doi.org/10.1186/1471-2156-10-11

Hrcek, J., Miller, S.E., Quicke, D.L.J., & Smith, M.A. (2011). Molecular detection of trophic links in a complex insect host-parasitoid food web. *Molecular Ecological Resources*, *11*(5), 786-94. https://doi.org/10.1111/j.1755-0998.2011.03016.x

Insect Barcode Informatica (IBIn). – Indian council of Agricultural Research, National Bureau of Agricultural Insect Resources. Available from http://www.cib.res.in/ibin/index.php (accessed on 30 July 2017)

Ivanova, N.V., Borisenko, A.V., & Hebert, P.D.N. (2009). Express barcodes: racing from specimen to identification. *Molecular Ecological Resources*, *9*, 35- 41. https://doi.org/10.1111/j.1755-0998.2009.02630.x

Jalali, S.K., Ojha, R., Venkatesan, T., & Chakravarthy, A.K. (2015). DNA Barcoding for Identification of Agriculturally Important Insects. *New Horizons in Insect Science: Towards Sustainable Pest Management:* Springer, India, pp. 13-23. https://doi.org/10.1007/978-81-322-2089-3_2

Jarman, S.N. & Elliott, N.G. (2000). DNA evidence for morphological and cryptic Cenozoic speciations in the Anaspididae, 'living fossils' from the Triassic. *Journal of Evolution Biology, 1*(3), 624-633. https://doi.org/10.1046/j.1420-9101.2000.00207.x

Jinbo U., Kato T., & Ito M. (2011). Current progress in DNA barcoding and future implications for entomology. *Entomological Science, 14*, 107–124. https://doi.org/10.1111/j.1479-8298.2011.00449.x

Jurado-Rivera, J.A., Vogler, A.P., Reid, C.A.M., Petitpierre, E., & Gomez-Zurita, J. (2009). DNA barcoding insect-host plant associations. Proceedings of the Royal Society of London B: *Biological Sciences*, *276*, 639-648. https://doi.org/10.1098/rspb.2008.1264

Kerr, K.C.R., Stoeckle, M.Y., Dove, C.J., Weigt, L.A., Francis, C.M., & Hebert, P.D.N. (2007). Comprehensive DNA barcode coverage of North American birds. *Molecular Ecology Notes*, *7*, 535–543. https://doi.org/10.1111/j.1471-8286.2007.01670.x

Kumara, G.A., Venkatesana Stouthamerb, R., Niranjanaa, P., Lalithaa, Y., & Jalalia, S.K. (2009). Internal transcribed spacer-2 restriction fragment length polymorphism (ITS-2-RFLP) tool to differentiate some exotic and indigenous trichogrammatid egg parasitoids in India. *Biological Control*, *49* (3), 207-213. https://doi.org/10.1016/j.biocontrol.2009.02.010

Lee, W., Kim, H., Lim, J., Choi, H.R., Kim, Y., Kim, Y.S., Ji, J.Y., Foottit, R.G., & Lee, S. (2010). Barcoding aphids (Hemiptera:Aphididae) of the Korean Peninsula: updating the global data set. *Molecular Ecological Resources*, *11* (1), 32-7. https://doi.org/10.1111/j.1755-0998.2010.02877.x

Lukhtanov, V.A., Sourakov, A., Zakharov, E.V., & Hebert, P.D.N. (2009). DNA barcoding Central Asian butterflies: increasing geographical dimension does not significantly reduce the success of species identification. *Molecular Ecological. Resources*, *9*, 1302-1310. https://doi.org/10.1111/j.1755-0998.2009.02577.x

Mayhew, P.J. (2007.). "Why there are so many insect species? Perspectives from fossils and Phylogenies". *Biological Reviews*, *82* (3), 425-454. https://doi.org/10.1111/j.1469-185X.2007.00018.x

Miller, K.B., Alarie, Y., Wolfe, G.W., & Whiting, M.F. (2005). Association of insect life stages using DNA sequences: the larvae of *Philodytes umbrinus* (Motschulsky) (Coleoptera: Dytiscidae). *Systematic Entomology*, *30*, 499-509. https://doi.org/10.1111/j.1365-3113.2005.00320.x

Munro, J.B., Heraty, J.M., Burks, R.A., Hawks, D., Mottern, J., Cruaud, A., Rasplus, J.Y., & Jansta, P. (2011). A Molecular Phylogeny of the Chalcidoidea (Hymenoptera), *PLoS One, 6* (11), e27023. https://doi.org/10.1371/journal.pone.0027023

Padial, J.M., & Riva, I.D.L. (2007). Integrative taxonomists should use and produce DNA barcodes. *Magnolia Press Zootaxa, 1586*, 67–68.

Pfenninger, M., Nowak, C., Kley, C., Steinke, D., & Streit, B. (2007). Utility of DNA taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. *Molecular Ecology*, *16* (9), 1957-68. https://doi.org/10.1111/j.1365-294X.2006.03136.x

Pinzon-Navarro, S., Jurado-Rivera, J.A., Gomez-Zurita, J., Lyal, C.H.C., & Vogler, A.P. (2010). DNA profiling of host–herbivore interactions in tropical forests. *Systematic Entomology*, *35* (Suppl 1), 18–32.

Pires, A.C., & Marinoni, L. (2010). DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: a view that challenges the debate questioning both methodologies. *Biota Neotropica*, *10* (2), 339-346. https://doi.org/10.1590/S1676-06032010000200035

Plaisance, L., Caley, M. J., Borisenko, A.V., Sones, J.E., & Hebert, P.D.N. (2009). The front-end logistics of DNA barcoding: challenges and prospects. *Molecular Ecological Resources*, *9*, 27-34. https://doi.org/10.1111/j.1755-0998.2009.02629.x

Pratheepa, M., Jalali, S.K., Arokiaraj, R.S., Venkatesan, T., Panda, M.N.M., & Pattar, S. (2014). Insect Barcode Information System Volume: *Bioinformation*, *10*(2), 98-100. https://doi.org/10.6026/97320630010098

Preethi, N., Lakshmi, P., & Menon, D. (2016). Diversity and Distribution of Chalcid Wasps in Kerala: Key Biological Control Agents in Cultivated Ecosystems. *Arthropod Diversity and Conservation in the Tropics and Sub-tropics,* Springer Singapore, pp. 213-226. https://doi.org/10.1007/978-981-10-1518-2_13

Ratnasingham, S., & Hebert, P.D.N. (2007). BOLD: The Barcode of Life Data System. *Molecular Ecological Notes*, *7*, 355-64. Available from (http://www.barcodinglife.org). https://doi.org/10.1111/j.1471-8286.2007.01678.x

Raupach, M.J., Astrin, J.J., Hanning, K., Peters, M.K., Stoeckle, M.Y., & Wägele, J.W. (2010). Molecular species identification of Central European ground beetles (Coleoptera: Carabidae) using nuclear rDNA expansion segments and DNA barcodes. *Frontiers in Zoology*, *7*(1), 26. https://doi.org/10.1186/1742-9994-7-26

Rivera, J., & Currie, D.C. (2009). Identification of Nearctic black flies using DNA Barcodes (Diptera: Simuliidae). *Molecular Ecological Resources*, *9*, 224-236. https://doi.org/10.1111/j.1755-0998.2009.02648.x

Rubinoff, D., Cameron, S., & Will, K. (2006). A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. *Journal of Heredity*, *97*(6), 581-594. https://doi.org/10.1093/jhered/esl036

Salokannel, J., Rantala, M.J., & Whalberg, N. (2010). DNA-barcoding clarifies species definitions of Finnish *Apatania* (Trichoptera: Apataniidae). *Entomologica Fennica*, *21*, 1-11.

Savolainen, V., Cowan, R.S., Vogler, A.P., Roderick, G.K., & Lane, R. (2005). Towards writing the encyclopedia of life: an introduction to DNA barcoding. *Philosophical Transactions of the Royal Society B. Biological Sciences, 360*, 1805–1811. https://doi.org/10.1098/rstb.2005.1730

Sheffield, K.A., Hebert, P.D.N., Kevan, P.G., & Packer, L. (2009). DNA Barcoding a regional bee (Hymenoptera: Apoidea) fauna and its potential for ecological studies. *Molecular Ecological Resources, 9*, 196-207. https://doi.org/10.1111/j.1755-0998.2009.02645.x

Shufran, K.A., & Puterka, G.J. (2011). DNA Barcoding to Identify All Life Stages of Holocyclic Cereal Aphids (Hemiptera: Aphididae) on Wheat and Other Poaceae. *Annals of the Entomological Society of America, 104*, 39-42. https://doi.org/10.1603/AN10129

Sithanantham, S., Chandish, R.B., Jalali, S.K., & Bakthavatsalam, N. Molecular Taxonomy of Trichogammatids . *Biological control of insect pests using egg parasitoids*., *1*, 39-66.

Smith, M.A, Fernandez-Triana, J., Roughley, R., & Hebert, P.D.N. (2009). DNA barcode accumulation curves for understudied taxa and areas. *Molecular Ecological Resources, 9*, 208-216. https://doi.org/10.1111/j.1755-0998.2009.02646.x

Smith, M.A., Fisher, B.L., & Hebert, P.D.N. (2005). DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society B. Biological Sciences*, *360*, 1825-1834. https://doi.org/10.1098/rstb.2005.1714

Smith, M.A., Woodley, N.E., Janzen, D.H., Hallwachs, W., & Hebert, P.D.N. (2006). DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proceedings of the National Acadamy of Sciences, USA, 103*, 3657-3662. https://doi.org/10.1073/pnas.0511318103

Song, H., Buhay, J. E., Whiting, M. F., & Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the national academy of sciences*, *105*(36), 13486-13491. https://doi.org/10.1073/pnas.0803076105

Strutzenberger, P., Brehm, G., & Fiedler, K. (2010). DNA barcoding-based species delimitation increases species count of *Eois* (Geometridae) moths in a well-studied tropical mountain forest by up to 50 %. *Insect Science*, *18*, 349-362. https://doi.org/10.1111/j.1744-7917.2010.01366.x

Tautz, D., Arctander P., Minelli, A., Thomas, R.H., & Vogler, A.P. (2003). A plea for DNA taxonomy. *Trends in Ecology and Evolution*, *18*, 70–74. https://doi.org/10.1016/S0169-5347(02)00041-1

Theron, J., & Cloete, T.E. (2000). Molecular techniques for determining microbial diversity and community structure in natural environments. *Critical Reviews in Microbiology*, *26*, 37–57. https://doi.org/10.1080/10408410091154174

Vaglia, T., Haxaire, J., Kitching, I.J., Meusnier, I., & Rougerie, R. (2008). Morphology and DNA barcoding reveal three cryptic species within the *Xylophanes neoptolemus* and *loelia* species-groups (Lepidoptera: Sphingidae). *Zootaxa, 1923*, 18-36.

Venkatesan, T., Prabhakar, R., Baskar, R., Jalalia, S.K., & Lalitha, C.R. (2016). Differentiation of some indigenous and exotic trichogrammatids

(Hymenoptera: Trichogrammatidae) from India based on Internal transcribed spacer-2 and cytochrome oxidase-I markers and their phylogenetic relationship. *Biological Control*, *101*, 130-137. https://doi.org/10.1016/j.biocontrol.2016.07.005

Ward, R.D., Zemlak, T.S., Innes, B.H., Last P.R., & Hebert, P.D.N. (2005). DNA barcoding of Australia's fish species. *Philosophical Transactions of the Royal Society B. Biological Sciences*, *360*, 1847–1857. https://doi.org/10.1098/rstb.2005.1716

Wheat, C.W., & Watt, W.B. (2008). A mitochondrial-DNA-based phylogeny for some evolutionary-genetic model species of Colias butterflies (Lepidoptera: Pieridae). *Molecular Phylogenetics and Evolution*, *47*, 893-902. https://doi.org/10.1016/j.ympev.2008.03.013

Wiemers, M., & Fiedler, K. (2007). Does the DNA barcoding gap exist?–a case study in blue butterflies (Lepidoptera: Lycaenidae). *Frontiers in zoology*, *4*(1), 8. https://doi.org/10.1186/1742-9994-4-8

Williams, S.T., & Knowlton, N. (2001). Mitochondrial pseudogenes are pervasive and often insidious in the snapping shrimp Genus *Alpheus*. *Molecular Biology and Evolution*, *18*, 1484-1493. https://doi.org/10.1093/oxfordjournals.molbev.a003934

Yoshitake, H., Kato, T., Jinbo, U., & Ito, M. (2008). A new *Wagnerinus* (Coleoptera: Curculionidae) from northern Japan: description including a DNA barcode. *Zootaxa*, *1740*, 15-27.

Zhang, D. X., & Hewitt, G. M. (1996). Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology & Evolution*, *11*(6), 247-251. https://doi.org/10.1016/0169-5347(96)10031-8

Zhou, X., Robinson, J.L., Geraci, C.J., Parker, C.R., Flint, J.R.O.S., Etnier, D.A., Ruiter, D., Dewalt, R.E., Jacobus, L.M., & Hebert, P.D.N. (2011). Accelerated construction of a regional DNA-barcode reference library: caddisflies (Trichoptera) in the great smoky mountains national park. *Journal of the North American Benthological Society, 30*, 131-162. https://doi.org/10.1899/10-010.1

In Memoriam - Prof. dr. Božidar Krajnčič
(1935–2018)

Božidar Krajnčič se je rodil 20. januarja 1935 v Mariboru kot najmlajši izmed šestih otrok. Po končani osnovni šoli je nadaljeval šolanje na nižji gimnaziji in nato na višji II. gimnaziji, kjer je leta 1954 maturiral. Istega leta se je vpisal na Prirodoslovno matematično fakulteto v Ljubljani in leta 1959 diplomiral iz biologije.

Sprva se je zaposlil na Ekonomski srednji šoli v Mariboru, v jeseni 1961 pa je sprejel mesto učitelja na I. gimnaziji v Mariboru, kjer je do leta 1978 poučeval biologijo in kemijo. V želji, da bi nudil svojim dijakom čim več, zlasti na področju raziskovalnega dela, se je vpisal na podiplomski študij biologije na Prirodoslovno-matematični fakulteti Vseučilišča v Zagrebu. V magistrski nalogi se je poglobil v fotoperiodizem pri vodolečevkah in v maju 1972 postal magister bioloških znanosti. Na isti fakulteti je nadaljeval z doktorskim študijem in raziskovalnim delom na področju fiziologije cvetenja vodolečevk. Pod mentorstvom akademika prof. dr. Zvonimira Devidé je bil leta 1976 promoviran v doktorja bioloških znanosti.

Od leta 1970 je predaval biologijo in rastlinsko fiziologijo na takratni Pedagoški akademiji, sedanji Fakulteti za naravoslovje in matematiko. Leta 1978 je nastopil službo na Višji agronomski šoli v Mariboru kot profesor biologije in rastlinske fiziologije in bil leta 1983 izvoljen v naziv rednega univerzitetnega profesorja.

V letih 1983–1985 je opravljal funkcijo prorektorja za raziskovalno dejavnost Univerze v Mariboru in bil aktiven član Sveta za znanost Republike Slovenije. V letih 1985–1989 je postal dekan Višje agronomske šole in v tej funkciji svoje delo usmerjal predvsem v kadrovsko krepitev, posodobitev šole in opremljanje laboratorijev. Leta 1991 je bil izvoljen za prorektorja za izobraževalno dejavnost Univerze v Mariboru. Po smrti dekana mag. Milana Erjavca v letu 1992 je ponovno prevzel funkcijo dekana. Pod njegovim vodenjem in z izjemnim osebnim prizadevanjem je omogočil, da je leta 1995 Višja agronomska šola napredovala v Fakulteto za kmetijstvo, današnjo Fakulteto za kmetijstvo in biosistemske vede, ki jo je prof. dr. Božidar Krajnčič vodil do leta 2003. Fakulteta je v tem času na prvotni lokaciji pod Kalvarijo opremila laboratorije za fiziologijo, agrokemijo, mikrobiologijo in vinarstvo, delno pa tudi za genetiko in tkivne kulture. Predvsem je potrebno izpostaviti njegova prizadevanja za pridobitev kmetijskega posestva v letu 1994 in ustanovitev Univerzitetnega kmetijskega centra, ki obsega 155 ha obdelovalnih površin in 240 ha gozdov.

Od samega začetka njegove raziskovalne poti se je

prof. dr. Božidar Krajnčič posvetil vodoležvekam. Na področju SV Slovenije in Istre je opisal več kot sto rastišč. Prvi je v Istri odkril rastišče navadne vodne lečice (Wolffia arrhiza (L.) Horkel ex Wimm.). Z rezultati raziskav je skupaj s prof. dr. Devidéjem veliko prispeval k opredelitvi filogenetskega položaja navadne žabje leče (Spirodela polyrhiza (L.) Schleid.). Uspel je tudi bistveno izpopolniti metode gojenja vodoležvek z namenom biološkega testiranja kakovosti voda. Njegovo raziskovanje je privedlo do nekaterih temeljnih dognanj o fotoperiodizmu teh rastlin. Proučeval je vpliv esencialnih aminokislin, kelatirajočih spojin in rastlinskih rastnih regulatorjev na rast, razvoj in indukcijo cvetenja vodoležvek.

Raziskovalno se je tudi ukvarjal s fiziologijo gozdnega drevja v povezavi z odpornostjo na bolezni in škodljivce, in pri tem znotraj Univerzitetnega kmetijskega centra pomembno vzpodbujal gozdarstvo. Pomembno je prispeval tudi k aplikativnim raziskavam v kmetijstvu in pri tem sodeloval s številnimi kmetijskimi podjetji.

Njegov bogat bibliografski opus obsega 25 izvirnih znanstvenih člankov, dve znanstveni in štiri strokovne monografije, 4 znanstvene priročnike, univerzitetne učbenike in gradiva ter številne druge znanstvene in strokovne prispevke.

Prof. dr. Krajnčič je mestu Maribor predvsem znan kot pobudnik izgradnje ter strokovni vodja Botaničnega vrta Univerze v Mariboru. Le-ta je od leta 1999 vključen v združenje ″Botanic Garden Conservation International″, za javnost pa je odprt od maja 2002. Z vso vnemo in predanostjo se je posvečal zasaditvi posameznih sklopov botaničnega vrta. Posebej je bil ponosen na pinetum, ki predstavlja bogato zbirko golosemenk, predvsem borov, smrek in jelk iz Evrazije in Amerike. Z izgradnjo botaničnega vrta in sistematičnim poučevanjem je pomembno sooblikoval svoje sodelavce in vrtnarski naraščaj. Botaničnemu vrtu je ostal zvest in predan tudi po svoji upokojitvi.

Prof. dr. Božidar Krajnčič je bil vsestransko strokovno aktiven kot član Zveze evropskih društev rastlinske fiziologije (Federation of European Societies of Plant Physiology) in član mednarodne ekspertne skupine za raziskave cvetenja (International Working Group on Flowering). Leta 2002 je Društvo za rastlinsko fiziologijo Slovenije (sedanje Slovensko društvo za biologijo rastlin) ob 20-letnici delovanja prof. dr. Božidarju Krajnčiču podelilo naziv častni član društva.

Potrebno je izpostaviti tudi izjemne zasluge na področju razvoja visokega šolstva in znanosti v Sloveniji. Bil je član Sveta za visoko šolstvo Republike Slovenije in ekspertne skupine Sveta za znanost in tehnologijo RS za področje biotehniških ved. Sodeloval je v komisiji za habilitacije Univerze v Mariboru in v komisijah Sveta za visoko šolstvo in Ministrstva za znanost.

Za svoje delo je prejel številna priznanja in nagrade, med katerimi velja izpostaviti: Red dela s srebrnim vencem (1978), Zlati znak biologije ob 60. letnici biologije na Univerzi v Ljubljani (1979), Republiško priznanje Zveze organizacij za tehniško kulturo Slovenije, dve Zlati plaketi Univerze v Mariboru, Mestni pečat Maribora (1997), Nagrado Republike Slovenije na področju visokega šolstva (1999), Zoisovo priznanje Republike Slovenije (1999), Častni znak svobode Republike Slovenije (2000), priznanje Kmetijsko gozdarske zbornice Slovenije (2004) in mnoga druga priznanja. Leta 2009 je prof. dr. B. Krajnčič postal zaslužni profesor Univerze v Mariboru ter leta 2016 častni občan Mestne občine Maribor.

Prof. Dr. Božidar Krajnčič je s svojo odločnostjo, vztrajnostjo in jasnimi vizijami začrtal prelomne mejnike Fakultete za kmetijstvo in biosistemske vede. Z vso vnemo se je posvetil izgradnji Botaničnega vrta Univerze v Mariboru. Svoje bogato botanično znanje in izkušnje je delil z mnogimi generacijami študentov in drugih, še posebej mladih. Njegovi kolegi in sodelavci na Fakulteti za kmetijstvo in biosistemske vede si bomo prof. dr. Božidarja Krajnčiča zapomnili kot poštenega, natančnega, delavnega in izredno sposobnega človeka. Hvaležno se zahvaljujemo za neizmerne prispevke k razvoju Univerze v Mariboru, Fakultete za kmetijstvo in biosistemske vede, Univerzitetnega kmetijskega centra in še posebej, Botaničnega vrta.

In memoriam Prof. Dr. Božidar Krajnčič (1935–2018)

Božidar Krajnčič was born in Maribor on 20thJanuary1935 as the youngest of six children. After completing the elementary school, he continued his education in the secondary school and at II. Gymnasium, where he graduated in 1954. In the same year, he enrolled into the Faculty of Natural Sciences and Mathematics University of Ljubljana, where he graduated in biology in 1959.

He began to work as a teacher at the High School for Economics in Maribor and later, in 1961, he pursued his career at the I. Gymnasium in Maribor, where he taught biology and chemistry until 1978. In his desire to offer his students as much as possible, especially in the field of research, he enrolled into postgraduate studies in biology at the Faculty of Science, University of Zagreb. In his master's thesis, he analysed photoperiodism of some species belonging to the duckweed family (Lemnaceae) and, in May 1972, became a Master of biological sciences. At the same faculty, he continued his doctoral studies under the supervision of prof. dr. Zvonimir Devidé and, in 1976, he was promoted to the doctor of biological sciences.

Since 1970, he lectured biology and plant physiology at the Pedagogical Academy Maribor, which later became Faculty of Natural Sciences and Mathematics. In 1978, he became professor of biology and plant physiology at the Higher Agricultural School in Maribor, and in 1983 he was awarded a title of an associate professor. In the years 1983 - 1985, he served as the Vice-Rector for Research at the University of Maribor. In 1985, he was elected Dean of the Higher Agricultural School, and in this function he directed

work primarily on human resources strengthening and modernization. In 1991, he was appointed as Vice-Rector for the educational activities of the University of Maribor. After the death of dean mag. Milan Erjavec in 1992, he re-assumed the dean's role. Under his leadership and strategic direction, and with exceptional personal efforts, he made it possible that the Higher Agronomy School progressed to the Faculty of Agriculture, now Faculty of Agriculture and Life Sciences. Prof. Dr. Božidar Krajnčič led the faculty until 2003. He helped to establish new laboratories for physiology, agrochemistry, microbiology, enology, genetics and tissue culture, and the University Agricultural Centre in 1994, which covers 155 ha of cultivated land and 240 ha of forests.

From the very beginning of his research path, Prof. Dr. Božidar Krajnčič was dedicated to the studies of species belonging to the Lemnaceae family (e.g., Lemna minor L., Wolffia arrhiza (L.) Horkel ex Wimm.) and Spirodela polyrhiza (L.) Schleid.). In SE Slovenian region and in Istria, he described more than hundred locations and contributed significantly to the knowledge of those species. He first described the location of an spotless water meal (Wolffia arrhiza) in Istria. Together with Prof. Dr. Devidé he defined the phylogenetic position of Spirodela polyrhiza. He also improved the methods of their cultivation, in order to use them in biological testing of water quality. With his research he contributed to the fundamental knowledge of photoperiodic responses of species within the Lemnaceae family. He studied the influence of essential amino acids, chelating compounds and plant growth regulators on the growth, development and flower induction of duckweeds.

Within the activities of the University Agricultural Centre he conducted agricultural based research by encouraging forestry and tree physiology. He investigated the resistance of different trees to pests and diseases. With the monitoring of bark beetles and by using biotic approaches of forest protection, he was able to reduce the damage caused by bark beetles in the forests of Pohorje. He also made an important contribution to applied research in agriculture and cooperated with many agricultural companies.

His rich bibliography comprises 25 original scientific articles, two scientific and four professional monographs, 4 scientific manuals, several university textbooks and numerous other publications.

Prof. Dr. Božidar Krajnčič is especially known as the initiator and the professional manager of the Botanical Garden of the University of Maribor. Since 1999, the garden has been included in the association "Botanic Garden Conservation International". It was officially opened to the public in May 2002. He established all crucial sectors of the garden and was especially proud of the pinetum, which represents a rich collection of gymnosperms, mainly pines, spruce and fir trees from Eurasia and America. During the establishment of the botanical garden and his teaching, he delivered his rich botanical knowledge and gardening practises to colleagues and gardeners. He remained faithful and committed to the botanical garden even after his retirement.

Prof. Dr. Božidar Krajnčič was an active member of the Federation of European Societies of Plant Biology and a member of the International Working Group of Physiology of Flowering. In 2002, The Slovenian Plant Physiology Society (currently the Slovenian Society of Plant Biology), celebrating the 20th anniversary, promoted him to the Honorary Member of the Society.

He was a member of several important government and university councils and expert groups, such as Council for Higher Education of the Republic of Slovenia and the expert group of the Council for Science and Technology of the Republic of Slovenia for the field of biotechnical sciences. For his work, he received numerous awards and prizes from the Slovenian government, various ministries, universities and public organizations:

Order of Work with Silver Wreath (1978), Golden Sign of Biology at the 60th anniversary of Biology at the University of Ljubljana (1979), Republic recognition of the Association of Technological Culture Organizations of Slovenia, two Golden Plaques of the University of Maribor (1997), City Seal of Maribor (1997), Award of the Republic of Slovenia in the field of higher education (1999), Zois Certificate of Recognition of the Republic of Slovenia (1999), The Honorary Sign of The Freedom of the Republic of Slovenia (2000), Acknowledgment of the Chamber of Agriculture and Forestry of Slovenia (2004) and many other recognitions. In 2009, Prof. Dr. Božidar Krajnčič became Professor Emeritus of the University of Maribor and, in 2016, the honorary citizen of the Municipality of Maribor.

Prof. Dr. Božidar Krajnčič, through his determination, perseverance, and clear visions, outlined the milestones of the Faculty of Agriculture and Life Sciences. With all his enthusiasm, he dedicated himself to the establishment of the Botanical Garden of the University of Maribor. He shared his rich botanical knowledge and experience with many generations of students and others, especially young people. The colleagues of the Faculty of Agriculture and Life Sciences will always remember and gratefully acknowledge his honest character, creativity, hard work and unmeasurable contributions to the development of the University of Maribor, Faculty of Agriculture and Life Sciences, University Agricultural Centre and, especially, the University Botanical Garden.

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dr. Andreja Urbanek Krajnc
Katedra za botaniko in fiziologijo rastlin / Botany and plant physiology unit
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NAVODILA AVTORJEM

UVOD

Acta agriculturae Slovenica je četrtletna odprtodostopna znanstvena revija z recenzentskim sistemom, ki jo izdaja Biotehniška fakulteta Univerze v Ljubljani. Revija sprejema izvirne in še neobjavljene znanstvene članke v slovenskem ali angleškem jeziku, ki se vsebinsko nanašajo na širše področje rastlinske pridelave in živalske prireje in predelave. Pokritost zajema širok razpon tem, kot so agronomija, hortikultura, biotehnologija, fiziologija rastlin in živali, pedologija, ekologija in okoljske študije, agrarna ekonomika in politika, razvoj podeželja, sociologija podeželja, genetika, mikrobiologija, imunologija, etologija, mlekarstvo, živilska tehnologija, prehrana, bioinformatika, informacijske znanosti in ostala področja, povezana s kmetijstvom. Pregledne znanstvene članke sprejemamo v objavo samo po poprejšnjem dogovoru z uredniškim odborom. Objavljamo tudi izbrane razširjene znanstvene prispevke s posvetovanj, vendar morajo taki prispevki zajeti najmanj 30 % dodatnih originalnih vsebin, ki še niso bile objavljene. O tovrstni predhodni objavi mora avtor obvestiti uredniški odbor. Če je prispevek del diplomske naloge, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Navedbe morajo biti v slovenskem in angleškem jeziku. Uredništvo revije zagotovi prevode izbranih bibliografskih elementov (naslova, izvlečka, opomb in ključnih besed) v primeru tujih avtorjev. Prispevke sprejemamo skozi celo leto.

PROCES ODDAJE PRISPEVKA

Avtorji lektorirane prispevke oddajo v elektronski obliki na spletni strani OJS Acta agriculturae Slovenica. Pred oddajo prispevka se mora avtor na spletni strani najprej prijaviti oziroma registrirati, če prvič vstopa v sistem (potrebno je klikniti na Registracija in izpolniti obrazec za registracijo). Bodite pozorni, da na dnu regis-

AUTHOR GUIDELINES

INTRODUCTION

Acta agriculturae Slovenica is an open access peer-reviewed scientific journal published quarterly by the Biotechnical Faculty of the University of Ljubljana, Slovenia. The Journal accepts original scientific articles from the fields of plant production (agronomy, horticulture, plant biotechnology, plant-related food-and-nutrition research, agricultural economics, information-science, ecology, environmental studies, plant physiology & ecology, rural development & sociology, soil sciences, genetics, microbiology, food processing) and animal production (genetics, microbiology, immunology, nutrition, physiology, ecology, ethology, dairy science, economics, bioinformatics, animal production and food processing, technology and information science) in Slovenian or English language. Review articles are published upon agreement with the editor. Reports presented on conferences that were not published entirely in the conference reports can be published. Extended versions of selected proceedings-papers can also be considered for acceptance, provided they include at least 30 % of new original content, but the editorial board must be notified beforehand. If the paper is part of BSc, MSc or PhD thesis, this should be indicated together with the name of the mentor at the bottom of the front page and will appear as foot note. All notes should be written in Slovenian and English language. Slovenian-language translation of selected bibliographic elements, for example the title, abstract, notes and keywords, will be provided by the editorial board. Manuscripts are accepted throughout the year.

SUBMISSION PROCESS

Manuscripts should be submitted to the Acta agriculturae Slovenica OJS site. The submitting author should be registered to the site. Click Register and fill in the registration form. Be sure to check in the Author

tracijskega obrazca ne pozabite odkljukati potrditvenega polja »Avtor«, sicer oddaja prispevka ne bo mogoča.

Proces oddaje prispevka poteka v petih korakih. Priporočljivo je, da se avtor pred oddajo najprej seznani s postopkom in se na oddajo prispevka pripravi:

Korak 1: Začetek oddaje prispevka
- izbrati je potrebno eno od sekcij,
- pri rubriki »Pogoji za oddajo prispevka« morate potrditi vsa potrditvena polja,
- dodatna pojasnila uredniku je mogoče vpisati v ustrezno polje.

Korak 2: Oddaja prispevka
- Naložite prispevek v formatu Microsoft Word (.doc ali .docx).

Korak 3: Vpis metapodatkov
- Podatki o avtorjih: ime, priimek, elektronski naslovi in ustanove vseh avtorjev v ustreznem vrstnem redu. Korespondenčni avtor mora biti posebej označen.
- Vpišite naslov in izvleček prispevka,
- Vpišite ključne besede (največ 8, ločeno s podpičjem) in označite jezik besedila,
- Vnesete lahko tudi podatke o financerjih,
- V ustrezno besedilno polje vnesite reference (med posameznimi referencami naj bo prazna vrstica).

Korak 4: Dodajanje morebitnih dodatnih datotek
- Grafično gradivo naj bo naloženo v eni ZIP datoteki. Grafične slike imenujte Slika1.jpg, Slika2.eps, in podobno,
- Za vsako dodatno naloženo datoteko je potrebno zagotoviti predvidene metapodatke.

Korak 5: Potrditev
- Potrebna je končna potrditev.

check box on the form. We advise you to check in also the Reader check box.

Submission process consists of 5 steps. Before submission, authors should go through the checklist and prepare for submission:

Step 1: Starting the submission
- Choose one of the journal sections.
- Confirm all the requirements of the Submission Preparation Checklist.
- Additional plain text comments for the editor can be provided in the relevant text field.

Step 2: Upload submission
- Upload full manuscript in the form of the Microsoft Word document file format (.doc or .docx).

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- First name, last name, contact e-mail and affiliation for all authors, in relevant order, must be provided. Corresponding author has to be selected.
- Title and abstract must be provided in plain text.
- Key words must be provided (max. 8, separated by semicolons) and enter the language of the text.
- Data about contributors and supporting agencies may be entered.
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- All graphic have to be uploaded in a single ZIP file. Graphics should be named Figure1.jpg, Figure2.eps, etc.
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Step 5: Confirmation
- Final confirmation is required.

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