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Asymbiotic seed germination of *Phalenopsis* Blume orchids after hand pollination

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ABSTRACT

Seven commercial hybrids of *Phalaenopsis* were included in an asymbiotic germination experiment. Plants differed in size and color of flowers and number of inflorescences and flowers. A total of 109 flowers were included of which 60 or 55% were cross-pollinated and reciprocally pollinated. Seed capsules developed on 46 (76.7%) fertilized flowers. Of these, 38 (82.6%) produced seeds as a result of 19 combinations of cross crossing and 19 combinations of reciprocal crossing. Seeds were surface sterilized using 1.6% dichloroisocyanuric acid and inoculated in Petri dishes on commercial media Sigma P1056, in two repeats. There were 23.7% contaminated Petri dishes with seeds after sterilization. The highest average percentage of protocorms (43.1%) developed in cross crossing of plants 3x7, which statistically significantly differed ($p < 0.001$) from all other combinations of cross crossing except cross crossing 2x6 (30.6%). Crossing 2x6, 1x6 (8.1%) and 1x5 (5.5%) overlap and there were no statistically significant differences between them. There were no statistically significant differences among all of the remaining crossings. There was an overlap of groups among combinations of cross crossing with small flowers x big flowers and reciprocal crossing of big flowers x small flowers. In successful crossing combinations, first plants with two leaves and one or two roots developed only 80 days after seed inoculation on media. Plant no. 4 with the smallest green-yellow flowers was not compatible with any test plants. Seed capsules without seeds developed when plant 4 was a female plant and flowers just fell off when plant 4 was a male plant.

Key words: *Phalaenopsis*, crossing, cross crossing, reciprocal crossing, seed, media, morphological stages

IZVLEČEK

ASIMBIOTSKA KALITEV ORHIDEJ *Phalaenopsis* Blume PO ROČNI OPRAŠITVI

V poskus asimbiotske kalitve je bilo vključenih 7 komercialnih orhidej iz rodu *Phalaenopsis*, ki so se razlikovale po velikosti in barvi cvetov ter številu socvetij in cvetov. Skupno je bilo na njih 109 cvetov, od teh je bilo 60 oz. 55% navzkrižno in recipročno oprašenih. Semenske glavice je oblikovalo 46 oz. 76,7% oplojenih cvetov. V 38 oz. v 82,6% semenskih glavicah so nastala semena, kot rezultat 19 kombinacij navzkrižnega in 19 kombinacij recipročnega križanja. Semena so bila površinsko razkužena z 1,6% dikloroizocianurno kislino in inokulirana v dveh ponovitvah na komercialno gojišče Sigma P1056 v petrijevke. Po razkuževanju je bilo okuženih 23,7% petrijevk s semeni. Največji odstotek nastalih protokormov (43,1%) je bil dobljen pri navzkrižnem križanju rastlin 3x7, ta odstotek se statistično značilno ($p < 0.001$) razlikuje od ostalih kombinacij navzkrižnih križanj, razen navzkrižnega križanja 2x6 (30,6%). Križanja 2x6, 1x6 (8.1%) in 1x5 (5.5%) se prekrivajo in med njimi ni statistično značilnih razlik. Prav tako ni statistično značilnih razlik med ostalimi kombinacijami križanja. Med skupinami navzkrižnih križanj rastlin z malimi cvetovi x rastlin z večjimi cvetovi in recipročnih križanj rastlin z velikimi cvetovi x malimi cvetovi pride do medsebojnega prekrivanja in med njimi ni statistično značilnih razlik. Pri uspešnih kombinacijah križanj so že po 80 dneh inokulacije semen na gojišče nastajale prve rastline z dvema listoma in eno ali dvema koreninama. Rastlina z oznako 4 z najmanjšimi, zeleno rumenimi cvetovi ni bila kompatibilna z nobeno od vključenih rastlin v križanja. V primerih, ko je bila materina rastlina, so nastale semenske glavice brez semen, v primerih, ko je bila očetova rastlina, so cvetovi po opraševanju odpadli.

Ključne besede: *Phalaenopsis*, križanje, navzkrižno, recipročno, seme, gojišče, morfološke faze

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1 INTRODUCTION

Phalaenopsis hybrids are a result of intensive breeding of plants of increasing market value as cut flowers and even more as potted plants. Because of the large, colorful and durable flowers, as well as their adaptability to room conditions, they are the most popular orchid genus in the horticultural industry (FloraHolland, 2010).

According to turnover data of Flora Holland flower auction *Phalaenopsis* were still the best-selling pot plants in Europe in 2010, as in previous years, with as many as 100 million pots sold (FloraHolland, 2010).

The Royal Horticultural Society, International Cultivar Registration Authority for Orchid Hybrids registered 643 new varieties in 2010, meaning that are altogether almost 45000 varieties of *Phalaenopsis* (RHS, 2010).

Orchid seeds are almost microscopic in size. Almost 400 years separate the time when orchid seeds were first seen and the development of a practical asymbiotic method for their germination by Knudson in 1921. Thereafter, orchid growing and hybridization became widespread. Hybrids that early growers could not even have imagined became possible (Wing Yam and Arditti, 2009).

Breeding for new varieties of *Phalaenopsis* is lengthy and time consuming. Modern hybrid seedlings are normally derived from crosses of two high-quality parental varieties. In general, breeding programs are designed to improve the size and color of the flowers, as well as other characteristics such as longevity, stalk length, leaf shape, ease of cultivation or disease resistance (Tang and Chen, 2007).

In order to study the relationship between fertility and male or female parents used in crosses, diploid *Phalaenopsis equestris* was used to cross with tetraploid commercial hybrids as either male or female parents and *vice versa* for commercial hybrids. The fertility of each cross was determined by measuring the viable seeds produced from each cross. The results showed that 50-57% of crosses produced viable seeds if *P. equestris* was used as male parent to cross with commercial hybrids. However, no viable seed was produced if *P. equestris* was used as female parent. This means failure of seed production when tetraploid plants were used as male parents to cross with diploid varieties (Tang and Chen, 2007).

On the other hand, Hicks (2000) states that a plant with smaller flowers should be used as a female plant and a plant with larger flowers as a male plant. Pollen of smaller flowers, when germinating on stigma of a larger flower, may not develop a long enough pollen tube to reach the egg cell in the ovarium of larger flowers.

In the present study, compatibility after cross pollination and reciprocal pollination of randomly chosen commercial varieties of *Phalaenopsis* and the importance of the designation of female and male plant was examined, as well as the *in vitro* growth and development of seedlings.

2 MATERIALS AND METHODS

2.1 Plant material

Seven commercial hybrids of *Phalaenopsis* were included in an asymbiotic germination experiment. Plants differed in color and size of flowers and number of inflorescences and flowers (Table 1). Flowers were cross pollinated and reciprocally pollinated by hand. Some flowers were fertilized after pollination and others fell off prematurely. Fertilized flowers developed into seed capsules, with or without seeds. Those seeds were used for asymbiotic germination. Seed capsules were collected gradually as they opened at maturity. The first one was collected in 6 months and the last one in 9 months after pollination. Seeds were air dried and stored at room temperature until sterilization and inoculation on media.

Table 1: Description of *Phalaenopsis* hybrids used in crossing

Plant label	Flower		Number	
	size	color	inflorescences	flowers
1	small	pink	2	16
2	small	pink	2	14
3	middle	purple	2	15
4	smallest	green-yellow	3	21
5	large	white	1	9
6	large	white	2	16
7	large	white	2	18

2.2 Seed sterilization and inoculation on media

Seed was sterilized and inoculated in laminar flow on commercial media P1056 (Sigma-Aldrich, St. Loius, MO, USA) with 2.6 g/l Gellan gum (Sigma-Aldrich) as gelling agent. The pH was set at 5.4 prior to autoclaving. Dichloroisocyanuric acid (Sigma-Aldrich) in a 1.6% solution was used for disinfection. Tween 20 (Sigma-Aldrich) was added for surfactant activity. Seed was transferred to microcentrifuge tubes using forceps, 0.8 ml disinfecting solution was added and the mixture was left for 8 minutes at room temperature. Disinfection proceeded for 2 minutes in a centrifuge (J2-HS, Beckman Coulter, Brea, CA, USA) at 5000 rpm (1900 x g) and 4 °C. The disinfecting solution was then removed with a micropipette and sterile bi-distilled water was added. Centrifuging and washing with water was repeated four times. After one hour, the seed was inoculated on media with a micropipette, together with the last round of washing water. The seed was spread on a Petri dish containing media using a sterile glass rod which had previously been bent to a 120° angle (Jevšnik, 2002). Seed of each combination of crossing was inoculated on media in two Petri dishes, i.e., in two repeats. Altogether there were 76 Petri dishes of 90 x 15 mm size.

2.3 Germination and growth

Germination and growth took place in a growing chamber (MPC 110, IZR, Škofja Loka, Slovenia) under a 16/8 photoperiod (16 hours of light, 8 hours of dark) at an illumination of 40 µmol m^{-2} s^{-1} and temperature of 23 ºC.

2.4 Evaluation

Cultures were examined with a stereomicroscope (C-PS, Nikon, Tokyo, Japan) at 20x magnification to assess germination (swollen and not-swollen seeds) and protocorms development. The development of leaves and roots was assessed visually. Nine evaluations were performed during the experiment. The first evaluation was performed 2 days after seed inoculation on media. Others followed 6, 14, 22, 40, 50, 65, 80 and 100 days after inoculation. Germinated and non-germinated seeds were counted, as well as vital protocorms with or without rhizoids and leaves and roots per square centimeter in each Petri dish.

Data were analyzed using the statistical program R (R Development Core Team, 2011). Treatments consisted of crossings: cross crossing (N) and reciprocal crossing (R) and of six plants: 1, 2, 3, 5, 6 and 7: 6 cross crossings with the following combinations of plants: N1x5, N1x6, N1x7, N2x6, N2x7, N3x7; and 6 reciprocal crossings: R5 x 1, R6 x 1, R6 x 2, R7 x 1, R7 x 2 and R7 x 3. The treatments N2x7 and N7x2 were excluded from statistical analysis because there was no repeat evaluation. There were 12 treatments altogether of which 10 were included in statistical analysis (Figure 2).

Equality of variance among treatments was tested using Levene's test of homogeneity of variance. For the percentage of protocorms, analysis of variance of random groups was performed on transformed data (*arcsin*(*sqrt*(portion of protocorms))). Duncan's multiple range test was used for multiple comparison between pairs of treatments ($\alpha = 0.05$).

3 RESULTS

3.1 Cross and reciprocal crossing

Table 2: Number of opened, pollinated, fertilized and detached flowers after cross and reciprocal crossing of *Phalaenopsis* orchids

Plant label	1. inflorescence				2. inflorescence				3. inflorescence				Total			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1	9	6	5	1	7	6	4	2	0	0	0	0	16	12	9	3
2	8	4	2	2	6	5	3	2	0	0	0	0	14	9	5	4
3	8	4	3	1	7	5	2	3	0	0	0	0	15	9	5	4
4	7	1	0	1	6	3	3	0	8	3	3	0	21	7	6	1
5	9	5	4	1	0	0	0	0	0	0	0	0	9	5	4	1
6	8	4	3	1	8	4	4	0	0	0	0	0	16	8	7	1
7	9	5	5	0	9	5	5	0	0	0	0	0	18	10	10	0
Total	58	29	22	7	43	28	21	7	8	3	3	0	109	60	46	14

Legend: A - opened flowers, B - pollinated flowers, C - fertilized flowers, D - detached flowers

There was a total of 109 flowers on 7 plants, 60 (55%) of which were pollinated. We did not fertilize all the flowers per plant because the load would be too heavy. Schwallier et al. (2011) suggest that a maximum of 3 flowers per plant or one flower per inflorescence should be pollinated. After pollination, 14 flowers (23.3%) fell

off prematurely. Seed capsules developed on 46 (76.7%) of fertilized flowers. Of those, 38 (82.6%) seed capsules produced seeds and 7 (15.2%) seed capsules dried prematurely. One seed capsule in the combination cross crossing with plant no. 4 was empty, without seeds. In the reciprocal crossing in which plant no. 4 was the male plant, all the flowers dropped off (Table 2). A higher percentage of fallen flowers after pollination could be a consequence of plant overload, although additional fertilizers were added during capsule development.

Thirty-eight crossings produced seed capsules with germinable seeds, of which 19 were combinations of cross crossing and 19 were combinations of reciprocal crossing. Out of 7 plants included in crossing, 6 plants produced an ovule (female plants) and pollen (male plants) (Table 3).

Flowers from plant 1 were included in crossing 9 times: 4 times with plant 5, 3 times with plant 7 and 2 times with plant 6. Plant 2 was included in crossing 5 times: 4 times with plant 6 and once with plant 7 (excluded from statistical analysis, no repetition). Plant 3 was included in crossing 5 times with plant 7. All these combinations were successful, i.e., seeds with embryo developed in seed capsules. Seed capsules were collected when they started to open, i.e., 200 to 270 days after pollination.

Plant 4 was excluded from statistical analysis because it was not compatible in crossing with any test plant.

3.2 Seed sterilization and inoculation on media

Seeds were inoculated on media after sterilization (using 1.6% dichloroisocyanuric acid) and being soaked in water for one hour at room temperature. Contaminated seeds were gradually excluded from the experiment until the contaminations stopped. There were 23.7% contaminated Petri dishes with seeds in total, of which 22.3% were with mould, 66.7% with bacteria and 11.1% with a combination of mould and bacteria. Neither crossing combination was completely lost due to contamination, only seeds from one Petri dish, i.e., one repeat was lost.

A combination of 1.6% dichloroisocyanuric acid and 10 minutes of sterilization was very effective. With the use of a centrifuge, the surface tension of seeds was neutralized, which enabled the sterilization solution to access the surface of seeds, thus adding to the effectiveness of sterilization. It is also possible to remove the sterilization solution much more easily with a micropipette, while seeds were collected at the bottom of the microcentrifuge tubes, as previously reported also by Jevšnik (2002). The combination of the selected concentration of sterilization solution, sterilization agent and sterilization time beneficially affected seed sterilization, since 76.3% of the seed combinations remained uncontaminated. Hicks (2000) recommended calcium hypochlorite as the best disinfecting agent for orchid seeds but notes more than 50% contamination after such sterilization.

3.3 Morphological stages

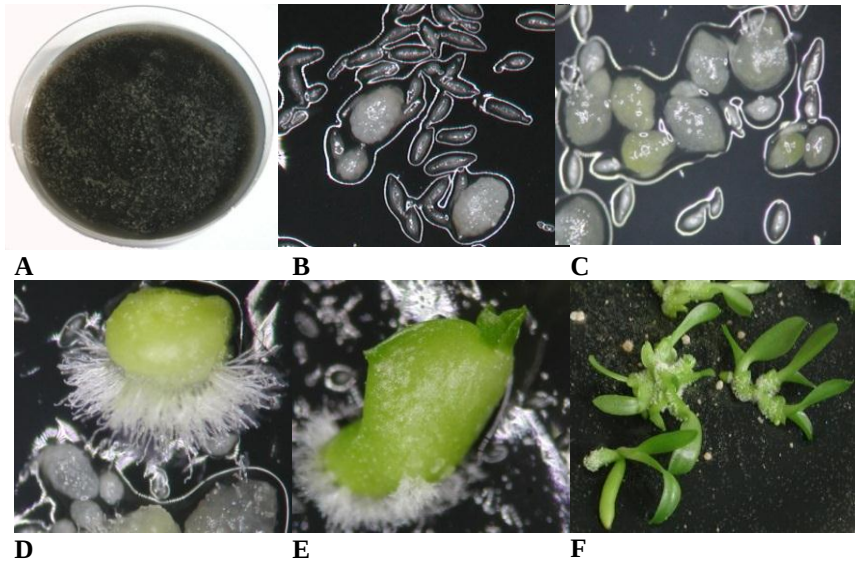

Fig. 1: *In vitro* germination, growth and development of *Phalaenopsis* hybrids: A - sterilized seeds inoculated on media; B - 6 days after inoculation the majority of seeds were unswollen, only a few seeds had developed into protocorms; C - 22 days after inoculation some white protocorms had started to produce chlorophyll; D - 40 days after inoculation green protocorms had formed rhizoids; E - 65 days after inoculation the first leaves had appeared; F - 80 days after inoculation there were plants with 1 - 2 leaves and one or more roots.

The beginning of germination was considered to be when embryos started to swell and to grow, which was clearly visible through transparent testa using a stereomicroscope. That stage was already noticeable after two days of inoculation of seeds on media, regardless of the cross combination. Bhattacharjee et al. (1999) reported seed swelling only 14 days after inoculation but those seeds had not previously been soaked. In our case, seeds were soaked for one hour in water prior to inoculation. In addition, media for inoculation and the microclimate in the Petri dish had a high relative humidity. Protocorms already started to form 6 days after inoculation and, after 20 days, chlorophyll in the protocorms started to multiply. In the period up to 40 days following inoculation, rhizoids and the beginnings of the first leaves were seen.

A lot of green protocorms with rhizoids and the beginnings of leaves started to decay after 40 days after inoculation. The reason for browning may have been an unsuitable media composition, an excess or deficit of a particular element. It is probably due to the use of full concentration P1056 media, which contained too many nutrients for normal development. Protocorms should probably be sub-cultivated on half strength media. It is known from the literature that many orchid species germinate well on less complex media, i.e., with fewer nutrients that are not required for further growth and development (Rasmussen, 1995). Protocorms that remained vital developed leaves and roots very quickly on this media.

Sixty-five days after inoculation, most protocorms had second leaves and the beginnings of roots. In the following 14 days (80 days after inoculation), plants with two leaves and one to two roots appeared. Hinnen et al. (1989) noted the importance of nitrogen in media, which influences growth. They also mentioned that strong shoot growth reflects weaker root development, which they define as the ratio shoot/roots. Media P1056 contains nitrogen in the form of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3). A nitrate form of nitrogen is more accessible to a plant, but only when the plant reaches the developmental stage where enzymes started to function. The addition of an ammonium form of nitrogen as well is therefore recommended (Hinnen et al., 1989).

The first plants started to appear 80 days after inoculation of the seeds on media. The development of *Phalaenopsis* seedlings *in vitro* can take from 50 to 724 days and for further *in vivo* development, another 4.2 to 31.5 months. Seedlings can flower for the first time after one year or after 11 years, depending on the species (Arditti, 1992).

The main problem in developing new varieties is the long time period needed for the first flowering induction. Selection of new varieties is based on the color and shape of the flowers and it is therefore very important for new plants to flower as soon as possible. It is possible to induce *in vitro* flowering in vegetatively propagated shoots of *Phalaenopsis* but flowers are often deformed, although they can indicate an interesting phenotype (Duan and Yazawa, 1995).

3.4 Percentage of developed protocorms 40 days after inoculation

The percentage of developed protocorms of all 12 treatments is shown in Figure 2. According to the data, they were transformed with the *arcsin*(*sqrt*(portion of protocorms)) function. The assumption of the analysis of variance test that variances among treatments are equal was accepted (Levene's test p = 0.3667).

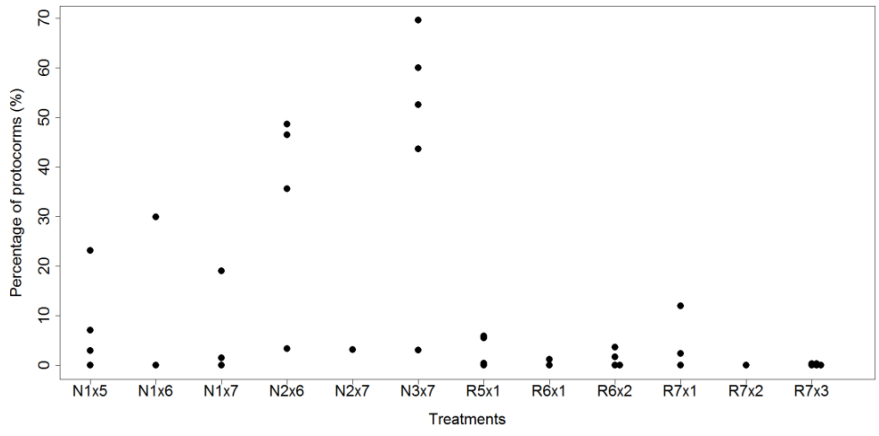

Fig. 2: Percentage of developed protocorms after cross and reciprocal crossing for each repetition of the same cross combination for 6 *Phalaenopsis* orchids.

Table 3: Crossing combination of 6 *Phalaenopsis* orchids and the average of developed protocorms 40 days after inoculation and the percentage of grown plants 80 days after inoculation. Averages of percentages followed by identical letters are not significantly different according to Duncan's multiple-range test ($p < 0.001$).

Crossing	Plant combination	Flower size	Number of repeti-tions	Standard error of mean (%)	Number of days after seed inoculation		
					40 days - average percentage of protocorms		80 days - % of plants
N	3♀ x 7♂	middle x large	5	0.9	43.1	a	2.8
N	2♀ x 6♂	small x large	4	1.1	30.6	ab	1.4
N	1♀ x 6♂	small x large	2	2.3	8.1	bc	1.3
N	1♀ x 5♂	small x large	4	1.1	5.5	bc	1.2
N	1♀ x 7♂	small x large	3	1.5	3.6	c	0.8
R	7♀ x 1♂	large x small	3	1.5	2.9	c	0.7
R	5♀ x 1♂	large x small	4	1.1	1.8	c	0.8
R	6♀ x 2♂	large x small	4	1.1	0.7	c	0.5
R	6♀ x 1♂	large x small	2	2.3	0.3	c	0.5
R	7♀ x 3♂	large x middle	5	0.9	0	c	0

Legend: N - cross crossing, R - reciprocal crossing, ♀ - female plant, ♂ - male plant

Analysis of variance showed that there are statistically significant differences between the average percentage of developed protocorms ($p = 0.0009$) among treatments (Table 3).

The largest average of protocorms (43.1%) developed in cross crossing of plants N3x7, which statistically significantly differed from all other crossing combinations, except cross crossing N2x6 (30.6%). There are no statistically significant differences between crossings N2x6, N1x6 (8.1%) and N1x5 (5.5%) and there are no statistically significant differences among all of the remaining crossings. There is an overlap of groups among combinations of cross crossing with small flowers x big flowers and reciprocal crossing of big flowers x small flowers (Table 3).

There is a statistically significant difference between cross crossing of plants N2x6 and reciprocal crossing of R6x2, in which a higher proportion of protocorms developed in cross crossing N2x6 (30.6%) compared to reciprocal crossing R6x2 (0.7%). Statistically significant differences in the average number of developed protocorms also appeared between cross crossing N3x7 and reciprocal crossing R7x3. In reciprocal crossing R7x3, protocorms did not develop at all while the proportion of developed protocorms was the highest in cross crossing (43.1%).

In general, more protocorms developed in cross combination when plant with smaller flowers was a female plant than when it was a male plant (Table 3).

Plant no. 4 was not compatible with any test plants. Seed capsules without seeds developed when plant 4 was a femaleplant and flowers just fell off when plant 4 was a male plant. Hicks (2000) notes that it is very important how the parental plants are chosen. If there are two plants, one with bigger flowers than the other, the plant with bigger flowers should be used as the pollen donor. The reason for this is that germinating pollen does not always develop a long enough pollen tube, capable of reaching the ovarium of the host plant. In our case, plant no. 4 had slightly smaller flowers than all other plants. In addition, it had green-yellow flowers,

which are usually poorly compatible in crossing, as was confirmed in our case.

4 ACKNOWLEDGEMENT

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Selenium supplementation stimulates vegetative and reproductive growth in canola (*Brassica napus* L.) plants

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ABSTRACT

Selenium (Se) is a beneficial element for higher plants and its positive effect on plants growth and performance has been reported. Effect of Se under non-stress conditions especially during reproductive phase has not been attracted enough attention. In this work effect of Se supplementation at 0, 10 and 20 µg Se plant^{-1} was studied in canola (*Brassica napus*'RGS') plants during vegetative and reproductive phase of growth under greenhouse conditions. Selenium addition resulted in a significant enhancement of dry matter production of vegetative parts as well as pod and seed dry weight. In addition, Se supplementation caused a considerable acceleration of reproductive events. In vegetative plants, higher photosynthesis rate, carbohydrates and protein content in the leaves was observed in Se treated plants compared with control. Our results suggested beneficial effect of Se on canola seed yield that may also contribute in improving nutritional value of canola for livestock and human.

Key words: flowering, pod dry weight, reproductive phase, seed yield

IZVLEČEK

DODATEK SELENA POSPEŠUJE RAST IN REPRODUKCIJO PRI RASTLINAH KANOLE (*Brassica napus* L.)

Selen (Se) je za višje rastline koristen element, znani so njegovi ugodni vplivi na rast rastlin. Pri rastlinah, ki rastejo v nestresnih razmerah, zlasti v reproduktivni fazi razvoja, učinki Se še niso bili ustrezno raziskani. V tem delu so avtorji raziskovali dodatek Se (0, 10 in 20 µg Se na rastlino) pri kanoli (*Brassica napus* L., cv. RGS) tekom vegetativne in reproduktivne faze. Dodatek Se je povzročil značilno povečanje sušine vegetativnih delov, luskov in semen. Dodatek Se je vplival tudi na pospešitev poteka reprodukcije. Pri rastlinah je bila ugotovljena tudi povečana fotosinteza, večja vsebnost ogljikovih hidratov in beljakovin v listih tretiranih rastlin v primerjavi s kontrolo. Rezultati kažejo na ugoden vpliv Se na pridelek kanole, kar lahko vpliva tudi na izboljšano hranilno vrednost te poljščine za živali in ljudi.

Ključne besede: cvetenje, sušina luskov, reproduktivna faza, pridelek semen

1 INTRODUCTION

Selenium (Se) has long been recognized as an essential micronutrient for animal and human nutrition, but the essentiality of Se to higher plants is still under debate (Terry *et al.,* 2000; Germ *et al.,* 2007). Growth stimulating effect of trace amounts of Se has been frequently reported in some plant species such as ryegrass (Hartikainen *et al.,* 2000), lettuce (Xue *et al.,* 2001), potato (Seppänen *et al.,* 2003) and different varieties of *Brassica oleracea* (Hajiboland and Amjad 2007). At proper levels it also delays some of the effects of senescence (Djanaguiraman *et al.,* 2005). The growth-promoting response of Se is mainly accompanied with the enhanced antioxidative capacity manifested in decreasing lipid peroxidation, marked increase in the activity of antioxidant enzymes and a peak concentration of antioxidant metabolites (Xue *et al.,* 2001). Selenium stimulates plants growth even under non-stress conditions. A considerable growth promotion up to 59% has been reported for cabbage plants in response to Se supplementation at 20 µM (Hajiboland and Amjad 2007).

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While much is known about the effect of Se on vegetative growth particularly under stressful conditions, published works on the effects of Se on reproductive events are rare. Effect of Se on seed yield was studied in soybean (Djanaguiraman *et al.,* 2004) plants. Spraying leaves with Se increased seed yield in soybean likely due to a better partitioning efficiency, as evidenced by greater number of pods per plant, seeds per pod and seed weight (Djanaguiraman *et al.,* 2004). However, effect of Se on the time of flowering and other characteristics of reproductive growth has not been investigated so far.

Species of Brassicaceae are able to take up more sulfur from medium and also need higher sulfur on dry weight basis compared with Graminae and Leguminosae species (Marschner 1995). Since Se (SeO_4^{2-}) is taken up and assimilated through uptake system and biochemical reduction pathway of sulfur (SO_4^{2-}) respectively (Terry *et al.,* 2000, White *et al.,* 2004; Germ *et al.,* 2007), it is likely to be more taken up and assimilated in the members of Brassicaceae compared with other species. It may also result in higher responsiveness to Se treatment in Brassicaceae compared with other plant families.

Canola (*Brassica napus*) (Brassicaceae) is considered a secondary accumulator of Se with concentrations of several hundred mg Se/kg dry weight when grown in soils with moderate levels of Se (Terry *et al.,* 2000). Agronomic biofortification of food and feed crops with Se can improve their nutritive quality (Vogrincic et al., 2009; Seppänen *et al.,* 2010; Stibilj *et al.,* 2011).

This study was aimed to investigate the effects of selenium on vegetative and reproductive growth in canola plants. Some physiological parameters during vegetative growth as well as timing of reproductive events and seed yield were studied using a spring canola cultivar grown hydroponically under greenhouse conditions.

2 MATERIALS AND METHODS

2.1 Plants culture and treatment

Seeds of canola (*Brassica napus* 'RGS', a spring cultivar) provided by Seed and Plant Improvement Institute (Karaj, Iran) were surface-sterilized with 1% active hypochlorite and germinated on perlite in the dark and moistened by distilled water. After germination, young seedlings were transferred to the light. One week-old seedlings were transferred to 10 L flat plastic container filled with washed perlite, 8 plants were cultivated in each container. Irrigation of plants was carried out with nutrient solution (Hoagland and Arnon 1945) or water at field capacity after daily weighing. The volume of nutrient solution was 500 ml per week in the first 4 weeks and 700 ml in the following growth period. Plants were grown in greenhouse conditions with a temperature regime of 25/18 °C day/night, a relative humidity of 70/80% and at a photon flux density of about 200-300 µmol $m^{-2}s^{-1}$.

Two separate groups of plants were cultivated in parallel in this work. The first group were cultivated for 19 weeks and foliarly treated with selenate with three levels of Se (0, 10 and 20 µg $plant^{-1}$ Se) in order to examine Se effect on reproductive growth of plants. Reproductive phase was demonstrated as four distinct stages and the time course of reproductive phase was divided into time intervals of 10 days. Reproductive events were monitored by visual daily inspection throughout 15 weeks growth in this group. Number of plants (% over total) at each stage and over the four divided time intervals was calculated. Thereafter, plants were irrigated daily with distilled water and let to grow for further 4 weeks for development of seeds. Nineteen weeks after sawing, plants were harvested. In addition of dry matter production of vegetative parts, pods weight and length and seed dry weight (DW) were determined. Results of this experiment were presented in Figures 1, 2 and Table 1.

The second group of plants was treated with two levels of Se including control (no Se addition) and 10 µg $plant^{-1}$ Se. This group was harvested 9 weeks after sowing (shortly before flowering) and samples were used for measurement of various physiological parameters at vegetative stage. Before harvest, chlorophyll (Chl) fluorescence and gas exchange parameters were determined in the attached leaves. Results of this experiment were presented in the Tables 2 and 3.

Both groups were cultured with four independent replications (four containers) per treatment. Selenium was added gradually between the 3rd and 7th weeks after sowing as sodium selenate (Na_2SeO_4, Fluka) dissolved in the nutrient solution.

Table 1. Effect of Se supplementation on shoot DW, pod number, length and DW, and seed DW in canola (*Brassica napus* L.) plants grown for 19 weeks in greenhouse. Data of each parameter followed by the same letter are not significantly different (P<0.05).

Treatments	**Shoot DW** (mg $plant^{-1}$)	**Pod number** ($plant^{-1}$)	**Pod length** (cm $plant^{-1}$)	**Pod DW** (mg $plant^{-1}$)	**Seed DW** (mg $plant^{-1}$)
–Se	2.09±0.75 ^{b}	55±12 ^{a}	5.19±1.08 ^{a}	2.44±0.92 ^{b}	1.05±0.29 ^{b}
10 µg $plant^{-1}$ Se	4.44±0.91 ^{a}	41±17 ^{a}	5.58±1.17 ^{a}	5.41±1.13 ^{a}	1.59±0.19 ^{a}
20 µg $plant^{-1}$ Se	3.17±0.60 ^{ab}	36±15 ^{a}	5.81±1.35 ^{a}	3.49±1.10 ^{ab}	1.20±0.16 ^{ab}

Table 2. Effect of Se supplementation (10 µg plant^{-1}) on the concentration of chlorophyll a, b, carotenoids (mg g^{-1} FW) and anthocyanins (mg cyanindin^{-3}-glucoside g^{-1} FW), chlorophyll fluorescence parameters including F_v/F_0 (the ratio of variable to initial fluorescence), F_v/F_m (photochemical efficiency of PSII), F′v/F′m (excitation capture efficiency of open PSII), qP (photochemical quenching) and qN (non-photochemical quenching) and leaf gas exchange parameters including net photosynthetic rate (A, µmol CO^2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and stomatal conductance to water vapor (gs, mol m^{-2} s^{-1}), in canola (*Brassica napus* L.) plants grown for 9 weeks in greenhouse. Data of each parameter followed by the same letter are not significantly different (P<0.05).

	Chl a	**Chl b**	**Carotenoids**	**Anthocyanins**
–Se	2.07±0.19 [a]	0.65±0.06 [b]	150±9 [b]	2.44±0.94 [a]
+Se	2.18±0.25 [a]	0.78±0.08 [a]	167±2 [a]	2.69±0.47 [a]
	F_v/F_0	F_v/F_m	F'_v/F'_m	*qP*
–Se	4.82±0.37 [a]	0.82±0.01 [b]	0.63±0.05 [b]	0.82±0.14 [a]
+Se	5.23±0.19 [a]	0.85±0.01 [a]	0.72±0.03 [a]	0.98±0.22 [a]
	qN	*A*	*E*	g_s
–Se	0.37±0.07 [a]	7.98±0.14 [b]	0.62±0.09 [b]	0.96±0.08 [a]
+Se	0.42±0.09 [a]	9.27±0.24 [a]	0.77±0.04 [a]	0.99±0.01 [a]

Table 3. Effect of Se supplementation (10 µg plant^{-1}) on the total soluble sugars and starch (mg g^{-1} FW), soluble proteins (mg g^{-1} FW) and total free α-amino acids (µmol g^{-1} FW) in canola (*Brassica napus* L.) plants grown for 9 weeks in greenhouse. Data of each parameter followed by the same letter are not significantly different (P<0.05).

	Soluble sugars		**Starch**		**Protein**		**Amino acids**	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
–Se	8.0±0.97 [b]	3.4±0.02 [a]	2.4±0.25 [a]	0.25±0.04 [a]	11.4±1.3 [b]	1.2±0.11 [a]	5.2±0.3 [b]	0.98±0.1 [b]
+Se	11.5±0.85 [a]	3.6±0.34 [a]	1.6±0.06 [b]	0.03±0.01 [b]	15.4±1.2 [a]	1.1±0.06 [a]	7.2±0.1 [a]	1.23±0.1 [a]

2.2 Determination of chlorophyll fluorescence and gas exchange parameters

Chlorophyll fluorescence parameters were recorded using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK) for both dark adapted and light adapted leaves. Measurements were carried out on the 3 youngest, fully-expanded leaves. An average of 4 records from different parts of each individual leaf was considered for each replicates. Leaves were acclimated to dark for 30 min using leaf clips before measurements were taken. Initial (F_0), maximum (F_m), variable ($F_v=F_m-F_0$) fluorescence, the ratio of variable to initial fluorescence (F_v/F_0) as well as maximum quantum yield of PSII (F_v/F_m) were recorded. Light adapted leaves (300 µmol m^{-2}s^{-1}) were used for measurement of initial (F_t) and maximum (F'_m) fluorescence. Calculations were made for F'_0 ($F'_0=F_0/[(F_v/F_m)+(F_0/F'_m)]$), excitation capture efficiency of open PSII (F'_v/F'_m), photochemical quenching, *qP* [$(F'_m-F_t)/(F'_m-F'_0)$] and non-photochemical quenching, *qN* (1-[$(F'_m-F'_0)/(F_m-F_0)$]) (Oxborough 2004).

CO_2 assimilation and transpiration rates were measured in parallel with Chl fluorescence measurements in the same leaf with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) between 10:00 and 13:00 at harvest. The measurements were conducted with photosynthetically active radiation intensity at the leaf surface of 300 µmol m^{-2} s^{-1}. measured by a quantum sensor attached to the leaf chamber of the gas exchange unit. The net photosynthesis rate by unit of leaf area (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and the stomatal conductance to water vapor (gs, mol m^{-2} s^{-1}) were calculated using the values of CO_2 and humidity variation inside the chamber, both measured by the infrared gas analyzer of the portable photosynthesis system.

2.5 Determination of chlorophyll, carotenoids, anthocyanins and carbohydrates

Leaf concentration of Chl a, b and carotenoids were determined after extraction of pigments in the cold acetone and allowing the samples to stand for 24 h in the dark at 4 ºC (Lichtenthaler and Wellburn 1985). Determination of anthocyanins was performed using a pH differential method at pH 1 and pH 4.5 in the methanol/HCl (98:2, v/v) extract (Giusti and Wrolstad 2001). Concentration of total anthocyanins was expressed as g of cyanidine-3-glucoside g^{-1} FW. For determination of carbohydrates, leaves were homogenized in 100 mM phosphate buffer (pH 7.5) at 4ºC, 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 (Magné *et al.*, 2006).

2.6 Determination of soluble proteins and total free aminoacids

Soluble proteins were determined according to the method of Bradford (1976) using a commercial reagent (Sigma) and BSA (Merck) as standard. Content of total free α-amino acids was assayed using ninhydrin colorimetric method. Glycine was used for production of standard curve (Hwang and Ederer 1975).

Experiments were undertaken in complete randomized block design with 4 replications. Statistical analyses were carried out using Sigma Stat (3.02) with Tukey test ($p<0.05$).

3 RESULTS AND DISCUSSION

Dry matter production of shoot increased by Se supplementation, this effect was significant for the lower Se level (10 μM). Pod DW (but not pod length) and seed DW were also significantly higher in Se supplemented plants. In contrast to other parameters, Se treatment influenced negatively the number of pods, though this effect was not statistically significant (Table 1). According to daily inspection of flowering plants, lower pod number was not due to reduction in the number of flowers or seed abortion. There is a known adverse relationship between the number of fruits and their size in plants, thus, an increase in pod and seed DW can reasonably be expected from reduction in the number of pods in this work. However, pod DW at harvest could be also the result of interaction of many other factors.

Number of leaves was slightly higher in the presence of 10 μM Se, while at higher Se concentration (20 μM) it did not differ from control plants. In contrast, in the reproductive growth phase, the length of inflorescence was higher in Se treated plants and 20 μM Se was more effective than 10 μM Se (Figure 1). Selenium accelerated all four stages of reproductive growth and the two applied Se concentrations did not differ considerably in this respect. The most responsive parameter was podding, that was mainly influenced by Se application in last three intervals (Figure 2). Other reproductive characters such as timing of side branches formation and total number of side branches were not influenced by Se addition. Difference between side and main branches in the reproductive characters as influenced by Se was not also detected, thus, data of side and main branches were combined.

In vegetative plants, leaf Chl b and carotenoids content increased by Se treatment, while Chl a and anthocyanins contents were not affected by Se supplementation. Maximum quantum yield of PSII (F_v/F_m) and excitation capture efficiency of open PSII (F'_v/F'_m) increased significantly by Se treatment. Net assimilation rate was enhanced by about 16% while transpiration and stomatal conductance were influenced only slightly by Se treatment (Table 2). Accordingly, significant enhancement of the net CO_2 assimilation rate appeared to be mainly caused by increase of non-stomatal parameters such as improved leaf photochemistry as was reflected in the significant rise of F_v/F_m and F'_v/F'_m. It is also likely that Se activates photosynthetic carbon metabolizing enzymes (see below). Regarding increase in the number of leaves upon Se treatment, a considerable enhancement in the photosynthesis of whole canola plants is expected in this work. Higher Chl b and β-carotene in the leaves of Se treated plants may support photochemistry of leaves via improved protection of reaction centers against active oxygen species produced inevitably by light (Demmig-Adams and Adams 1992).

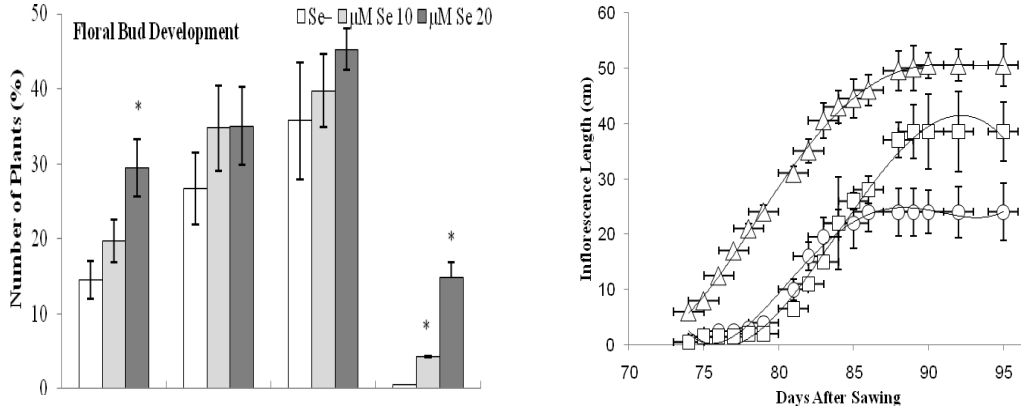

Figure 1. Effect of Se supplementation on the number of leaves (a) and length of stem (b) in canola (*Brassica napus*) plants grown in greenhouse.

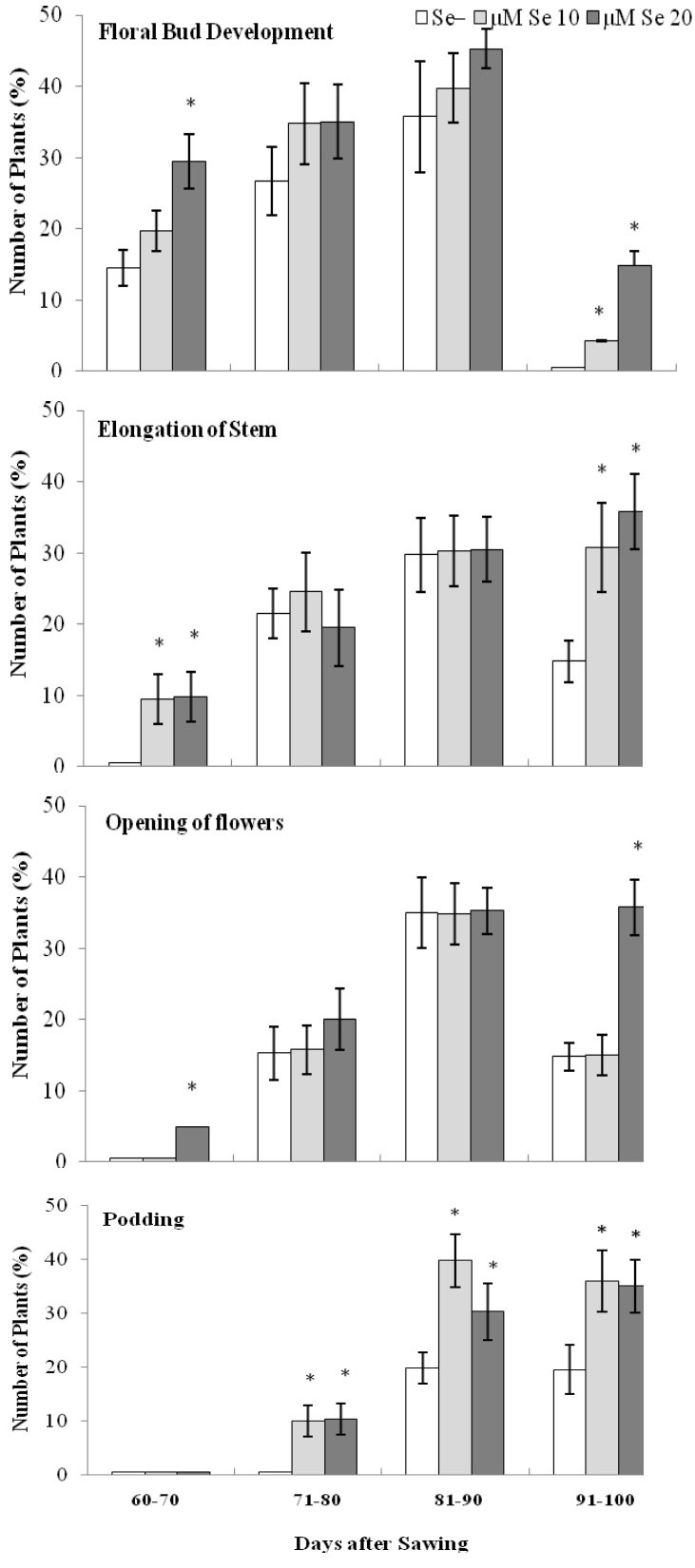

Figure 2. Effect of Se supplementation on the timing of reproductive events in canola (*Brassica napus*) plants grown in greenhouse.

In vegetative plants, leaf Chl b and carotenoids content increased by Se treatment, while Chl a and anthocyanins contents were not affected by Se supplementation. Maximum quantum yield of PSII (F_v/F_m) and excitation capture efficiency of open PSII (F'_v/F'_m) increased significantly by Se treatment. Net assimilation rate was enhanced by about 16% while transpiration and stomatal conductance were influenced only slightly by Se treatment (Table 2). Accordingly, significant enhancement of the net CO_2 assimilation rate appeared to be mainly caused by increase of non-stomatal parameters such as improved leaf photochemistry as was reflected in the significant rise of F_v/F_m and F'_v/F'_m. It is also likely that Se activates photosynthetic carbon metabolizing enzymes (see below). Regarding increase in the number of leaves upon Se treatment, a considerable enhancement in the photosynthesis of whole canola plants is expected in this work. Higher Chl b and β-carotene in the leaves of Se treated plants may support photochemistry of leaves via improved protection of reaction centers against active oxygen species produced inevitably by light (Demmig-Adams and Adams 1992).

Improved reproductive characters, acceleration of reproductive stages and seed yield increase of canola plants in this study appeared to be the result of an increased vegetative growth of plants following improved photosynthetic capacity of whole plant and enhanced carbohydrates and protein synthesis. However, effect of Se on phytohormones balance and/or polyamine content could not be excluded. Selenium treated potato plants had higher putrescine content (Turakainen *et al.,* 2008). Polyamines have been implicated in various plant growth and developmental processes including stimulation of cell division, embryogenesis, senescence, floral development, and fruit ripening (Kakkar and Sawhney 2002).

Effect of Se on the stimulation of canola flowering in this work was not accompanied by accelerating the aging process as could be judged by the higher leaf Chl concentration measured shortly before flowering. In addition, during the 4-weeks period of seed development, Se treated plants showed a considerable delay in the aging of pods. The pods had been remaining green for a longer time in Se treated plants while seed development appeared not to be influenced. It implies likely the effect of Se on delaying senescence without affecting seed development. Se addition delayed monocarpic senescence in soybean plants (Djanaguiraman *et al.,* 2004).

Timing of flowering is a critical factor in canola production. Cultivars are preferred that flower at a time before the onset of severe drought stress and high temperatures because it enables plants to complete seed development (Robertson *et al.,* 2002). In addition, Se enrichment of canola not only could accelerate flowering and improve its yield, but also contributes in the improvement of nutritional quality of canola seed and oil for livestock and human, respectively.

Although a considerable effect of Se enrichment on reproductive growth of canola was demonstrated in this work, more detailed studies are needed on the effect of Se on carbohydrates partitioning in vegetative and reproductive plants, polyamines metabolism, seed development and fruit ripening. In addition, studies are needed for finding optimum concentrations and application methods in the field in order to improve canola seed yield by Se supplementation.

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Induction of drought tolerance with seed priming in wheat cultivars (*Triticum aestivum* L.)

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ABSTRACT

Delay in sowing and low precipitation (<300mm annual) in wheat (*Triticum aestivum* L.) farming is the major problem in the irrigated and rainfall lands of Iran. A factorial experiment for evaluating the effects of seed priming on wheat cultivars was carried out under laboratory, greenhouse and at two field conditions during seasons of 2008-2010. Arrangement of treatments were Zarrin, Shariar, Sardary and Azar cultivars as A factor, and priming treatments including distilled water (DW), osmotic solutions (10% PEG, 2.5% KCl, 4% MN, 10% Urea, 5% NaCl W/V) and plant growth inducers (20 ppm IAA, 1000 ppm CCC) with non-primed seed as a control established B factor. During the second year of field experiment two separate treatments were done under drought stress and well watered conditions. Drought stress was withheld by irrigation at booting stage of plants. Maximum amount of absorbed water was determined in cultivar Shariar, 15.5 g DW. Seed weight of all cultivars increased the most when primed with CCC and IAA. Irrespective of the cultivar seedlings related traits revealed that treatment with CCC increased plumule and radical dry weights (11.5 and 8.0 mg) and their lengths (17.2 and 17.8 cm). In opposite, urea pretreatment had negative effects on seedlings growth. All priming treatments increased grain yield and its components, chlorophyll content and nitrogen absorbed under field and green house conditions in four cultivars in comparison to control. Plants arising from seeds primed with potassium chloride under drought stress had the lowest percentage of variation for traits such as relative water content (-9.3%), total dry matter (-10.7%) and grain yield (-4.0%) in comparison with well watered plants. Potassium chloride improved drought tolerance at all wheat cultivars. There were significant correlations between grain yield at primed with KCl and following wheat traits: number of spikes per square meter (0.91**), number of grains per spike (0.92**) and total dry matter (0.79*). Therefore, it seems that these traits could be used as indirect criteria for selection of high grain yield of cultivars for primed seed.

Key words: drought stress, hydro and osmo priming, plant growth inducers, common bread wheat

IZVLEČEK

INDUKCIJA TOLERANCE NA SUŠO S PREDSETVENIM TRETIRANJEM SEMEN PRI IZBRANIH SORTAH PŠENICE (*Triticum aestivum* L.)

Zakasnitev v setvi in majhna količina padavin (<300mm letno) sta glavna problema pri pridelavi pšenice (*Triticum aestivum* L.) v namakanih in nenamakanih območjih Irana. Izvrednotenje učinka predsetvenega tretiranja semena izbranih sort pšenice je bilo narejeno s faktorskim poskusom v laboratoriju, rastlinjaku in v dveh poljskih poskusih v sezonah 2008-2010. Poskus je bil zastavljen s štirimi sortami pšenice (Zarrin, Shariar, Sardary in Azar) kot faktorjem A in predsetvenimi tretmaji, ki so obsegali destilirano vodo (DW), raztopine osmotikov (0% Urea, 5% NaCl W/V) in rastlinske rastne regulatorje (20 ppm IAA, 1000 ppm CCC) primerjalno z netretiranimi semeni, kar je bila kontrola in faktor B. V drugem letu poljskega poskusa sta bili opravljeni še obravnavi s sušo in zadostnim zalivanjem. Sušni stres je bil preprečen z zalivanjem v fazi bilčenja. Največ absorbirane vode je bilo izmerjeno pri sorti Shariar, 15.5 g DW. Teža semen vseh sort se je povečala najbolj, kadar so bila semena pred setvijo tretirana s CCC in IAA. Ne glede na sorto se je pokazalo, da sta se suha teža mladega poganjka in korenine (11.5 in 8.0 mg) pri kalicah povečali kot tudi njuni dolžini (17.2 in 17.8 cm) kadar je bilo seme pretretirano s CCC. Nasprotno je imelo predtretiranje z ureo negativni učinek na rast kalic. Vsa predtretiranja so povečala pridelek zrnja in njegove komponente, vsebnost klorofila in privzetje dušika v poskusih v rastlinjaku in poljskem poskusu pri vseh sortah v primerjavi s kontrolo. Rastline, ki so zrasle iz semen predtretiranih s KCl v razmerah sušnega stresa so imele najmanjši odstotek variabilnosti v znakih kot so reletivna vsebnost vode (-9.3%), celokupna suha snov (-10.7%) in pridelek zrnja (-4.0%) v

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primerjavi z dobro zalivanimi rastlinami. Natrijev klorid je pri vseh sortah pšenice izboljšal prenašanje suše. Ugotovljene so bile značilne korelacije med pridelkom zrnja pšenice, ki je bila predtretirana s KCl in naslednjimi znaki pridelka: število klasov na kvdratni meter (0.91**), število zrn na klas (0.92**) in celokupno suho snov (0.79*). Zato izgleda, da bi lahko te znake uporabili kot posredni kriterij za izbor visokoproduktivnih sort pšenice, pri katerih se seme predtretira.

Ključne besede: sušni stres, vodno in osmotsko predtretiranje semen, rastlinski rastni regulatorji, krušna pšenica

1 INTRODUCTION

In irrigated lands, winter wheat and sugar beet fallow is the dominant rotation in 130.000 hectares of West Azerbaijan province of Iran. Planting of winter wheat is delayed after harvesting of sugar beet. In addition, low precipitation and inadequate moisture of seed zone under rainfall conditions reduces grain yield potential. Therefore, seed priming is a technology that enhances rapid (7-10 d) emergence and early establishment of wheat. Rapid and uniform field emergence is an essential prerequisite at two irrigated and rainfall conditions to reach the yield potential, quality, and ultimately profit in annual crops. Seed priming has been common pretreatment that reduces the time between seed sowing until emergence and synchronizes seedling emergence (Parera and Cantliffe 1994).

Seed priming can be accomplished through different methods such as hydro-priming (soaking in DW), osmo-priming (soaking in osmotic solutions such as PEG, potassium salts, e. g., KCl, K_2SO_4) and plant growth inducers (CCC, Ethephon, IAA) (Capron *et al.,* 2000; Chiu *et al.,* 2002; Harris *et al.,* 1999; Chivasa *et al.,* 1998).

Several investigations confirmed that seed priming has many benefits including early and rapid emergence, stand establishment, higher water use efficiency, deeper roots, increasing in root growth, uniformity in emergence, germination in wide range of temperature, break of seed dormancy, initiation of reproductive organs, better competition with weed, early flowering and maturity, resistance to environmental stresses (such as drought and salinity) and diseases (*Sclerotium rolfsii* L.): Higher grain yield in wheat (*Triticum aestivum* L.) (Ghana and Schillinger 2003), corn (*Zea mays* L.) (Subedi and Ma 2005) canola (*Brassica napus* L.) (Farhoudi and Sharifzadeh 2006), pearl millet (*Pennisetum glaucum* L.), chickpea (*Cicer arietinum* L.), rice (*Oriza sativa* L.) (Harris *et al.,* 1999 and 2005) lettuce (*Lactuca sativa* L.) (Cantliffe *et al.,* 1984) is reported from field and laboratory studies. Inversely, longevity of primed seed can be decreased (Bruggink *et al.,* 1999).

Singh and Agrawal (1977) found out that wheat which seeds were treated with DW for 12h increased nitrogen uptake for 11 kg/ha. Misra and Dwivedi (1980) reported that seed soaking in 2.5% KCl for 12 h before sowing increased wheat grain yield for 15%. Paul and Choudhury (1991) observed that seed soaking with 0.5 to 1% solutions with KCl or K_2SO_4 significantly increased plant height, grain yield and its components in wheat genotypes. Kulkarni and Eshanna (1988) stated that pre-sowing seed treatment with IAA at 10 ppm improved root length, rate of germination, and seedling vigor.

The objective of this study was to evaluate the effect of several priming solutions on early growth, grain yield and its components under laboratory and field conditions. Specific objective was to determine the effect of seed priming on improving the response of winter wheat cultivars to drought stress under field conditions.

2 MATERIALS AND METHODS

Responses of four wheat cultivars, Sardary and Azar for rainfall conditions and Zarrin and Shariar for irrigated conditions, to hydro, osmo-priming and plant growth inducers were studied. Seed from latest harvest was used. and treated with eight priming media: 1- hydropriming (DW), 2- osmopriming (2.5% KCl, 10% urea, 5% NaCl, 4% MN and 10% PEG 8000 W/V), 3- plant growth inducers (20 ppm IAA, and 1000 ppm CCC). Non-treated seeds of each cultivar were used as control. All priming media were prepared in distilled water and seeds soaked at 25°C. The duration of soaking for hydro, osmopriming and plant growth inducers were 16 h and 30 min, respectively. 500 g of seeds of each cultivar was placed in 36 one liter capacity bashers and immersed in liquid priming media. After soaking weight of seeds was recorded and rinsed three times with tap water. All seed sets were surface sterilized with 10% sodium hypochlorite solution for 10 minutes, then rinsed with sterilized water and air dried at room temperature (25°C) for 20 days. After air-drying, the weight of seed sets was recorded again, and amount of water absorbed during soaking was determined (Subedi and Ma 2005, Ghana and Schillinger 2003).

2.1 Laboratory experiment

Germination test of dried seed was measured in laboratory using a factorial experiment based on Completely Randomized Design for 36 combination treatments with five replications. Factor A and B included four wheat cultivars and nine priming media+control, respectively. For each treatment 100 seeds were placed on five 90 mm diameter petri dish. Two filter

papers of Whatman No. 2 were moistened with 10 mL of distilled water. Seed was kept at germinator in 20°C for 10 days under 16/8 h day/night light. After this period plumule and radical lengths, and dry weights of them were measured.

2.2 Greenhouse experiment

Plants were grown in 0.5 L plastic pots (5 cm diameter) filled with a mixture of soil, peat moss, Vermiculite and Perlite (3:1:5:1 v/v). Decision for greenhouse treatments was based on germination performance from the laboratory experiment. Urea and NaCl had negative effect on germination therefore these treatments were removed. The experimental design was the same as in laboratory experiment. Three uniform seed sets of each treatment were sown on 25 February 2008. At seedling emergence (10 days after planting), one gram of NH4NO3 fertilizer was applied per pot at each irrigation. Pots were regularly watered. The temperature inside the greenhouse was maintained at 25/15°C (day/night regime ± 3°C) with 10 h photoperiod. At 60 days after planting, when plants were at five leaves stage, plants were removed and oven dried at 80°C for 24 h and then nitrogen uptake was measured (Bremner and Mulvaney 1982). Leaf chlorophyll content was measured with using SPAD-502.

2.3 Field Experiments

Field experiment was carried out in West Azerbaijan agricultural research center in 2009-10. The experimental field station was located in latitude 45º 22'N, 75º 32' 36° 58´, longitude 46° 6´ and altitude 1371 m, by a typical silty loam texture.

At the first year, seed lots used in the laboratory experiment were planted with factorial experiment based on randomized complete blocks design with five replications. Chemical fertilizers were applied pre-planting according soil analysis, therefore 100 kg per hectare NH_4NO_3 was applied before planting. At the booting stage, 1.5 $L.ha^{-1}$ of 2- 4-D was used for weed control.

In the second year the same treatments were carried out at two separate factorial experiments based on randomized complete blocks designs under drought and well-watered conditions. In drought experiment water was withheld at the booting stage and irrigation was done after 150 ± 5 mm evaporation from the Class A Pan. Well watered plots were irrigated after 75 ± 5 mm evaporation from the Class A Pan (Table 1). To determine above ground biomass, four central rows were harvested upon maturity. Total dry matter, grain yield, 1000-kernel weight, spike/m2, grain per spike, relative water content (Gonzalez 1999) and plant height were measured.

Analyses of variance for all data's of laboratory, greenhouse and field experiments were conducted by Mstat-c software. Treatments were considered significantly different at $p \leq 0.05$.

Table 1. General characteristics, summary of water inputs (rainfall and irrigation), class A pan evaporation and maximum and minimum temperatures in 2009-10 at field conditions.

		Month	Tmax (°C)	Tmin (°C)	Rain (mm)	Irr. (mm)	Evap. (mm)
Number of cultivars	4						
Number of pretreatments	7						
Number of combination treatments	28	October	26.1	9.2	5.5		124.1
Total plots	280	November	15.6	9.3	27.7		45.2
Density of plants	400	December	8.9	1.5	32		
Internals between blocks	1.3m	January	7.7	-0.7	38.4		
Intervals between rows	0.2m	February	5.9	-3.8	16.6		
Rows per plot	6	March	14.4	-0.4	44.6		
Plot size	$1.2\times2m^2$	April	15.9	5.3	63.6	25	57.7
Harvest area per plot	$1m^2$	May	20.2	6.9	34.1	33	130.9
Replications per experiment	5	June	28.6	10.7	3.8	110	232.3
Ec of water irrigation	0.024ds/m	July	33	15.7	4.4	130	314.6

3 RESULTS AND DISCUSSION

3.1 Seed Soaking

The greatest amount of absorbed water within cultivars was observed for Shariar with DW and the lowest amount corresponded to Zarrin and Shariar with CCC pretreatment (Table 2). Priming with CCC and IAA pretreatments had the shortest time of imbibition and the lowest absorbed water to the other types, but the most increased seed weight. In general, increased weight of primed seed lots was due to activation of cell respiration (Bewley and Black 1994), repairs of macromolecules (Osborn 1993), movements of acquired materials (Gallardo *et al.*, 2001), activation of cell cycling (Vasquez-Ramos and Sanchez 2004) and weakening of seed coat structure for root emergence (Cantliffe *et al.*, 1984). Water absorption is the first stage of germination, at the second stage or retardation stage, seeds start the replication of DNA (Bray *et al.*, 1989), increasing of protein and RNA synthesis (Gallardo *et al.*, 2001), availability of more ATP (Mazor *et al.*, 1984), rapid embryo growth (Dahal *et al.*, 1990) than control seeds.

Table 2. Changes in weight and moisture content of wheat seed cultivars after soaking and air-drying for 20 days in 25°C.

Cultivar	Seed weight (g)	Pretreatment DW	NaCl 5%	Urea 10%	KCl 2.5%	PEG 10%	MN 4%	IAA 20ppm	CCC 1000ppm
Sardary	water absorbed	9.68	2.43	3.42	10.44	11.48	12.04	3.60	2.28
	Increased seed weight	0.42	0.33	0.32	0.96	1.12	1.02	1.20	1.12
Azar	water absorbed	13.32	2.55	2.96	9.52	7.76	12.40	4.4	3.68
	Increased seed weight	0.16	0.17	0.05	0.20	0.40	0.28	0.76	0.84
Zarrin	water absorbed	10.56	2.78	3.1	8.88	6.32	11.20	2.68	1.92
	Increased seed weight	0.52	0.22	0.25	0.80	1.16	0.52	0.96	1.20
Shariar	water absorbed	15.12	3.35	3.77	13.32	9.88	13.32	4.88	1.96
	Increased seed weight	0.4	0.12	0.15	0.60	0.88	0.44	0.96	1.24

3.2 Seedling Vigor and Plant Stand

Root lengths of Shariar and Zarrin seedlings at pretreatments with IAA and CCC were 22.3, 22.0, 22.0 and 22.5 cm, respectively (Fig. 1-A), which is much more than in other treatments. This effect could be related to CCC and IAA enhanced cell divisions at root tip (Farooq *et al.*, 2006; Fu *et al.*, 1988). The trend of variation between cultivars and pretreatments for plumule length was similar with root length, however, at urea pretreatment length of both decreased (Fig. 1-B). Irrespective of cultivar, pretreatments with CCC, IAA and DW with 8.1, 8.0, 8.1 g had bigger effect on radical dry weights (Fig. 1-C). Within pretreatments, potassium chloride had the most positive effect on plumule dry weight, 12.6 g, and urea treatment had the most negative effect, 7.1 g (Fig. 1-D). Increased plumule dry weight due to osmopriming was reported by Harris *et al.*, (2004). It caused rapid establishment of plants during germination and ultimately higher production of dry matter. Rapid germination in primed seeds was caused by increased enzyme activity, including of alfa amylase, higher levels of ATP, increased synthesis of RNA and DNA and increased number and efficiency of mitochondria (Bittencourt *et al.*, 2005).

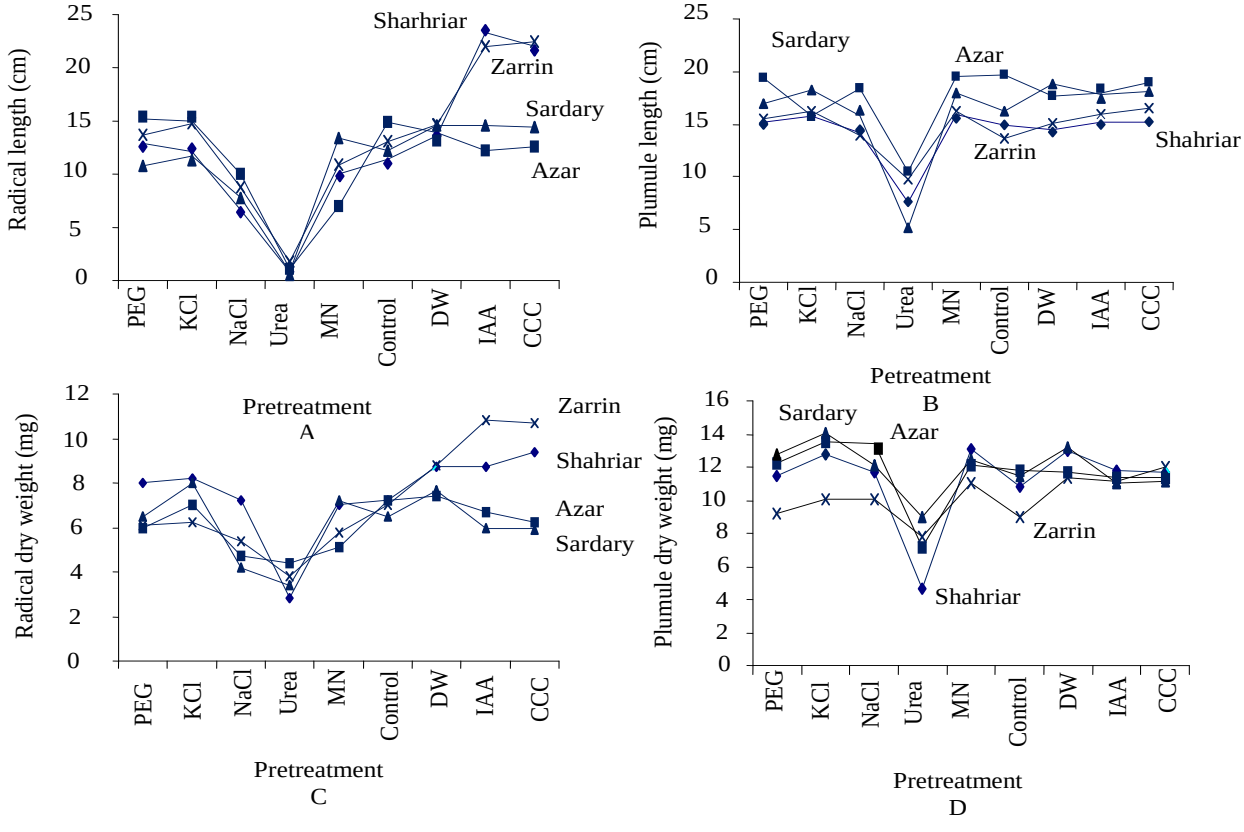

Figure 1. The effects of different seed pretreatments in wheat cultivars on seedling related traits.

3.3 Response to Nitrogen

Cultivars Azar and Sardary had the highest chlorophyll content at IAA pretreatment while the highest content of chlorophyll in cultivars Zarrin and Sharriar was determined in DW treatment (Fig. 2-B). All pretreatments in four cultivars had higher nitrogen absorption than control. The highest nitrogen absorption, 57.3% was measured in cultivar Shariar and the lowest in cultivar Sardary, 47.6% (Fig. 2-A). In all cultivars priming with CCC and IAA resulted in higher nitrogen absorption in comparison to the other pretreatments. The reason behind that is probably the increase of the root length, which was seen in the laboratory evaluation. The increase of nitrogen absorption at priming with plant growth inducers may finally cause improvement of grain yield. Absorbed nitrogen directly effects on leaf chlorophyll content, and, in turn, improves metabolism and photosynthesis (Kulkarni and Eshanna 1988).

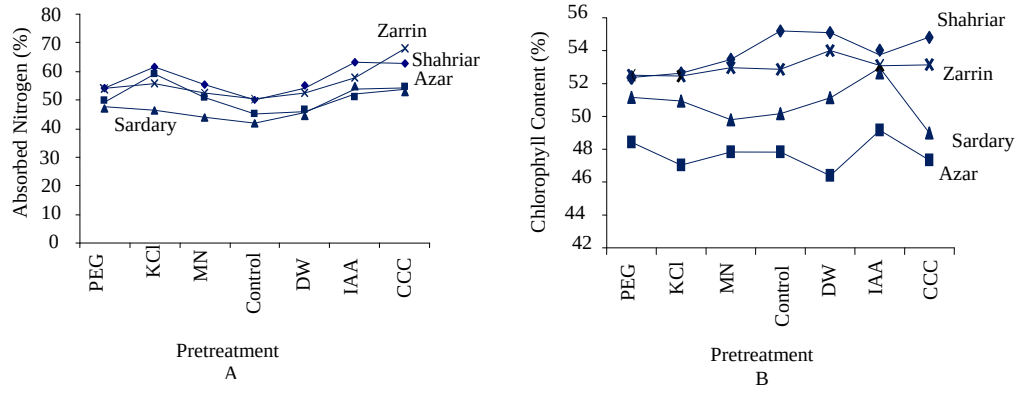

Figure. 2. The effects of different seed pretreatments in wheat cultivars on traits of chlorophyll content and absorbed nitrogen under green house conditions

3.4 Morpho-physiological traits

Under field conditions, all of pretreatments at four cultivars gave bigger grain yield than control and among them CCC treatment gave the highest yield, 591 g/m^2. Responses of cultivars varied with the type of pretreatments. Therefore, the grain yields of cultivar Shariar were 635, 625 and 613 g/m^2 for hydropriming, CCC and MN, respectively. Cultivar Zarrin treated with IAA and CCC had 628 and 620 g/m^2 grain yield, respectively. Pretreatment with CCC gave the grain yield for cultivars Sardary and Azar 590 and 520 g/m^2, respectively (Fig. 3-A). The increase of grain yield with pretreatments was due to the expansion of leaves, which resulted in higher photosynthesis, assimilation and ultimately higher production of total dry matter. Accumulated priming materials in plants were effective during seed set and grain filling (Haris *et al.*, 1999 and 2004). Many researchers reported the increase of grain yield in wheat cultivars due to pretreatments, as 37% in (Misra and Dwibedi 1980), and 15% (Haris *et al.*, 1999 and 2004). Success in seed priming depends on type of cultivar, osmotic potential of solution, time of priming, temperature environment, seed vigor, the rate of seed re-drying and the conditions during primed seed storage (Parera and Cantliffe 1994).

The highest number of grains per spike, 66 grains, was counted for cultivars Shariar and Zarrin, and the lowest values was determined in cultivars Azar and Sardary, with 36 and 30 grains respectively (Fig. 3-B). Irrespective of cultivar, pretreatments with IAA, CCC, DW and MN had 54, 53, 52 and 51 grains per spike, respectively. Pretreatments with CCC for 1000-Kernel weight of 45 g and treatments by CCC, DW and MN which gave 372, 371 and 366 spikes per square meter were the highest values of these parameters (Fig. 3-C and D). The range of variations for number of spikes per square meter was between 277 and 392, related to cultivars Sardary and Zarrin, respectively.

The highets total dry matter was measured for pretreatments DW, IAA and CCC, with values of 1198, 1185.3 and 1196.6 g/m^2, respectively (Fig. 3-E). The increased number of spikes per square meter at all pretreatments is a reason for higher total dry matter production. The smallest plant height was obtained for IAA and CCC pretreatments (Fig. 3-F).

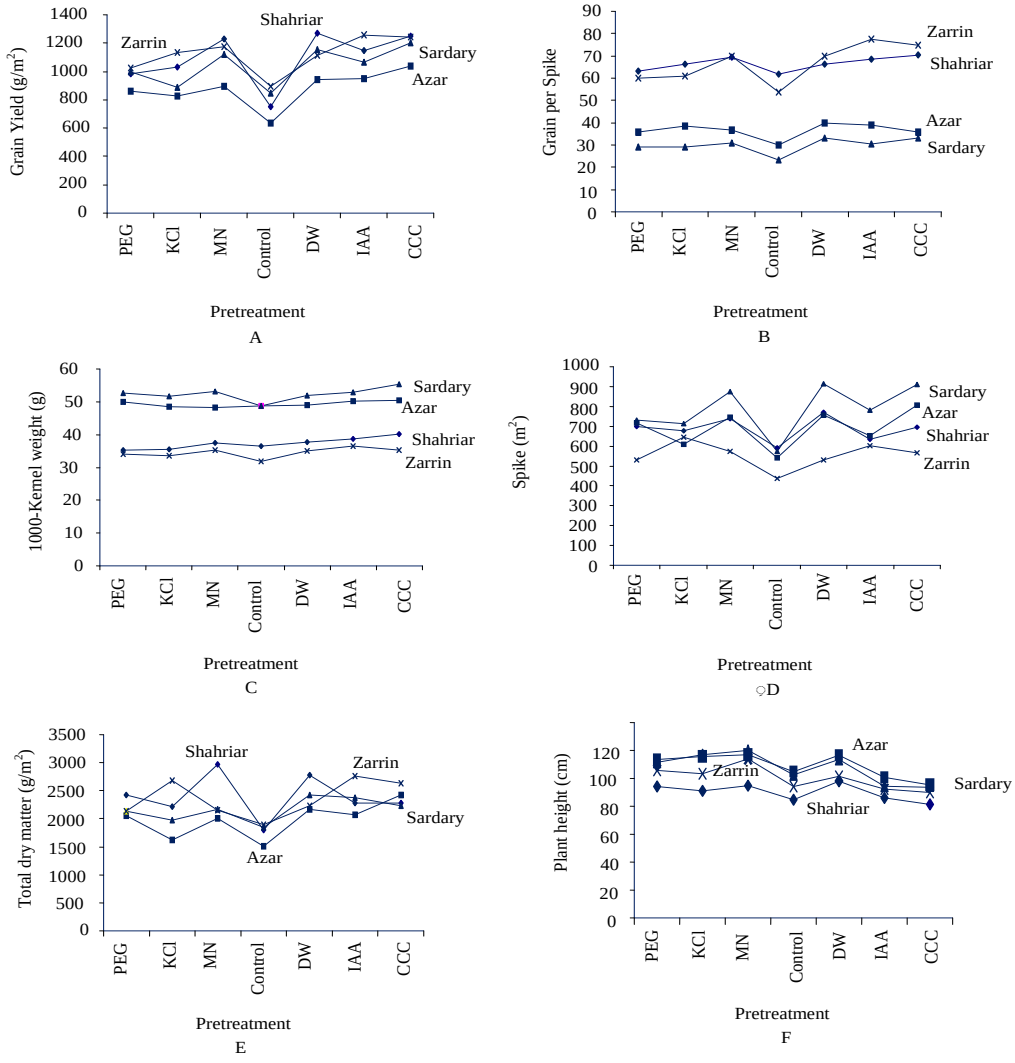

Figure. 3. The effects of different seed pretreatments in wheat cultivars on morpho-physiological traits under field conditions.

3.5 Inducing of tolerance to drought stress

The highest variation in grain yield under drought stress compared to well watered plants were measured in cultivar Shahriar (39 %), primed with IAA, cultivar Azar (23 %), primed with CCC, cultivar Sardary (32%) primed with PEG and cultivar Zarrin (45%) primed with CCC. In contrast, pretreatment with potassium chloride in all four cultivars had the lowest variations in grain yield (Table 3). Potassium ion induced tolerance to drought stress under drought treatment (Khajeh-hosseini *et al.*, 2003). Depending on the cultivar responses of grain yield components to pretreatments were different. The percentage of variation in grain yield components within primed seeds with potassium chloride at four cultivars showed that under drought stress 1000-kernel weight had the lowest value. In contrast, except cultivar Sardary, maximum percentage variation was seen for number of spikes per square meter (Fig. 4). Saha *et al.*, (1990) reported that performance of grain yield at primed seeds of soybean had differed and depended on cultivar type. The increase of grain yield at primed seeds in wheat, barley, rice, sorghum, chickpea and millet were stated by different researchers (Harris *et al.*, 2001; 2004; Misra and Dwibedi 1980; Paul and Choudhury 1991).

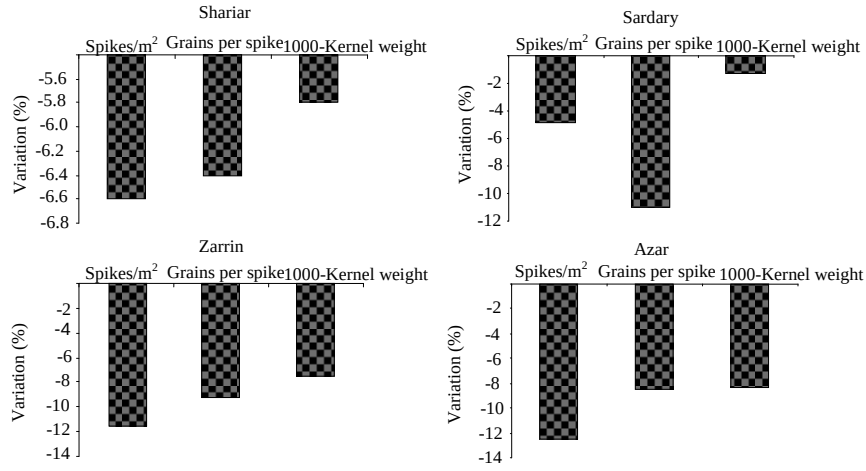

Figure. 4. Variations percentage of grain yield components of primed seed with using of potassium chloride in wheat cultivars under drought stress in comparison with well watered.

All pretreatments in our experiment had lower variation of total dry matter under drought stress compared to well-watered treatments (Table 3). Among them potassium chloride had the lowest value, which could be related to the fact that potassium induces higher tolerance to drought stress. Pretreatments resulted in high total dry matter production through effects on growth period. Primed seeds had after sowing faster germination, rapid establishment, and uniform growth.

Such a plant expands root system at shorter time compared to the control and by uptaking more water and nutrients produces photosynthetic organs rapidly and reaches earlier autotrophic stage (Duman 2006). Relative water content of wheat flag leaf primed with potassium chloride has minimal variation (Table 3).

Table 3. Variations percentage for traits of grain yield, total dry matter and relative water content in primed seeds of wheat cultivars under drought stress with comparison well watered.

	Grain yield (g/m^2)						
Cultivar	PEG 10%	KCl 2.5%	MN 4%	Control	DW	IAA 20ppm	CCC 1000ppm
Shariar	-17.6	-7.1	-25.1	-36.1	-33.0	-39.0	-32.0
Azar	-22.5	-1.9	-7.4	-15.6	-9.1	-16.6	-23.9
Sardary	-32.4	-2.3	-8.8	-27.7	-16.5	-16.7	-17.1
Zarrin	-27.3	-4.3	-37.7	-33.4	-32.9	-40.8	-45.4
	Total dry matter (g/m^2)						
Shariar	-14.2	-12.3	-11.0	-18.1	-14.3	-15.5	-13.3
Azar	-12.7	-10.1	-13.8	-17.4	-17.2	-12.4	-14.0
Sardary	-13.9	-9.8	-10.3	-15.8	-10.7	-11.7	-12.2
Zarrin	-15.7	-10.4	-18.1	-20.2	-11.0	-13.6	-12.3
	Relative water content (%)						
Shariar	-28.0	-9.1	-24.9	-37.1	-19.6	-22.7	-11.7
Azar	-25.4	-10.5	-17.3	-25.6	-14.6	-18.9	-12.3
Sardary	-19.5	-9.6	-16.2	-27.1	-11.0	-17.9	-13.6
Zarrin	-37.0	-8.0	-34.0	-35.0	-21.8	-34.5	-9.7

Variation (%)=[(Mean cultivar under stress-Mean cultivar under well watered)/Mean cultivar under well watered]×100

3.6 Correlation coefficients traits

Traits of wheat such as number of spikes per square meter, number of grains per spike, total dry matter has with grain yield positive but significantly different correlation (Table 4). With increasing value of these traits, grain yield increases as well. Number of spikes per square meter and number of grains per spike at potassium chloride pretreatment had the highest percentage of variation. Therefore, these traits could be used as an indirect criterion for the selection for high grain yield. Chimenti and Hall (1994) observed positive correlation between leaf area and grain yield in sunflower under drought stress, and used it as an indirect selection in screening tolerant genotypes under drought stress.

Table 4. Correlation coefficients of wheat cultivars traits of primed seed using of different pretreatments.

Trait	Grain yield (g/m^2)	Spikes/m^2	Grains per spike	1000-Kernel weight (g)	Total dry matter (g/m^2)
Spikes/m^2	0.91**				
Grains per spike	0.92**	0.83*			
1000-Kernel weight (g)	0.54	0.74	0.59		
Total dry matter (g/m^2)	0.79*	0.79*	0.76*	0.29	
Relative water content	0.50	0.66	0.29	0.33	0.55

* and **: Significant differences at $p \leq 0.05$ and 0.01 probability levels, respectively.

4 CONCLUSION

Responses of wheat cultivars were different to pretreatments. Seed priming with IAA and CCC for 30 minutes had positive effects on seedling regarding nitrogen absorption and grain yield traits. It also increased the components of grain yield more than other pretreatments for 18 hours. In opposite, urea pretreatment had negative effect on seedling related traits compared to control. Therefore, we excluded urea pretreatment in green house and field experiments. The biggest percentages of variation for grain yield under drought stress compared to well-watered treatment were found for cultivars Shariar, Azar, Sardary and Zarrin with IAA 39%, CCC 23%, PEG 32% and CCC 45%, respectively. In contrast, potassium chloride pretreatment showed at all four cultivars the minimum variations for grain yield, total dry matter and relative water content, probably due to induction of higher drought tolerance This pretreatment, except in cultivar Sardary, had the strongest effect on trait number of spikes per square meter. The trend of variations for plumule length at laboratory experiment was similar with plant height at field conditions. In the case of CCC pretreatment, the pretreatment decreased internodial lenght and subsequently plant height, but in other pretreatments it was increased. Seed priming improved grain yield up to 40 percent. Increase of 25% in absorbed nitrogen causes better vegetative growth and total dry matter compared to control. Pretreatments increased seed vigor and rapid growth at seedling stage under field conditions what had direct effect on grain yield. In addition, improving the germination percentage and uniformity emergence, pretreatment results in suitable density with increased tiller number and grains per spike. Under drought stress conditions, it is recommended that seeds be primed with potassium chloride. It is suggested that proteomic techniques should be used to identify molecular mechanisms under drought stress for primed seeds.

5 ACKNOWLEDGEMENTS

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Influence of NaCl treatments on growth and biochemical parameters of castor bean (*Ricinus communis* L.)

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ABSTRACT

Castor bean (*Ricinus communis* L.) is widely cultivated throughout the world for use as a medicinal plant and oil consumption purposes; however its salt tolerance has not been clarified. To investigate the effect of salt stress on its growth and on activity of antioxidative enzymes in different organs, castor bean plants at the 4-leaf stage were subjected to 50, 100 and 200 mM NaCl admixed to Hoagland's solution for 10 days under greenhouse conditions. The results showed that salt stress inhibited plant growth (root and shoot length, fresh root and shot weight) but root growth was more affected then shoot. Relative water content of leaves and the membrane stability of the leaves were decreased with increasing NaCl concentration. The activity of guaiacol peroxidase (GPX) and catalase (CAT) was sharply decreased by escalation of salt stress. However activity of ascorbate peroxidase (APX) was enhanced under moderate salt stress (100 mM NaCl) in both root and shoot but then decreased with increased NaCl concentration. The activity of superoxide dismutase (SOD) increased with the increase of the concentration of NaCl in shoots and root. However alternation in enzymatic antioxidant activity was noticed in shoot compared to root. Increased H_2O_2, total soluble protein, proline content and malondialdehyde (MDA) concentration in both plant's organs was linearly and positively correlated with increasing NaCl concentration. The results of this study suggest that the salt sensitivity of Castor bean plant under salt stress conditions is probably due to a lack of efficient activity of CAT and GPX probably lead to imperfect H_2O_2 scavenging.

Key words: Castor bean, salt stress, oxidative stress, APX, CAT, H_2O_2, lipid peroxidation, MDA

IZVLEČEK

VPLIV TRETMAJA Z NaCl NA RAST IN BIOKEMIČNE PARAMETRE NAVADNEGA KLOŠČEVCA (*Ricinus communis* L.)

Navadni kloščevec (*Ricinus communis* L.) se v svetovnem merilu pogosto goji kot zdravilna rastlina ali zaradi uporabnega olja, njegova toleranca na solni stres pa še ni bila raziskana. Za preučevanje vpliva solnega stresa na rast in aktivnost antioksidacijskih encimov smo navadni kloščevec izpostavili v razvojni fazi 4 listov koncentracijam NaCl 50, 100 in 200 mM, raztopljenih v Hoaglandovi raztopini in rastline gojili deset dni v rastlinjaku. Rezultati so pokazali, da je solni stres inhibiral rast rastlin (dolžino korenin in poganjkov in njihovo svežo težo), vendar je bila rast korenin bolj inhibirana. Relativna vsebnost vode in integriteta membran listnega tkiva sta upadali z naraščajočo koncentracijo NaCl. Aktivnost gvajakol peroksidaze (GPX) in katalaze (CAT) je močno upadla po povečanju solnega stresa. Aktivnost askorbat peroksidaze (APX) se je povečala v razmerah zmernega solnega stresa (100 mM NaCl) v koreninanh in poganjku, vendar je s povečevanjem koncentracije NaCL potem upadla. Aktivnost superoksid dismutaze (SOD) se je s povečevanjem koncentracije NaCl povečevala v koreninah in poganjku. Kljub temu je bilo v poganjku opaziti večje spremembe v encimatski antioksidacijski aktivnosti v primerjavi s koreninami. Vsebnost H_2O_2, celokupnih topnih proteinov, prolina in malondialdehida (MDA) se je v obeh organih linearno povečevala z naraščajočo koncentracijo NaCl. Rezultati te raziskave kažejo, da je občutljivost navadnega kloščevca na solni stres posledica nezadostne aktivnosti encimov CAT in GPX, kar verjetno vodi do nepopolne presnove H_2O_2.

Ključne besede: navadni kloščevec, solni stres, oksidacijski stres, APX, CAT, H_2O_2, peroksidacija lipidov, MDA

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1 INTRODUCTION

Salty soils extensively exist in arid and semi-arid climate regions of the world and cause salt stress in plants (Khan et al., 2001). Salinity is an important abiotic stress factor seriously affecting plant productivity and survival. The ability of vegetation to survive under higher salinity conditions is important for the distribution of plants and agriculture around the world. Enhancing the salt tolerance of plants is an important breeding objective in areas, which are affected by soil salinity (Flowers and Flowers, 2005). A plant's ability to acclimate to salt stress includes alterations at the leaf level, associated with morphological, physiological and biochemical characteristics whereby many plants adjust to high salinity and the consequent low soil water availability (Munns, 2002). Morphologically the most typical symptom of saline injury to plant is reduction of growth (Azooz et al., 2004), which is a consequence of several physiological responses including modification of ion balance, water status, mineral nutrition, photosynthetic efficiency, carbon allocation and utilization (Ismail 2003; Taylor et al., 2004).

From physiological and metabolic aspects salt stress can also stimulate formation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals. ROS injure the cellular components of proteins, membrane lipids and nucleic acids (Foyer et al., 1994). Evidence suggests that membranes are the primary sites of salinity injury to cells and organelles (Candan and Tarhan, 2003) because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasma membrane or in the membranes of organelles (Karabal et al., 2003). Malondialdehyde (MDA), a product of lipid peroxidation indicates well the oxidative stress and can be used as a tool for determining salt tolerance in plants (Yildirim et al., 2008). To scavenge ROS, plants possess specific mechanisms, which include activation of antioxidant enzymes and non-enzymatic antioxidants such as, carotenoids and ascorbic acid (Mittler, 2002). The enzymatic antioxidant system is including superoxide dismutase (SOD: EC 1.15.1.1), which can be found in various cell compartments and catalyses the disproportion of two O_2^- radicals to H_2O_2 and O_2 (Scandalios, 1993). H_2O_2 is eliminated by various antioxidant enzymes such as catalases (CAT: EC 1.11.1.6) (Scandalios 1993) and guaiacol peroxidase (GPX: EC 1.11.1.7) (Gara et al., 2003) which convert H_2O_2 to water. Ascorbate peroxidase (APX: EC 1.11.1.11) also play a key role by reducing H_2O_2 to water through the Halliwell–Asada pathway (Noctor and Foyer, 1998). However, under salt stress, the mechanisms developed by Castor bean plants to scavenge ROS are still poorly understood.

Castor bean (*Ricinus communis* L) is a crop plant of commercial relevance since its oil is used for manufacturing surfactants, coatings, greases, fungistats, pharmaceuticals, cosmetics, and many other products. Most part of castor bean is grown in semiarid regions where salinity stress may affect germination and plant growth intensively (Pinheiro et al., 2008). Plant with high constitutive and induced antioxidant levels has better resistance to damage (Parida and Das, 2005). The degree of damage by ROS depends on the balance between the product of ROS and its removal by these antioxidant scavenging systems (Khan and Panda, 2008). However, to the best of our knowledge, the antioxidant responses of Castor bean to salt stress have not been reported.

The aim of this work was to study the effects of different concentrations of salt on the growth, lipid peroxidation and antioxidant enzyme activities (e.g., CAT, POX, GPX & APX) of Castor bean plant and to analyze the trends of these parameters under different levels of salinity stress. This may be helpful in developing a better understanding of tolerance threshold and provide additional information on the mechanisms of salt tolerance.

2 MATERIAL AND METHODS

2.1 Trials protocol

Seeds of castor bean (*Ricinus communis* L.) were obtained from local seed mass, Isfahan, Iran. Homogenous seeds of were surface sterilized using 5% sodium hypochlorite solution for 5 min and then rinsed 3 times with sterile distilled water. Seeds were germinated in 120 mm covered Petri dishes on two layers of filter paper moistened with 15 ml of distilled water and then approximately 20-25 seedlings were planted onto plastic trays covered with cheesecloth containing half-strength Hoagland's solution. Nutrient solution was permanently aerated and renewed 2-3 times a week to minimize a pH shifts and nutrient depletion. Hydroponics were kept in a growth room (approximately 14h light/10 h dark) providing white fluorescent light and natural light with an photosyntheticaly active radiation of 250 μmol $m^{-2}s^{-1}$, day/night temperature of 25 ± 2 °C / 15 ± 2 °C and 60 ± 5 % relative humidity. The plants were grown in normal growth conditions until 4-5 leaf stage. Then, twenty-three-day old plants were treated with Hoagland's solution containing 50, 100 and 200 mM NaCl and maintained for 10 days in these conditions. Control plants were kept in Hoagland solution without NaCl. After treatment for 12 days, the castor bean plants were sampled (leaf and root). The plants were first washed with tap water, and then with distilled water. Roots and shoots were separated and the fresh weight was determined for each plant. Portion of the fresh samples were taken to measure the physiological indices and some of them transferred to liquid nitrogen and maintained at -80°C for future extraction.

2.2 Measurement of plant water status

Five fresh leaves of same size and same age of five plants from each treatment were collected and weighted (Fw). Leaf segments were kept immersed in distilled water for 24 h at room temperature in the dark. The turgid weight (Tw) of leaves were measured and then oven-dried at 80°C for 72 h until constant weight and reweighting (Dw). The fresh weights, turgidity and dry weights of the leaf segments were used to determine the hydratation and relative water content according to Sangakkara et al. (1996). Hydration was determined as H (%) = 100 - 100 (Dw / Fw). The relative water content (RWC) was determined as RWC (%) = [(Fw-Dw) / (Tw-Dw)] x 100.

Leaf Membrane Stability Index (MSI) was measured as ion leakage, the washed leaves were cut into 1 cm pieces and placed in a glass beaker containing 10 mL deionised water. The beakers were kept at 30°C for 3 h and the conductivity of solution was measured by a conductivity meter. The same samples were boiled for 2 min and then their conductivity was measured again, when the solution was cooled to room temperature. The percentage of Membrane Stability was calculated as follows, MSI (%) = {1- (C1/C2)} x 100. Where C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively.

2.3 Total soluble protein and proline content assay

Total soluble protein content was measured according to Bradford (1976) using bovine serum albumin (BSA) as a protein standard. Fresh leaf samples (1 g) were homogenized with 4 ml Na-Phosphate buffer (pH 7.2) and then centrifuged at 4°C. Supernatants and dye were pipetting in spectrophotometer cuvettes and absorbance was measured using a UV-vis spectrophotometer (PG instruments T80) at 595 nm. Proline was extracted and its concentration determined by the method of Bates et al. (1973). Leaf tissue was homogenized in sulfosalicylic acid, and the homogenate was centrifuged at 3000 × g for 20 min. The supernatant was treated with acetic acid and acid-ninhydrin, boiled for 1 h, and then absorbance at 520 nm was determined.

2.4 Enzyme extraction

For SOD, CAT and GR extraction, leaf and root samples (0.5 g) were homogenized in ice cold 0.1 M phosphate buffer (pH=7.5) containing 0.5 mM EDTA with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in Beckman refrigerated centrifuge for 15 min at 15000×g. The supernatant was used for enzyme activity assay (Esfandiari et al., 2007).

2.5 Enzyme activity assay

SOD activity was estimated by recording the decrease in absorbance of superoxidenitro blue tetrazolium complex by the enzyme (Sen-Gupta et al., 1993). About 3 ml of reaction mixture, containing 0.1 ml of 200 mM methionine, 0.01ml of 2.25 mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1 ml distilled water and 0.05 ml of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 ml riboflavin (60 µM) and placing the tubes below a light source of two 15 W florescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal color. A non-irradiated complete reaction mixture which did not develop color served as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

CAT activity was measured according to Aebi (1984). About 3 ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH=7), 0.5 ml of 75 mM H_2O_2, 0.05 ml enzyme extraction and distilled water to make up the volume to 3 ml. Reaction started by adding H_2O_2 and decrease in absorbance recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed. APX activity was measured according to Yoshimura et al. (2000) by monitoring the rate of ascorbate oxidation at 290 nm (E=2.8mM-1cm-1). The reaction mixture contained 25 mM phosphate buffer (pH=7), 0.1 mM EDTA, 1 mM H_2O_2, 0.25 mM AsA and the enzyme sample. No change in absorption found in the absence of AsA in the test medium.

Guaiacol peroxidase was determined by measuring the oxidation of guaiacol. The assay mixture contained 10 mM/L potassium phosphate (pH 6.4), 8 mM/L guaiacol, and 2.75 mM/L H_2O_2. The increase in absorbance was recorded at 470 nm within 2 min (linear phase) after the addition of H_2O_2 (Huang et al., 2006). Malondialdehyde (MDA) was measured by colorimetric method (Stewart and Bewley, 1980). 0.5 g of leaf samples were homogenized in 5ml of distilled water. An equal volume of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid solution was added and the sample incubated at 95°C for 30 min. The reaction stopped by putting the reaction tubes in the ice bath. The samples then centrifuged at 10000×g for 30 min. The supernatant removed, absorption read at 532 nm, and the amount of nonspecific absorption at 600 nm read and subtracted from this value. The amount of MDA present calculated from the extinction coefficient of 155 $mM^{-1}cm^{-1}$. Enzyme activity and MDA content of samples were recorded with duplication.

2.6 Statistical analysis

Primary statistical analyses such as frequency distribution and normality tests were conducted. Normality test of the data was assessed using the Anderson-Darling normality test (Minitab, 14.0) and homogeneity test of variances with Levene test. Data were determined by analysis of variance and differences between treatment means were separated by the least significant difference (LSD) at a 0.05 probability level. All the statistical analyses were carried out using version 14 (SPSS Institute, 2004) and MINITAB version 14 (2005) software.

3 RESULTS

Relative to control plants, growth of castor bean plant was drastically reduced due to NaCl at all tested concentrations (Table 1). The dry matter production of the different organs (root and shoot) differed in their response to salinity stress. The reduction percentage in dry weight was averagely higher in root (about 78%) than of shoot (56%) as compared with control plants displayed a highly significant reduction in dry matter of different organs at the most salinization levels as compared with the control. Leaf area was also significantly smaller than control at higher NaCl concentration by 81%. In addition, the relative water content (RWC) of leaf was decreased from 96.4% in control to 78.6, 74.3 and 66.5% at 50, 100 and 200 mM NaCl treatment, respectively (Table 1). In this connection at the end of the experimental period, the leaves of NaCl treated plant showed a visual symptoms (chlorosis and necrotic). To examine the biochemical responses to salt stress, we first determined the effect of NaCl on soluble protein content in castor bean plants. As a result to the exposure to NaCl, soluble protein content showed a significant increase trend with the increase of the concentration of NaCl (Fig. 1). Soluble protein increased by 23.4, 54.6 and 65% in shoots, 14.53, 45.15 and 73% in roots, at 50, 100 and 200 mM NaCl, respectively.

Table 1.: Dry weight (Dw) of root and shoot (g plant^{-1}), percentage water content (Wc%), leaf area (cm^{-2}), percentage relative water content (RWC%) and membrane stability index of castor bean (*Ricinus communis* L) in response to different NaCl concentrations

NaCl concentration	Root		Shoot		Leaf		
	Dw	WC%	Dw	WC%	Area	RWC	MSI
Control	0.60±0.045	93.0±5.25	2.75±0.254	92.8±5.24	22.57±1.41	96.4±7.71	73±7.42
50	0.39±0.050	88.1±6.85	2.43±0.243	87.6±8.10	13.41±1.25	78.6±7.09	59±8.07
100	0.35±0.045	73.5±10.68	1.64±0.237	79.4±5.74	8.77±1.29	74.3±6.04	41±8.54
200	0.13±0.037	69.8±6.45	1.22±0.177	76.2±8.63	4.29±1.03	66.5±7.96	39±7.69
LSD at 5%	0.154	19.56	0.691	13.24	4.38	8.22	27.41

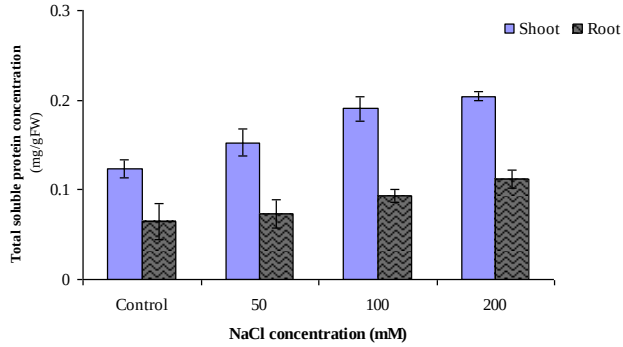

Figure 1.: Effect of NaCl stress on total protein concentration in root and shoot of castor bean (*Ricinus communis* L.) plant. The values and standards errors (vertical bars) of three replications are shown.

For better understanding the interrelationships among the mentioned traits, simple correlation coefficients were computed (Table 2). The Dw had significant positive correlation with the other traits. Also, similar results were seen for all of studied traits which were correlated with each other. Due to low numbers of dataset, some of these high correlations were not significant. However it seems that there were good associations among stress tolerance indices in slat stress conditions.

Table 2.: Dry weight (Dw) of root and shoot (g plant^{-1}), percentage water content (Wc%), leaf area (cm^{-2}), percentage relative water content (RWC%) and membrane stability index of castor bean (*Ricinus communis* L) in response to different NaCl concentrations

NaCl concentration	Root		Shoot		Leaf		
	Dw	WC%	Dw	WC%	Area	RWC	MSI
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200	0.13±0.037	69.8±6.45	1.22±0.177	76.2±8.63	4.29±1.03	66.5±7.96	39±7.69
LSD at 5%	0.154	19.56	0.691	13.24	4.38	8.22	27.41

The activity of antioxidant enzymes such as APX, GPX, CAT and SOD was differentially affected by salinity. The activity of APX in shoots and roots of castor bean showed increase at low and medium NaCl concentrations but decreased at higher concentration. It considerably increased in 100 mM NaCl treated plants, with 35.14% (roots) and 74% (shoots) increase compared to control ones. The trends in APX activity were similar in root and shoot tissues. The increase of NaCl concentration up to 200 mM caused and 24.14% and 21% decrease in APX activity compared with 100 mM NaCl treated plants, respectively in root and shoot (Fig. 2A). The GPX activity in roots and shoots decreased significantly with rising NaCl concentration. Results showed similar level if GPX activity in shoots and roots under stress condition, but under control condition GPX activity in shoots was significantly higher than in roots (Fig. 2B). In case of CAT, a significant decrease was noticed in castor bean cells under salt stress. The activity of CAT nearly halved in shoots at 200 mM of NaCl (Fig. 2C). By contrast, in root tissues the decline in CAT activity was not so sharp; e.g., it was 31% at 200 mM. SOD activity in shoot increased significantly with increasing NaCl concentration compared to the control, reaching increase of 66% at 200 mM NaCl. Similarly, the highest SOD activity in the roots increased by 81% at NaCl concentration of 200 mM compared to control (Fig. 2D).

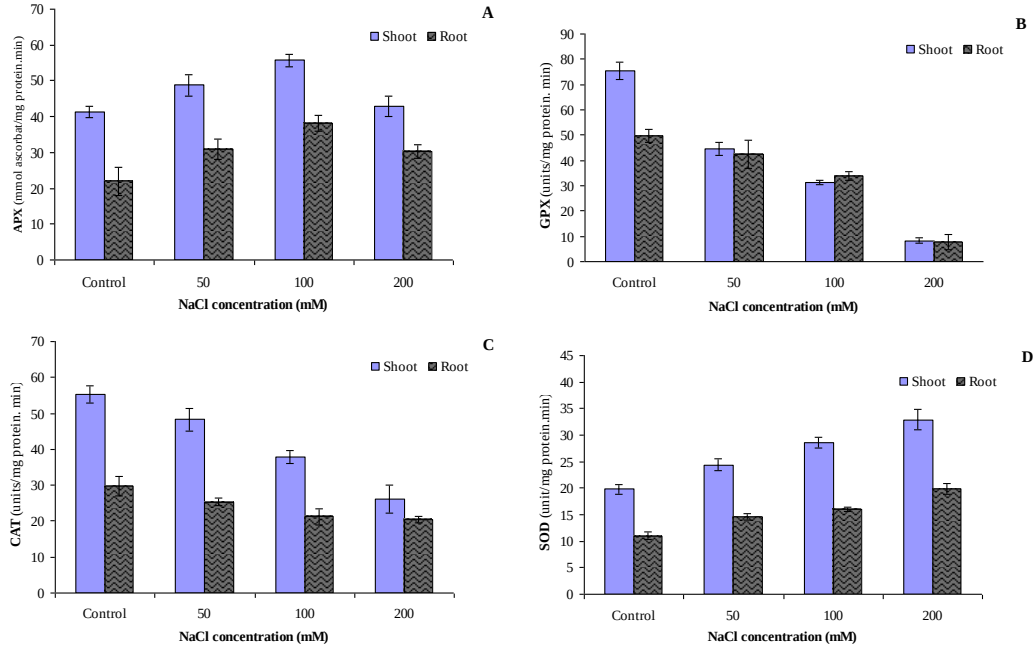

Figure 2.: Effect of NaCl stress on antioxidant enzymes (A) ascorbate peroxidase (APX); (B) guaiacol peroxidase (GPX); (C) catalase (CAT); and (D) Superoxide dismutases (SOD) activities in root and shoot of castor bean (*Ricinus communis* L.) plant. The values and standards errors (vertical bars) of three replications are shown

According to Table 2, the APX activity in shoot had significant positive correlation with the APX activity in root a. In contrast the APX activity in shoot did not show any significant positive or negative correlations with the other enzymes. The GPX activity in root indicated relatively high negative correlation with CAT activity in root, and relatively moderate negative correlation with CAT activity in shoot while showed relatively moderate positive correlation with SOD activity in shoot. It is mentioned that these correlations were not significant due to low numbers of observations. The GPX activity in shoot indicated significant positive correlation with GPX activity in root, CAT activity in shoot and CAT activity in root while showed significant negative correlation with SOD activity in root and shoot (Table 2). The GPX activity in root indicated significant positive correlation with CAT activity in shoot while showed significant negative correlation with SOD activity in root and shoot. The CAT activity in shoot and root had significant negative correlation with SOD activity in root and shoot while both of SOD activity in root and shoot were associated with each other (Table 2).

The result revealed that increased salt concentration caused a significantly higher MDA production in shoot compared to root. Changes in lipid peroxidation in shoots and roots of castor bean at different treatments were shown in Fig. 3. For different concentrations of NaCl in shoots it increased by 1.2-, 5.1- and 5.6-fold and in roots it increased by 3.3-, 5.2- and 6.2-fold, respectively. MDA response of shoot and root at 50 mM were not significantly different. The amount of hydrogen peroxide (a product of SOD and GPX reaxtions) showed a significant increase in shoots and roots of castor bean plant treated with NaCl particularly at higher concentrations compared to untreated plants and were more pronounced in shoots than in roots (Fig. 4). The level of H_2O_2 in roots of NaCl treated plants drastically increased by 110, 236.4 and 319% compared to the controls. Similar trend also occurred in shoot with a significantly lower increase that observed in roots.

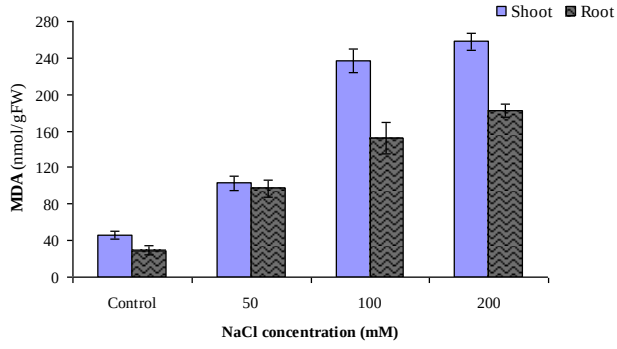

Figure 3.: Effect of NaCl stress on malondialdehyde (MDA) concentration in root and shoot of castor bean (*Ricinus communis* L.) plant. The values and standards errors (vertical bars) of three replications are shown.

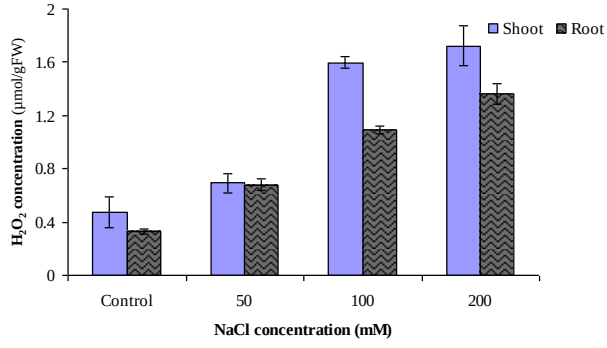

Figure 4.: Effect of NaCl stress on H_2O_2 concentration in root and shoot of Ricinus communis L. plant. The values and standards errors (vertical bars) of three replications are shown.

Proline content of the shoots was significantly enhanced with NaCl at all tested concentrations; by contrast, the roots appeared less sensitive to salt exposure and only at 100 mM an increase of 26% was compared to control (Fig. 5). The increase of proline concentration in the shoots is dose dependent, and it increases by 19.6, 69.3 and 137% in the shoots treated with 50, 100 and 200 mM of NaCl, respectively, compared to the controls.

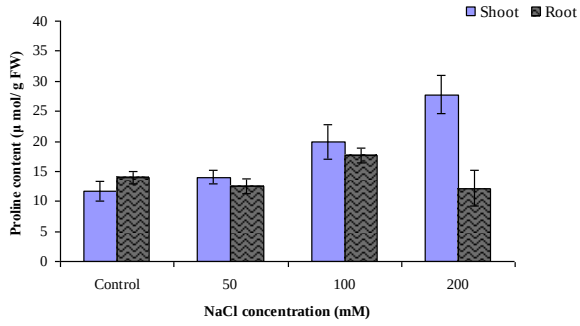

Figure 5.: Effect of NaCl stress on proline content in root and shoot of castor bean (*Ricinus communis* L.) plant. The values and standards errors (vertical bars) of three replications are shown.

4 DISCUSSION

The effect of salinity on plant growth is due to an osmotic effect and/or ion toxicity. However, variation of adaptive mechanisms exists in different species (Rehman et al., 1996). In present study, 50 mM NaCl treatment resulted in a significant decrease in the root dry weight and leaf area. In a 200 mM NaCl concentration, growth reduction of roots was much higher than that of shoots. Our finding suggested that different changes are related to effects of different NaCl concentrations on different organs of plants. The results suggested that the roots were more susceptible to salt stress; perhaps it is result of roots vicinity to Na^+ and Cl^- ions. Differences between shoots and roots response to same level of salinity stress might result from pattern of sodium partitioning between them. Investigation of castor bean plants during late vegetative growth under salt stress revealed that high selectivity exist for Na^+, so that sodium cations noticeably retained in the root (Jeschke and Pate, 1991). Furthermore, lateral uptake of Na^+ from xylem by hypocotyl, stem internodes and petioles can result in low intake by young leaf laminae and substantial cycling from older leaves back to the root may causing more inhibition of root growth.

Plants according to their sensitivity to different levels of salt content are divided into four groups which including halophytes, halophilic crop species, Salt tolerant crop species and Salt sensitive crop species. Although In the literature the coaster bean plant is considered moderately salt tolerant (Jeschke and Pate, 1991; Pinheiro et al., 2008), the result obtained here showed that even high level of applied salinity (200 mM) was not lethal. However, with increasing of the salinity level growth was strongly inhibited. Comparison of current result with previous experiments suggests there is a high diversity of salt tolerance in different varieties or local masses of coaster bean.

Like to other stresses, salt stress is also expected to alter activities of antioxidative enzyme and thereby creating an oxidative stress situation. Superoxide dismutase, CAT, GPX and APX are important antioxidative enzymes that function in the cells to prevent the build-up of ROS (Elster 1982; Halliwell and Gutteridge, 1988). Because of NaCl induction to generation of oxidative stress, this study showed that membrane stability index decreased in leaves tissue as a result of oxidative damage induced by NaCl treatments (Table 1).

Under salt stress the protection against H_2O_2 becomes weakened due to the decline in activities of GPX and CAT which may favor the elevated steady state level of H_2O_2. This ROS can react in presence of transition metal ions to produce the hydroxyl radical (Elster, 1982). Result indicated that in the case of castor bean, even though there was some protection against superoxide, the H_2O_2 detoxification mechanism was insufficient. In aerobic cells, hydroxyl radicals the most potentially toxic species - are known to be formed from H_2O_2 in presence of transition metal ions (Halliwell and Gutteridge, 1988).

Generally, peroxidases catalyse the oxidoreduction between hydrogen peroxide and reductants. As reported by Lobarzewski et al. (1991) peroxidases are involved in auxin and ethylene metabolism, redox reactions in plasma membranes, cell wall modifications (lignification and suberinization) as well as in developmental and defense processes. It present study GPX and APX differently responded to NaCl stress, the activity of GPX sharply decreased while APX showed dose dependent increase in shoot and roots of castor bean. Plant peroxidase isozymes are differentially expressed during plant cell development (Ros-Barcelo and Sabater, 1986) thus observed result might be due to different sensitivity of GPX to salt stress or its expression in other growth stages.

Among the antioxidant enzymes SOD was the one which showed highest activity increase. At organ level shoots showed highest activity when compared to roots. In this study, salt-induced increase in SOD activity suggests that due to imposition of NaCl stress, *de novo* synthesis of enzymatic protein may have occurred which would be more pronounced in shoot tissues. A decreased superoxide concentration is thus to be expected, but parallel with an increased production of H_2O_2. A positive correlation between salt stress and the abundance of SOD formerly reported by Cavalcanti et al., (2007) in cowpea cells. In our case the lack of efficient activity of CAT and GPX probably lead to imperfect H_2O_2 scavenging. Accumulation of peroxide is a general stress response, which has been observed in plants exposed to various biotic and abiotic stresses. A similar increase in H_2O_2 level was marked under salinity stress as seen for other plants (Esfandiari et al., 2007; Cavalcanti et al., 2007).

Lipid peroxidation is a process by which the functionality and integrity of the membrane is affected and can produce irreversible damage to cell function. Lipid peroxidation gets initiated by ROS or by lipooxygenases. Salt stress enhanced the MDA level in castor bean, which is an index of lipid peroxidation and oxidative stress and represent a balance of oxidative stress that induced production of MDA in relation to NaCl treatments. Thus the increased MDA indicates the prevalence of oxidative stress and perhaps this may be one the possible mechanisms by which toxicity due to NaCl stress could be manifested in plant tissues. Probably this oxidative stress situation might have occurred due to alternation in activity of antioxidative enzymes.

Proline concentration showed a remarkable increase at shoots with the increase of NaCl, also its increase in root was in salt concentration dependent manner. Higher concentrations of proline in shoot might be the reason of higher salt tolerance or prevention of NaCl effects when compared to roots. Proline accumulation, accepted as an indicator of environmental stress, is also considered to have important protective roles. Environmental stress may lead to proline accumulation. Proline accumulation in plant tissues has been suggested to result from (a) a decrease in proline degradation, (b) an increase in proline biosynthesis, (c) a decrease in protein synthesis or proline utilization, and (d) hydrolysis of proteins (Charest and Phan, 1990). In Arabidopsis, the gene of A'-pyrroline-5-carboxylate synthetase, as a key enzyme in proline biosynthesis, is induced by salt stress and other forms of osmotic stress (Yoshiba et al., 1995).

In conclusion, growth parameters and cell membranes of castor bean plants were drastically affected under salinity stress. Despite of the significant increase in activity of some antioxidant enzyme under salt stress conditions, it might not be enough for ROS scavenging and no recovery of root and shoot growth as well as membrane integrity was observed, suggesting the tested castor bean is sensitive to long-term salt stress at least during the investigated stage. But since all seedlings survived even under the highest stressful conditions at the end of experiment it seems that salt tolerance in local masses (especially from arid and semi arid regions) still remain poorly understood.

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Water and nitrogen use efficiency of common ragweed (*Ambrosia artemisiifolia* L.) at different nitrogen and water levels

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ABSTRACT

Common ragweed (*Ambrosia artemisiifolia* L.) spread across Europe and other regions is becoming a serious health and economic threat. A pot experiment was conducted in 2011 to determine effect of various nitrogen (N) (10, 100 kg/ha) and water supply regime on resource use efficiency of ragweed. Ragweed plants increased their dry matter production with increased water and N availability. Nitrogen use efficiency (NUE) was decreased with N addition and was not influenced by water availability. Mean nitrogen residence time (MRT) was longer at low N and water levels. In contrast, nitrogen productivity (NP), NUE and water use efficiency (WUE) were all increased with enhanced water supply. A trade-off between parameters of NUE was attributed to differential response of NP and MRT to soil fertility and water supply. Our results confirmed that ragweed displayed high adaptation to unproductive sites. However, ragweed's greater plasticity in response to water availability compared to N availability suggest, that water supply plays important role in its invasion success and in combination with disturbance ragweed might further spread into more productive environments.

Key words: *Ambrosia artemisifolia*, invasivity, water use efficiency, nitrogen use efficiency, pot experiment, Slovenia

IZVLEČEK

UČINKOVITOST IZRABE VODE IN DUŠIKA PRI PELINOLISTNI AMBROZIJI (*Ambrosia artemisiifolia* L.) OB RAZLIČNIH RAVNEH DUŠIKA IN VODE

Pelinolistna ambrozija (*Ambrosia artemisiifolia* L.) postaja s svojim nezadržnim širjenjem po Evropi in drugih regijah sveta resen ekonomski in zdravstveni problem. Za določitev učinkovitosti izrabe virov je bil v letu 2011 zasnovan lončni poskus z dvema obravnavanjema z dušikom (N) (10 in 100 kg/ha) in vodo (veliko, malo vode). Pelinolistna ambrozija je povečala produkcijo suhe mase pri večjih odmerkih dušika in vode. Učinkovitost izrabe dušika (NUE) se je pri večjem odmerku dušika statistično značilno zmanjšala, preskrba z vodo pa ni imela vpliv na NUE. Srednji čas zadrževanja dušika v rastlini (MRT) je bil daljši pri manjših odmerkih dušika in manj vode. Nasprotno so se produktivnost dušika (NP), NUE in učinkovitost izrabe vode (WUE) pri večji dostopnosti vode povečali. Kompromis med faktorji NUE je bil pripisan različnemu odzivu NP in MRT na preučevane dejavnike dušika in vode, pri čemer je bil odziv pelinolistne ambrozije pri različnih odmerkih vode bolj plastičen v primerjavi z različnimi odmerki dušika. Naši rezultati so potrdili, da je pelinolistna ambrozija prilagojena na neproduktivna rastišča, vendar nakazujejo, da ima voda velik vpliv pri invazivnem uspehu pelinolistne ambrozije in bi se, glede na izkazano plastičnost, v motenih okoljih lahko razširila tudi v bolj produktivna rastišča.

Ključne besede: *Ambrosia artemisifolia*, invazivnost, učinkovitost izrabe vode, učinkovitost izrabe dušika, lončni poskus, Slovenija

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1 INTRODUCTION

The ecology of plant resource use is one of the most important topics as plants differ considerably in their resource use and adaptive strategies to varying growing conditions. Determination of plants resource use efficiency is widely used approach to study significant adaptive traits. Resource use efficiency is defined as the ratio between output parameter (biomass production, seed production, photosynthetic carbon gain) and a resource input parameter (light absorption, nutrient uptake, water uptake). Successful invasive plants tend to have morphological and physiological traits that enable them to acquire resources in the limiting environments (Funk and Vitousek, 2007) or respond plastic with morphological and physiological adjustment to contrasting resource availabilities (Claridge and Franklin, 2002).

Common ragweed (*Ambrosia artemisiifolia* L.) (hereafter referred to as ragweed) is an annual monoecious weed (*Asteraceae*), introduced to Europe in the eighteenth century (Bonnot, 1967). Since then, it has spread over many European countries (Chauvel et al., 2006; Vogl et al., 2008). In Europe, as in North America, ragweed is considered a troublesome weed and a pioneer plant of abandoned and degraded areas, such as roadsides, fallow fields and other disturbed habitats, where the species takes advantage of the reduced competitiveness from native species or tolerates severe growing conditions (DiTommaso, 2004; Fumanal et al., 2008a). Lastly, ragweed represents a major human health problem because its pollen is a strong allergen that causes ocular and respiratory allergies that often develop into asthma (Dahl et al., 1999; Ziska, 2001). In North America, ragweed pollen is known as one of the main causes of hay fever (allergic rhinitis) (Mitich, 1996) or a form of dermatitis in some people.

Although alien plant invasion, as experienced in the case of ragweed, have become increasingly frequent, plant resource use efficiency and ecological traits underlying the invasion are still poorly understood. High resource use efficiency as well as enhanced phenotypic plasticity have been hypothesized as decisive plant traits allowing the species to become invasive in newly colonized habitats (Dukes and Mooney, 1999), comparing also ecophysiological traits of species growing in invaded and native stands (Durand and Goldstein, 2001). It has been documented that some invasive species posses significantly higher photosynthetic nitrogen use efficiency (PNUE), water use efficiency (WUE), and higher specific leaf area (SLA) than native species (Durand and Goldstein 2001; Niinemets et al. 2003; Feng et al., 2007). Performance of plant species in terms of dry matter production is limited by the availability of nutrients in many semi-natural communities (Vitousek and Howarth, 1991). As nitrogen is one of the main limiting growth resources, the nitrogen use efficiency (NUE) of plant species has been widely subjected to studies. NUE is generally determined as productivity per unit N uptake or loss (Vitousek, 1982). NUE is a measure of how plant responds to soil nitrogen, defined as the amount of organic matter lost or produced in a plant per amount of nitrogen used. Berendse and Aerts (1987) further divided NUE to two components: nitrogen productivity (NP) and the mean residence time of nitrogen in the plant (MRT). NP is the rate of dry matter production per unit of N content in the plant and MRT is the period during which the absorbed nitrogen can be used for carbon fixation. Most studies on NUE have tried to discover patterns in the NUE of species in relation to nitrogen availability in their natural habitat. Soil water supply also affects nutrient transport, uptake, and transformation. Furthermore, it may be the underlying cause of variation in habitat fertility. Plants developing in environments with different soil water supply regimes can exhibit differences in traits such as photosynthetic capacity, growth rate, leaf N content (Lambers et al., 1998) which are correlated with NUE and could thus potentially affect N use strategy. Identifying plant traits associated with invasiveness is necessary for possible control of existing invasive species or potentially to predict new invasive species, their traits and target habitats before introduction. Most of the invasive species occur in highly disturbed resource rich environments (Daehler, 2003; Gross et al., 2005; Huenneke et al., 1990), but increasing evidence indicates that invasion is also a danger in resource-poor communities, though the mechanisms of low-resource invasion are less well understood (Mack et al., 2000; Funk and Vitousek, 2007). Altough ragweed is adapted to low nutrient environments, it is likely that in combination of better nutrient supply and disturbance, it can spreads into more productive habitats and it can be found on variety of plant communities, soil types and site productivities (Fumanal, 2008b). Despite increased research efforts in past decade have been undertaken, still little information is available on ragweed resource use efficiency.

Our study was therefore oriented on determing ragweed's resource use efficiency under different nitrogen and water levels. We hypothesized that ragweed plants would increase its dry mater production with addition of nitrogen and water. Conversely, with increased N and water supply its NUE and WUE will decrease. We were also interested in variations and plasticity of the species underlying NUE components of nitrogen economy (NP and MRT) in the response to resource supply. Finally, we studied the existence of the hypothesized trade-off between NP and MRT, components of NUE.

2 MATERIAL AND METHODS

A pot experiment was conducted in the experimental field of Biotechnical Faculty, University of Ljubljana, over three months from July to September 2011. The experiment consisted of a randomised factorial design with four replications. The experimental units (pots) were randomly assigned to each combination of N availability (two levels), water availability (two levels) and sampling time (growth stage; three levels) with four replicates per combination. In total, 96 pots were used in the experiment. The total N addition levels were 0.08 g/pot (10 kg/ha) and 0.8 g/pot (100 kg/ha) over the growing season. Seeds of ragweed were sown on July 8 th 2011 at a depth of 2 cm in each pot; the pots without the drainage holes were 15 cm tall and 19 cm wide. To ensure homogeneity of growing conditions, pots contained a mixture of peat (70%), perlite (10%), vermiculite (10%) and sand (10%). Pots were watered to field capacity once after sowing to stimulate germination, later on 100 mL and 200 mL of water were applied every 3-5 days for low and high water level, respectively. After sowing, 30 % of the total N treatment was uniformly applied as aqueous NH_4NO_3, after which the remaining N was divided and applied in three equal rates before ragweed reached V4 stage (4-leaves; BBCH 12) (Hess et al. 1997). At this growth stage ragweed was thinned to achieve the desired density three plants per pot and the pot surface was covered with aluminium membrane and attached with the rubber band to prevent soil evaporation (Clifton-Brown and Lewandowski, 2000).

To ensure that plant growth was not limited by elements other than N, other macro- and micronutrients were added separately on a schedule similar to that of N application. At each application single pot received 100 mL of modified nitrogen-free Hoagland solution containing 0.2 mM KH_2PO_4, 1 mM KCl, 1 mM $CaCl_2.2\ H_2O$, 0.4 mM $MgSO_4.7\ H_2O$, 1 mM H_3BO_3, 1 mM $MnCl_2.4\ H_2O$, 1 mM $ZnSO_4.7\ H_2O$, 1 mM $CuSO_4.5\ H_2O$, 1 mM $H_2MoO_4.H_2O$ and 1 mM Fe-EDTA. Ragweed plants at each temporal block were harvested at following stages: V4, V10 and full flowering.

Half of the pots were used to determine the leaf relative water content (LRWC) of ragweed at each sampling according to Turner (1981). Between 10 and 14 h, the youngest fully expanded leaves were selected and leaf tissue without major veins was excised, placed in pre-weighed vials and stored in a cooler. The vial weight with fresh ragweed leaves was recorded before hydration for 6 h at 10 °C in distilled water to ensure full turgidity. Fully turgid leaves were placed on filter paper to remove excess water and weighed. The samples were then oven-dried at 80 °C to constant mass and the weighed to determine the dry weight. The LRWC of the fresh leaves was calculated using the following equations (Kirkham, 2005):

$$LRWC\ [\%] = [(FW - DW) / (TW - DW)] \times 100 \quad (2),$$

where FW is the leaf fresh weight, TW is the leaf turgid weight and DW is the leaf dry weight (data not presented).

The plants in remaining pots were clipped at ground level, dry weights were determined after drying at 45 °C until a constant mass was achieved. Plant aboveground N content of each harvest was determined using modified Kjeldahl procedure.

Nitrogen use efficiency (NUE) was determined according to Berendse and Aerts (1987) using following equations:

$$NUE = NP \times MRT\ [g\ DM\ g^{-1}\ N]$$

$$NP = (DW_2 - DW_1) / N_A)\ [g\ DM\ g^{-1}\ N\ day^{-1}]$$

where DW_2 and DW_1 are dry weights at the time t_2 (final sampling) and t_1 (first sampling) and N_A is the mean plant total nitrogen content averaged over three sampling intervals. MRT was determined as:

$$MRT = N_A \times \Delta T / N_T\ (day),$$

where N_T is the total N uptake in the period ΔT. Calculation of mean residence time (MRT) was based on the redefined concept from Hirose (2011), where MRT with respect to their N uptake and N losses can equally be applied to steady and non-steady state systems, therefore N losses were not estimated. NUE, NP and MRT represent mean values over the vegetative growth period.

Water-use efficiency (WUE) determination was based on the transpiration method (Clifton-Brown and Lewandowski, 2000), where the amount of dry matter produced, relative to the amount of water used during a growing period was determined using the following equation:

$$WUE = DW_R/DW_W,$$

where DW_R is the total dry weight of the ragweed plants and the DW_W is the amount of water applied in the growing period. The total water was expressed as the sum of the applied water and the difference betweeen the initial and the final pot weight.

Statistical analysis

Statistical analysis was performed using the STATGRAPHICS Centurion XVI (2011, Statpoint Technologies, Warrenton, VA). To test assumptions of ANOVA data were tested for homogeneity and normality of variances with Levene's test and with Shapiro-Wilk's test. Two-way ANOVA was performed to test the significance of the main treatment effects, their replications and interactions. No interactions between nitrogen and water was observed for tested variables, thus the data were presented for each nitrogen and water levels. Means were compared with post-hoc Tukey's HSD test at $P<0.001$.

3 RESULTS

As expected, ragweed final dry matter production was significantly influenced by nitrogen and water level (P<0.001) (Table1).

Table 1. Significance levels in two-way ANOVA of the effects of nitrogen level (0 and 100 kg/ha) and water levels (low and high) on dry matter production, MRT (mean residence time, day), NP (nitrogen productivity, g DM g^{-1} day^{-1}), NUE (nitrogen use efficiency, g DM g^{-1} N), WUE (water use efficiency, g DM g^{-1} water) of *Ambrosia artemisiifolia* L.

Terms	Dry matter production (g)	Mean residence time (MRT)	Nitrogen productivity (NP)	Nitrogen use efficiency (NUE)	Water use efficiency (WUE)
Nitrogen (N)	***	ns	ns	***	ns
Water (W)	***	***	***	ns	*
N × W	ns	ns	ns	ns	ns

***P<0.001; **P<0.01; *P<0.05; ns = not significant.

Higher N level significantly increased ragweed dry matter production from 1.18 g to 1.86 g compared to low nitrogen treatment. Similarly higher water level increased ragweed dry matter production from 1.26 g to 1.87 g compared to low water level (Table 2, Fig 1a).

Mean residence time (MRT) was affected only by water level (P<0.001) and significantly different values of 5.75 to 9.24 day were observed for low and high water level, respectively (Table 2).

Table 2. Dry matter production, MRT (mean residence time, day), NP (nitrogen productivity, g DM g^{-1} day^{-1}), NUE (nitrogen use efficiency, g DM g^{-1} N), WUE (water use efficiency, g DM g^{-1} water) at two levels of nitrogen-N (10 kg/ha-low and 100 kg/ha-high), and two water levels (low and high water level) of *Ambrosia artemisiifolia* L. Data presented are means with SE.

Parameter	Nitrogen level		Water level	
	Low	High	Low	High
Dry matter	1.18 ± 0.14	1.86 ± 0.08	1.26 ± 0.15	1.87 ± 0.12
MRT	58.33 ± 8.53	49.98 ± 4.72	67.83 ± 6.88	40.49 ± 3.18
NP	4.45 ± 0.50	5.07 ± 0.51	3.58 ± 0.27	5.95 ± 0.38
NUE	248.71 ± 3.1	222.10 ± 3.2	234.68 ± 4.75	236.11 ± 6.1
WUE	0.45 ± 0.05	0.52 ± 0.05	0.41 ± 0.03	0.57 ± 0.05

Nitrogen productivity (NP) was influenced only by water (P<0.001), it significantly increased from 3.58 to 5.95 g DM g^{-1} N with higher water availability (Table 1-2, Fig 1b). Nitrogen use efficiency (NUE) was affected only by nitrogen level (P<0.001); it significantly decreased with nitrogen addition (Table 1-2, Fig 1c). Conversely, water use efficiency (WUE) significantly increased from 0.41 g DM g^{-1} to 0.57 g DM g^{-1} with higher water availability, compared to low water level (Table 1-2, Fig 1d).

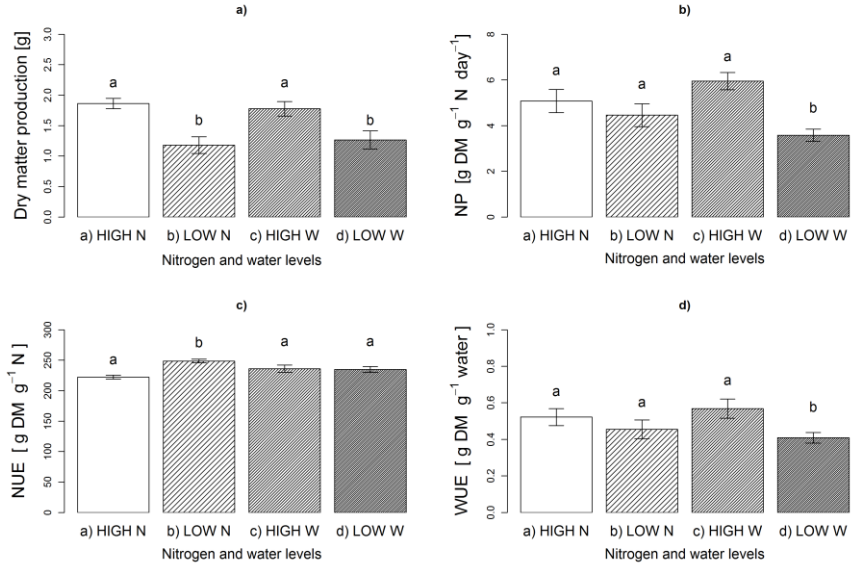

Figure 1. Dry matter production, MRT (mean residence time, day), NP (nitrogen productivity g DM g^{-1} day^{-1}), NUE (nitrogen use efficiency, g DM g^{-1} N), WUE (water use efficiency, g DM g^{-1} water) at two levels of nitrogen-N (10 kg/ha-low N and 100 kg/ha-high N), and two water levels (low W and high W) of *Ambrosia artemisiifolia* L. Data presented are means with SE. (Table1). Different letters present significant diferences for each nitrogen and water level at $P<0.05$.

4 DISCUSSION

Results of our study confirmed report of ragweed's increased dry matter production with nitrogen addition (Lehoczky, 2008). Additionally, ragweed's dry matter production increase at higher water supply was rather expected. Beside dry matter production MRT, NP and NUE were also strongly affected by water availability. Our findings partially counter Yuan and Li (2007); they found that the species at higher water availability had higher MRT, whereas our results are displaying opposite pattern. At the same time, in both studies, similar NUE at various water supplies were observed. Moreover, in their study plants at low N supply had lower NP values than plants with high nitrogen supply , which is consistent with our results. MRT as a component of NUE was similar at both nitrogen levels, consistent with Aerts and De Caluwe (1989) report; they also found no differences in MRT at different N supplies. Higher MRT means also longer time of nitrogen presence in the plant and thus better use efficiency of the nitrogen absorbed by the plant.

The NUE of ragweed in our study was relatively high compared to results determined in previous reports, as NUE is both habitat and species dependent (Vázquez de Aldana and Berendse 1997; Nakamura et al., 2002). Our data are more similar to those obtained by Pajević et al. (2010) who report for NUE 224 g DM g^{-1} N, when averaged across ragweed vegetative growth period. Increased NUE in our study was not achieved through higher NP, but higher MRT was observed, due to higher N content in the plants (data not shown). A higher NP is associated with rapid growth, a relatively large investment of N in photosynthetic tissue, an efficient photosynthetic N use in leaves and relatively small proportion of carbon used for respiration (Garnier and Aronson, 1998; Lambers et al., 1998). Results of increased WUE at higher water supply was surprising, most authors have reported opposite pattern with reports of high WUE as a potential mechanism by which this invasive plant may increase the efficiency of resource capture (Pajević et al, 2010). We assume that our low water level was set to high, although our results of the ragweed leaf relative water content (LRWC) displayed substantial difference with values of 49.7% and 62.6% for low and high water level, respectively. Better explanation of these data would be possible if measurements of transpiration and photosynthesis would be done, and WUE could be determined from ratio photosynthesis/transpiration. Our results are in line with several authors reporting, that there is a trade-off between NUE and NP (Yuan and Li, 2007; Yuan et al, 2008). Similar NUE values were observed, with low NP and high MRT values or conversely with high NP and low MRT values. A high NUE is generally considered to be an adaption to a habitat with low soil nitrogen availability (Chapin, 1980).

In conclusion, our results showed that ragweed exhibit different N use strategies at various N and water levels. High NUE, which was not contributed to high NP but rather by low nutrient loss rate with high MTR values, confirmed that ragweed displayed high adaptation to less fertile environments consistently with previous reports (Yuan et al., 2005). However, with increasing water supply ragweed altered its conservative strategy for low resource systems and increased NP and decreased MRT, although NUE remain similar. We observed fare more plastic response of the NUE underlying components in the response to water supply compared to nitrogen supply. The obtained data also supports hypothesis, that there is a trade-off between the NP and MRT. Our results suggest that water availability may play important role in the ragweed invasion success, as ragweed displayed high plasticity to water supply. Based on our observations, it can be presumed that ragweed will further spread into more productive habitats in combination with disturbance and other environmental stressors which decrease the competition intensity, but further focused studies on ragweed resource and adaptive traits are needed to explain its invasion und underlying plant traits.

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Pesticide residues in samples of apples, lettuce and potatoes from integrated pest management in Slovenia from 2005-2009

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ABSTRACT

In the period from 2005 to 2009 225 samples of apples, lettuce and potatoes from Slovene producers included in integrated pest management (IPM) were analysed for plant protection product (PPP) residues. The samples were analysed for the presence of more than 200 different active compounds using four analytical methods. In 38.7% of apple samples residues were not detected, 58.6% of apple samples contained residues lower than or equal to Maximum Residue Levels (MRLs) while 2.7% of apple samples exceeded MRLs. In 84.6% of lettuce samples residues were not detected, 12.3% of lettuce samples contained residues lower than or equal to MRLs while 3.1% of lettuce samples exceeded MRLs. In 98.0% of potato samples residues were not detected, 2.0% of potato samples contained residues lower than or equal to MRLs and no potato samples exceeded MRLs. Multiple residues were found only in apples and lettuce. The trend observed during the years was the decrease of sample portion of samples containing multiple residues in apples from 2005 to 2008. The most frequently found active substance in apples and lettuce was dithiocarbamates. In potato only phosalone was found.

Key words: apples, lettuce, potatoes, integrated pest management, plant protection product residues, GC/MS, LC/MS/MS

IZVLEČEK

OSTANKI PESTICIDOV V VZORCIH JABOLK, SOLATE IN KROMPIRJA V INTEGRIRANI PRIDELAVI V SLOVENIJI V LETIH 2005-2009

V obdobju od 2005 do 2009 smo na ostanke fitofarmacevtskih sredstev (FFS) analizirali 225 vzorcev jabolk, solate in krompirja slovenskih proizvajalcev vključenih v integrirano pridelavo (IP). Vse vzorce smo analizirali s štirimi analitskimi metodami na prisotnost več kot 200 različnih aktivnih spojin. V 38,7% vzorcev jabolk ostankov nismo določili, 58,6% vzorcev jabolk je vsebovalo ostanke manjše ali enake maksimalnim dovoljenim količinam ostankov (MRL) medtem ko je 2,7% vzorcev jabolk preseglo MRL vrednosti. V 84.6% vzorcev solate ostankov nismo določili, 12,3% vzorcev solate je vsebovalo ostanke manjše ali enake MRL vrednostim medtem ko je 3,1% vzorcev solate preseglo MRL vrednosti. V 98,0% vzorcev krompirja ostankov nismo določili, 2,0% vzorcev krompirja je vsebovalo ostanke manjše ali enake MRL vrednostim in nobeden vzorec krompirja ni presegel MRL vrednosti. Ostanke dveh ali več aktivnih spojin smo določili le v jabolkih in solati. Trend, ki smo ga opazili tekom let je, da delež vzorcev, ki vsebujejo ostanke dveh ali več aktivnih spojin v jabolkih, pada od leta 2005 do 2008. Najpogosteje najdena aktivna snov v jabolkih in solati je ditiokarbamati. V krompirju smo določili le fosalon.

Ključne besede: jabolka, solata, krompir, integrirana pridelava, ostanki fitofarmacevtskih sredstev, GC/MS, LC/MS/MS

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1 INTRODUCTION

The main objective of conventional farming is elimination of the pest with at least 90% effectiveness, while IPM aims to maintain a population level or balance below the tolerance threshold, intervening only when the population density exceeds an action treshold (Oliva et al., 1999). Unfortunately, conventional farming poses a negative impact on the environment, agriculture, and human health. PPP may kill pests, disease, and weed, but they also end up as residues on our food. The largest negative impact of conventional farming is its contamination of our freshwater supply, soil erosion and decreased soil fertility. PPP also enter the air and can be transported to other areas where no PPPs are used. The PPP use has a negative impact on natural predators, because they do not only kill the targeted pests but also other beneficial organisms. Regular application of PPP ends up breeding a stronger, more resistant community to PPPs (Kaul et al., 2009; Turgut et al., 2011; Vasileiadis et al., 2011)

Concerns about the negative effects of PPPs led to research and promotion of alternative crop production such as IPM.

IPM is a careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of PPPs and other forms of intervention to levels that are economically and ecologically justified and reduce or minimise risks to human health and the environment (Vasileiadis et al., 2011). In other words, the IPM is a system that includes measures required for a good agricultural practice, for the safety and hygiene of workers, for the safety of products, for the full traceability of measurements and for the preservation of the environment (Danis et al., 2011).

In spite of that, IPM itself is not a guaranty that PPP residues will not be found in the environment. Also PPP residues used in IPM are found in water, soil and are transported to other areas. But with measures taken in IPM their content in water, soil and air will be better controlled and/or reduced to minimum required for food production than in conventional farming.

In Slovenia the PPPs allowed in IPM are yearly published in the Technical Guidelines for IPM in fruit, grape, vegetables and field crop growing. The main benefits are lower incidence and lower levels of PPP residues in agricultural products. To control this type of production surveillance monitoring is required. This is why Agricultural Institute of Slovenia determined PPP residues in apple, lettuce and potato of the Slovene producers included in IPM prior to the market in accordance to the Slovenian legislation (RS 2004a, 2004b, 2007a, 2007b, 2009).The samples were taken randomly in eight production areas in Slovenia: Celje, Koper, Kranj, Nova Gorica, Novo mesto, Murska Sobota, Maribor, and Ljubljana. Apples, lettuce and potatoes were chosen because they are the most frequently consumed agricultural products in Slovenia (the Slovene Food Basket has not yet been demarcated). The results are intended to:

- Determine the conformity with the legally prescribed MRLs
- Determine the conformity of the production with good agricultural practice
- Determine the sources and/or causes of residues found

For the monitoring purposes quick and reliable multiresidual methods which enable simultaneous determination of a wide spectrum of active substances are needed. Extraction is performed by ethylacetate (Berrada et al., 2006; Čajka and Hajšlová, 2004; Ferrer et al., 2005; Sharif et al., 2006), acetonitrile (method also known as QuEChERS method) (Lehotay, 2007; Maštovská et al., 2005) or acetone (Díez et al., 2006; Pizzutti et al., 2009; Stan and Linkerhägner, 1996). We used the multiresidual method in which extraction was performed with acetone, petroleumether and dichlorometane and determination with gas chromatograph coupled to mass spectrometer (GC-MS) (Baša Česnik and Gregorčič, 2003; Baša Česnik et al., 2006). The same extraction procedure was also used for another multiresidual method in which liquid chromatography was coupled to tandem mass spectrometry (LC/MS/MS) (Bossi et al., 2002; Ortelli et all., 2004; Lehotay et al., 2005). Single residue methods were also used for the determination of maneb group and benzimidazoles. Extraction of the maneb group (dithiocarbamates) was performed with isooctane and determination with GC-MS (Baša Česnik and Gregorčič, 2006). Benzimidazoles were extracted by acetone, petroleumether and dichloromethane and determined by LC with UV and fluorescent detector (van Zoonen, 1996).

2 MATERIALS AND METHODS

From 2005 to 2009, 225 samples of apples, lettuce and potatoes from IPM were analysed. The sampling is presented in Table 1.

Table 1. Number of IPM samples of apple, lettuce and potato.

	2005	**2006**	**2007**	**2008**	**2009**	**sum**
apples	15	30	37	29	/	111
lettuce	12	12	17	12	12	65
potatoes	3	12	6	8	20	49

For the determination of PPP residues we used four different testing methods:

- method for the determination of benzimidazoles: tiabendazol and the sum of benomyl and carbendazim (in 2005-2007) (van Zoonen, 1996),
- method for the determination of the maneb group: maneb, mancozeb, metiram, propineb, thiram, ziram and zineb, the sum is expressed as carbon disulfide (in the years 2005-2009) (Baša Česnik and Gregorčič, 2006)
- multiresidual GC/MS method (in 2005-2009) (Baša Česnik and Gregorčič, 2003; Baša Česnik et al., 2006)
- multiresidual LC/MS/MS method (in 2006-2009) (Bossi et al., 2002; Ortelli et al., 2004; Lehotay et al., 2005).

In 2005 the scope was: acephate, aldrin, azinphos-methyl, azoxystrobin, bifenthrin, bromopropylate, bupirimate, captan, carbaryl, carbofuran, chlorothalonil, chlorpropham, chlorpyriphos, chlorpyriphos-methyl, cyhalotrin-lambda, cypermethrin, cyprodinil, DDT, deltamethrin, diazinon, dichlofluanid, dimethoate, diphenylamine, endosulfan, endrin, fenitrothion, fenthion, fludioxonil, folpet, HCH-alpha, heptachlor, heptenophos, imazalil, iprodione, kresoxim-methyl, lindane, malathion, mecarbam, metalaxyl, methamidophos, methidathion, myclobutanil, omethoate, oxydemeton-methyl, parathion, permethrin, phorate, phosalone, pirimicarb, pirimiphos-methyl, procymidone, propargite, propyzamide, pyridaphenthion, pyrimethanil, quinalphos, spiroxamine, thiabendazole, tolclofos-methyl, tolylfluanid, triadimefon triazophos, triadimenol and vinclozolin.

In 2006 the scope was extended with the following active substances: cyromazine, penconazole, trifloxystrobin.

In 2007 the scope was extended with the following active substances: boscalid, dichlorvos, fenamidone, quinoxyfen, tebuconazole. Cyromazine was removed.

In 2008 the scope was extended with the following active substances: carboxin, chloridazon, clomazone, cyproconazole, diniconazole, fenbuconazole, indoxacarb, metconazole, methacrifos, metribuzin.

In 2009 the scope was extended with the following active substances: acrinathrin, dazomet, desmethylpirimicarb, dimethachlor, esfenvalerate, fenvalerate, flonicamid, fluquinconazole, HCH-betha, HCH-deltha, hexachlorobenzene, metalaxyl-M, metrafenone, oxadixyl, parathion-methyl, profenofos, quinoclamine, tetraconazole, tetradifon.

In 2006 the scope of analyses was: aldicarb, bentazone, cymoxanil, difenoconazole, fenazaquin, fenhexamid, fluroxypyr, imidacloprid, methiocarb, methomyl, phoxim, pymetrozine, spirodiclofen, tebufenozide, thiacloprid, thiamethoxam and zoxamide.

In 2007 the scope was extended with the following active substances: acetamiprid, amidosulfuron, benalaxyl, bitertanol, clofentezine, cyromazine, dimethomorph, epoxiconazole, ethofumesate, famoxadone, fenpropidin, fenpropimorph, fenpyroximate, flufenacet, fluquinconazole, hexythiazox, iprovalicarb, lufenuron, metosulam, pendimethalin, prochloraz, propamocarb, propiconazole, pyridate, spinosad, terbuthylazine, thiophanate-methyl and trichlorfon.

In 2008 the scope was extended with the following active substances: aldicarb sulfon, aldicarb sulfoxid, buprofezin, carbendazim, clopyralid, clothianidin, cycloxydim, desmedipham, flutriafol, foramsulfuron, iodosulfuron-methyl-sodium, isoxaflutole, linuron, malaoxon, metamitron, metazachlor, methiocarb sulfon, methiocarb sulfoxid, methoxyfenozide, napropamide, phenmedipham, prosulfocarb, prosulfuron, pyraclostrobin, rimsulfuron, tetraconazole, thifensulfuron-methyl, thiodicarb, triasulfuron, trifluralin and triflusulfuron-methyl.

In 2009 the scope was extended with the following active substances: 2,4-D, amitrole, azinphos-ethyl, beflubutamid, benalaxyl M, bromoxynil, carbosulfan, chlortoluron, cyazofamid, demeton-S-methyl sulphone, dichloprop-P, diflufenican, dimethenamid-P, fenarimol, fenoxaprop-P-ethyl, fenoxycarb, fenthion sulfone, fenthion sulfoxide, fipronil, florasulam, fluazifop-P-butyl, fluazinam, fluorochloridone, flusilazole, hexaconazole, isoproturon, mandipropamid, MCPA, monocrotophos, nicosulfuron, oxamyl, paraoxon-methyl, phorate sulfone, phorate sulfoxide, propaquizafop, pyrazophos, teflubenzuron, tribenuron-methyl and trinexapac-ethyl. Fluquinconazole and tetraconazole were removed.

The trueness of testing methods was verified by recoveries which had to be from 70% to 120%.

The trueness was additionally verified by participation in the French inter-laboratory proficiency testing scheme BIPEA (Bureau interprofessionnel d´etudes analytiques) and CRL European Proficiency Tests.

In January 2005 determination of PPP residues was accredited by the French accreditation body COFRAC.

3 RESULTS AND DISCUSSION

During the period from 2005 to 2009, 225 IPM samples were analysed. Sample portions below the reporting level (RL), sample portions below or equal to MRLs and sample portions above MRLs are presented in Table 2.

Table 2. Sample portions of PPP residues for each analysed matrix from 2005 to 2009

	Sample portion <RL (%)	Sample portion ≤MRL (%)	Sample portion >MRL (%)
apples	38.7	58.6	2.7
lettuce	84.6	12.3	3.1
potatoes	98.0	2.0	0.0

The highest portion of PPP residues exceeding MRLs was found in lettuce (3.1%). The highest portion of PPP residues found but not exceeded, i.e. 58.6%, was found in apples. The farmers have to protect apples against rot, mould and insects otherwise they would not be able to grow them. Potatoes were an agricultural product with very little residues found.

Annual results for apples are presented in Table 3.

Table 3. PPP residues in apple samples for the period from 2005 to 2008

	Sample portion <RL (%)	Sample portion ≤MRL (%)	Sample portion >MRL (%)
2005	0.0	93.3	6.7
2006	6.7	86.7	6.7
2007	10.8	89.2	0.0
2008	27.6	72.4	0.0

The highest percentage of apple samples with detected but not exceeding PPP residues was found in 2005 (93.3%) and the lowest in 2008 (72.4%). The highest MRL exceedances in apple samples were found in 2005 and 2006 (6.7%). The trend observed during the years was exceedances that were no longer determined at the end.

Annual results for lettuce are presented in Table 4.

Table 4. PPP residues in lettuce samples for the period from 2005 to 2009

	Sample portion <RL (%)	Sample portion ≤MRL (%)	Sample portion >MRL (%)
2005	100.0	0.0	0.0
2006	91.7	8.3	0.0
2007	76.5	17.6	5.9
2008	83.3	8.3	8.3
2009	75.0	25.0	0.0

In comparison to apples lettuce is an agricultural product with less PPP residues found. The highest percentage of lettuce samples with detected but not exceeding PPP residues was found in 2009 (25.0%) and the lowest in 2005 (0.0%). The highest MRL exceedances in lettuce samples were found in 2008 (8.3%).

The least PPP residues were found in potato. In 2005, 2007, 2008 and 2009 no residues were found in potato samples. In 2006 the percentage of samples with determined but not exceeding PPP residues was 8.3%.

Multiple residues were found only in apples and lettuce. The results for apples are presented in Figure 1. The highest number of active substances found per one apple sample was 7 in 2006. The trend observed during the years was that the sample portion of samples containing multiple residues in apples decreased from 2005 to 2008

in spite of the larger number of active substances sought.

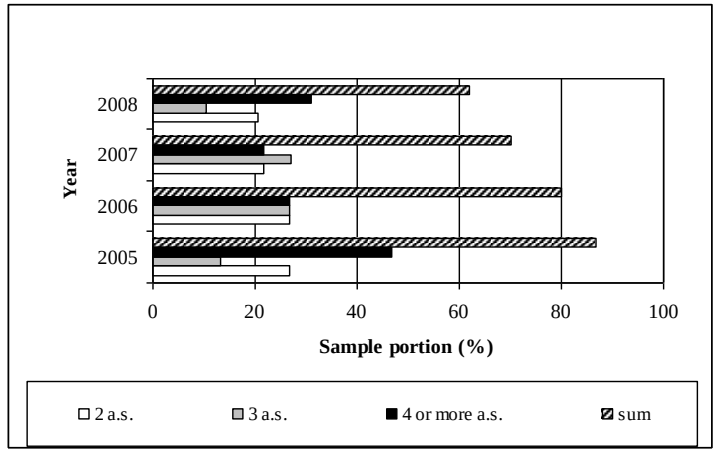

Figure 1.: Multiple residues found in apples from 2001 to 2008

In lettuce, maximum 2 active substances were found per sample: in 2007 in 5.9% of samples and in 2009 in 16.7% of samples.

Active substances found in apples are presented in Table 5. The most frequently found active substance was dithiocarbamates (maneb group).

Table 5. Active substances found in apples in the years from 2005 to 2008

	Sample portion (%)				
Active substance	**2005**	**2006**	**2007**	**2008**	**average**
Acetamiprid	n.a.	n.a.	**2.7**	**24.1**	**7.2**
Bitertanol	n.a.	n.a.	**5.4**	n.d.	**1.8**
Boscalid	n.a.	n.a.	**8.1**	**31.0**	**10.8**
Captan	**33.3**	**26.7**	**27.0**	**17.2**	**25.2**
Chlorpyrifos	**26.7**	**16.7**	**40.5**	**13.8**	**25.2**
Chlorpyrifos-methyl	**6.7**	n.d.	n.d.	n.d.	**0.9**
Cyprodinil	**13.3**	**3.3**	**2.7**	n.d.	**3.6**
Diazinon	**40.0**	**30.0**	**24.3**	n.d.	**21.6**
Difenoconazole	n.a.	n.d.	n.d.	**3.4**	**0.9**
Diphenylamine	n.d.	**3.3**	n.d.	n.d.	**0.9**
Dithiocarbamates	**93.3**	**73.3**	**32.4**	**37.9**	**53.2**
Fenazaquin	n.a.	**3.3**	n.d.	n.d.	**0.9**
Fluquinconazole	n.a.	n.a.	n.d.	**3.4**	**0.9**
Folpet	**6.7**	n.d.	n.d.	n.d.	**0.9**
Lufenuron	n.a.	n.a.	**5.4**	n.d.	**1.8**
Methoxyfenozide	n.a.	n.a.	n.a.	**13.8**	**3.6**
Phosalone	**20.0**	**46.7**	**45.9**	n.d.	**30.6**
Pirimicarb	**6.7**	n.d.	n.d.	**3.4**	**1.8**
Pyraclostrobin	n.a.	n.a.	n.a.	**31.0**	**8.1**
Pyrimethanil	n.d.	**20.0**	**10.8**	**10.3**	**11.7**
Spirodiclofen	n.a.	**16.7**	**21.6**	**10.3**	**14.4**
Tebufenozide	n.a.	**3.3**	n.d.	**10.3**	**3.6**
Thiacloprid	n.a.	**6.7**	**2.7**	**6.9**	**4,5**
Tolylfluanid	**73.3**	**30.0**	n.d.	n.d.	**18.0**
Trifloxystrobin	n.a.	n.a.	**2.7**	n.d.	**0.9**

n.a. means not analysed, n.d. means not detected

PPPs allowed in IPM of apples, which contained active substances from maneb group in years 2006-2008 are presented in Table 6.

Table 6. PPPs allowed in IPM of apple which contained active substances from maneb group from 2006 to 2008

year	PPP	active substance
2006	Bakreni dithane	mancozeb
2006 - 2008	Dithane Dg neotec	mancozeb
2006-2008	Dithane M-45	mancozeb
2006-2008	Kor DG	mancozeb
2006	Mancozeb 80 WP	mancozeb
2006	Mazeb	mancozeb
2006-2007	Penncozeb 75 DG	mancozeb
2006-2007	Penncozeb 80 WP	mancozeb
2006-2008	Polyram DF	metiram
2006-2008	Thiram 80 WG	thiram
2006-2007	Triscobal DG	ziram
2006-2007	Ziram 76 WG	ziram

Active substances found in lettuce are presented in Table 6. The most frequently found active substance was dithiocarbamates (maneb group).

Table 7. Active substances found in lettuce in the years from 2005 to 2009

	Sample portion (%)					
Active substance	**2005**	**2006**	**2007**	**2008**	**2009**	**average**
Chlorothalonil	n.d.	n.d.	**5.9**	n.d.	n.d.	**1.5**
Difenoconazole	n.a.	n.d.	**5.9**	n.d.	n.d.	**1.5**
Dithiocarbamates	n.d.	**8.3**	**5.9**	**8.3**	**8.3**	**6.2**
Iprodione	n.d.	n.d.	n.d.	n.d.	**16.7**	**3.1**
Pendimethalin	n.a.	n.a.	n.d.	**8.3**	n.d.	**1.5**
Propyzamide	n.d.	n.d.	**5.9**	n.d.	n.d.	**1.5**
Thiamethoxam	n.a.	n.d.	**5.9**	n.d.	**16.7**	**4.6**

n.a. means not analysed, n.d. means not detected

PPPs allowed in IPM of lettuce, which contained active substances from maneb group in years 2006-2009 are presented in Table 8.

Table 8. PPPs allowed in IPM of lettuce which contained active substances from maneb group from 2006 to 2009

year	PPP	active substance
2006-2009	Aliette flash	metiram
2006-2009	Aviso DF	mancozeb
2006-2009	Polyram DF	metiram
2006-2009	Ridomil gold MZ Pepite	mancozeb

In potato only one active substance was found: phosalone in one sample in 2006 (8.3% in 2006 and average for 2005-2009 2.0%). Only one PPP with active substance phosalone was allowed in IPM of potatoes in 2006 and 2007: Zolone.

Active substances that exceeded MRLs werethe following: tolylfluanid in 2005 (1 apple sample, residue=0.73 $mgkg^{-1}$, MRL=0.21 $mgkg^{-1}$) and in 2006 (2 apple samples, residue=0.24 and 0.26 $mgkg^{-1}$, MRL=0.21 $mgkg^{-1}$), chlorothalonil in 2007 (1 lettuce sample, residue=0.05 $mgkg^{-1}$, MRL=0.01 $mgkg^{-1}$) and

pendimethalin in 2008 (1 lettuce sample, residue= 0.06 $mgkg^{-1}$, MRL=0.05 $mgkg^{-1}$).

Considering the non-conformities in IPM (the use of PPP which was not allowed in IPM) we observed only one violation: in 2005 the use of PPP with the active substance folpet on apples (1 sample).

4 CONCLUSIONS

The levels of PPP residues in IPM samples of agricultural products in Slovenia in 2005 to 2009 do not give any cause for alarm. We compared our results with those of monitoring of PPP residues in the products of plant origin in 27 European Union Member States (EU MS) and 2 European Free Trade Association (EFTA) States (Norway and Iceland) (http://www.efsa.europa.eu/cs/) in 2007 (apples and lettuce) and in 2008 (potatoes). The type of production in EU is unknown. In EU the MRL exceedances in apples in 2007 match the average exceedances in IPM apples in Slovenia from 2005 to 2008 (2.7%). In EU, the MRL exceedances in lettuce in 2007 (2.9%) are slightly lower than the average exceedances in IPM lettuce in Slovenia from 2005 to 2009 (3.1%). In EU, the MRL exceedances in potatoes in 2008 (0.5%) are higher than the average exceedances in IPM potatoes in Slovenia from 2005 to 2009 (0.0%).

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Compatibility of selected herbicides with entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill

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ABSTRACT

The *in vitro* effect of five commonly used herbicides *viz*., pyridate, fluazifop-P-butyl, foramsulfuron, tembotrione and S-metolachlor on mycelial growth of entomopathogenic fungus *Beauveria bassiana* (ATCC 74040) was evaluated each at different concentrations: 100, 75, 50, 25, 12.5, 6.25 and 0% of recommended field application rate on PDA agar plates at 15 and 25°C. The herbicides tested were classified in 1-4 scoring categories based on reduction in mycelial growth: 1 = harmless (<25% reduction), 2 = slightly harmful (25-50%), 3 = moderately harmful (51-75%), harmful (>75%) in toxicity tests. All the five herbicides had fungistatic effect to *B. bassiana* at varying intensities depending on their concentrations in medium. The present study showed that *B. bassiana* is very sensitive to the herbicides tested, particularly at recommended as well as lower field dosage. The selected herbicides foramsulfuron, tembotrione and S-metolachlor have strong fungistatic effect on mycelial growth (> 75% inhibition) at 15 °C and concentrations from 50 to 100%. Foramsulfuron has fungicidal effect at 100 % concentration. Foramsulfuron, tembotrione and S-metolachlor were less inhibitory at 25 than at 15 °C, but the temperature had no influence on reduction of mycelial growth at pyridate and fluazifop-P-butyl. Of the herbicides tested, pyridate and fluazifop-P-butyl showed less adverse effects and are probably compatible with *B. bassiana* in the field. However, extensive field studies complemented by parallel laboratory experiments should consider assessing the interaction between selected herbicides and *B. bassiana* isolates to evaluate their ecological impact in cropped environments.

Key words: *Beauveria bassiana*, herbicides, inhibition, mycelial growth, compatibility

IZVLEČEK

KOMPATIBILNOST IZBRANIH HERBICIDOV Z ENTOMOPATOGENO GLIVO *Beauveria bassiana* (Bals.) Vuill

V *in vitro* poskusih smo na PDA agarnih ploščah in temperaturah 15 ter 25 °C preučevali učinek petih pogosto uporabljanih herbicidov, in sicer piridata, fluazifop-P-butila, foramsulfurona, tembotriona in S-metolaklora na rast micelija entomopatogene glive *B. bassiana* (ATCC 74040) pri različnih koncentracijah: 100, 75, 50, 25, 12,5, 6,25 in 0 % priporočenega poljskega odmerka. Glede na inhibicijo rasti micelija smo po toksikoloških testih preučevane herbicide razvrstili v štiri razrede: 1 = neškodljiv (<25 % inhibicija), 2 = malo škodljiv (25 – 50 %), 3 = zmerno škodljiv (51 – 75 %), 4 = škodljiv (> 75 %). Vseh pet herbicidov ima fungistatičen učinek na glivo *B. bassiana*, na obseg pa vpliva njihova koncentracija v gojišču. Raziskava je pokazala, da je gliva *B. bassiana* zelo občutljiva na preizkušane herbicide, posebej pri priporočenih poljskih koncentracijah, pa tudi pri manjših odmerkih. Pri temperaturi 15 °C in koncentracijah od 50 do 100 % imajo herbicidi na podlagi foramsulfurona, tembotriona in S-metolaklora izrazit fungistatičen učinek (> 75% inhibicija), pri 100 % odmerku pa ima foramsulfuron celo fungicidni učinek. Foramsulfuron, tembotrion in S-metolaklor so bili manj inhibitorni pri 25 kot 15 °C. Temperatura ni vplivala na inhibicijo rasti micelija pri piridatu in fluazifop-P-butilu. Od vseh preizkušanih herbicidov sta imela piridat in fluazifop-P-butil najmanj zaviralnih učinkov in bi jih lahko uporabljali na pridelovalnih površinah skupaj z glivo *B. bassiana*. Poleg laboratorijskih testov s herbicidi bi morali izvajati vzporedne poskuse na pridelovalnih površinah, da bi dejansko izvrednotili njihov ekološki vpliv na glivo *B. bassiana*

Ključne besede: *Beauveria bassiana*, herbicidi, inhibicija, rast micelija, kompatibilnost

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1 INTRODUCTION

Entomopathogenic fungi have an important role in biological control of various harmful insects and mites (Keller, 1991). *Beauveria bassiana* (Bals.) Vuill. is the most studied and well known entomopathogenic fungus and is frequently used in commercially available mycoinsecticides (Inglis *et al*., 2001).

Numerous organisms, physical and chemical factors of soil ecosystem and agrochemicals that are commonly used in crop production, influence entomopathogenic fungi present in soil. Their diversity and number is also affected by plant species present in crop rotation, cultural practices, intensity of soil use and fertilization with mineral and organic fertilizers (Hummel *et al*., 2002; Klingen and Haukeland, 2006).

Pesticides are anthropogenic factor with synergistic or antagonistic influence on pests as well as their pathogens (entomopathogenic fungi) and through that on their efficiency (Benz, 1987). Optimally chosen pesticides can minimize their harmful effect on entomopathogenic fungi (Luz *et al*., 2007; Sterk *et al*., 1999).The efficacy of entomopathogenic fungus *B. bassiana* incorporated in the soil is affected by regular pesticide usage in agronomical practice, because it often leads to their accumulation in soil. It is important to know the compatibility of pesticides (including herbicides) with entomopathogenic fungi to be able to include mycoinsecticides with *B. bassiana* in integrated crop protection (Ambethgar *et al*., 2009).

Non-target effects of pesticides on beneficial organisms are gaining their importance in developing active substances for new insecticides and in re-registration of existing ones in European community. Side effects of pesticides (especially of fungicides) on entomopathogenic fungi and the influence of entomopathogenic fungi on other useful microorganisms are tested from microbiological point of view (Sterk *et al*., 2003).

Due to the complexity of natural environment, expenses and duration of field experiments it is important to make some preliminary test *in vitro* where it is possible to control all the factors. Despite all that, the obtained results can not be directly used in agronomical practice. *In vivo* field test must follow in order to finally confirm or reject the outcome of laboratory tests (Mietkiewski *et al*., 1997; Moorhouse *et al*., 1992).

Data obtained by *in vitro* and some *in vivo* experiments suggest a general sensitivity of entomopathogenic fungi to some herbicides (Ambethgar *et al*., 2009; Gardner and Storey, 1985; Harrison and Gardner, 1992; Keller, 1986; Mietkiewski *et al*., 1989; Poprawski and Majchrowicz, 1995; Todorova *et al*., 1998; Wardle and Parkinson, 1992).

We have observed a strong inhibitory effect of some tested herbicides on mycelium growth of *B. bassiana* in our previous laboratory experiments. Herbicides even had a stronger detrimental effect in comparison to fungicides (Celar *et al*., 2011). Based on these results we have extended our survey to some other herbicides commonly used in Slovenian crop production and the results of this study are presented in this paper.

2 MATERIALS AND METHODS

Our test method is based on the guidelines for testing side-effects of pesticides on *B. bassiana* (Coremans-Pelseneer, 1994), but small plugs of mycelium were placed on the treated medium with different herbicide concentrations, instead of spore suspension inoculation. Five herbicides were used in our essay (Table 1).

Table 1: Basic data about herbicides used in laboratory essay.

Preglednica 1: Osnovni podatki o herbicidih, uporabljenih v laboratorijskem preizkušanju.

HERBICIDE	Active ingedient	a.i. %	Dose rate/ ha	Water per ha (l) (recommended)	FD* ml(g)/l	Manufacturer
Lentagran WP	pyridate	45	2 kg	200-400	2	Belchim Crop Protection
Fusilade Forte	fluazifop-P-butyl	15	2 l	200-300	2	Syngenta
Equip	foramsulfuron	2,25	2,5 l	200-300	2,5	Bayer CS
Laudis	tembotrione	4,4	1,5 l	200-300	1,5	Bayer CS
Dual Gold 960 EC	S-metolchlor	96	1,5 l	200-500	1,5	Syngenta

*100 % field dosage used in essay – herbicide concentration in medium
*100 % poljski odmerek v poskusu - koncentracija herbicida v gojišču

To isolate entomopathogenic fungus *B. bassiana* from a product Naturalis® (INTRACHEM Bio Italia S.p.A.) in pure culture, a standard dilution method on potato dextrose agar (PDA – Merck) medium was used. This mycoinsecticide has permission for use in Slovenia and contains *B. bassiana* isolate ATCC 74040.

Just before solidification of sterile PDA medium, herbicide was added in different concentrations; 100 % of recommended field dosage rate, 75 %, 50%, 25%, 12.5 %, and 6.25 %. Treated medium was than poured in sterile Petri dishes (9 cm diameter), 15 ml in each and cooled. Recommended water consumption for herbicides is between 200 and 500 L/ha, but for our essay common water consumption of 1.000 L/ha was used to prepare agar plates. This means that initial laboratory concentrations in agar plates were 2 to 5-times lower that it would be in actual field application suspension. E.g. dose rate of Lentagran is 2 kg/ha, our initial (100 %) concentration was 0,2 %. Control was sterile PDA medium without herbicides added. A small plug (Ø 5 mm) of *B. bassiana* inocubated in the dark on PDA plates at 25 °C for 14 days, was inverted in the center of prepared PDA plates with herbicide and control. Three repetitions were made for each treatment. Inoculated agar plates were then incubated in dark in growth chambers at 15 and 25 °C with 60 % relative air humidity.

After 7 and 14 days, mycelial growth was measured with image analyzer (Nikon NIS Elements BR 2.30).

The fungus-herbicides compatibility data were analyzed according to IOBC classification scheme (Sterk et al., 2003). The replicated fungus radial growth data were averaged and were expressed as percentage of growth inhibition in comparison to corresponding control following Hokkanen and Kotiluoto (1992).

$$I(\%) = \frac{C - H}{C} \cdot 100$$

Where: I, C, H stand for percentage of growth inhibition, growth of fungus in control and growth of fungus in herbicidal medium, respectively. The herbicides were further classified into the toxicity categories proposed by the IOBC working group: Class 1: harmless (<25% inhibition), Class 2: slightly harmful (25%-50%), Class 3: moderately harmful (51%-75%) and Class 4: harmful (>75%).The effect of the herbicides was scored as fungicidal if growth dropped totally, as otherwise it was taken as fungistatic.

All data were analysed using Student-Newman-Keuls test, $P < 0.05$ (Statgraphics Plus Professional 5.1; StatPoint Technologies, Inc.).

3 RESULTS AND DISCUSSION

Average relative mycelium growth rates of *B. bassiana* on agar plates containing different herbicides in six concentrations are presented in Figure 1 and 2. Herbicides based on foramsulfuron, tembotrione and S-metolachlor at 15°C and 50–100% concentration have a strong fungistatic effect (>75% of growth inhibition). Foramsulfuron at the highest tested concentration (100%) even had a fungicidal effect (the mycelium did not grow). The inhibitory effect of these three herbicides on mycelium growth is decreasing with the decreasing concentration in agar plates, but there are no statistically significant differences in concentrations higher or equal to 50 %, where the inhibition rate reaches 90 to 100% (Figure 1, Table 2). Even 25% concentration of herbicides results in high growth inhibitory effect (82–90%). Similar, 12.5% concentration inhibits mycelium growth for 71-74%. Even at the lowest tested concentration (6.25%) the

inhibitory effect of these three herbicides is so strong, that they are assigned to class 2 (25–50%, slightly harmful). Other two tested herbicides, i.e. pyridate and fluazifop-P-butyl, had a much lower inhibitory effect on mycelium growth in comparison to previously mentioned three herbicides. Pyridate and fluazifop-P-butyl were moderately harmful (51–75 % of inhibition, class 3) at higher concentrations (50–100%). However, at lower tested concentrations (6.25–25%) herbicides can by assigned to class 2 (slightly harmful) or class 1 (harmless) based on the inhibition rates, as in the case of fluazifop-P-butyl.

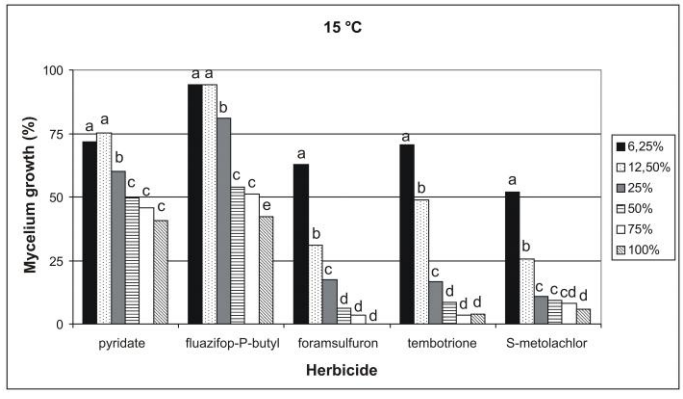

Figure 1: Mean relative mycelium growth of *B. bassiana* at different herbicides and concentrations according to control treatment in % (control treatment 100 %) at 15 °C after 14 days (different lowercase letters show significant differences in mean relative mycelial growth among different concentrations within each herbicide).

Slika 1: Povprečni relativni prirasti micelija glive *B. bassiana* pri različnih herbicidih in koncentracijah v primerjavi s kontrolo izraženi v % (kontrola je 100 %) pri 15 °C po 14 dneh (različne črke pomenijo statistično značilne razlike v priraščanju micelija pri različnih koncentracijah v okviru enega herbicida).

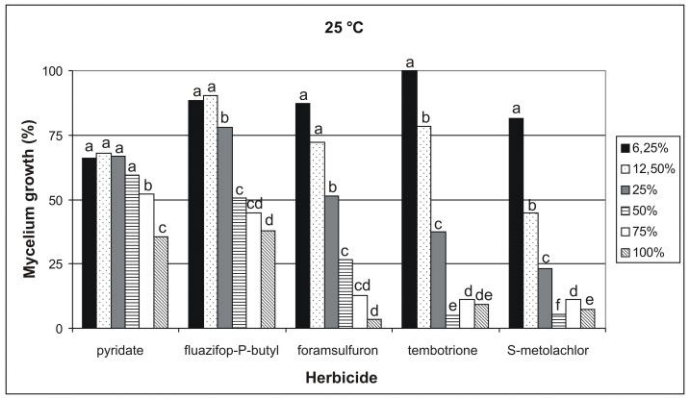

Figure 2: Mean relative mycelium growth of *B. bassiana* at different herbicides and concentrations according to control treatment in % (control treatment 100 %) at 25 °C after 14 days (different lowercase letters show significant differences in mean relative mycelial growth among different concentrations within each herbicide).

Slika 2: Povprečni relativni prirasti micelija glive *B. bassiana* pri različnih herbicidih in koncentracijah v primerjavi s kontrolo izraženi v % (kontrola je 100 %) pri 25 °C po 14 dneh (različne črke pomenijo statistično značilne razlike v priraščanju micelija pri različnih koncentracijah v okviru enega herbicida).

Similar results were obtained at 25ºC, where herbicides based on foramsulfuron, tembotrione, and S-metolachlor had a significant growth-inhibitory effect, especially at 50–100% concentrations (Figure 2, Table

2). All three previously mentioned herbicides are classified as harmless (class 1) at lowest tested concentration based on growth inhibition rates. The mycelium growth inhibition rates at lower tested concentrations (6.25–25%) are much lower at 25ºC in comparison to those at 15ºC.

Lower fungistatic effect of herbicides at higher temperature could be explained with the better vitality of *B. bassiana* at higher temperatures (its optimum temperatures for development are between 20 and 26ºC). Average growth rates of *B. bassiana* at 25ºC are up to 5-times higher compared to 15ºC. However, this does not explain the results for pyridate and fluazifop-P-butyl, where the temperature had no significant effect on mycelium growth inhibition (Figure 1 and 2).

100% concentration of tested herbicides was calculated based on the 1000 L of water used for treatment of one hectare. It can be seen in Table 1, that the manufacturer recommends 2- to 5-times less water use (200–500 L). This means 2- to 5- times higher herbicide concentration for the treatment. This decision for amount of water used was made in order to make concentrations of individual herbicides more comparable. *B. bassiana* incorporated in the soil is never exposed to the herbicide concentration used for plant treatment. Our highest concentration of herbicide on agar plates (100%), means only 20–50% of the concentration used for plant treatment on the field (depends on the type of herbicide).

Table 2: Percent of mycelium growth inhibition of *B. bassiana* at different herbicides and concentrations according to control treatment at 15 and 25 °C after 14 days and classification in scoring categories based on reduction in mycelial growth.point mutations.

Preglednica 2: Odstotek inhibicije rasti micelija glive *B. bassiana* pri različnih herbicidih in koncentracijah v primerjavi s kontrolnim obravnavanjem pri 15 in 25 °C po 14 dneh ter razdelitev v posamezne razrede glede na obseg inhibicije.

		pyridate	X	fluazifop-p-butyl	X	foramsulfuron	X	tembotrion	X	S-metolachlor	X
100 %	15°C	59,17 d[1]	3	57,60 e*	3	100 e*	4	95,95 e	4	94,09 e	4
	25°C	**64,65 d[2]**	**3**	**62,29 e***	**3**	**96,46 e***	**4**	**90,77 de**	**4**	**92,65 f**	**4**
75 %	15°C	54,34 d	3	48,75 d	2	96,38 e*	4	96,53 e	4	92,02 de*	4
	25°C	**47,97 c**	**2**	**55,23 de**	**3**	**87,11 de***	**4**	**88,70 d**	**4**	**88,82 e***	**4**
50 %	15°C	50,44 d	3	46,06 d	2	93,76 e	4	91,44 e*	4	90,78 d*	4
	25°C	**40,36 b**	**2**	**49,43 d**	**2**	**73,29 d**	**3**	**94,98 e***	**4**	**94,46 g***	**4**
25 %	15°C	40,00 c	2	18,89 c	1	82,58 d*	4	83,47 d*	4	89,13 d*	4
	25°C	**33,23 b**	**2**	**22,08 c**	**1**	**48,52 c***	**2**	**62,68 c***	**3**	**76,64 d***	**4**
12,5 %	15°C	24,72 b	1	5,66 b	1	69,07 c*	3	51,06 c*	3	74,25 c*	3
	25°C	**31,98 b**	**2**	**9,81 b**	**1**	**27,79 b***	**2**	**21,46 b***	**1**	**55,20 c***	**3**
6,25 %	15°C	28,26 b*	2	5,84 b	1	37,17 b	2	29,32 b*	2	47,96 b*	2
	25°C	**34,03 b***	**2**	**11,57 b**	**1**	**12,55 ab**	**1**	**0,00 a***	**1**	**18,66 b***	**1**
0 %		0,00 a		0,00 a		0,00 a		0,00 a		0,00 a	

Legend: X - scoring categories of inhibition: 1. class: harmless (<25%), 2. class: slightly harmful (25-50%), 3. class: moderately harmful (51-75%), and 4. class: harmful (>75%)
* significant difference of percent of mycelial growth inhibition between two temperatures (15 and 25 °C)
[1, 2] different lowercase letters show significant differences in mycelial growth inhibition among different concentrations within each herbicide at one temperature

Legenda: X – razred inhibicije: 1. razred: neškodljiv (<25 %); 2. razred: malo škodljiv (25-50 %); 3. razred: zmerno škodljiv (51-75 %) in 4. razred: škodljiv (>75 %)
* statistično značilna razlika v % inhibicije med temperaturama (15 in 25°C)
[1, 2] različne črke pomenijo statistično značilne razlike v inhibiciji med posameznimi koncentracijami pri enem herbicidu in eni temperaturi

Conditions established in soil after herbicide application (partial binding to clay and hummus particles, microbiological and chemical decomposition, rinsing, dissolving due to precipitation etc.) were simulated in this way. Beside all that, we were interested to see the effect of small amounts of herbicide residuals on entomopathogenic fungus *B. bassiana*. The later is very important to be able to evaluate the influence of residuals of herbicides used in previous crop on efficacy of mycoinsecticide based on *B. bassiana* used in current

crop. Obtained results have confirmed that our decision was correct, since all of the studied herbicides had a significant fungistatic effect even at low concentrations.

If we take a look at the harmfulness classification (Table 2) of individual treatments we see that S-metolachlor, tembotrione and foramsulfuron are harmful for *B. bassiana* at 50% of field concentration (class 4), S-metolachlor even at 25% concentration. Herbicides pyridate and fluazifop-P-butyl are moderately harmful (class 3) only at the highest tested concentrations. Laboratory experiments *in vitro* showed higher acceptability of pyridate and fluazifop-P-butyl usage together with the use of *B. bassiana* in integrated crop production in comparison to S-metolachlor, tembotrione and foramsulfuron, that are inhibitory for mycelium growth even at low concentrations.

After collecting published results of many different experiments on *B. bassiana* and pesticides Klingen and Haukeland (2006) observed that fungicides have the highest mycelium growth inhibitory effect, while insecticides and herbicides have a fungistatic effect. Detrimental effects of herbicides (especially terrestrial ones) on growth and sporulation of fungus *B. bassiana* was confirmed by many different researchers (Ambethgar, 2009; Gardner and Storey, 1985; Harrison and Gardner, 1992; Mietkiewski *et al.*, 1989; Poprawski in Majchrowicz, 1995; Todorova *et al*., 1998; Wardle and Parkinson, 1992). In our study, effects of herbicides not commonly tested by other researchers were evaluated. Only Poprawski and Majchrowicz (1995) also observed fungicidal effect of metolachlor on *B. bassiana*. Similar herbicide from our study, S-metolachlor, also inhibited mycelium growth (93%). We have to stress, that it is a similar kind of herbicide, not exactly the same one and that concentrations used in our study were lower compared to those by Poprawski and Majchrowicz (1995).

4 CONCLUSIONS

Based on the results of this essay, conclusions that entomopathogenic fungus *B. bassiana* is very sensitive to the herbicides tested can be made. Particularly at recommended, but as well as at lower field dosage, herbicides have strong fungistatic or even fungicidal effect. Preliminary tests on agar plates (*in vitro*) in laboratory conditions have limited application value and therefore can not be directly transformed in field practice. These results must be examined also in field trials (*in situ*). Besides of active ingredients the formulation of herbicide can also have fungistatic or fungicidal effect (Morjan *et al.*, 2002) meaning that results can not be generalized for all the products containing the same active ingredient.

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In vitro plant regeneration of Indian jujube (*Ziziphus mauritiana* Lamk.) cv. Zaytoni via indirect organogenesis

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ABSTRACT

In vitro plant regeneration was achieved in *Ziziphus mauritiana* Lamk., through *de novo* formation of meristems in callus obtained from the shoot tip cultured on MS medium supplemented with either IBA (10.0 mg.l^{-1}) or NAA (15.0 mg.l^{-1}) and 5.0 mg.l^{-1} BA. Organogenic callus was obtained when the primary callus was cultured on MS medium supplemented with BA at 5.0 mg.l^{-1} .Adventitious shoots were obtained when the organogenic callus was incubated on MS medium supplemented with BA at 1.0 mg.l^{-1} and NAA at 0.1 mg.l^{-1}. Whole plants were developed when the adventitious shoots were transferred to half strength MS medium supplemented with NAA at 0.2 mg.l^{-1}.

Key words: *Ziziphus mauritiana* Lamk., adventitious shoots, shoot tip explants

IZVLEČEK

***IN VITRO* REGENERACIJA INDIJSKE ŽIŽOLE (*Ziziphus mauritiana* Lamk.) CV. ZAYTONI S POSREDNO ORGANOGENEZO**

In vitro regeneracija poganjkov je bila dosežena pri indijski žižoli (*Ziziphus mauritiana* Lamk.) iz meristemskih izsečkov preko kalusa na MS gojišču z dodatkom bodisi hormona IBA (10,0 mg l^{-1}) ali NAA (15,0 mg l^{-1}) in 5.0 mg l^{-1} BA. Organogeni kalus je bil dobljen, ko se je primarni kalus prestavilo na MS gojišče z dodatkom 5,0 mg l^{-1} hormona BA. Adventivni poganjki so nastali iz organogenega kalusa, ki je bil gojen na MS gojišču z dodatkom hormonov BA (1.0 mg l^{-1}) in NAA (0.1 mg l^{-1}). Adventivne poganjke se je koreninilo na polovični koncentraciji MS gojišča z dodatkom hormona NAA (0.2 mg l^{-1}).

Ključne besede: žižola, *Ziziphus mauritiana* Lamk., meristemski izsečki, adventivni poganjki

1 INTRODUCTION

The jujube belongs to the genus *Ziziphus* Mill., which is in the *Rhamanaceae* or bulk thorn family. The genus include about 40 species of plants in tropical and sub-tropical regions of the northern hemisphere (Lyrene,1979), of which the species *Z. jujaba* Mill. and *Z. mauritiana* Lamk. were the most important in the terms of distribution and economic significance. Four species are native to Iraq, but only two, namely *Ziziphus spina-christi* (L.) Wild. and *Z. mauritiana* Lamk. are of economic importance and are grown mainly in the Basrah region (Abbas,1997).

NAA: α- naphthalene acetic acid.
IBA: Indole-3- butyric acid.

Jujube fruits have a sponge, sweet-testing pulp, and are an excellent source of ascorbic acid and carotenoids (Abbas,1997).

Although, it is possible to multiply Indian jujubes through budding of selected genotypes on seedling rootstocks, the rate of multiplication is very low and therefore is not suitable for mass production to meet the demands of planting materials. Thus to meet the demand of planting material, it is necessary to obtain a true to the type plants through a method of rapid vegetative propagation.

Plant tissue culture is in efficient method of vegetative propagation of various perennial trees. Various protocols of regeneration through shoot tip *in vitro*

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culture and nodal stem segments in Indian jujubes have been reported (Goyal & Arya,1985; Mathur *et al*.,1995; Rathore *et al*.,1992; Sudhersan *et al*.,2001; Sudhersan & Hussain,2003). So far, no information is available on the *in vitro* propagation of Indian jujubes through organogenesis (direct or indirect).

The present paper describes the induction of organogenic callus from shoot tip, organogenesis and subsequent plantlet regeneration in Indian jujube cv. Zaytoni which is an important commercial cultivar and its fruits are of excellent quality.

2 MATERIALS AND METHODS

The experiment to be described was carried out at Plant Tissue Culture Laboratories, Date Palm Research Centre, Basrah University, Basrah, Iraq.

Shoot tips (1.0 cm) of *Ziziphus mauritiana* Lamk. cv. Zaytoni was obtained from a healthy and well-established fruit yielding mature trees growing in a private orchard. The shoot tips were then kept in antioxidant solution containing 100 mg l^{-1} ascorbic acid and 150 mg l^{-1} citric acid for 24 hours to avoid phenolic compounds exudation during explants culturing. The shoot tips were then rinsed with sterile distilled water for 3 times and surface sterilized with 20% commercial Chlorax solution containing 1.05% sodium hypochlorite, and a drop of tween 20 for 15 minutes. The shoot tips were rinsed in sterile distilled water 3 times.

2.1 Callus induction

Shoot tips were cultured on full strength MS basal media, supplemented with either IBA (10 mg l^{-1}) or NAA (15 mg l^{-1}) and 5 mg l^{-1} BA. The pH of the media was adjusted to 5.7 with 0.1 N NaOH or HCl after adding 0.7% agar, and before autoclaving at 1.04 Kg cm^{-2} for 15 minutes. All media were dispensed in 25X150 mm test tubes containing 25 ml medium. Cultures were incubated under 1000 lux light intensity provided by white fluorescent lamps for 16 hrs photoperiod at 26 ± 1°C. After 60 days, white globular callus were formed at the base of the shoot tip (Fig.1 A and B).

2.2 Organogenic callus induction

The white globular callus was divided and incubated on full strength MS medium supplemented with BA at 5.0 mg l^{-1} for callus proliferation. This process continued for 120 days to obtain sufficient amount of organogenic callus (Fig.1 C), with sub-culturing every six weeks.

2.3 Adventitious shoot proliferation

The organogenic callus obtained from previous step was incubated on half strength MS supplemented with BA at 1.0 mg.l^{-1} and NAA at 0.1 mg.l^{-1} for the induction of adventitious shoots on the surface of the callus. New shoots were developed on the surface of the callus within six weeks of culture (Fig.1 D, E, F, G).

2.4 Induction of rooting

The newly formed shoots obtained in the previous step were separated with a small amount of callus and transferred to a rooting medium consisting of half strength MS supplemented with NAA at 0.2 mg.l^{-1}. Rooted shoots were obtained with in six weeks of culture on this medium (Fig.1 H, I).

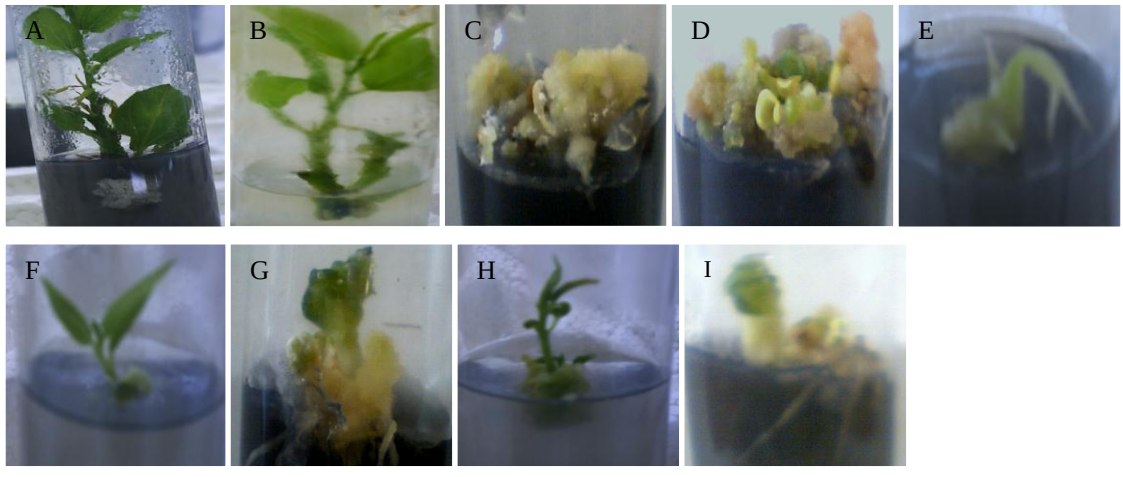

Figure 1: Indirect organogenesis and plantlet regeneration in Indian jujube (*Ziziphus mauritiana* Lam. cv. Zaytoni): A, B: Callus formation; C: Callus proliferatio; D,E,F,G: Adventitious shoot proliferation; H,I: Rooted shoot.

3 RESULTS AND DISCUSSION

It is evident from Fig. 1, that *Ziziphus mauritiana* Lamk. cv. Zaytoni can be clonally mass propagated *in vitro* using indirect organogenesis from shoot tip derived callus. To our knowledge, this is the first report on the use of indirect organogenesis for *in vitro* propagation of Indian jujube.

Earlier several authors have used shoot tip culture and auxiliary meristem culture for *in vitro* propagation of Indian jujube of several cultivars, but the major problem was low rooting efficiency (Goyal & Arya,1985; Mathur *et al*.,1995; Rathore *et al*., 1992; Sudhersan *et al*.,2001). In the closely related species, *Ziziphus sativa* L. (wild jujube) and *Z. jujuba* Mill. (Chinese jujube), *in vitro* plant regeneration have been achieved using indirect and direct organogenesis but the source of explants were zygotic embryos and seedling trees (Kim *et al*.,1987; Gu & Zhang,2005).

Callus initiation occurred at the cut end of the shoot tip explants, as white globular mass on MS medium containing 5.0 mg l^{-1} BA + 10.0 mg l^{-1} IBA within 60 days. Profuse callus was obtained on MS medium supplemented with BA at 5.0 mg l^{-1}. The results obtained in the present work are similar to those reported for other jujube cultivars, regarding the importance of auxins for callus induction and cytokinins for callus proliferation (Kim *et al*.,1987; Rathore *et al*.,1992; Mitrofanova *et al*.,1997; Gu & Zhang,2005). It is obvious from the present work, that half strength MS medium supplemented with BA at 1.0 mg l^{-1} and NAA at 0.1 mg l^{-1} promoted adventitious shoot regeneration (Fig.1). Similar results were obtained by Kim *et al*.(1987) for *Z. sativa* L. and Gu & Zhang (2005), for *Z. jujuba* Mill., who demonstrated the importance of cytokinins/auxins ratio in adventitious shoot proliferation during *in vitro* indirect and direct organogenesis. The importance of appropriate ratio of cytokinins: auxins for the production of adventitious shoots *in vitro* is well documented for a wide range of plant species (Collins & Edwards,1998).

Regenerated shoots rooted (100%) when they transferred to half strength MS medium supplemented with NAA at 0.2 mg l^{-1} (Fig. 2).

In conclusion, the results obtained in the present work demonstrate efficient cloning of *Z. mauritiana* Lamk. cv. Zaytoni via indirect organogenesis.

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Agris category code: F02, F62

Acclimatization of terrestrial orchid *Bletilla striata* Rchb.f. (Orchidaceae) propagated under *in vitro* conditions

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ABSTRACT

Bletilla striata is a terrestrial sympodial orchid. Substrates used for outdoor growing, differing in the mixture of added components and nutrients, were chosen for acclimatization of the asymbiotic propagated plants. A total of 651 *Bletilla striata* orchids were planted in 3 commercial substrates: ″Tonsubstrat″ (Ton), ″Baltisches substrat″ (Baltski) and ″Royal-Garden″ (Royal). Prior to acclimatization, the plants were 2.5 cm on average, with at least 2 leaves and 2 - 3 cm long root or roots. Fewest plants (3.1%) died in Ton substrate, 3.2% in Baltski substrate and 5.0% in Royal substrate. There were no statistically significant differences among substrates (p = 0.558) in the percentage of plants that died. Substrates used in combination with the chosen phenophase and established conditions were suitable for acclimatization of *Bletilla striata* orchids, whereby 95-97% of plants successfully adapted from heterotrophic to autotrophic conditions in a very short period of two months. The basic conditions for success are that plants are large and vital enough prior to acclimatization, that the substrate is appropriate and that appropriate conditions of relative humidity, temperature, light and ventilation without major fluctuations of these factors are ensured during acclimatization.

Key words: ornamental orchid, *Bletilla striata*, tissue culture, acclimatization, substrate, growth, development

IZVLEČEK

AKLIMATIZACIJA TERESTIČNE ORHIDEJE *Bletilla striata* Rchb.f. (Orchidaceae) RAZMNOŽENE V *in vitro* RAZMERAH

Bletilla striata je simpodialno razraščajoča se, v tleh rastoča orhideja. Za aklimatizacijo asimbiotsko razmnoženih rastlin smo izbrali substrate, ki se uporabljajo za gojenje rastlin na prostem ter se razlikujejo glede mešanice dodanih komponent in hranil. Skupno je bilo posajenih 651 orhidej v 3 komercialne substrate, ″Tonsubstrat″ (Ton), ″Baltisches substrat″ (Baltski) in ″Royal-Garden″ (Royal). Pred aklimatizacijo so imele rastline v povprečju 2,5 cm velik nadzemni del z vsaj dvema listoma in 2 do 3 cm dolgo korenino oz. korenine. Najmanj (3,1%) rastlin je propadlo v postopku aklimatizacije v Ton substratu, 3,2% v Baltskem substratu in največ 5,0% v Royal substratu. Med substrati ni bilo statistično značilnih razlik (p = 0,558) v odstotku propadlih rastlin med aklimatizacijo. Uporabljeni substrati v kombinaciji z izbrano fenofazo in vzpostavljene razmere so zelo primerni za aklimatizacijo te orhideje saj se je 95 do 97% rastlin uspešno prilagodilo iz heterotrofnih na avtotrofne razmere in to v zelo kratkem obdobju dveh mesecev. Osnovni pogoj uspeha je, da so *in vitro* rastline pred aklimatizacijo dovolj velike in vitalne, primeren substrat ter ustrezne razmere, vlaga, temperatura, svetloba in kroženje zraka, brez večjih nihanj v obdobju aklimatizacije.

Ključne besede: okrasna orhideja, *Bletilla striata*, tkivna kultura, aklimatizacija, substrat, rast, razvoj

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1 INTRODUCTION

Bletilla striata Rchb.f. (Orchidaceae) is a sympodial, terrestrial orchid originating from China and Japan. It has short rhizomes that develop corm-like pseudobulbs at ground level. Each pseudobulb bears several lance-shaped pleated leaves and is of annual duration only. On established plants, almost every new growth shoot has a flower spike before leaves fully develop. Inflorescences are terminal racemes, with up to 12 bell-shaped magenta flowers, with a ruffled lip, 3 cm in diameter (Brickell, 1996).

Bletilla is very easy orchid to cultivate. It was the second tropical orchid to be cultivated in Europe in the 18th century and is still often labeled simply 'Hardy Orchid' or 'Chinese Ground Orchid'. It is semi-hardy and needs protection from severe frost. It is deciduous, dying back to ground level in autumn. The blooms usually last a few weeks if conditions are good (Strong, 2000).

Bletilla is used in both traditional Chinese and in modern medicine. When employed in herbal remedies, the tuber is peeled and dried in the sun, then cut into slices or ground into a powder (Yeung, 1985). Among modern applications, *Bletilla* is often used in antibacterial, anti-inflammatory, anti-phlogistic, demulcent, pectoral, skin, styptic and vulnerary treatment and because of its astringent properties also to stop bleeding, heal wounds, reduce swelling and promote tissue regeneration (Singh and Duggal, 2009). Recent studies indicate that *Bletilla* can have an important role in the treatment of liver tumor (Qian et al., 2003). Another study (Diaoa et al., 2008) claims that *Bletilla striata* polysaccharide enhances the wound healing mechanism with an influence on macrophages.

The ultimate success of micropropagation on a commercial scale depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates (Chandra et al., 2010). Environmental conditions for *ex vitro* growth are quite different from those used for *in vitro* cultivation (Kozai et al., 1997). The acclimatization of *in vitro* plants is the last phase of micropropagation and is essential for the survival and successful establishment of plantlets. In other words, the survival percentage is determined by the hardening of the plantlets (Deb and Imchen, 2010). The conditions during *in vitro* culture result in the formation of plantlets of abnormal morphology, anatomy and physiology. After *ex vitro* transfer, plantlets endure shock because of sudden changes in environmental conditions. They need a period of acclimatization to correct the abnormalities (Pospišilova et al., 1999).

It is well documented that *in vitro* grown plantlets exhibit a low capacity for inorganic carbon assimilation because of their heterotrophic metabolism (Premkumar et al., 2001). The use of air-tight vessels in order to prevent contamination in tissue culture decreases air turbulence and limits the inflow of CO_2. The culture conditions also have very high air humidity and low irradiance, and the cultivation media are supplemented with saccharides (sucrose, glucose) as carbon and energy sources (Pospišilova et al., 1999). Under standard tissue culture conditions, the relative humidity is usually greater than 95%. *In vitro* leaves may not develop a waxy cuticle and functional stomata to the same extent as found in *ex vitro* plants (Seelye et al., 2003). Acclimatization of regenerates overcomes this threat by gradual lowering the air humidity (Bolar et al., 1998). Ventilation using loosely fitting closures or vents reduces the relative humidity, which leads to increases in plant transpiration and the development of functional stomata for controlling plant water loss (Seelye et al., 2003). During the acclimatization process, seedlings must overcome the critical phase when the heterotrophic behavior of the *in vitro* plants is shifted to autotrophic functioning.

The aim of our work was to optimize the acclimatization of *Bletilla striata* orchids as the final stage of successful micropropagation. We tested three different substrates and used mini-greenhouses for the procedure.

2 MATERIAL AND METHODS

2.1 Plant material

A *Bletilla striata* plant has been growing for some years outdoors in a garden in Ljubljana. Flowers were pollinated in summer, seed capsules were collected in October when fully mature and were stored in a refrigerator at 4 °C until use.

2.2 *In vitro* and *in vivo* propagation

Seeds were surface sterilized using dichloroisocyanuric acid (Sigma-Aldrich, St. Loius, MO, USA) in a 1.6% solution with Tween 20 (Sigma-Aldrich) added as surfactant. Seeds were then inoculated on commercial media Sigma P1056. Seedlings of a size of 1 to 2 cm, with second leaf indicated and at least one root, were transferred to sub-cultivation media consisting of macro elements of B5 media (Gamborg et al., 1968), micro elements of MS media (Murashige in Skoog, 1962) and other components, except banana powder, summarized from Hinnen et al. (1989). Plants 2 to 3 cm large with at least 2 leaves and 2 roots from the sub-cultivation media were randomly planted in 3 different substrates. Prior to transplantation, media was carefully cleaned or washed from the roots with distilled water to prevent pathogenic microorganisms developing. The cleaned plants were left in distilled water for protection against desiccation. They were then planted in substrate and immediately covered with a mini-greenhouse cover (Figure 1A, B and C). During acclimatization, the plants were watered with distilled water to prevent calcium carbonate deposition on plants, which could block stomata on leaves.

2.3 Substrate

Three commercial organic substrates were used in the acclimatization experiment: ″Tonsubstrat″, ″Baltisches substrat″ and ″Royal-Garden″. They are used for outdoor growing and are poor to middle-rich with nutrients

In our experiment, ″Tonsubstrat″ was labeled ″Ton″ and is a product of Klasmann-Deilmann GmbH, Germany. It consists of a mixture of poorly to medium decomposed white peat and very decomposed black peat and clay grains. The electrical conductivity of the substrate is 40 mS/m (+/- 25 %), pH value is 5.5 - 6.5 and the amount of added fertilizer is 1.5 kg/m^3 NPK 14:16:18.

″Baltisches substrat″ was labeled ″Baltski″ and is of Baltic origin, producer Hawita-Grupe GmbH, Germany. It consists of a mixture of clay grains, white peat, bark, humus, perlite and other components which are not specified. The pH value and salt content varies.

″Royal-Garden″ was labeled ″Royal″ and is a product of Humko Bled, Slovenia. It consists of a mixture of siliceous fine sand, vermiculite and clay. The pH value is 6, soluble nutrient content 180 - 350 mg/l N, 200 - 400 mg/l P_2O in 200 - 450 mg/l K_2O.

After random planting of seedlings in substrate, the process of acclimatization, i.e., adaptation to autotrophic metabolism started.

2.4 Growing place and acclimatization procedure

Orchids were acclimatized in plastic mini-greenhouse-like seed trays, consisting of two parts. The bottom part was dark green, made from more flexible plastic, while top part (cover) was harder and transparent. There were two openings on the cover, i.e., vents for ventilation of the growing area. The size of the bottom part was 36 × 22 × 6 cm and the size of cover 36 × 22 × 12 cm (Figure 1C).

After planting, the plants were moderately watered with distilled water. The substrate should not be too moist, because the plants have unhardened (thin and tender) cell walls in the hypocotyl part and can quickly become infected and die. We therefore placed two 50 ml beakers with water in each mini-greenhouse to establish a high relative humidity as soon as possible. The mini-greenhouses were placed in a shaded part of a greenhouse. After one week, the beakers with water were removed. The vents on the covers of the mini-greenhouses stayed closed for two weeks (Figure 1C) but the growing area was ventilated by removing the covers for a few minutes every day and then re-closed. The vents on the covers were gradually opened in the third week. In the fourth week, the covers were gradually lifted and, at the end of the week, completely removed. The orchids in the opened mini-greenhouses were watered at least once a week or as required depending on the moistness of the substrate.

2.5 Evaluation of data

The number of surviving and dead plants was monitored every two weeks. The first data were collected after the 2nd week of acclimatization, the second data collection was after the 5th week and the third in the 8th week, when the covers of the mini-greenhouses were removed and the plants were exposed to greenhouse conditions for two weeks. Data were processed using a logistic regression model.

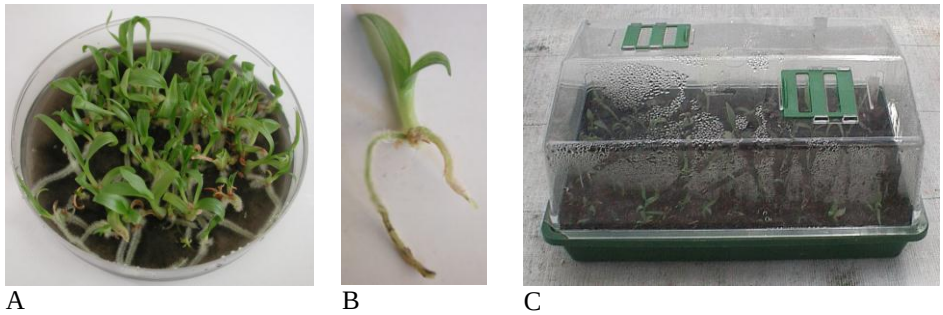
3 RESULTS AND DISCUSION

A total of 651 *Bletilla striata* orchids were planted in three commercial substrates, Ton, Baltski and Royal. They were asymbiotically propagated and grown *in vitro* until acclimatization (Figure 1A and B). All plants were subject to the same growth conditions except for the substrate. Mini-greenhouses were used for easier maintenance of conditions. Very high relative humidity was maintained in the growing area of the mini-greenhouses at the beginning, from 95-100%, which was later gradually reduced (Figure 1C). It is important for the substrate to maintain aeration in spite of the high relative humidity in the growing area. It is possible to establish a high relative humidity in mini-greenhouses quickly and successfully, which is very important for heterotrophic plant survival. Gordon (1991) considered five factors to be very important and they must be provided and controlled in tropical orchid acclimatization. In addition to humidity, these are substrate, temperature, light and air circulation. It is most important to maintain permanent conditions, i.e., to avoid stress.

Bletilla striata is a sympodial ground growing orchid, so we chose substrates that are used for growing outdoor plants and differ in the mixture of added components and nutrients.

No plants died in the first two weeks of acclimatization. During that period, the plants grew at high relative humidity, which varied from 95 to 100%, except during daily ventilation (Figure 1C). Such humidity was maintained at the beginning with water in beakers and, during the second week, by watering with distilled water only, in order not to block the stomata on leaves. Conditions during that period were very similar to *in vitro* conditions, except for the type and concentration of available nutrients and the light, which was suitable for photosynthesis.

In the third week, the vents on the covers of the mini-greenhouses were gradually opened and the relative humidity in the growing area was lowered. That influenced the function of the stomata and the formation and hardening of a wax cuticle on the leaves and stems.



A B C

Figure 1: Acclimatization of *Bletilla striata*: A - *in vitro* cultivated plants before acclimatization; B - media removed from roots before planting in substrate; C - acclimatization in mini-greenhouse.

The highest number of plants died during the 5th week of acclimatization, when the covers of the mini-greenhouses were totally removed and the plants were directly exposed to conditions in a greenhouse. The highest percentage of plants died during the fifth week was 2.2%, in Ton substrate. Percentage of plants died in Baltski substrate and Royal substrate was 1.8% and 1.1%, respectively (Table 1). Over the course of 5 weeks of acclimatization, the plants hardened and established stomata function and, consequently, transpiration. They did not therefore wilt after removal of the covers, although a few plants died during the next phase.

During the 8th week of acclimatization, the plants had already been exposed to greenhouse conditions for two weeks and dying was reduced, except in Royal substrate. Fewest plants (0.9%) died in Ton substrate, 1.4% in Baltski substrate but dying increased in Royal substrate to 3.9% of plants (Table 1). By that time, the plants had adapted from heterotrophic to autotrophic conditions and acclimatization was completed.

Table 1: Number of planted and dead plants of *Bletilla striata* during the acclimatization procedure and the results of the logistic regression model.

Substrate	**Number of planted plants**	**Number and percentage of dead plants**				**Total**	**Logistic model estimates**	
		5th week		**8th week**			**% of died**	**95% Conf. Int.**
Ton	198	5	2.2%	1	0.9%	6	3.1	1.2-6.4
Baltski	223	4	1.8%	3	1.4%	7	3.2	1.3-6.4
Royal	230	2	1.1%	9	3.9%	11	5.0	2.6-8.8

The acclimatization procedure was very successful in all substrates, 95-97% of plants survived and the number of dead plants was insignificant. There were no statistically significant differences among substrates (p = 0.558) in the percentage of dead plants (Table 1).

The acclimatized plants developed new leaves and roots, some of them also a pseudobulb and new shoots (Figure 2A, B and C).

A B C

Figure 2: *Bletilla striata* plants after acclimatization: A - plant with new leaves and roots after acclimatization; B plant with a new shoot after acclimatization; C - plant with pseudobulbs and a new shoot after acclimatization.

It is also important for plants to be exposed to light during acclimatization, which enables them to establish the process of photosynthesis. Other important conditions are an appropriate temperature without major fluctuations or air circulation. This was achieved by planting the plants in mini-greenhouses at the end of March, when the day is longer, and they were then placed in a greenhouse. When the period of hot weather started, the mini-greenhouses were moved to a shaded part of the greenhouse. Every day during the first two weeks, when the mini-greenhouses were completely closed, we removed the covers for a few minutes and exchanged the air in the growing area. Gordon (1991) recommended artificial light, whereby it is easier to control the length and intensity of illumination. Artificial light avoids seasonal differences or longer periods of cloudy weather and, in addition, partly solves the heating problem.

In addition to the listed factors and appropriate substrate, the size and developmental stage and vitality of *in vitro* cultivated plants are very important. The orchids included in the experiment were an average size of 2.5 cm, with at least 2 leaves and 2 - 3 cm long root or roots (Figure 1A and B). Croezen (2002) reported that the appropriate size for orchid acclimatization is when their leaves are at least 5 cm, while Park et al. (2003) recommended a shoot size of 3 - 4 cm with two leaves and 3 - 4 roots. Nayak et al. (1997) recommended for the monopodial orchid *Acampe praemorsa* (Roxb.) Blatt. & McCann a shoot with an average of 4.5 roots, 3.7 cm long. Chang and Chang (1998) state that the appropriate size of regenerants for the sympodial orchid *Cymbidium ensifolium* (L.) Swartz is 5 cm. In other literature, there are different data depending on the genus or species of orchid.

Our plants were smaller and with fewer roots than stated in the aforementioned literature. No data are available in the literature for *Bletilla striata* orchid, so we decided the appropriate size and phenophase ourselves, based on when the plants have at least the minimum of nutrients stored in the leaves and roots required for the acclimatization process. By using the minimum plant size possible to acclimatize we shortened the period of *in vitro* cultivation, which is very important for mass market production.

The percentage of acclimatized plants was high (95 - 97%) and was obtained in two months, which is very fast according to the literature. All factors were not optimal, as recommended for other genera and species of tropical orchids. Chen et al. (2002) reported 90% successful acclimatization for the sympodial orchid *Epidendrum radicans* Lindl. Chen and Chang (2000) reported 100% success for the sympodial orchid *Oncidium*. Murthy and Pyati (2001) reported 84% acclimatization after 3 months for the monopodial orchid *Aerides maculosum* Lindl.

4 CONCLUSIONS

The use of mini-greenhouses was very handy for the acclimatization of a smaller to medium number of plants. It allows optimal relative humidity, temperature and ventilation to be maintained easily without much investment. Some processes that were included in acclimatization - appropriate ventilation of the growing area, temperature, no exposure to direct sunlight and the provision of shade significantly affected plant survival. We also observed that acclimatization success depended on the interaction of the aforementioned factors, on avoiding major fluctuations of these factors and of suitable phenophase and vitality of the *in vitro* cultivated plants. Plants with 2.5 cm average size with at least 2 leaves and 2 - 3 cm long root or roots were

very suitable. Substrates used in combination with the chosen phenophase and established conditions were suitable for the acclimatization of a smaller number of *Bletilla striata* orchids, whereby 95-97% of plants successfully adapted from heterotrophic to autotrophic conditions in a very short period of two months.

It can be concluded on the basis of the presented data that the fundamental conditions for success are size, vitality and an appropriate stock of nutrients in the leaves and roots of *in vitro* propagated plants, as well as appropriate measures during the acclimatization period.

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Agris category code: H20

Antraknoza pri ameriških borovnicah (*Vaccinium corymbosum* L): povzročitelji in epidemiologija bolezni

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IZVLEČEK

Antraknoza sodi med najpomembnejše glivične bolezni gojenih borovnic. Prizadene predvsem plodove: v času zorenja in med skladiščenjem se značilno zgubajo in zmehčajo, prekrijejo jih oranžne sluzaste gmote trosov. V letih 2005 - 2009 smo v nasadih ameriških borovnic na Ljubljanskem barju zbrali 33 primerkov simptomatičnih rastlin. S standardnimi mikroskopsko morfološkimi in molekulskimi tehnikami smo ugotovili, da je povzročiteljica antraknoze pri ameriških borovnicah gliva *Colletotrichum fioriniae*. S sukcesivno izolacijo iz naravno okuženih ameriških borovnic smo ugotovili, da je gliva *C. fioriniae* navzoča tekom vse rastne dobe. Izolirali smo jo tako iz organov in tkiv z izraženimi bolezenskimi znamenji kot tudi iz navidezno zdravih. Potrdili smo, da gliva prezimi v poganjkih z odmrlimi vršički ter v ostankih pecljevine, pa tudi v navidezno zdravih poganjkih in brstih. Brsti, zlasti rodni, so poleg poganjkov z odmrlimi vršički in ostankov pecljevine najpomembnejši vir primarnega inokuluma.

Ključne besede: ameriška borovnica, antraknoza, *Colletotrichum fioriniae*

ABSTRACT

ANTRACNOZE IN AMERICAN BLUBERRY (*Vaccinium corymbosum* L): FUNGUS AND EPIDEMIOLOGY OF DISEASE

Anthracnose is one of the most important fungal diseases of cultivated blueberries. It mainly affects fruits and causes rotting of ripe fruit both before harvest and during storage. Infected blueberries become wrinkled, soft and covered with slimy orange conidial masses. In the years 2005 -2009 we collected 33 samples of symptomatic plants from high-bush blueberry plantations in the Ljubljana Wetland. Using standard morphological and molecular methods we identified *Colletotrichum fioriniae* as the causative agent of the disease. Successive isolations from naturally infected high-bush blueberry bushes revealed the presence of *C. fioriniae* during the entire growing season. It was consistently isolated from symptomatic as well as from symptomless tissues. We confirmed that the fungus overwinters in canes with dead tips and fruit spurs and also in symptomless canes and buds. Buds, particularly flower buds, appear to be the most important source of primary inoculum apart from canes with dead twigs and fruits spurs.

Key words: high-bush blueberry, anthracnose, *Colletotrichum fioriniae*

1 UVOD

Antraknoza je pomembna glivična bolezen gojenih borovnic. Prizadene številne vrste iz rodu *Vaccinium*, predvsem severnoameriške vrste *V. corymbosum*, *V. angustifolium* in *V. ashei*. Bolezen je razširjena in gospodarsko pomembna v številnih pridelovalnih območjih borovnic v ZDA in Kanadi (Milholland, 1995; Verma in sod. 2006). Tamkajšnji pridelovalci borovnic ocenjujejo, da zaradi nje vsako leto propade 10 - 20 % pridelka, med skladiščenjem pa se obseg škod znatno poveča in doseže tudi do 80 % (Milholland 1995).

Znamenja antraknoze so najbolj izrazita na dozorevajočih borovnicah: okužene jagode se zmehčajo in zgubajo, prekrijejo jih gmote trosov, ki so videti kot oranžno rjave kapljice na površju plodov. Prizadeti plodovi odpadejo z grmov, so neokusni in neuporabni. Pogosto se bolezenska znamenja pokažejo šele med skladiščenjem borovnic ali prevozom na tržišče. Bolezen prizadene tudi poganjke in liste, na katerih povzroča nekroze (Yoshida in Tsukiboshi, 2002); večja gospodarska škoda pa nastane le na plodovih.

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Bolezen povzročajo glive iz rodu *Colletotrichum*. Rod je taksonomsko zelo zapleten. Opravljenih je bilo več temeljitih revizij (von Arx, 1957; Sutton, 1992; Hyde in sod., 2009), vendar število vključenih vrst in njihov status še nista dorečena. Na osnovi taksonomskih in filogenetskih raziskav, opravljenih v zadnjih letih predvidevajo, da rod *Colletotrichum* obsega 66 vrst (Hyde in sod., 2009). Številne so gospodarsko zelo pomembne in povzročajo nevarne bolezni na kmetijskih rastlinah, pa tudi na okrasnem in gozdnem drevju ter grmičevju. Razširjene so v zmernem, tropskem in subtropskem pasu. Zmožnost, da povzročijo latentno ali skrito okužbo jih uvršča tudi med pomembne povzročiteljice skladiščnih bolezni. Bolezenska znamenja, ki jih povzročajo, imenujemo antraknoza in se kažejo kot uleknjene, bolj ali manj okrogle temne pege na katerih se razvijejo trosišča (acervuli) in rožnati do oranžni skupki trosov. Nekatere vrste rodu *Colletotrichum* imajo zelo širok spekter gostiteljev, druge so ozko specializirane na posamezne družine, rodove, vrste ali celo kultivarje.

V starejših virih navajajo kot povzročiteljico antraknoze pri ameriških borovnicah vrsto *C. gloeosporioides* (Hartung in sod. 1981; Daykin in Milholland, 1984), v novejših pa vrsto *C. acutatum* (Verma in sod., 2006; Yoshida in Tsukiboshi, 2002, Talgø in sod., 2007). Gliva *C. acutatum* je do nedavnega veljala za glavno povzročiteljico antraknoze pri sadnem drevju in jagodičevju. Prvič je bila najdena v Avstraliji leta 1965. Kasneje so jo zasledili na več kot štiridesetih gostiteljih po vsem svetu. Pri različnih gostiteljih okuži različne organe in tkiva in povzroča različne bolezni: črno pegavost jagod, sušenje poganjkov, defoliacijo in propadanje plodov pri mandlju, gnitje plodov pri jablani, oljki, češnji in citrusih ter druge bolezni (Brown in sod., 1996; Talhinhas in sod., 2005; Børve in Stensvand, 2006; Freeman in Katan, 1997). Zaradi velikega gospodarskega pomena so jo v Evropski zvezi uvrstili med karantenske škodljive organizme, pred nekaj leti pa umaknili s seznama zaradi njene vsesplošne razširjenosti v naravnem okolju. V zadnjih letih ugotavljajo, da *C. acutatum* ni enotna vrsta, temveč kompleks, ki vključuje več različnih vrst in molekularnih skupin. V populaciji glive so sprva identificirali osem molekularnih skupin in jih poimenovali A1 do A8 (Sreenivasaprasad in Talhinhas, 2005), kasneje pa štiri molekularne skupine opisali kot samostojne vrste: *C. simmondsii, C. fioriniae, C. clavatum in C. acutatum* sensu stricto (Shivas in Tan, 2009; Faedda in sod., 2011).

V nasadih ameriških borovnic na Ljubljanskem barju je antraknoza ena najpomembnejših bolezni. V letih, ki so za njen razvoj ugodna, povzroči pri občutljivih sortah velik izpad pridelka. O bolezni smo poročali leta 2006 in kot povzročiteljico identificirali vrsto *C. acutatum* s.l. (Munda in Žerjav, 2006). Namen sedanje raziskave je bil identificirati povzročitelje bolezni glede na novejša taksonomska spoznanja o rodu *Colletotrichum* ter raziskati vire okužbe in druge ključne dejavnike bolezenskega cikla v naših pridelovalnih razmerah.

2 MATERIAL IN METODE

Primerke ameriških borovnic (*Vaccinium corymbosum* L) z znamenji antraknoze smo nabrali v nasadih na Ljubljanskem barju, v okolici Borovnice in na Drenovem griču. V letih 2005 – 2009 smo zbrali in analizirali 33 vzorcev simptomatičnih rastlin. Iz okuženega rastlinskega materiala smo izolirali povzročitelje bolezni po standardnem postopku: obolele rastlinske dele smo površinsko razkužili, izrezali tkivo na robu med zdravim in okuženim in ga prenesli na krompirjevo dekstrozno gojišče z dodanim antibiotikom (PDA+). Pri nadaljnjih morfoloških in molekulskih analizah ter umetnih inokulacijah smo uporabili enotrosne izolate, ki smo jih pridobili z osamitvijo posameznih kalečih trosov. Izolate smo shranili na poševnem krompirjevem dekstroznem gojišču (PDA) pri temperaturi 4° C, rastlinske dele z znamenji antraknoze pa kot herbarijski material.

Za identifikacijo dobljenih izolatov smo uporabili standardne mikroskopsko morfološke in molekulske tehnike. Izmerili smo velikost konidijev, ki so se razvili v deset dni starih kulturah na gojišču PDA, pri temperaturi 25° C in v temi. Konidije smo obarvali z anilinskim modrilom v laktoglicerolu. S prekrivanjem micelija s krovnimi stekelci smo pospešili tvorbo apresorijev, nato pa zabeležili njihovo velikost in obliko. Izmerili smo prirast kolonije med petim in devetim dnem rasti na PDA gojišču, pri temperaturi 25° C in v temi. Pri deset dni starih kolonijah smo zabeležili barvo micelija in pigmentacijo podlage ter jakost sporulacije. Iz micelija, namnoženega v tekočem gojišču, smo izolirali DNA s pomočjo komercialnega kompleta BioSprint 15 DNA Plant Kit (Qiagen) in robota KingFisher mL (Thermo). ITS predel ribosomske DNA smo v verižni reakciji s polimerazo (PCR) namnožili z začetnima oligonukleotidoma ITS1 in ITS4 (White in sod., 1990). Dobljenim produktom smo določili nukleotidno zaporedje (Macrogen, Koreja), jih uredili in primerjali z drugimi zaporedji v javnih bazah s pomočjo orodja BLAST (Altschul in sod., 1997). V primerjavo smo vključili zaporedja referenčnih izolatov vrst *C. acutatum* (AF411700, FJ788417), *C. fioriniae* (EF464594), *C. simmondsii* (GU183331), *C. clavatum* (JN121126) in *C. gloeosporioides* (AJ749693, AJ749682), ki so bila objavljena v študijah Talhinhas in sod. (2002, 2005), Shivas in Tan (2009), Vinnere in sod. (2002) ter Faedda in sod. (2011).

Virulentnost izbranih izolatov smo preverili z umetno inokulacijo plodov. Poleg ameriških borovnic smo okužili še jagode, češnje in jabolka. Inokulum smo pripravili iz deset dni starih kultur, ki smo jih prelili s sterilno destilirano vodo in postrgali trose s površja kolonije. Zrele plodove smo površinsko razkužili z natrijevim hipokloritom (1-3 % raztopina), ranili povrhnjico in nanjo nanesli inokulum (7 µl suspenzije trosov v koncentraciji $1x10^6$ konidijev / ml). Na kontrolne plodove smo po enakem postopku nanesli sterilno destilirano vodo. Inokulirane plodove smo deset dni inkubirali pri sobni temperaturi (22 – 25° C) in 100 % relativni zračni vlagi, nato pa preverili pojav bolezenskih znamenj in opravili reizolacijo glive iz okuženih plodov.

V letih 2007 in 2008 smo v nasadu ameriških borovnic na Drenovem Griču, v katerem je antraknoza že dlje časa navzoča, naključno izbrali tri grme sorte Coville in z njih jemali vzorce za analizo. Vzorčili smo enkrat mesečno, od začetka maja do konca avgusta 2007 ter v marcu in aprilu 2008. Vsakokrat smo na izbranih grmih odrezali po dva poganjka, dolga 15 – 25 cm. Posamezne rastlinske dele smo ločili, razrezali na nekaj mm velike segmente, površinsko razkužili v etilnem alkoholu (70 %, 1 minuta) in raztopini natrijevega hipoklorita (3 % aktivnega klora, 4 minute) ter položili na gojišče PDA+. Liste in poganjke smo razrezali na 20 – 30 delov, pecljevino na 5 -10, brste in plodove pa na dva do štiri dele. Ker so glive iz rodu *Colletotrichum* pogosto navzoče v latentni obliki, smo opravili izolacijo tako iz simptomatičnih kot tudi iz navidez zdravih rastlinskih delov. Spomladi smo za izolacijo uporabili brste, ostanke pecljevine in poganjke, kasneje med rastno dobo pa liste (po dva lista na poganjek), plodove, pecljevino in poganjke (preglednica 1). Glive iz rodu *Colletotrichum*, ki so se po sedmih dneh inkubacije pri temperaturi 20° C in v temi razvile na obravnavanih rastlinskih delih, smo osamili v čisti kulturi in identificirali po zgoraj opisanem postopku. Okužbo posameznih organov in tkiv smo ovrednotili glede na pogostost izolacije gliv iz rodu *Colletotrichum* iz obravnavanih vzorcev.

3 REZULTATI IN DISKUSIJA

3.1 Povzročiteljica antraknoze pri ameriških borovnicah

Analizo morfoloških in molekularnih značilnosti smo opravili pri 33 izolatih iz ameriških borovnic. Vsi izolati so pripadali rodu *Colletotrichum*. Na podlagi morfoloških karakteristik in primerjave nukleotidnih zaporedij dobljenih izolatov z zaporedji referenčnih izolatov smo ugotovili, da je povzročiteljica antraknoze pri ameriških borovnicah vrsta *C. fioriniae* (Marcelino & S. Gouli) R.G. Shivas & Y.P. Tan. Nukletotidna zaporedja ITS predela ribosomske DNA slovenskih izolatov so bila identična nukleotidnim zaporedjem tipskega primerka te glive (*C. fioriniae* EHS58).

Z umetnimi inokulacijami plodov različnih sadnih vrst smo potrdili virulentnost izolirane glive. Pri vseh inokuliranih plodovih je povzročila nastanek nekroz, velikost nekroz pa se ni razlikovala glede na sadno vrsto, kar kaže, da gliva *C. fioriniae* ni specializirana na posameznega gostitelja in je zmožna navzkrižnih okužb različnih sadnih vrst.

Opis morfoloških značilnosti glive *C. fioriniae*:

- Kolonije so v sredini svetlo do temno sive, ob robu svetlejše, mestoma prekrite s kompaktnim sivim zračnim micelijem. Na spodnji strani so karminsko rdeče pigmentirane, opazne so tudi posamezne črne pege. Prirast kolonije med petim in devetim dnem rasti na gojišču PDA in pri temperaturi 25° C je 22,5 do 28 mm. Sporulacija ni obilna, posamezni acervuli z oranžnimi gmotami trosov se oblikujejo le na robovih kolonije.
- Konidiji so enocelični, ozko eliptični, brezbarvni gladki in na konceh priostreni. Merijo 8,8 - 14,2 x 3,6 - 4,3 µm.
- Sete se ne pojavljajo.
- Apresoriji so kroglasti do rahlo nepravilni, veliki 6,5 – 8 x 5,5 – 7 µm.

Glivo so leta 2008 odkrili v ZDA; Marcelino in sodelavci (2008) so poročali o pojavu entomopatogene glive na kaparju *Fiorinia externa*, ki je povzročal obsežno sušenje čug na severovzhodu države. Iz mumificiranih kaparjev so izolirali glivo, ki je po morfoloških značilnostih ustrezala vrsti *C. acutatum*, a so jo zaradi njenega entomopatogenega značaja opisali kot posebno varieteto *C. acutatum* var. *fioriniae*. Hkrati so ugotovili, da gliva živi tudi kot endofit v 28 rastlinskih vrstah, ki so rasle v bližini napadenih čug. Kasneje sta Shivas in Tan (2009) glivo opisala kot samostojno vrsto *C. fioriniae*. Ekologija glive še ni podrobneje raziskana. Shivas in Tan (2009) navajata, da so njeni gostitelji avokado, mango in akacija, pri katerih povzroča gnilobo plodov ter ožige na poganjkih in listih. Pri nas gliva *C. fioriniae* okuži poleg ameriških borovnic še domači oreh, lesko, jablano, hruško, gozdno borovnico, veleplodno mahovnico (*Vaccinium macrocarpon*), pa tudi rododendron in nekatere druge okrasne vresovke (Munda in Gerič, 2011). Pri vseh povzroča za antraknozo značilna bolezenska znamenja na plodovih ali listih.

Zaradi zapletene taksonomije rodu *Colletotrichum* in podobnosti morfoloških značilnosti pri sorodnih vrstah je identifikacija povzročiteljev antraknoze težavna in zahteva uporabo tako morfoloških kot molekulskih tehnik. Velikost in oblika konidijev se pri posameznih vrstah oz. molekularnih skupinah prekrivata in nista uporabni za njihovo razlikovanje (Shivas in Tan, 2009). Najbolj zanesljivo jih lahko prepoznamo po nukleotidnem zaporedju ITS predela rDNK ter dela gena za β tubulin (Shivas in Tan, 2009). Razlikujejo se tudi po tvorbi pigmentov na gojišču PDA, vendar je pigmentacija pri posameznih izolatih iste vrste spremenljiva in odvisna od gojitvenih razmer, zlasti temperature (Shivas in Tan, 2009; Marcelino in sod., 2008).
Ekologija posameznih vrst, spekter njihovih gostiteljev in specializacija na posamezne gostiteljske vrste še nista podrobneje raziskana. Razumevanje pomena teh gliv pa otežuje tudi spoznanje, da žive posamezne vrste na

svojih gostiteljih kot epifiti, endofiti ali paraziti (Freeman in sod., 2001).

3.2 Epidemiologija bolezni

Življenjski cikel povzročiteljice antraknoze pri ameriških borovnicah in epidemiologija bolezni sta bila v zadnjih desetih letih predmet številnih študij (DeMarsay in Oudemans, 2002, 2003, 2004; Wharton in Diéguez-Uribeondo, 2004; Wharton in Schilder, 2003). V teh še niso upoštevali sodobnega taksonomskega koncepta rodu *Colletotrichum* in so kot povzročiteljico bolezni obravnavali vrsto *C. acutatum* s. l. Namen naše raziskave je bil ugotoviti mesto prezimovanja in identificirati vire okužbe z glivo *C. fioriniae*, ki v naših pridelovalnih razmerah povzroča antraknozo pri ameriških borovnicah. Oba dejavnika sta ključnega pomena za razumevanje bolezenskega cikla in načrtovanje varstvenih ukrepov zoper bolezen.

Z izolacijo glive *C. fioriniae* iz različnih rastlinskih delov smo ugotovili, da gliva okuži vse nadzemne dele gostitelja, razen listov (preglednica 1). Spomladi, še pred odganjanjem rastlin, smo glivo najpogosteje in v največjem odstotku izolirali iz ostankov pecljevine in iz poganjkov z odmrlimi vršički. Ugotovili smo jo tudi v navidez zdravih poganjkih, vendar je bila pogostost izolacije iz le-teh manjša kot iz poganjkov z izraženimi bolezenskimi znamenji. V visokem odstotku smo glivo *C. fioriniae* izolirali tudi iz navidezno zdravih brstov. Skupno je bilo okuženih 36 odstotkov pregledanih brstov, na posameznem poganjku pa od 0 do 75 odstotkov. Cvetne in vegetativne brste smo obravnavali ločeno in ugotovili znatno večjo stopnjo okuženosti generativnih brstov v primerjavi z vegetativnimi.

V obdobju rasti smo glivo *C. fioriniae* izolirali iz vseh rastlinskih delov, ki so kazala znamenja okužbe, pa tudi iz navidez zdravih. V največjem odstotku smo jo izolirali iz simptomatičnih zrelih plodov. Izolirali smo jo tudi iz poganjkov z znamenji bolezni in iz porjavele pecljevine, vendar je bila pogostost izolacije iz teh vzorcev manjša kot pri plodovih. V tem obdobju smo glivo izolirali tudi iz navidezno zdravih poganjkov, pecljevine in zelenih jagod. Izolirali pa smo jo tudi iz navidezno zdravih brstov; okuženih je bilo 26 odstotkov pregledanih brstov.

Preglednica 1: Izolacija glive *C. fioriniae* iz okuženih ameriških borovnic
Table 1: Isolation of *C. fioriniae* from infected high-bush blueberry bushes

	Datum vzorčenja	Simptomi niso izraženi	Simptomi izraženi
Pecljevina	28. 8. 2007	12 %	18 %
	14. 3. 2008	NT	53 %*
Poganjki	8. 5. 2007	12 %	NT
	31. 7. 2007	10%	18 %
	28. 8. 2007	9 %	37 %
	14. 3. 2008	30 %	45 %
	10. 4. 2008	9 %	NT**
Generativni brsti	14. 3. 2008	35 %	NT
	10. 4. 2008	11 %	NT
Vegetativni brsti	14. 3. 2008	0 %	NT
	10. 4. 2008	8 %	NT
Listi	8. 5. 2007	0 %	NT
	10. 6. 2007	0 %	NT
	31. 7. 2007	0 %	NT
	28. 8.2007	0 %	NT
Zeleni plodovi	10. 6. 2007	27 %	NT
Zreli plodovi	31. 7. 2007	11 %	100 %

* delež segmentov posameznega organa oz. tkiva, iz katerih je bila izolirana gliva *C. fioriniae*
** ni podatka

Iz rezultatov sukcesivne izolacije glive *C. fioriniae* iz naravno okuženih ameriških borovnic lahko sklenemo, da je gliva v gostitelju navzoča tekom vse rastne dobe. Pogosteje smo jo izolirali iz organov in tkiv z izraženimi bolezenskimi znamenji kot iz navidezno zdravih. V poganjkih z nekrozami in suhimi vršički ter v ostankih pecljevine gliva prezimi in se ohrani do naslednje rastne dobe. Pomembno mesto prezimovanja glive in vir primarnih okužb mladih tkiv pa so poleg

simptomatičnih rastlinskih delov tudi navidezno zdravi organi, zlasti poganjki in rodni brsti.

Kljub temu, da se rezultati naše raziskave nanašajo na glivo *C. fioriniae*, so primerljivi z rezultati raziskav okuženosti borovnic z glivo *C. acutatum* s. l., ki so jih pri ameriških borovnicah opravili DeMarsay in Oudemans (2002, 2003, 2004), Yoshida in sodelavci (2007) ter Verma in sodelavci (2006). V teh ugotavljajo, da se gliva *C. acutatum* s. l. v zimskem času ohrani v odmrlih poganjkih in pecljevini, poleg tega pa še v navidez zdravih brstih in poganjkih. Izolirali so jo iz lusk cvetnih brstov in skorje poganjkov, ni pa bila navzoča v notranjosti brstov in v ksilemu poganjkov (Yoshida in sod., 2007). Tudi v teh raziskavah so ugotovili, da je delež navidez zdravih, a okuženih cvetnih brstov velik in lahko pri občutljivih sortah doseže celo 73 odstotkov (DeMarsay in Oudemans, 2004). Nasprotno pa so vegetativni brsti okuženi v veliko manjši meri in zato manj pomembni pri širjenju okužbe na mlada tkiva (Verma in sod., 2006). Podobno tudi rezultati raziskav antraknoze pri drugih sadnih vrstah kažejo, da so generativni brsti pomemben vir okužb v naslednji rastni dobi (Børve in Stensvand, 2006; Børve in Stensvand, 2007). Tako na primer ugotavljajo, da je pri češnjah okuženih kar 55 % generativnih brstov in le 32 % vegetativnih (Børve in Stensvand, 2006). Spomladi se na okuženih delih oblikujejo trosišča (acervuli) v katerih so trosi (konidiji), ki širijo okužbo na zdrava tkiva. Čas in trajanje sproščanja trosov sta odvisna od vremenskih razmer in od razvojne faze gostitelja. Opazni so trije viški sproščanja trosov, ki sovpadajo z obdobji močnejših okužb: prvi v času cvetenja, drugi v fazi zelenih plodov, tretji pa v času zorenja plodov (Verma in sod., 2007). Taka dinamika sporulacije glivi omogoči, da zanesljivo okuži plodove in naseli tkiva, v katerih nato prezimi. Okužbe so pogostejše, če je vreme deževno in so temperature zmerne; po tujih podatkih zadošča za okužbo že 11° C, če so občutljiva tkiva omočena vsaj 10 ur (Verma in sod., 2007). V zelenih plodovih ter navidezno zdravih poganjkih in brstih ostane okužba latentna in se bolezenska znamenja ne izrazijo. Pojavijo se šele, ko se spremene razmere v okolju in fiziološko stanje gostitelja. Na plodovih se pokažejo šele ob zorenju. Tedaj gliva pride iz biotrofične v nekrotrofično fazo, agresivno preraste plod ter oblikuje trosišča in trose, ki širijo okužbo na plodove in druge občutljive organe in tkiva (Wharton in Diéguez-Uribeondo, 2004). Simptomatični plodovi se osujejo z grmov na tla, kjer kmalu razpadejo. Po podatkih tujih raziskovalcev gliva v odpadlih plodovih ne more prezimiti, zato ti niso pomembni za širjenje okužb v naslednji rastni dobi (Verma in sod., 2006).

4. SKLEPI

Povzročiteljica antraknoze v nasadih ameriških borovnic na Ljubljanskem barju je gliva *C. fioriniae*. Je ena izmed novo opisanih vrst iz kompleksa *C. acutatum*. s.l. Sprva so jo opisali kot entomopatogeno glivo, kasneje pa ugotovili, da živi kot endofit in parazit v številnih rastlinskih vrstah. Pri nas je razmeroma pogosta povzročiteljica antraknoze na sadnem drevju in jagodičevju.

Iz številnih tujih epidemioloških študij antraknoze pri ameriških borovnicah je znano, da so odmrli vršički poganjkov in ostanki pecljevine primarna mesta prezimovanja povzročiteljice bolezni in poglavitni viri spomladanskih okužb mladih tkiv. Obseg bolezni v naslednji rastni dobi je v veliki meri odvisen od preživetja glive v času mirovanja. Od načina prezimovanja glive pa je odvisna tudi strategija varstva pred boleznijo. Rezultati naše raziskave potrjujejo, da povzročiteljica antraknoze tudi v naših pridelovalnih razmerah najpogosteje prezimuje v simptomatičnih poganjkih in ostankih pecljevine, kjer se spomladi oblikuje inokulum za okužbo cvetov. Ugotovili smo še, da gliva naseli navidezno zdrave poganjke in brste ter prezimi tudi v njih. Na podlagi dobljenih rezultatov ugotavljamo, da so brsti, zlasti rodni, skrit, a zelo pomemben vir primarnih okužb mladih tkiv ameriških borovnic. Kako se brsti okužijo in v kakšni obliki gliva v brstih prezimi, še ni podrobneje znano in ostaja predmet nadaljnjih raziskav.

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Agris category code: F30

Analiza EST klonov križancev *Coffea arabica* X *Coffea canephora* in *Coffea canephora* X *Coffea congensis*

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IZVLEČEK

EST ali oznake izraženih zaporedij so DNA zaporedja, dolga od 100 do 800 baznih parov, pridobljena z eno reakcijo določanja nukleotidnega zaporedja cDNA molekulam iz 5' ali 3' smeri. Vsebujejo prepisana, ne nujno pa tudi prevedena, zaporedja genov ter pogosto tudi elemente vektorjev. Predstavljajo presežen nabor izraženih genov v nekem vzorcu in se uporabljajo za študije izražanja genov, iskanje novih genov, raziskave alternativnega izrezovanja intronov idr. Mnogokrat predstavljajo prvo orodje funkcionalne genomike manj raziskanih organizmov. Zaradi presežnosti se jih mnogokrat združuje v gruče. Največjo zbirko EST zaporedij vzdržuje NCBI, imenuje se dbEST in ima več kot 70 milijonov zaporedij. V omenjeni bazi smo poiskali klone EST dveh križancev kave, *Coffea arabica* X *Coffea canephora* ter *Coffea canephora* X *Coffea congensis*, ter s pomočjo BLAST algoritma poiskali katere proteine kodirajo. Najdenim proteinom smo nato določili ontologijo.

Ključne besede: EST, *Coffea arabica* X *Coffea canephora*, *Coffea canephora* X *Coffea congensis*, BLAST, ontologija, bioinformatika

ABSTRACT

EST CLONE ANALYSIS OF TWO COFFEE HYBRIDS (*Coffea arabica* X *Coffea canephora* and *Coffea canephora* X *Coffea congensis*)

Expressed sequence tags (ESTs) are short (from 100 to 800 base pairs) 5' or 3' sequences that are acquired with single pass sequencing of cDNA molecules. They contain transcribed, but not necessarily translated regions of genes and often also vector elements. They represent a redundant set of expressed genes in a given sample and are used in gene expression studies, finding new genes, alternative splicing research etc. ESTs often represent primary tool of functional genomic of orphan crops. They are often clustered due to their redundancy. The largest EST collection named dbEST, it is maintaned by NCBI and contains more than 70 million sequences. In this database, we have searched for EST clones of two coffee hybrids, *Coffea arabica* X *Coffea canephora* and *Coffea canephora* X *Coffea congensis*, and used BLAST algorithm to find out which proteins they are encoding. We have also determined gene ontology of protein hits.

Key words: EST, *Coffea arabica* X *Coffea canephora*, *Coffea canephora* X *Coffea congensis*, BLAST, ontology, bioinformatics

1 UVOD

Oznake izraženih zaporedij, EST (angl. expressed sequence tag) so kratki deli DNA zaporedja, ki nastanejo iz določanja nukleotidnega zaporedja enega ali obeh koncev izraženega gena. Zaporedje določimo delom DNA, ki predstavljajo izražene gene določene celice, tkiva ali organa različnih organizmov in uporabimo te oznake za iskanje genov iz kopice kromosomske DNA. EST zaporedja in zaporedja komplementarne DNA (cDNA, angl. complementary DNA) nam omogočajo pregled vzorcev transkriptov, in so pomemben vir transkriptomskih raziskav. EST so presežna zaporedja dolga od 200 do 800 nukleotidnih baz v primeru Sangerjeve tehnologije, dobljena z eno reakcijo sekvenciranja (angl. single pass sequencing) iz cDNA knjižnic. Že za relativno nizko ceno lahko pridobimo večje število EST cDNA klona in tako

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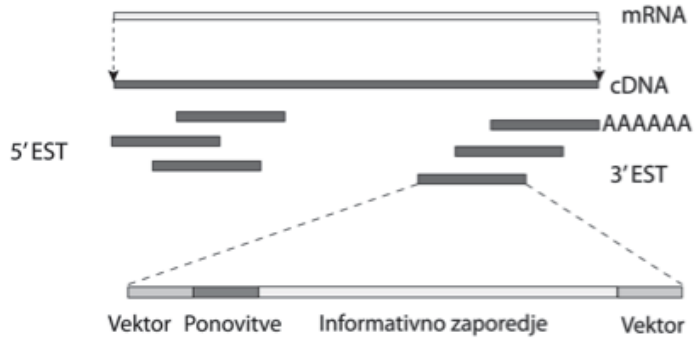
dobimo informacijo o prepisanih regijah posameznega organizma.
Kopije izraženih genov dobimo iz zaporedij informacijske RNA (mRNA) v celici. Ker RNA ne moremo direktno klonirati, jih z encimom reverzno transkriptazo prepišemo v cDNA. Dobljeno cDNA kloniramo in ustvarimo knjižnico, ki predstavlja set prepisanih genov prvotne celice, tkiva ali organizma. Tem cDNA klonom naključno določimo nukleotidno zaporedje z eno samo reakcijo določanja nukleotidnega zaporedja iz obeh koncev, da dobimo 5' ali 3' zaporedje. Rezultati so lahko presežni EST-ji, saj so lahko cDNA matrice sekvencirane delno, v celotni dolžini ali pa so v knjižnici že same po sebi presežne.

Dias Neto in sod. (2000) so ustvarili novo, cenovno ugodno metodo za pridobivanje velikega števila EST, oznake izraženega okvirja z odprtim bralnim okvirjem, imenovane ORESTES (angl. open reading frame expressed sequence tags). Ta metoda se razlikuje od konvencionalnega pridobivanja EST tako, da pridobimo zaporedje iz sredinske kodirajoče regije, ki je najbolj informativna. ORESTES nukleotidne podatke lahko prav tako najdemo v dbEST bazi.



Slika 1: Osnovne značilnosti klonov EST. Gre za krajše fragmente cDNA, ki lahko poleg kodirajočega zaporedja vsebujejo tudi neprevedena zaporedja (5' ali 3' UTR, angl. untranslated region) ter zaporedja vektorjev.

Figure 1: Basic characteristics of the EST clones. This is a short fragment of cDNA containing coding sequences, and can also contain 5' and 3' untranslated regions and sequences of vectors.

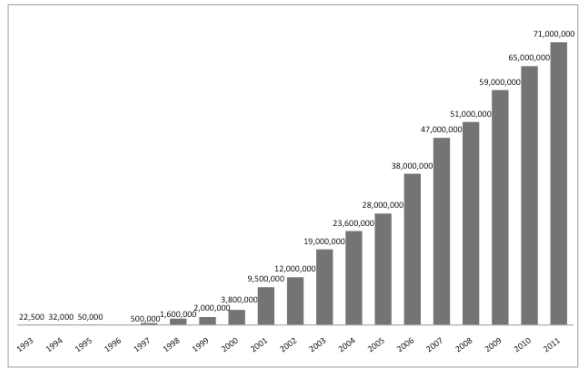
EST zaporedje (Slika 1) je le kratka kopija mRNA, ki je sekvencirana samo enkrat in je zelo podvržena napakam, še posebno na koncih. Kvaliteta zaporedja je ponavadi večja na sredini. Vektorje in ponovljena zaporedja izrežemo v postopku pred-procesiranja EST-jev. Pred-procesiranje zmanjšuje skupne motnje, ki nastanejo pri EST podatkih in tako izboljša učinkovitost nadaljnjih analiz. Splošno, je kvaliteta odčitavanja baz v posameznih EST zaporedjih na začetku slaba (do 20 % v prvih 50 do 100 baznih parih), nato se izboljša in ponovno poslabša proti koncu (Aaronson in sod., 1996). Presežnost in preveč ter premalo zastopani transkritpi so dejanski problemi pri EST podatkih. Razlog je predvsem v različni stopnji izražanja določenih genov v različnih tkivih, deloma pa tudi v neenotnosti protokolov uporabljenih pri pridobivanju EST. Pogosto opažene napake EST so tudi artefakti zaporedij, skupno tudi do 5 % (Aaronson in sod., 1996), ponavljanje baz, še posebno G in T, in slaba kvaliteta zaporedij. Pride lahko tudi do pogoste kontaminacije iz vektorjev, adapterjev in himernih zaporedij, kot tudi iz genomskih DNA fragmentov. Slaba kvaliteta značilnih zaporedij, kratka zaporedja, ponovitve in napake v anotaciji lahko predstavljajo probleme za nadaljnjo analizo. Tudi naravne variacije v procesih, kot je alternativno RNA procesiranje in genomske variacije, nastale zaradi SNP (angl. single nucleotide polymorphism) lahko predstavljajo izzive, saj je težko razlikovati med artefaktnimi in naravno prisotnimi zamenjavami in insercijami ter delecijami v danem podatkovnem setu EST.

Največja in prosto dostopna baza EST podatkov (71,276,166 EST iz 2325 organizmov, december 2011) je dbEST (http://www.ncbi.nlm.nih.gov/dbEST/). UniGene iz National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/UniGene), Združene države Amerike, shranjuje edinstvene gene in predstavlja nepresežen set gensko orientiranih gruč nastalih iz EST. Drugi specializiran vir EST ustvarjen za specifične organizme pa je na Dana Farber Cancer Institute, predhodno urejen in vzdrževan na The Institute for Genome Research (TIGR; http://compbio.dfci.harvard.edu/tgi/).

Kontaminacija z vektorji je pri EST široko razširjena, in pogosto se del vektorja ali adaptorja, ki smo ga uporabili pri kloniranju sekvencira skupaj z EST zaporedjem. Takšni vektorji morajo biti odstranjeni preden so EST-ji zbrani v gruče. Kontaminacije lahko tako identificiramo in jih izločimo, če primerjamo EST z nepresežnimi bazami vektorjev. Primerni viri za pred-procesiranje EST klonov so podatkovna baza UniVec (http://ncbi.nlm.nih.gov/VecScreen/UniVec.html), programsko orodje za primerjavo EMVec (http://www.ebi.ac.uk/Tools/sss/ncbiblast/vectors.html) in tudi orodje RepeatMasker (http://repeatmasker.org/cgi-bin/WEBRepeatMasker).

Ker so EST podatki presežni in vsako zaporedje vsebuje le majhno informacijo o zaporedju gena, se jih na podlagi identičnosti združuje v gruče. Deloma to delamo zaradi zmanjšanja števila ponovljenih transkriptov, deloma pa tudi zato, da transkripte istega gena združimo v isto gručo, s čimer smo korak bližje celotnemu zaporedju gena. Enostaven način za zbiranje EST je z merjenjem podobnosti sekvenčnih parov med njimi. Te razdalje so potem pretvorjene v binarne enote, glede na to, ali se značilno ujemajo ali ne, in tako je sekvenčni par sprejet v nastajajočo gručo ali izločen iz nje. Dva pristopa zbiranja sta opisala Ptitsyn in Hide (2005) kot zaostreno (angl. stringent) in ohlapno (angl. loose) zbiranje. Zaostren tip zbiranja je konzervativen in temelji na enkratnem zbiranju EST, kar da relativno natančne gruče, vendar so nastala zaporedja krajša z manjšim številom izraženih genov. V nasprotnem primeru, nam ohlapno zbiranje s ponavljanjem poravnav EST zaporedij slabše kvalitete ustvari manj natančna zaporedja, ki pa so daljša in imajo tako večjo pokritost izraženega gena ter podajo boljšo informacijo o alternativnem izrezovanju intronov, vendar obstaja nevarnost da se v gručo vključi tudi paralogna zaporedja. Pristop, ki ga uporablja TIGR je zaostreno zbiranje, UniGene pa je med zaostrenim in ohlapnim zbiranjem.

Najbolj pogosto uporabljeni programi za zbiranje in združevanje EST zaporedij, pridobljenih s Sangerjevo tehnologijo, so Phrap (Ewing in Green, 1998) (http://www.phrap.org), CAP3 (Huang in Madan, 1999) (http://pbil.univ-lyon1.fr/cap3.php) in zelo popularno orodje izdelano na TIGR TGICL (Lee in sod., 2005) (angl. TIGR gene indices clustering tools), ki združuje programa megablast in CAP3. Primerjava teh treh programov (Liang in sod., 2000) je pokazala, da je CAP3 najbolj optimalen za uporabo.



Slika 2: Število zaporedij v bazi dbEST po letih. Od ustanovitve leta 1992 je število klonov EST strmo naraščalo. Večina zaporedij je bilo humanega izvora, saj so bili EST pomembno orodje pri odkrivanju novih genov v človeškem genomu.

Figure 2: Number of sequences in the dbEST database through the years. Since its beginnings in 1992, the number of ESTs has been growing rapidly. The majority of sequences comes from human, since the ESTs have been an important tool in finding new genes in the human genome.

Ko pridobimo skupno zaporedje iz sestavljenih EST, jim lahko pripišemo funkcije, do katerih pridemo s pomočjo iskanja podobnosti z že znanimi zaporedji v podatkovnih bazah. Najbolj univerzalno in znano orodje za iskanje podobnosti med zaporedji v bazah je BLAST (Altschul in sod., 1997) (angl. The basic local alignment search Tool) na strežniku NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) ali v obliki namizne različice programa. BLAST primerja nukleotidna, proteinska ali prevedena nukleotidna zaporedja z zaporedji iz baze podatkov in izračuna statistično značilnost med ujemanji. BLAST lahko uporabljamo za iskanje funkcionalnih in evolucijskih relacij med zaporedji in tudi za pomoč pri identifikaciji članov iz določene družine genov. Obstajajo različni algoritmi primerjav, BLASTN za iskanje nukleotidov v bazah nukleotidnih zaporedij, BLASTP za iskanje proteinov v proteinskih bazah, BLASTX prav tako za iskanje v proteinski bazi vendar za primerjavo uporabi prevedeno nukleotidno zaporedje, TBLASTN išče proteinska ujemanja s prevedeno nukleotidno bazo in TBLASTX išče podobnosti prevedenih nukleotidnih zaporedij s prevedeno nukleotidno bazo na aminokislinskem nivoju.

Zgodovina EST-jev sovpada z začetki avtomatiziranega določanja nukleotidnega zaporedja (približno leta 1990). EST-ji so igrali pomembno vlogo pri odkrivanju genov v projektu človeškega genoma, saj je bilo v začetku identificiranih in fizično kartiranih zelo malo človeških genov (Adams in sod., 1991). Uporabljali so jih za iskanje novih genov, za kartiranje genov na kromosome in za raziskovanje profila izražanja genov. EST zaporedja so uporabna tudi za študije strukture genov, alternativnega izrezovanja intronov ter diferencialno izraženih genov (na primer primerjava med zdravim in bolnim tkivom). Podatki o EST-jih so hitro postali množično uporabljeni in popularni, kar se odraža tudi v

hitrem naraščanju le-teh v bazi dbEST (Slika 2). V več kot 19 letih, odkar obstaja omenjena podatkovna zbirka, je število EST zaporedij iz dobrih 22.000 zraslo na več kot 71 milijonov. Prvi objavljeni EST so prihajali iz sedmih organizmov, danes je v dbEST zastopanih preko 2.000 različnih organizmov. V vrhu po številu EST zaporedij so: človek, miš, koruza, prašič, navadni repnjakovec, govedo, zebrica, soja, Xenopus, riž, pšenica in podgana. Danes EST predstavljajo večino (približno 60 %) zaporedij v podatkovni zbirki GenBank. Tudi v zadnjih letih, ko se močno uveljavljajo nove in hitrejše metode določanja nukleotidnega zaporedja celih genomov, število EST vztrajno narašča. Vzrok za to je verjetno dejstvo, da se nove metode določevanja nukleotidnega zaporedja celotnih genomov še uveljavljajo, za manjše laboratorije pa je za enkrat še vedno enostavnejše in cenejše pridobivanje EST zaporedij. Sčasoma se bodo EST verjetno umaknili in jih bodo nadomestile novejše, hitrejše in zanesljivejše metode, kot je na primer RNA-Seq (Ozsolak in sod, 2009).

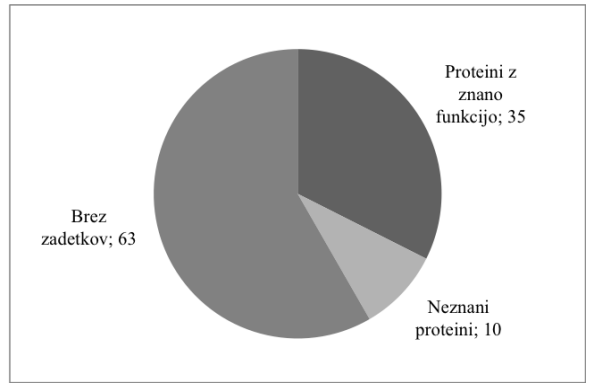
Namen prispevka je predstaviti EST, njihovo pridobivanje ter napake in rešitve napak, ki se pri tem lahko pojavijo. Zastavili smo si tudi praktični primer uporabe EST z analizo klonov dveh medvrstnih križancev rodu kave (*Coffea* sp.). Na tem primeru smo analizirali pridobljena EST zaporedja in primerjali kodirane proteine obeh križancev med seboj z uporabo genske ontologije.

2 MATERIAL IN METODE

V podatkovni bazi dbEST smo poiskali 108 EST zaporedij križanca *Coffea arabica* X *Coffea canephora* ter 145 zaporedij križanca *Coffea canephora* X *Coffea congensis*. Obe skupini zaporedij smo v FASTA obliki zbrali v dveh ločenih tekstovnih datotekah. Nato smo uporabili NetBlast program, ki omogoča iskanje po NCBI bazah brez internetnega vmesnika preko ukazne vrstice na lastnem računalniku. Program smo uporabili zato, ker omogoča iskanje z več zaporedji hkrati in omogoča delo z večimi zaporedji naenkrat, brez posredovanja uporabnika. Za iskanje zaporedij, podobnih našim EST zaporedjem, smo uporabili BLASTX algoritem, ki primerja prevedeno nukleotidno zaporedje z aminokislinskimi zaporedji. Izpis rezultatov smo omejili z vrednostjo e manjšo od 0,01. Vrednost e (angl. expected value) je pri BLAST analizi parameter, ki označuje pričakovano število naključnih zadetkov v dani bazi. Manjši kot je e, manj naključnih ujemanj lahko pričakujemo in bolj signifikanta je poravnava. Tekstovno datoteko z rezultati smo spremenili v tabelarično obliko z doma napisano PERL skripto, za kar smo uporabili BioPerl paket, modul BIO:searchIO (skripta je na voljo pri avtoricah). Ker smo delali v okolju Windows, smo potrebovali tudi nameščen Perl jezikovni program (ActivePerl). V tabeli z rezultati smo imeli izpisane akcesije EST zaporedij, njihove dolžine, akcesije njihovih zadetkov, opise zadetkov, aminokislinska zaporedja, dolžino poravnave, e vrednost in rezultat poravnave. Iz najboljšega anotiranega zadetka smo sklepali kateri protein kodira posamezno EST zaporedje. Za vsak kodiran protein smo nato s pomočjo baz UniProt (http://www.uniprot.org/) in Gene Ontology (http://www.geneontology.org/) določili vse tri ontologije, torej kje v celici se protein nahaja, kakšna je njegova molekularna funkcija in v kakšnem biološkem procesu sodeluje.

3 REZULTATI IN RAZPRAVA

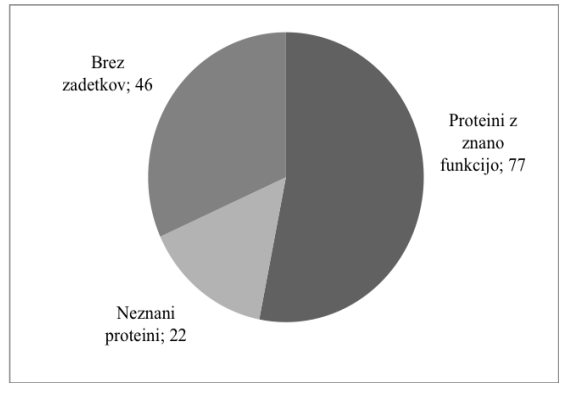
Izmed 108 klonov EST iz križanca *C. arabica* X *C. canephora*, ki smo jih našli v bazi dbEST, jih je imelo zadetke po izvedbi BLAST algoritma le 45 (Slika 3). Petintrideset od teh zadetkov so proteini, ki imajo znano funkcijo, 10 zadetkov pa so proteini z neznano funkcijo ali pa hipotetični in predvideni proteini. Do napovedi za hipotetičen protein ponavadi pride pri analizi genoma, kjer se najde dovolj velik odprt bralni okvir, za katerega se predvideva, da verjetno kodira nek proteinski produkt, vendar pa ni eksperimentalnih dokazov za obstoj proteina *in vivo*. Včasih imajo zaporedja predvidenih proteinov značilne regije, kot npr. določene funkcionalne domene, na podlagi katerih se lahko sklepa, kakšno funkcijo bi protein imel, če bi se dejansko izražal.



Slika 3: Rezultati uporabe algoritma BLAST na 108 EST iz *C. arabica* X *C. canephora*.

Figure 3: Results of BLAST algorithm used on 108 ESTs from *C. arabica* X *C. Canephor.*

Izmed 145 klonov EST križanca *C. canephora* X *C. congensis* iz baze dbEST, je imelo zadetke po uporabi BLAST algoritma 99 zaporedij (Slika 4). Kar 77 je bilo proteinov z znano funkcijo, neznani ali napovedani proteini pa so predstavljali 22 zadetkov (Slika 4).

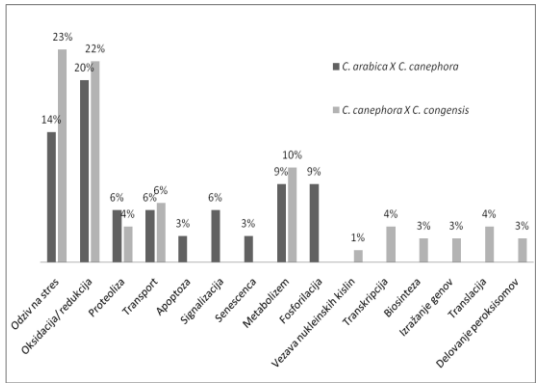


Slika 4: Rezultati uporabe algoritma BLAST na 145 EST iz *C. canephora* X *C. congensis.*

Figure 4: Results of BLAST algorithm used on 108 ESTs from *C. canephora* X *C. congensis.*

Na splošno je imela večina EST iz *C. arabica* X *C. canephora* zadetke z visokimi vrednostmi e, dolžine poravnav so bile krajše kot pri EST iz *C. canephora* X *C. congensis*. Vzrok za zaporedja brez podobnih zadetkov iz baz je lahko v tem, da smo iskali po proteinski bazi, EST pa lahko vsebujejo tudi neprevedene (UTR) regije. Možen vzrok, vendar manj verjeten, je tudi da gre za nova, specifična zaporedja, ki jih še ni v bazi. Med pridobivanjem EST pa je lahko prišlo tudi do genomskih kontaminacij, ki se prav tako kažejo v zaporedjih brez podobnih zadetkov.

Proteine, ki jih kodirajo kloni EST obeh križancev, smo razporedili glede na lokacijo v celici, njihovo molekularno funkcijo in biološki proces, v katerem sodelujejo.

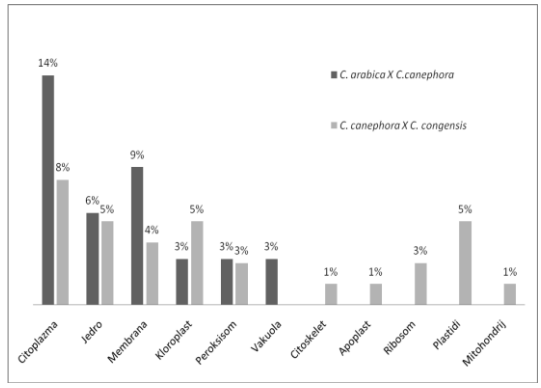


Slika 5: Biološki procesi v katerih sodelujejo proteini, ki jih kodirajo EST zaporedja obeh križancev. Predstavljeni so odstotki vseh proteinov z znano funkcijo.

Figure 5: Biological processes that involve proteins, coded by EST sequences of both hybrids. Shown in the figure are percentages of all proteins with known function.

Od 35 določenih proteinov iz križanca *C. arabica* X *C. canephora* jih je podatke o biološki vlogi vsebovalo 24. Iz drugega križanca smo našli tovrstne podatke za 60 od 77 proteinov. Slika 5 prikazuje odstotek določenih proteinov iz vsakega križanca, ki nastopajo v določenem biološkem procesu. Nekateri proteini nastopajo v več kot enem procesu. Največ proteinov ima vlogo v odzivu na stres – tu gre predvsem za veliko število proteinov toplotnega šoka, ki so se pojavili kot zadetki po uporabi algoritma BLAST. Naslednjo veliko skupino prestavljajo proteini, ki sodelujejo v redukciji ali oksidaciji. Vzrok za velik delež teh proteinov je verjetno v tem, da so redoks procesi osnovni procesi v celicah, mnogi izmed proteinov pa niso imeli natančneje določene vloge v tem procesu (na primer oksidacija točno določenih spojin). Tretja večja skupina bioloških procesov je metabolizem – v tej kategoriji so prisotni proteini, ki sodelujejo v metabolizmu ogljikovih hidratov in drugih bioloških molekul ter proteini, ki nimajo natančneje določene vloge v metabolizmu. Težko je narediti primerjavo proteinov obeh križancev, ker je že samo število zadetkov in določenih proteinov zelo različno (križanec *C. canephora* X *C. congensis* ima določenih dvakrat več proteinov kot primerjani križanec). Razumljivo je, da se v obeh rastlinah pojavljajo temeljni proteini, nujni za preživetje celice, kot so metabolni encimi, redoks encimi in transportni proteini. Zanimivo je, da so v križancu *C. arabica* X *C. canephora* prisotni tudi proteini, ki sodelujejo v senescenci in apoptozi.

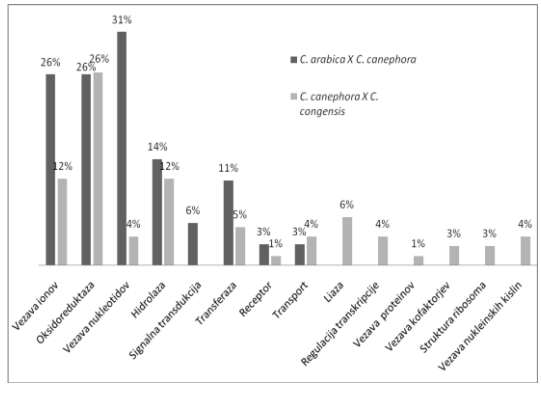
Podatke o celični lokaciji proteinov smo našli za 12 proteinov iz *C. arabica* X *C. canephora* in za 28 proteinov iz C. canephora X C. congensis (Slika 6). Po pričakovanjih je večina proteinov locirana v citoplazmi, jedru ali membrani. *C. arabica* X *C. canephora* ima manjši delež jedrnih proteinov, kar sovpada z zgornjo ugotovitvijo, da ima tudi manj proteinov, ki sodelujejo pri izražanju genov.



Slika 6: Lokacija proteinov, ki jih kodirajo EST obeh križancev. Predstavljeni so odstotki vseh proteinov z znano funkcijo.

Figure 6: Location of proteins, coded by EST sequences of both hybrids. Shown in the figure are percentages of all proteins with known function.

Lokacije proteinov v celici so bile bolj natančno določene v križancu *C. canephora* X *C. congensis*, saj smo našli primere proteinov citoskeleta, ribosomalnih proteinov ter več plastidnih proteinov.



Slika 7: Molekularne funkcije v katerih sodelujejo proteini, ki jih kodirajo EST zaporedja obeh križancev. Predstavljeni so odstotki vseh proteinov z znano funkcijo.

Figure 7: Molecular functions involving proteins, coded by EST sequences of both hybrids. Shown in the figure are percentages of all proteins with known function.

Podatke o molekularni funkciji proteinov smo našli za 26 od 35 proteinov iz *C. arabica* X *C. canephora* in za 46 od 77 proteinov iz *C. canephora* X *C. congensis* (Slika 7). Tudi pri tej ontologiji se je pojavljal problem različne mere natančnosti, s katero so bili anotirani proteini. Po pričakovanjih glede na rezultate, prikazane na sliki 5, ima veliko število proteinov oksidoreduktazno aktivnost. Za križanca *C. arabica* X *C. canephora* so značilni še proteini, ki vežejo ione, tako kovinske kot tudi druge, ter proteini, ki vežejo nukleotide, ni pa prisotnih proteinov, ki so značilni za procese nadzorovanja transkripcije. V obeh rastlinah so prisotne hidrolaze (proteaze, peptidaze, fosfataze, esteraze idr.), saj sodelujejo v pomembnih metabolnih procesih. Tudi v tem primeru je težko primerjati najdene proteine med obema križancema, ker je najdeno število proteinov z določeno funkcijo zelo različno. Kot v obeh prej omenjenih primerih, je za križanca *C. canephora* X *C. congensis* značilna večja pestrost molekularnih funkcij proteinov. Za obe rastlini pa velja, da ima mnogo proteinov več različnih molekularnih vlog (na primer: kinaza ima istočasno tranferazno aktivnost, veže pa tudi določeno molekulo).

4 SKLEPI

EST kloni so primerno orodje za analizo izražanja genov v nekem vzorcu, iskanje novih genov ter raziskovanje alternativnega izrezovanja intronov. Njihovo pridobivanje je relativno cenovno ugodno in enostavno, vendar pa uporabo EST v zadnjem času nadomeščajo nove tehnike masovnega paralelnega sekvenciranja RNA (angl. RNA-seq). V raziskavi smo analizirali 108 EST zaporedij križanca *C. arabica* X *C. canephora*, ter 145 EST zaporedij križanca *C. canephora* X *C. congensis* iz baze dbEST. Po uporabi BLAST algoritma smo pri prvem križancu našli 35 zadetkov, ki predstavljajo proteine z znano funkcijo, pri drugem križancu pa je bilo takih zadetkov 77. Večina najdenih proteinov je locirana v citoplazmi, jedru in membrani. Najbolj pogoste molekularne funkcije, ki jih opravljajo identificirani proteini, so vezava nukleotidov, vezava ionov ter oksidoreduktazne funkcije. Na podlagi analize je razvidno, da sta si križanca različna tako v številu EST klonov najdenih v bazi, kot tudi v karakteristiki proteinskih zaporedij, ki jih EST kloni kodirajo.

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Večji del izdelka je bil pripravljen kot seminarska naloga pod vodstvom prof. dr. Gregorja Anderluha, prof. dr. Blaža Zupana in prof. dr. Uroša Petroviča pri predmetu Bioinformatika na doktorskem študiju Biomedicina, smer Genetika (Tina Svetek) in doktorskem študiju Bioznanost, smer Biologija (Nataša Šibanc).

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COBISS Code 1.02

Agrovoc descriptors: noxious plants, aphalaridae, biological control, control methods, biological control organisms, natural enemies, beneficial organisms, ecosystems, nature conservation, damage

Agris category code: H60, P01

Japonski dresnik (*Fallopia japonica* [Houtt.] Ronse Decraene) in njegovo zatiranje z bolšico *Aphalara itadori* Shinji

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IZVLEČEK

Evropska agencija za okolje je sestavila seznam 163 najpomembnejših invazivnih organizmov, ki ogrožajo ekosisteme v Evropi. Med njimi je tudi rastlinska vrsta japonski dresnik (*Fallopia japonica*), ki uspeva na različnih tipih tal. Najhitreje poseljuje ruderalna rastišča, vendar postaja zaradi njegove izredne konkurenčnosti vse pomembnejši člen ekosistemov, kjer izpodriva samonikle rastlinske vrste. Mehansko odstranjevanje japonskega dresnika s košnjo je le začasna rešitev, saj ga na takšen način ne zatremo. Ker se ta rastlinska vrsta razrašča v urbanih območjih in ob vodah, številni strokovnjaki menijo, da predstavlja dolgoročno rešitev le biotično zatiranje tega plevela, in sicer z vnosom njegovega naravnega sovražnika iz okolja, od koder izvira tudi omenjena invazivna vrsta. Na Japonskem ima japonski dresnik okoli 180 različnih naravnih sovražnikov, a le bolšici *Aphalara itadori* pripisujejo gospodarski pomen. Največjo škodo s sesanjem rastlinskega soka na japonskem dresniku povzročajo najmlajše nimfe. Napadene rastline sicer ne propadejo, vendar je njihova rast omejena. V Veliki Britaniji, ki je prvo ozemlje v Evropi, kamor so vnesli omenjeno bolšico, so potrdili zmožnost njenega preživetja na prostem tudi pozimi. Vnos bolšice *Aphalara itadori* z namenom zatiranja japonskega dresnika je tako v Evropi kot v svetu prvi zgled klasičnega biotičnega zatiranja plevelov.

Key words: japonski dresnik, *Fallopia japonica*, škoda, klasično biotično varstvo, *Aphalara itadori*

ABSTRACT

DAMAGE POTENTIAL OF JAPANESE KNOTWEED (*Fallopi japonica*) AND ITS BIOLOGICAL CONTROL WITH PSYLLID *Aphalara itadori* SHINJI

European Environment Agency composed the list of 163 most important invasive organisms, that are threatening European ecosystems. One of above mentioned invasive species is also the plant Japanese knotweed (*Fallopia japonica*), which grow on different soil types. In ruderal habitats the plant is most prevalent, however because of its competitive position the Japanese knotweed is becoming more and more important part of the ecosystems, because it is superseding indigenous species. Mechanical removal is only temporary solution of its extermination. While this plant species grows in urban areas and near the water, numerous researchers see the long-term solution of its extermination in biological control of this weed with introduction of natural enemy from its origin. In Japan Japanese knotweed has about 180 different natural enemies but only the psyllid *Aphalara itadori* has been prooved to be effective. Young larvae are the most damaging stage of the insect. With sucking of the plant juice, plant is not destroyed, it only develops slower. In Great Britain, which is the first area in Europe, where the above mentioned psyllid was introduced, species also overwinters in the open. The introduction od *Aphalara itadori* represent the first example of classical biological control of weeds not only in Europe but also worlwide.

Ključne besede: Japanese knotweed, *Fallopia japonica*, damage, biological control, *Aphalara itadori*

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1 UVOD

Evropski flora in favna sta se razvijali milijone let. Populacije so bile ločene z različnimi geografskimi pregradami (gorske verige, morja, reke), kar je omogočilo razcvet neverjetne biotske raznovrstnosti. S širitvijo mednarodne trgovine in potovanj pa so te ovire po vsem svetu padle, tako da so različne vrste prišle v neposreden stik. Tako se je začelo tekmovanje za hrano in življenjski prostor (Van Driesche et al., 2002).

Invazivne rastline, kot skupine neofitov, so tujerodne rastline, ki so v naše okolje prišle v bližnji preteklosti. Te rastline je človek prinašal nehote ali namenoma zaradi lastnih koristi, kot so hrana, okras, les... V Evropo je vsako leto zanesenih na tisoče tujerodnih organizmov, vendar pa vsi ne postanejo invazivni. Pravzaprav jih večina v novem okolju ne preživi, ker se ne morejo prilagoditi ali pa je zastopanih premalo osebkov za uspešno razmnoževanje. Nekatere vrste, imenujemo jih prehodne tujerodne vrste, lahko v okolju ostanejo dlje, bodisi zaradi dolge življenjske dobe, občasnega razmnoževanja ali pa ponavljajočih se naselitev. Posamezne vrste pa se postopoma, lahko tudi v obdobju več let, prilagodijo na novo okolje in se začnejo razmoževati. Tujerodne vrste, ki se v naravi redno razmnožujejo in se populacije vzdržujejo brez posredovanja človeka, imenujemo naturalizirane vrste. Te vrste še ne povzročajo zaznavnih sprememb v naravi. Sčasoma pa lahko nekatere naturalizirane vrste postanejo invazivne, zlasti takrat, ko se število osebkov invazivnih tujerodnih vrst (invazivk) poveča. Mnoge tujerodne vrste se najprej pojavijo v degradiranih, antropogeno vplivanih, okoljih. Naravno zastopane vrste sobivajo in so v nekakšnem ravnovesju. V degradiranem okolju je to ravnovesje porušeno, zato se pojavljajo možnosti za naselitev novih vrst. Za mnoge invazivne vrste je značilno hitro razmnoževanje, ki jim omogoča izjemno hitro osvojitev novega okolja, pri čemer izrinejo mnoge domorodne vrste (Jogan, 2000).

Stroški nadzora invazivnih vrst in odprave škode zaradi njihovega delovanja so v letu 2008 v EU znašali od 9,6 do 12 milijard evrov. Od leta 1992 je bilo v EU porabljenih več kot 38 milijonov evrov za 180 projektov, tako v mreži zavarovanih območij Natura 2000 kot zunaj nje. V ZDA ocenjujejo, da na leto porabijo približno 80 milijard evrov za zatiranje invazivnih vrst (Shaw et al., 2009). V popisu invazivnih vrst DAISE je bilo v Evropi ugotovljenih 10.822 tujerodnih vrst. Vse med njimi niso invazivne, a se ocenjuje, da jih približno od 10 do 15 % lahko ogroža evropsko biotsko raznovrstnost (Shaw et al., 2009). Evropska agencija za okolje je sestavila seznam 163 najpomembnejših invazivnih organizmov, ki ogrožajo ekosisteme v Evropi, in med njimi je tudi japonski dresnik.

2 JAPONSKI DRESNIK (*FALLOPIA JAPONICA* [HOUTT.] RONSE DECRAENE)

Vrsta *Fallopia japonica* izvira iz Japonske, Koreje, Tajvana in severne Kitajske. Zanesena je bila v Avstralijo in Novo Zelandijo, močno invazivna pa je v Severni Ameriki in Evropi (Conolly, 1977; Seiger, 1997). V Veliki Britaniji za preprečevanje širjenja japonskega dresnika vsako leto namenijo približno 153 milijonov funtov oziroma 180 milijonov evrov (Shaw et al, 2009). V Evropo so japonski dresnik zanesli leta 1823 (Synge, 1956). Rastlino so začeli saditi v vrtovih in parkih kot okrasno rastlino in sicer iz potomcev rastline, ki so jo v 20. letih 19. stoletja iz Japonske uvozili Nizozemci (Beerling et al., 1993). V naravi je bila ta vrsta prvič ugotovljena leta 1892. Sadili so ga tudi za utrjevanje brežin in preprečevanje erozije, pa tudi kot krmno oziroma medonosno rastlino.

Japonski dresnik, ki spada v družino dresnovk (Polygonaceae), je hitro rastoča, širokolistna trajnica, ki oblikuje goste, kompaktne skupine, zaradi česar je močno konkurenčna rastlinska vrsta. V njeni gosti senci praktično ne morejo uspevati druge rastline in z takšnih rastišč kmalu izrine naravno rastlinstvo. Korenike, ki so zelo razrasle in lahko segajo več metrov stran od materinske rastline, prezimijo. Če rastlino kosimo, iz njenih korenik na različnih mestih vsakič znova poženejo do nekaj decimetrov visoka stebla, na katerih se navadno ne oblikujejo cvetovi. Podobno kot številne druge invazivne rastlinske vrste, je japonski dresnik pozno poleti in v začetku jeseni cvetoča rastlina (slika 1), ki oblikuje drobne belkaste do zelenkaste cvetove, združene v pokončna latasta socvetja.

Slika 1: Cvetenje japonskega dresnika na Viču v Ljubljani oktobra 2011 (foto: J. Rupnik)

Najpomembnejši način širjenja japonskega dresnika je z rizomi. Vrsta ima namreč veliko sposobnost obraščanja, tudi iz zelo majhnih delov podzemnega stebla (Beerling et al., 1993). V raziskavah so iz 40 % rizomov, dolgih 1 cm, težkih 0,7 g, pognala stebla. Glede na ogromno biomaso rizomov, ki v 25 cm plasti tal na 1 ha znaša tudi 14 ton (Bailey, 1994), je potencial tega plevela za širjenje ogromen. Japonski dresnik najraje naseljuje zmerno vlažna rastišča, najpogosteje ob rekah in potokih. Uspeva na različnih tipih tal, tako kislih, bazičnih kot tudi na nekoliko slanih tleh. Najhitreje poseli ruderalna rastišča, vendar se zaradi svoje izredne konkurenčnosti vse bolj vključuje v naravno rastje, kjer izpodriva samonikle rastlinske vrste. Kot poročajo nekateri raziskovalci, se predvsem tam, kjer ni premikov tal (ob rekah), japonski dresnik širi tudi z oblikovanjem novih poganjkov iz stebelnega tkiva. Bailey (1994) poroča, da je regeneracija rastlin iz odrezanih stebel najuspešnejša v vodi in to iz stebel, pobranih v jesenskem času.

Urbančič-Zemljič in Škerlavaj (1999) poročata, da japonski dresnik pri nas oblikuje kalivo seme, saj je seme, ki so ga jeseni nabrali na enem od rastišč, v rastlinjaku kalilo. Nasprotno poročevalci iz Anglije ugotavljajo, da japonski dresnik na njihovem ozemlju redko oblikuje fertilne cvetove in je zato razmnoževanje s semenom v teh predelih manj pomembno (Hawke in Williamson, 1995). Podobno velja tudi za nekatere predele v Severni Ameriki (Locandro, 1984). Novejše raziskave (Forman in Kesseli, 2003; Wang et al., 2007) potrjujejo ugotovitve Urbančič-Zemljičeve in Škerlavaja, da se japonski dresnik širi tako z rizomi kot tudi s semenom.

V Sloveniji so japonski dresnik prvič opazili v začetku 20. stoletja v okolici Celja. Strgar (1981, 1982) je poročal o njegovem širjenju po Sloveniji, saj je naštel že več kot 100 nahajališč, raztresenih ob rekah Dravi, Meži, Sotli, Savinji in Savi s pritoki. Jogan (2006) navaja, da se je v Pomurju, Slovenskih Goricah, Halozah in okolici Gorice ta plevel razširil šele v zadnjih 20 letih, medtem ko se že več desetletij japonski dresnik uspešno širi po Ljubljanjski kotlini (slika 2) ter vzdolž Save in Drave. Danes je japonski dresnik z izjemo submediteranskega fitogeografskega območja pogost po vsej Sloveniji, kjer ga srečamo zlasti ob rekah in potokih. Večina tujerodnih vrst v alpskem fitogeografskem območju je omejena na montanski pas (500–1000 m), s povečanimi antropogenimi dejavnostmi (z gradnjo cestnih omrežij, stavb, s sečnjo, z odstranjevanjem obrežnega rastlinstva) pa se le-te vse pogosteje širijo tudi v višje predele. Ta pojav je posebej izrazit pri japonskem dresniku, ki so ga našli že na Pokljuki (1184 m nad morjem) (Jogan, 2006). Ker japonski dresnik za svoj razvoj potrebuje minimalno vsoto toplih dni ≥ 2505 ºC kot tudi vsoto hladnih dni ≥ -30,2 ºC, Beerling (1993) v svoji raziskavi navaja, da je moč pričakovati širjenje japonskega dresnika tako v smeri geografske širine kot tudi nadmorske višine, ob uresničitvi podnebnih scenarijev v prihodnosti.

Poleg japonskega dresnika v Sloveniji uspevajo tudi naslednje vrste iz rodu *Fallopia: F. convolvulus* (L.) Löve (navadni slakovec), *F. dumetorum* (L.) Holub (hostni slakovec), *F. baldschuanica* (Regel) Holub (grmasti slakovec) in *F. sachalinensis* (F. Schmidt) Ronse Decraene (sahalinski dresnik); ter dva križanca: *F.* x *convolvuloides* (Brügger) (Holub) in *F.* x *bohemica* (Chrtek & Chrtkova) J. P. Bailey (češki dresnik). Grmasti slakovec, japonski dresnik, sahalinski dresnik in češki dresnik predstavljajo invazivne tujerodne vrste (Strgulc-Krajšek in Jogan, 2011). Taksoni *F. japonica* var. *japonica, F. sachalinensis* in *F.* x *bohemica* so v naravi že precej razširjeni. Ker se po podatkih z različnih nahajališč v Sloveniji sahalinski dresnik ne razrašča zelo hitro, s stališča invazivnosti ni tako problematičen, kot to velja za japonski dresnik in češki dresnik (Strgulc-Krajšek in Jogan, 2011). Japonski dresnik in češki dresnik sta v Sloveniji najpogostejša predstavnika obravnavanega rodu. Ker nekateri raziskovalci (Bailey in sod., 2009) poročajo, da je češki dresnik predvsem ob rekah zelo pogost in po podatkih iz literature veliko uspešnejši pri vegetativnem razmnoževanju in širjenju, bo potrebno v prihodnje veliko pozornosti nameniti tudi tej vrsti in ne samo japonskemu dresniku (Strgulc-Krajšek in Jogan, 2011).

Slika 2: Razraščanje japonskega dresnika na obrežju Gradaščice na Viču v Ljubljani oktobra 2011 (foto: J. Rupnik)

3 NAČINI ZATIRANJA JAPONSKEGA DRESNIKA

Zatiranje japonskega dresnika je zelo težavno zaradi podzemnih stebel, iz katerih poganjajo novi poganjki. Mehansko odstranjevanje s košnjo je le začasna rešitev, saj rastline na tak način ne zatremo (Shaw et al., 2009). Beerling et al. (1993) navaja, da z rezjo spodbudimo tvorbo novih poganjkov, saj so med triletnim opazovanjem med rastno dobo japonski dresnik večkrat kosili in v primerjavi z nekošenimi rastišči to tudi potrdili. Nekateri raziskovalci kot edino možno metodo zatiranja navajajo košnjo v kombinaciji s kemičnim zatiranjem (Child et al., 1992; Kabat et al., 2006), a je tudi tovrstna metoda v številnih primerih neučinkovita (Zemljič-Urbančič in Škerlavaj, 1999).

Zemljič-Urbančičeva in Škerlavaj sta preučevala vpliv škropljenja s herbicidoma glifosat in 2,4-D na obraščanje rizomov japonskega dresnika. Škropljenje z 2,4-D v odmerku 3 L/ha ni bistveno zmanjšalo rasti rizomov v primerjavi s kontrolo, glifosat pa je pri večjih odmerkih (7, 10 in 12 l/ha) vplival na zmanjšano rast vseh rizomov, a je ni popolnoma onemogočil. Predvsem zaradi dejstva, da se rastlina razrašča na urbanih območjih in ob vodah, Holden in Fowler (1992) nakazujeta, da bo na dolgi rok edina možna rešitev le v obliki biotičnega zatiranja z vnosom naravnega sovražnika iz okolja, od koder izhaja omenjena invazivna rastlinska vrsta. V letu 2003 so v Veliki Britaniji začeli s projektom preučevanja možnosti biotičnega zatiranja japonskega dresnika. Pri preučevanju literaturnih virov in opazovanj na Japonskem so ugotovili, da ima japonski dresnik okoli 180 naravnih sovražnikov, a je le bolšica *Aphalara itadori* Shinji dosegla zadovoljive rezultate pri zmanjševanju širjenja japonskega dresnika.

4 BOLŠICA *APHALARA ITADORI* SHINJI

Bolšica *Aphalara itadori* (Homoptera: Psyllidae) je bila prvič najdena leta 1938. Najprej so jo uvrstili v rod *Psylla* (Shinji, 1938), od koder so jo premestili v rod *Aphalara* (Miyatake, 1964). Literatura navaja, da vrsta *A. itadori* zaključi svoj življenski krog, iz jajčeca prek 5 stopenj nimf do odraslega osebka, pri 23 ºC v 33 dneh. Jajčeca so velika okoli 0,5 mm in jih samice odlagajo na liste. Iz jajčeca se razvije nimfa, ki je v svojem zadnji, peti stopnji, velika okoli 1,5 mm. Iz nimfe se razvije odrasel osebek, ki lahko doseže do 2,5 mm. Največjo škodo s sesanjem rastlinskega soka na japonskem dresniku povzročijo najmanjše nimfe (Shaw et al., 2009).

Nekatere predhodne raziskave so pokazale, da ima vrsta *A. itadori* izjemno ozek krog gostiteljski rastlin, na katerih lahko prezimi oz. razvije svoj razvojni krog. Shaw s sod. (2009) poročajo, da so v laboratorijskem poskusu ugotavljali sposobnost razvoja bolšice *A. itadori* na 87 različnih gostiteljskih rastlin. Rezultati so pokazali, da je bilo le 1,58 % jajčec (od 146.885) odloženih na druge rastlinske vrste, vendar se nobeno od teh jajčec ni uspelo razviti v stadij nimfe. V nadaljevanju poskusa so ugotovili, da so nimfe, ki so jih naknadno dali na različne gostiteljske rastline, svoj razvoj v odrasel osebek uspele razviti le v 7 %, in to vedno le na rastlinah iz družine Polygonaceae. Ob koncu poskusa so ugotovili, da je bilo hranjenje odraslih osebkov vrste *A. itadori* na drugih rastlinskih vrstah zanemarljivo, zato omenjena vrsta bolšice velja za ustreznega biotičnega agensa pri zatiranju japonskega dresnika. Literatura navaja, da bo uspeh zatiranja japonskega dresnika z bolšico *A. itadori* viden šele v obdobju od 5 do 10 let po vnosu na določeno območje. Za omenjeno vrsto bolšice doslej še niso ugotovili ali prenaša viruse in fitoplazme (Shaw et al., 2009).

5 ZAKLJUČKI

Bolšico *A. itadori* zaenkrat še ni mogoče kupiti, s čimer bi bila uporabnikom omogočena lažja uporaba. V Veliki Britaniji so to koristno vrsto vnesli iz Japonske in jo v laboratorijskih razmerah namnoževali na japonskem dresniku. V gojitvenih komorah so (pri 22 ºC, relativni zračni vlagi med 50 in 80 % ter razmerju med dnevom in temo 13:11) na japonskem dresniku, ki je bil posajen v cvetlične lončke, namnoževali omenjeno bolšico. Na gostiteljske rastline so položili 50 odraslih osebkov vrste A. itadori. V obdobju enega meseca je žuželka zaključila svoj razvoj. Literatura navaja, da bodo osebki, ki jih bodo izpustili v okolje, predstavljali trideseti rod izvirno vnesenega para iz Japonske (Shaw et al., 2009).

Ker so predhodne raziskave (Velika Britanija) pokazale, da je vrsta *A. itadori* specializirana le na japonski dresnik, zaključujejo, da bi bil vpliv na samonikle rastlinske vrste zanemarljiv. Izsledki omenjene raziskave so pokazali, da bolšica lahko odloži svoja jajčeca tudi na nekatere druge vrste iz rodu *Fallopia*, ki rastejo tudi pri nas (češki dresnik, sahalinski dresnik in hostni slakovec), vendar je delež odloženih jajčec na omenjenih rastlinah v primerjavi z japonskim dresnikom

zanemarljiv (Shaw et al., 2009). Kot potencialno ustrezna gostiteljska vrsta za bolšico *A. itadori*, se sicer poleg japonskega dresnika navaja tudi češki dresnik, a je število jajčec, ki jih bolšica *A. itadori*, odloži na to vrsto, v primerjavi z japonskim dresnikom, manjša, poleg tega pa se iz tako odloženih jajčec ne razvijejo ličinke. Do podobnih izsledkov so prišli tudi nekateri drugi raziskovalci (Miyatake, 2001).

Pri morebitni odločitvi za vnos bolšice *A. itadori* v Slovenijo, bi sprva poskusi potekali v laboratorijskih razmerah, kjer bi bilo potrebno preučevati vpliv žuželke na nekatere domače gojene in samonikle rastlinske vrste, ki se pojavljajo na območju, kjer je razširjen japonski dresnik. Če bi tudi naši laboratorijski rezultati pokazali, da je delovanje bolšice *A. itadori* specifično (le za japonski dresnik), bi omenjeno bolšico izpustili v mrežnjak in določeno obdobje preverjali njeno delovanje v naravnih razmerah. Glede na razširjenost češkega dresnika pri nas, pa bi bilo potrebno natančno preučiti tudi možnost zatiranja te rastlinske vrste z bolšico *A. itadori*, saj bi bilo lahko njeno delovanje v novem okolju drugačno od tistega, v katerem so ugotovili njeno gostiteljsko neustreznost za omenjeno bolšico.

Za vrsto *Aphalara itadori* je sicer značilno, da prezimi na rastlinah iz rodu *Pinus* (Miyatake, 2001), vendar ne smemo izključiti dejstva, da bi bilo lahko v naših podnebnih razmerah preživetje omenjenega biotičnega agensa pozimi vprašljivo. V Veliki Britaniji, prvi evropski državi, kamor so vnesli omenjeno bolšico, so sicer potrdili zmožnost njenega preživetja na prostem tudi pozimi, a so podnebne razmere v Veliki Britaniji, v primerjavi s Slovenijo, bolj podobne tistim na Japonskem (Peel et al., 2007). Prav zato bi bilo potrebno natančno preučiti bionomijo omenjene bolšice in jo, če bodo temu v prid pokazali rezultati raziskave (ciljno delovanje in sposobnost preživetja na prostem), vnesti v naše okolje.

6 ZAHVALA

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Agrovoc descriptors: diatomite, adsorbents, storage losses, stored products pests, antibiotic properties, toxicity, biopesticides, biological control, pest control

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Značilnosti diatomejske zemlje kot naravnega insekticida za zatiranje skladiščnih škodljivcev

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IZVLEČEK

Diatomejsko zemljo uporabljajo v številne namene, med drugim tudi kot bioinsekticid za varovanje uskladiščenih pridelkov. Ta zemlja nastane z mletjem sedimentnih kamnin, imenovanih diatomiti. Obstaja veliko vrst diatomejske zemlje, a le zemlje z manj kot 7 % kristalnega SiO_2 so primerne za zatiranje škodljivcev. Diatomejska zemlja ni toksična za sesalce, dolgotrajno varuje živež pred škodljivimi žuželkami, za njen nanos se uporablja približno enaka tehnologija kot pri klasičnem nanosu insekticidov in se med predelavo zlahka odstrani z živeža. Med nekaj negativnimi lastnostmi gre izpostaviti dejstvo, da zmanjša hektolitrsko maso zrnja (zniža nasipno gostoto zrnja), ki je glavno merilo kakovosti zrnja. Diatomejska zemlja ima velik absorpcijski potencial in se veže na epikutikularne voske žuželk, zato deluje praktično na vse škodljivce, ki imajo kutikulo zaščiteno z voski.

Ključne besede: diatomejska zemlja, insekticidno delovanje, skladiščni škodljivci, uskladiščeni pridelki

ABSTRACT

CHARACTERISTICS OF DIATOMACEOUS EARTH AS BIOPESTICIDE FOR CONTROL OF STORED PESTS

Diatomaceous earth is used for many purposes, including as a bioinsecticide for protection of stored crops. This material is produced by milling the sedimentary rock called diatomite. There are many types of diatomaceous earth but only diatomaceous earths with less than 7% of crystalline silica are suitable for pest control. Diatomaceous earth is not toxic to mammals, it provides the protection of food on a long-term against harmful insects, and for its application it is used around the same technology as in the conventional application of insecticides and is easily removed during processing. Among some of the negative characteristics is that reduces bulk density of grain, which is the main criterion of assessing the quality of grain. Diatomaceous earth has a high absorption potential and is bound to insect epicuticular waxes, and act practically on all pests that have cuticula protected by wax.

Key words: diatomaceous earth, insecticidal activity, stored pests, stored products

1 UVOD

Za varovanje uskladiščenih pridelkov pred skladiščnimi škodljivimi žuželkami se najpogosteje uporabljajo neposredno na živež naneseni insekticidi. Zaradi več negativnih lastnosti sintetičnih insekticidov, kot so toksičnost za sesalce, ostanki insekticidov na živežu in pojav odpornosti nekaterih škodljivcev na insekticide (Arthur, 1996), so zaželene alternativne metode zatiranja. Mednje prištevamo zatiranje skladiščnih škodljivcev z diatomejsko zemljo (Athanassiou in sod., 2005a), ki je klasificirana kot okoljsko sprejemljiva metoda (Arthur, 1996) in ki jo v nizkih koncentracijah zmešamo z zrnjem (Rojht, 2010ab). V praksi se navadno uporablja v 0,1 % koncentraciji. V Sloveniji je trenutno registriranih pet fitofarmacevtskih sredstev (FFS) za zatiranje skladiščnih škodljivcev: žitni žužek (*Sitophilus granarius*), žitni kutar (*Rhizopertha*

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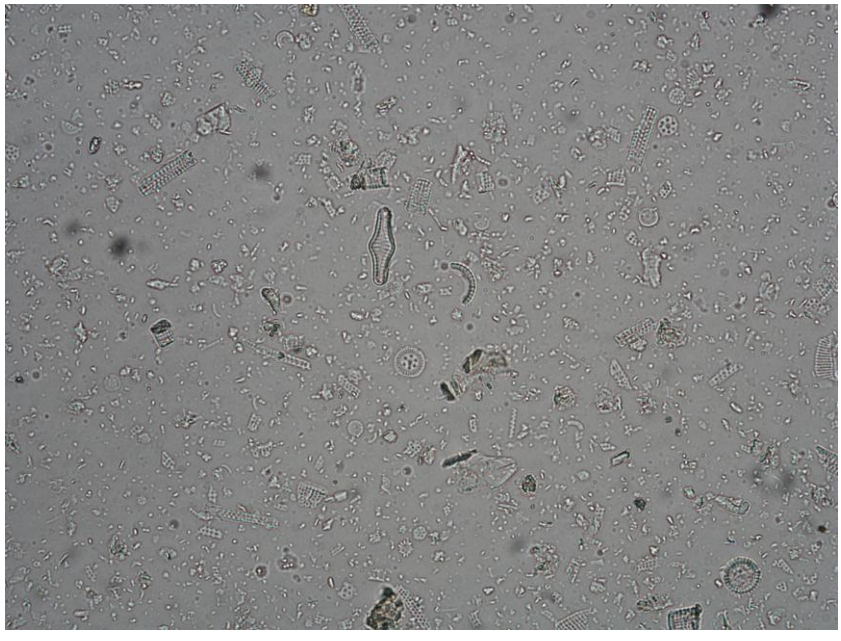
dominica), rižev žužek (*Sitophilus oryzae*), mokarji (*Tribolium* spp.) in krhljev molj (*Plodia interpunctella*), pri čemer se nekaterih pripravkov, zaradi možnega vpliva na kalitev, ne sme uporabljati v praznih prostorih, v katerih se bo kasneje skladiščilo semenski material (Seznam registriranih fitofarmacevtskih sredstev, 2012).

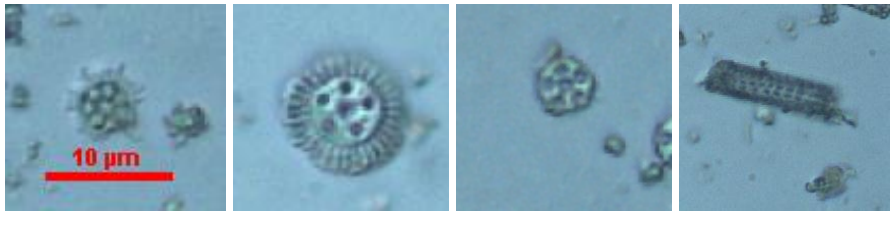
1.1 Kaj je diatomejska zemlja

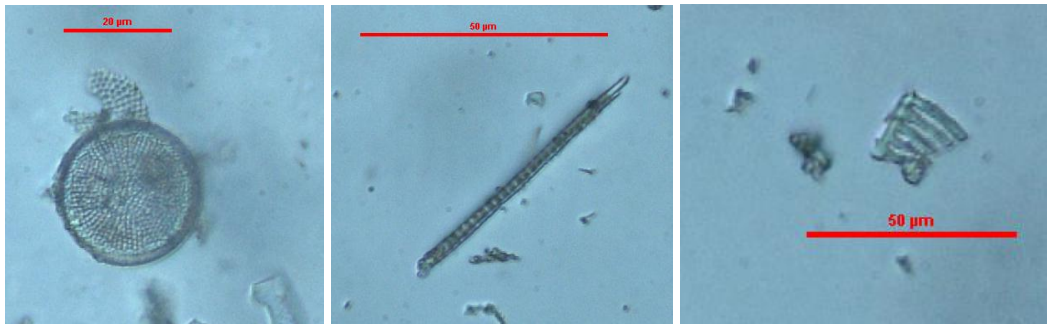
Diatomejska zemlja (DZ) je lahka, porozna biogena klastična sedimentna kamnina, ki je nastala z nakopičenjem skeletnih ostankov kremeničnih alg ali diatomej (Bacillariophyta), odloženih na dnu morij in jezer (Round in sod. 1990). Od 35.000 vrst alg je na svetu kar 12.000 kremeničnih, ki živijo v slani in sladki vodi ter v tleh. Skeleti odmrlih alg se nabirajo na dnu voda in na leto tvorijo od 0,2 do 0,5 mm debele lamine sedimentov (Hauptman in sod. 2006) ter v različnih geoloških obdobjih tvorijo več sto metrov debele sekvence kamnin (Ross, 1981). Kremenične alge so različnih oblik (slika 1, 2 in 3). Njihove lupine sestavljajo številne pore in so zgrajene iz hidratizirane amorfne kremenice oziroma opala ($SiO_2 \times nH_2O$). Tako je diatomejska zemlja sestavljena predvsem iz amorfne kremenice (SiO_2), z redkimi primesmi elementov in spojin, kot so aluminij, železov oksid, kalcijev hidroksid, magnezij in natrij (Round in sod. 1990).



Slika 1: Ostanki diatomej v vzorcu diatomita iz Grčije po mletju v diatomejsko zemljo. Fotografija je posneta pod optičnim mikroskopom Nikon Eclipse 80i (foto: F. A. Celar).

Figure 1: Remains of diatoms from Greek diatomite sample after the milling into diatomaceous earth. Image was taken by using optical microscope Nikon Eclipse 80i (photo: F. A. Celar).





Slika 2: Raznolikost diatomej v testiranih diatomejskih zemljah iz več Evropskih nahajališč. Fotografije so posnete pod optičnim mikroskopom Nikon Eclipse 80i (foto: H. Rojht).

Figure 2: The diversity of diatoms in tested diatomaceous earth from several European locations. Image was taken by using optical microscope Nikon Eclipse 80i (photo: H. Rojht).

Največje proizvajalke diatomejske zemlje so ZDA (620.000 t na leto), Kitajska (390.000 t), Danska (233.000 t) in Japonska (130.000 t), ki pa morajo za proizvodnjo izpolnjevati določene pogoje. Najstarejša nahajališča sedimentov kremenastih alg so v Burgerheimu, vendar so izkopavanje zaradi predragega postopka pred desetimi leti opustili (Hauptman in sod. 2006). V Sloveniji so do sedaj odkrili le diatomejske sedimente iz srednjega miocena, in sicer v Krški kotlini in Tuhinjskem gričevju. Prave diatomejske zemlje v Sloveniji ne poznamo. Glede na mineraloške analize jih uvrščamo v tri skupine (Horvat in Mišič, 2004). V prvo skupino se uvrščajo diatomejski meljevci z visoko vsebnostjo kremena (nad 30 %), opala-A (okoli 30 %) in glinenih mineralov (53-64 %), so brez karbonatov, vsebujejo pa tudi tufsko komponento. V drugo skupino se uvrščajo diatomejski sedimenti z visoko vsebnostjo karbonatov (nad 60 %), nizko vsebnostjo kremena (pod 15 %) in opala A (pod 4 %) ter so brez tufske komponente. Zaradi klastične strukture kamnin Horvat in Mišič (2004) za to skupino predlagata poimenovanje diatomejski laporovec oz. diatomejski karbonatni meljevec. Tretja skupina predstavlja diatomejski laporovec oz. diatomejski karbonatni meljevec z dokaj visoko vsebnostjo kremena (15-20 %) in opala-A (okoli 25 %), relativno nizko vsebnostjo karbonatov in v nekaterih primerih z visoko vsebnostjo tufske komponente. Znotraj te skupine najdemo vzorec, kjer delež tufske komponente preseže 20%, zato ga Horvat in Mišič (2004) opredelita kot tufski diatomit.

1.2 Uporaba diatomejske zemlje

Glede na nastanek delimo DZ na morske in sladkovodne. Obdelano DZ uporabljajo kot filtracijsko sredstvo za pripravo pijač (vino, pivo, sadni sokovi, rastlinska olja) ter kot polnilo pri izdelovanju avtomobilskih gum, papirja, zdravil, stenskih barv, kartona (daje mu trdnost in stabilnost), zobnih in polirnih past, kozmetičnih pripravkov. Alfred Nobel je z njeno pomočjo stabiliziral nitroglicerin in tako naredil dinamit. DZ sladkovodnega izvora so v preteklosti uporabljali za sijaj živalske dlake in čiščenje konjskih podkev (Hauptman in sod. 2006). Nekateri kmetje prisegajo na razglistevanje domačih živali z DZ (Fernandez, 1998). DZ, ki je registrirana kot insekticid, mora vsebovati manj kot 7 % kristalnega SiO_2. Kristaliziran SiO_2 namreč povzroča silikozo in je tudi rakotvoren, če se ga inhalira (IARC, 1997). Trenutno jo proučje veliko znanstvenikov z namenom njene implementacije v skladišča z uskladiščenimi pridelki.

2 PREGLED DELOVANJA DIATOMEJSKE ZEMLJE NA SKLADIŠČNE ŠKODLJIVCE

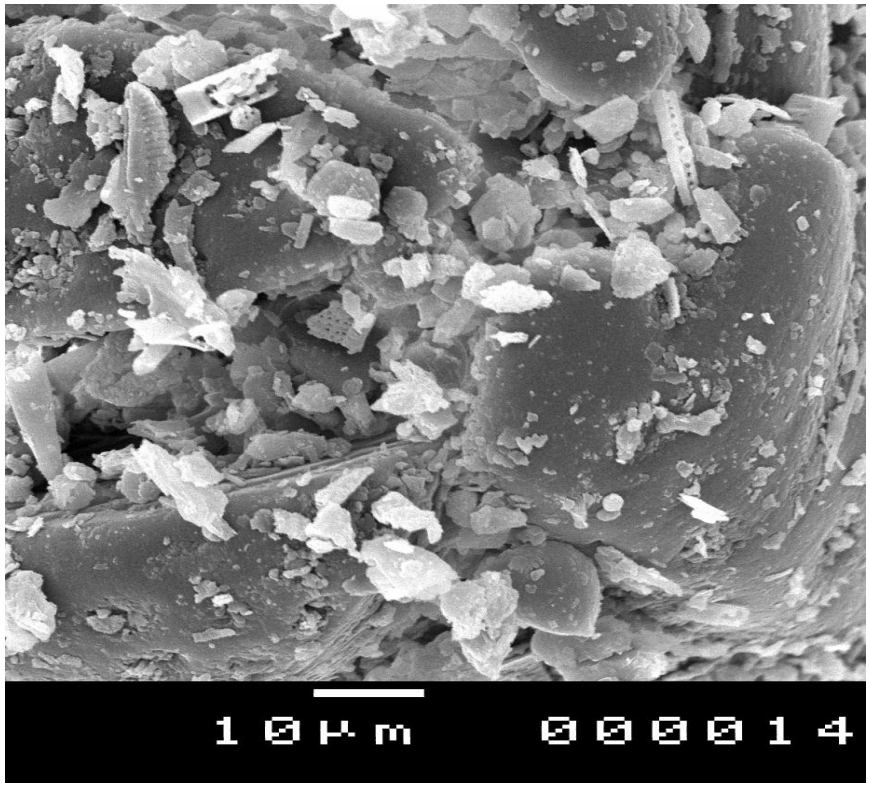
2.1 Insekticidne lastnosti diatomejske zemlje

Splošno znano je, da DZ deluje na zaščitno povoskano plast kutikule žuželk (delci DZ absorbirajo vodoodporne epikutikularne voske), kar rezultira v povečani izgubi vode in smrti zaradi izsušitve (Korunić, 1998; Subramanyam in Roesli, 2000). DZ deluje tudi abrazivno na kutikulo, vendar je to delovanje pri DZ z veliko vsebnostjo SiO_2 manj pomembno. Žuželke poginejo, ko izgubijo približno 60 % vode oz. 30 % telesne teže (Ebeling, 1971). Nekateri avtorji navajajo še druge teorije o delovanju na žuželke. Webb (1945) trdi, da delci DZ zamašijo stigme in traheje, Smith (1969) pa, da DZ poškoduje prebavni trakt. V vseh primerih gre za fizikalno delovanje DZ, zato je verjetnost za pojav rezistence žuželk nanjo majhna (Golob, 1997; Korunić, 1998; Subramanyam in Roesli, 2000). Diatomejska zemlja ima več prednosti pred drugimi insekticidi: ni toksična za sesalce, med predelavo se zlahka odstrani z živeža (Golob, 1997; Korunić, 1998; Subramanyam in Roesli, 2000), za njen nanos se uporablja približno enaka tehnologija kot pri klasičnem nanosu insekticidov, predstavlja dolgotrajno varstvo živeža pred škodljivci (Stathers in sod. 2004, Athanassiou in sod. 2005a). Na insekticidne lastnosti DZ vpliva več dejavnikov. Med najpomembnejše prištevamo: geološki izvor DZ, vsebnost SiO_2, zbito gostoto, absorbcijo za olje, velikost delcev in pH (Golob, 1997; Korunić, 1998). Insekticidno učinkovite DZ imajo vsebnost amorfnega SiO_2 nad 80 %, vsebnost kristalnega SiO_2 pod 7 %, pH pod 8.5 in zbito

gostoto pod 300 g/l (Korunić, 1997). Prav tako pa na učinkovito delovanje DZ vplivajo temperatura (Subramanyam in Roesli, 2000; Arthur, 2000), vlaga (Fields in Korunić, 2000), ciljni škodljivci (Fields in Korunić, 2000) in vrsta živeža, ki ga želimo zavarovati (Athanassiou in sod., 2003).

2.2 Primeri zatiranja skladiščnih škodljivcev z diatomejsko zemljo

Rižev žužek (*Sitophilus oryzae* [L.] Coleoptera, Curculionidae) in žitni kutar (*Rhyzopertha dominica* [F.], Coleoptera, Bostrychidae) sta najbolj škodljiva in najbolj razširjena škodljivca uskladiščenega žita (Aitken, 1975; Maceljski, 1999; Athanassiou et al., 2001ab). Spadata med primarne škodljivce, kar pomeni, da sta sposobna napasti zdrava, cela zrna. Ličinke obeh vrst se razvijajo v semenu, zato so zaščitene pred sredstvi za zatiranje. Vrsti sta razvili precejšno odpornost na insekticide (Champ in Dyte, 1976; Arthur, 1996; Benhalima et al., 2004) in ju ni mogoče zatreti z odmerki FFS, ki so učinkoviti za druge skladiščne škodljivce (Samson in Parker, 1989; Arthur, 1999). Rezultati več raziskav so pokazali, da je tega škodljivca mogoče zatirati z različnimi vrstami diatomejske zemlje, in sicer s komercialnimi pripravki kot so SilicoSec (Athanassiou in sod., 2003; Athanassiou in sod., 2004; Kavallieratos in sod., 2005; Shabbir, 2005), Protect-It (Athanassiou in sod., 2008) Insecto (Athanassiou in sod., 2004; Athanassiou in sod., 2005b; Kavallieratos in sod., 2005) in PyriSec (Athanassiou in sod., 2004; Athanassiou in sod., 2005b) in tudi nekomercialnimi lokalnimi diatomejskimi zemljami, ki vsebujejo manj SiO_2 (Athanassiou in sod., 2010; Rojht in sod., 2010ab). S komercialnimi pripravki diatomejske zemlje se lahko uspešno zatira vse pomembnejše skladiščne škodljivce (Arnaud in sod., 2005; Arthur, 2001; Athanassiou in sod, 2006; Athanassiou in sod, 2007; Islam in sod., 2010; Kavallieratos in sod., 2007). Na DZ so najbolj tolerantni mokarji (Arthur, 2000), med katere štejemo malega mokarja (*Tribolium confusum* du Val), ki lahko preživi odmerke DZ, ki so za druge skladiščne škodljivce letalni (Korunić, 1998).



Slika 3: Ostanki skeletov diatomej na vzorcu slovenske DZ na ustnem aparatu riževega žužka (*Sitophilus oryzae*) 21 dan po tretiranju. Posneto pod elektronskim mikroskopom JEOL JSM - T 300A na PIIR ZRC SAZU (foto: H. Rojht).

Figure 3: Remains of diatoms skelets from slovenian diatomaceous earth on mouthparts of rice weevils. Image was taken by using Scanning Electron Microscope JEOL JSM - T 300A at PIIR ZRC SAZU (photo: H. Rojht).

2.3 Omejitve pri uporabi diatomejske zemlje

Glavne omejitve pri uporabi DZ za zatiranje skladiščnih škodljivcev so: zmanjšana sipkost zrnja, neprijetno tretiranje zaradi velikega prašenja in zmanjšana hektolitrska masa zrnja. DZ se namreč zalepi na površino zrn in poveča trenje med zrni. To povzroči povišanje kota zdrsa zrn in zmanjša nasipno gostoto zrnja oziroma hektolitrsko maso zrnja (Fields, 1999).

Ker DZ deluje tako, da žuželke izsuši, ne deluje tako dobro v vlažnih razmerah (pri uskladiščenih pridelkih z višjo vlago) kot v suhih (le Patourel, 1986; Aldryhim, 1993). Vendar pa ta trditev ne velja za DZ z večjim deležem glinenih mineralov (in posledično z

manjšim deležem SiO_2), saj je laboratorijska raziskava pokazala signifikantno boljše delovanje na odrasle osebke riževega žužka pri 75 % relativni vlagi kot pri 55 % (Rojht in sod., 2010b). Za razliko od kemičnih fumigantov, DZ ne vpliva na razvojne stopnje škodljivcev, ki se dogajajo znotraj zrnja (Fields, 1999). Čeprav se za nanos DZ uporablja ista oprema, kot za nanos drugih FFS, pa je lahko tretiranje uskladiščenih pridelkov z DZ zelo neprijetno zaradi velike količine prahu, ki nastaja ob nanosu (Fields, 1999). V Avstraliji so zato uporabljali vodno aplikacijo DZ, vendar se je učinkovitost DZ zmanjšala (Maceljski in Korunić, 1972).

3 ZAKLJUČKI

Čeprav prvi zapisi o uporabi DZ za namene varovanja uskladiščenih pridelkov datirajo 4000 let nazaj, še danes ne vemo natančno, kako bi DZ najučinkoviteje uporabljali v skladiščih. Da bi premostili vse negativne lastnosti DZ, bi jo lahko nanašali le po površju uskladiščenih pridelkov ali po plasteh, kot jo uporabljajo v ZDA. Prav tako bi lahko DZ mešali z nizkimi koncentracijami sintetičnih insekticidov (Fields, 1999). Vsekakor je DZ primerna za varovanje uskladiščenega živeža, vendar pa jo je smiselno vključiti med ostale ukrepe integriranega varstva uskladiščenih pridelkov, prav tako pa je za uspešno varovanje živeža potrebno upoštevati tudi posredne in neposredne varstvene ukrepe.

4 ZAHVALA

Prispevek, ki ima korenine v mednarodnem projektu SEE-ERA.NET "Development of a non-toxic, ecologically compatible, natural-resource based insecticide from diatomaceous earth deposits of South Eastern Europe to control stored-product insect pests" (2007-2008), je nastal v okviru CRP projekta V4-1067, financiranega s strani Javne agencije za raziskovalno dejavnost RS, Ministrstva za kmetijstvo, gozdarstvo in prehrano RS in Ministrstva za okolje in prostor RS. Avtorji se zahvaljujemo Milošu Bartolu, Špeli Goričan in Adrijanu Koširju iz Paleontološkega inštituta Ivana Rakovca ZRC SAZU za pomoč pri delu na elektronskem mikroskopu, Franciju Acu Celarju za fotografije diatomej pod optičnim mikroskopom ter Nickolasu Kavallieratosu za posredovanje vzorcev diatomejske zemlje.

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CONTENT ANALYSIS OF THE PAPERS IN THE ACTA AGRICULTURAE SLOVENICA

VSEBINSKA OBDELAVA PRISPEVKOV V ACTA AGRICULTURAE SLOVENICA let. 99 št. 1

Tomaž BARTOL[a], Karmen STOPAR[b],

SUBJECT INDEX BY AGROVOC DESCRIPTORS
PREDMETNO KAZALO PO DESKRIPTORJIH AGROVOC

acquired characters	21-29, 69-75
adaptation	69-75
adsorbents	99-105
adventitious roots	65-67
ambrosia	41-47
anthracnosis	77-83
antibiotic properties	99-105
aphalaridae	93-98
apples	49-56
arabica coffee	85-92
artificial pollination	5-11
beauveria bassiana	57-63
beneficial organisms	93-98
biological control	93-98, 99-105
biological control organisms	93-98
biological development	69-75
biopesticides	99-105
blueberries	77-83
brassica napus	13-19
breeding methods	5-11
callogenesis	65-67
callus	65-67
castor beans	31-40
catalase	31-40
chemical composition	31-40
chemical reactions	31-40
coffea arabica	85-92
coffea canephora	85-92
coffea congensis	85-92
colletotrichum	77-83
congusta coffee	85-92
control methods	93-98
crop yield	13-19
crossbreeding	5-11
damage	93-98
developmental stages	69-75
diagnosis	77-83
diatomite	99-105
drought resistance	21-29
drought stress	21-29
ecosystems	93-98

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Acta agriculturae Slovenica, 99 - , marec 2012 str. 107 - 109

efficiency	41-47
entomogenous fungi	57-63
enzymes	31-40
explants	65-67
flowering	13-19
food safety	49-56
fungal diseases	77-83
fungal morphology	57-63
gene expression	85-92
genetic code	85-92
germination	5-11
growing media	69-75
growth	13-19, 99-105, 69-75, 57-63
growth control	21-29
herbicides	57-63
hybridization	85-92
hybrids	85-92
identification	77-83
in vitro regeneration	57-59
inhibition	65-67
integrated control	57-63
integrated pest management	49-56
lactuca sativa	49-56
lettuces	49-56
lipid peroxidation	31-40
malus pumila	49-56
maximum residue limits	49-56
meristem culture	65-67
mycelium	57-63
natural enemies	93-98
nature conservation	93-98
nitrogen	41-47
noxious plants	41-47, 93-98
nucleotide sequence	85-92
nucleotides	85-92
orchidaceae	5-11, 69-75
osmotic stress	31-40
peroxidases	31-40
pest control	99-105
pesticides	49-56, 57-63
phalaenopsis	5-11
physiological functions	13-19
plant anatomy	5-11
plant developmental stages	5-11, 13-19
plant physiology	31-40, 41-47
plant propagation	69-75
plant protection	49-56
plant vegetative organs	31-40
plant water relations	41-47
potatoes	49-56
pregermination	21-29
proximate composition	31-40, 41-47
rapeseed	13-19
reciprocal crossing	5-11
regulations	49-56

reproduction	13-19
residues	49-56
ricinus communis	31-40
robusta coffee	85-92
salt tolerance	31-40
seed treatment	21-29
seeds	5-11, 13-19
selenium	13-19
soft wheat	21-29
solanum tuberosum	49-56
stems	65-67
storage losses	99-105
stored products pests	99-105
stress	31-40
symptoms	77-83
tissue culture	69-75
toxicity	99-105
triticum aestivum	21-29
vaccinium corymbosum	77-83
varieties	5-11
water	31-40, 41-47
water metabolism	41-47
water uptake	41-47
water use	41-47
weeds	41-47
ziziphus	65-67

SUBJECT INDEX BY AGRIS CATEGORY CODES
VSEBINSKO KAZALO PO SKUPINAH ZNANJA (PREDMETNIH KATEGORIJAH)

F02 Razmnoževanje rastlin	69-75
F03 Semenarstvo	21-29
F30 Rastlinska genetika in žlahtnjenje rastlin	5-11, 85-92
f50 Zgradba rastlin	5-11
f60 Fiziologija rastlin in biokemija	21-29, 31-40, 41-47
F62 Fiziologija rasti in razvoja	5-11, 13-19, 31-40, 41-47, 65-67, 69-75
F63 Fiziologija reprodukcije	13-19
H10 Škodljivci rastlin	49-56, 99-105
H20 Bolezni rastlin	77-83, 57-63
H60 Plevel	41-47, 93-98
P01 Varstvo narave in varstvo zemljišče	93-98
P33 Kemija tal in fizika tal	99-105
Q01 Živilska tehnologija	99-105

Recenzija

Jože MAČEK

50 godina (1960-2010) univerzitetskog obrazovanja, naučne i stručne delatnosti u oblasti zaštite bilja - fitomedicine. Univerzitet u Novom Sadu - Poljoprivredni fakultet, Departman za fitomedicinu i zaštitu životne sredine. Novi sad 2010, 326 str.

Obravnavano knjigo je pripravil kolektiv avtorjev pod uredništvom akademika prof. dr. Dušana Čampraga in prof. dr. Stevana Jasniča, prof. dr. Tatjane Kereši, prof dr. Branka Konstantinovića, prof dr. Sanje Lazić, prof. dr. Stevana Maširevića, prof. dr. Dušana Petrića, prof. dr. Radoslava Sekulića. prof dr. Radmile Šovljanski in mag. Sladane Medić-Pap. Knjiga je posvečena akademiku prof. dr Pavlu Vukasoviću, ki je bil pravzaprav utemeljitelj fitomedicine kot znanosti in študija na novo osnovani Agronomski fakulteti Univerze v Novem Sadu. Namenjena je v znamenje zahvale za njegov veliki prispevek k znanosti, pedagoškemu in strokovnemu delu na področju fitomedicine. Ker je bil prof. dr. Pavle Vukasović slovenskega rodu, se toliko bolj spodobi, da knjigo predstavimo tudi v slovenski agronomski znanstveni publikaciji *Acta Agriculturae Slovenica*. Razen tega mi ni znana nobena zgodovinska publikacija, ki bi tako vsestransko in izčrpno obdelala kakršno koli agronomsko disciplino na univerzah našega kulturnega kroga, bodisi za krajše ali daljše zgodovinsko obdobje.

Iz zapisa poglavij bo razvidna bogata vsebina. Poleg predgovora je v knjigi 12 poglavij. 1. Od katedre do Oddelka (str 11-25), 2. Biografije znanstveno pedagoškega osebja (str. 27-100), 3. Pedagoška dejavnost (str. 101-150), 4. Od diplomiranih inženirjev do doktorjev znanosti (str. 151-193), 5. Znanstveno-raziskovalna dejavnost (str. 195-233), 6. Strokovna dejavnost (str. 235-263). 7. Izdajateljska dejavnost (str. 265-273), 8. Seznam objavljenih knjig (str. 275-300), 9. Nagrade in priznanja (str. 301- 305), 10. Sklep (str. 307-314), 11. Literatura (str. 315-319) in povzetek v angleščini (str. 321-326).

Knjiga v velikem obsegu temelji na prejšnjih publikacijah, biografijah in bibliografijah, letnih poročilih o znanstveno-raziskovalnem, strokovnem in pedagoškem delu, in drugih virih, prikaz diplomskih in magistrskih del ter doktorskih disertacij pa je gotovo prispevek biotehniške informatike.

Od novosadskih profesorjev bomo izčrpneje predstavili predvsem utemeljitelja tamkajšnjega fitomedicinskega študija, akademika Pavla Vukasoviča, ki je bil slovenskega rodu, vendar o njem v Sloveniji ni ničesar zapisanega. Rojen je bil v Zaječaru 15. julija 1893, umrl pa je v Beogradu 21. novembra 1973. Bil je šesti otrok častnika Janka, ki je bil po mojem spominu doma nekje z Goriškega. Kako je postal častnik srbske armade ni znano. Ena od možnosti, ki je precej verjetna je, da je bil častnik avstrijske armade, ki je sodeloval pri avstrijski okupaciji Bosne leta 1878, kamor so zaradi sorodnosti jezika radi pošiljali slovenske častnike. Ker pa je bil zelo slovansko usmerjen je dezertiral in vstopil v srbsko armado. V znamenje srbstva je opustil slovenski priimek in prevzel srbskega, po kom ni znano, morebiti po ženi Jeleni, ki je bila doma iz Zaječara. Sin Pavle Vukasović je obiskoval osnovno šolo v Požarevcu, gimnazijo pa v Beogradu, kjer je maturiral leta 1912. Njegov nadaljnji študij je bil prekinjen, ker je sodeloval v balkanski vojni 1912-1913, kjer je bil prostovoljec bolničar. S študijem medicine je začel v Nancyju v Franciji 1913-1914. Hitro se je vrnil v domovino, da je sodeloval v prvi svetovni vojni kot medicinec v sanitetni službi srbske armade. Doživel je vse strahote vojne in znameniti umik preko Albanije. Bil je nosilec Albanske spomenice.

Po prvi svetovni vojni je v Franciji nadaljeval s študijem. Leta 1921 je kot štipendist Ministrstva za kmetijstvo in vode dokončal študij na Agronomskem inštitutu univerze v Toulouseu z diplomo inženirja agronomije. Hkrati je študiral na biološki skupini tamkajšnje Filozofske fakultete, kjer je leta 1922 dobil diplomo "licencé ès sciences". V letih 1921-1924 je na isti fakulteti pripravljal doktorsko disertacijo, hkrati je bil asistent na katedri za zoologijo. Disertacija je imela naslov "Contribution a l'étude de l'Eudemis (*Polychrosis botrana* Schiff.) de la Pyrale de la Vigne (*Oenophthira pilleriana* Schiff.) et de leurs parasites." Dobil je naziv doktor zooloških znanosti. Kot asistent je tam dve leti delal na področju hidrobiologije in dobil diplomo za hidrobiologijo in ribištvo. V Parizu je leta 1924 opravil strokovni izpit iz parazitologije. V letih 1926-1927 se je v raznih ustanovah v Parizu kot Rockefellerjev štipendist izpopolnjeval iz področij parazitologije, medicinske in kmetijske entomologije. Na medicinski fakulteti je opravil tečaj iz parazitologije in malariologije.

Za asistenta na Katedri za sadjarstvo in vinogradništvo Agronomske fakultete beograjske agronomske fakultete v Zemunu je bil izvoljen leta 1925, vendar zaradi pomanjkanja proračunskih sredstev odobrenega službenega mesta ni mogel nastopiti. Nato je približno tri desetletja delal v

Centralnem higienskem inštitutu v Beogradu, najprej kot predstojnik Fitopatološkega, nato Parazitološkega in Biološkega oddelka. Predaval je na raznih tečajih za zdravnike. Sodeloval je s Prirodoslovnim muzejem v Beogradu in delovno skupino za preučevanje favne v Srbiji Srbske akademije znanosti in umetnosti. Ob ustanovitvi Agronomske fakultete v Novem Sadu ga je Komisija matičarjev 27. septembra 1954 izbrala za rednega profesorja za predmete Zoologija in Entomologija. V svojem službovanju na fakulteti je opravljal vse vodilne funkcije. Podrobnejši prikaz dela akademika Pavleta Vukasoviča, presega namen tega zapisa.

Med profesorji fitomedicine je bilo nekaj vodilnih strokovnjakov v jugoslovanskem merilu, iz starejše generacije npr akademik Dušan Čamprag, ter pokojni prof. dr. Momčilo Arsenijević, prof, dr. Adam Marič, prof. dr. Dušan Nikolić, prof. dr. Aleksandar Stankovič in drugi. Znano je bilo, da je specialni fitomedicinski študij na Agronomski fakulteti v Novem Sadu med najboljšimi v prejšnji, pa najbrž tudi v sedanjih novih državah, toda številčni prikaz je naravnost impozanten. Iz fitomedicine je v 50 letih diplomiralo 1054 univerzitetnih diplomiranih inženirjev, 135 magistrov, doktorski naziv pa je doseglo 97 kandidatov. objavljeno je bilo okoli 240 knjig (učbenikov, skript, monografij, priročnikov, barvnih atlasov) in več tisoč znanstvenih in strokovnih člankov. Njihova strokovna revija "Biljni lekar", ki jo pošiljajo v 20 držav, je v 96 številkah dosegla več kot 10.000 strani. V tem obdobju so dosegli odlično sodelovanje s kmetijskimi organizacijami vseh profilov, z živilsko industrijo in tudi s tovarnami in trgovino s fitofarmacevtskimi sredstvi, ki se je odražalo tudi v njihovem širokogrudnem financiranju strokovnega dela in sponzoriranju publikacij in nakupov raziskovalne opreme, o čemer lahko v Sloveniji le sanjamo. Oddelek za fitomedicino je dolgo časa uspešno vodil tudi pokrajinsko poročevalsko-prognozno službo.

Množice zanimivih podrobnosti moramo v tem prikazu pustiti vnemar, kogar zanima še veliko drugega si bo moral pač oskrbeti knjigo. Ta knjiga je in bo izvrsten zgodovinski vir. Z njo bo tako, kakor z arheološkimi ostalinami, čim starejše so, tem bolj so dragocene. Oddelku za fitomedicino Agronomske fakultete v Novem sadu in uredniku akad. prof. dr. Dušanu Čampragu velja čestitati za to knjigo.

Jože Maček

POPRAVEK/ CORRIGENDUM

Za zvezek 97-3 pravilno DOI številko za članek Rural women's attitudes toward their participation in the decision-making process and production of potato crops in Shoushtar, Iran: DOI: 10.2478/v10014-011-0014-6 (na strani 207)

In issue 97-3, correct DOI number for article Rural women's attitudes toward their participation in the decision-making process and production of potato crops in Shoushtar, Iran: DOI: 10.2478/v10014-011-0014-6 (on page 207).

NAVODILA AVTORJEM

Prispevki

Sprejemamo izvirne znanstvene članke, predhodne objave in raziskovalne notice s področja agronomije, hortikulture, rastlinske biotehnologije, raziskave živil rastlinskega izvora, agrarne ekonomike in informatike ter s sorodnih področij v slovenskem, angleškem in nemškem jeziku, znanstveno pregledne članke samo po poprejšnjem dogovoru. Objavljamo prispevke, podane na simpozijih, ki niso bili v celoti objavljeni v zborniku simpozija. 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.

Pri prispevkih v slovenskem jeziku morajo biti preglednice, grafikoni, slike in priloge dvojezični, povsod je slovenščina na prvem mestu. Naslovi grafikonov in slik so pod njimi. Slike in grafikoni so v besedilu. Priloženi morajo biti tudi jasno označeni izvirniki slik. Na avtorjevo željo jih vračamo, s tem da je želja pisno sporočena ob oddaji gradiva in ponovno v teku 30 dni po izidu. Latinske izraze pišemo ležeče. V slovenščini uporabljamo decimalno vejico, v angleščini decimalno piko. Prispevki v angleščini morajo imeti povzetek v slovenščini in obratno. Prispevki v nemščini morajo imeti tudi povzetka v slovenščini in angleščini.

Prispevki naj bodo strnjeni, kratki, praviloma največ 12 strani. Uporabljamo Microsoft Word 97 (Windows); pisava Times New Roman, velikost strani 16,2 x 23,5 cm, velikost črk besedila 10, v obsežnih preglednicah je lahko 8; izvlečki in metode dela Arial velikost 8, levi in desni rob 2,1 cm, zgornji rob 1,3 cm, spodnji rob 1,6 cm,

Prva stran

Na prvi strani prispevka na desni strani označimo vrsto prispevka v slovenščini in angleščini, sledi naslov prispevka, pod njim avtorji. Ime avtorjev navedemo v polni obliki (ime in priimek). Vsak avtor naj bo označen z indeksom, ki ga navedemo takoj pod avtorji, in vsebuje polni naslov ustanove ter znanstveni in akademski naslov; vse v jeziku prispevka. Navedemo sedež ustanove, kjer avtor dela. Če je raziskava opravljena drugje, avtor navede tudi sedež te inštitucije. Na željo avtorjev bomo navedli naslov elektronske pošte.

Pod naslovi avtorjev je datum prispetja in datum sprejetja prispevka, ki ostaneta odprta. Sledi razumljiv in poveden izvleček z do 250 besedami. Vsebuje namen in metode dela, rezultate, razpravo in sklepe. Sledijo ključne besede.

Izvlečku v jeziku objave sledi naslov in izvleček s ključnimi besedami v drugem jeziku.

Viri

V besedilu navajamo v oklepaju avtorja in leto objave: (priimek, leto). Če sta avtorja dva, pišemo: (priimek in priimek, leto), če je avtorjev več, pišemo: (priimek in sod., leto). Sekundarni vir označimo z "navedeno v" ali "cv.". Seznam virov je na koncu prispevka, neoštevilčen in v abecednem redu. Vire istega avtorja, objavljene v istem letu, razvrstimo kronološko z a, b, c. Primer: 1997a. Navajanje literature naj bo popolno: pri revijah letnik, leto, številka, strani; pri knjigah kraj, založba, leto, strani. Za naslove revij je dovoljena uradna okrajšava, za okrajšanimi besedami naj bodo vedno pike. Navedbo zaključimo s piko. Za primere upoštevajte objave v Zborniku BFUL.

Oddaja

Avtorji prispevke oddajo v dveh izvodih, enega z dvojnim razmakom med vrsticami in največ 35 vrst na strani, in na disketi. Priložijo tudi izjavo s podpisi vseh avtorjev, da avtorske pravice v celoti odstopajo reviji.

Prispevke recenziramo in lektoriramo. Praviloma pošljemo mnenje prvemu avtorju, po želji lahko tudi drugače. Če uredniki ali recenzenti predlagajo spremembe oz. izboljšave, vrne avtor popravljeno besedilo v 10 dneh v dveh izvodih, enega z dvojnim razmakom. Ko prvi avtor vnese še uredniške pripombe, odda popravljeno besedilo v enem izvodu in na disketi ter vrne izvod z uredniškimi popravki.

Prispevke sprejemamo vse leto.

NOTES FOR AUTHORS

Papers

We publish original scientific papers, preliminary communications and research statements on the subject of agronomy, horticulture, plant biotechnology, food technology of foods of plant origin, agricultural economics and informatics; in Slovenian, English and German languages while scientific reviews are published only upon agreement. Reports presented on conferences that were not published entirely in the conference reports can be published. If the paper is a part of diploma thesis, master of science thesis or dissertation, it should be indicated at the bottom of the front page as well as the name of the supervisor. All notes should be written in Slovenian and English language.

Papers in Slovenian language should have tables, graphs, figures and appendices in both languages, Slovenian language being the first. Titles of graphs and figures are below them. Figures and graphs are part of the text. Clearly marked origins of figures should be added; they can be returned if author desires. Latin expressions are written in italics. Decimal coma is used in Slovenian and decimal point in English. Papers in English should contain abstract in Slovenian and *vice versa*. Papers in German should contain abstracts in German, Slovenian and English.

The papers should be condensed, short and usually should not exceed 12 pages. Microsoft Word 97 (Windows) should be used, fonts Times New Roman, paper size 16.2 x 23.5 cm, font size in main text 10; in large tables size 8 could be used, abstracts and material and methods Arial size 8, right and left margin 2.1 cm, upper margin 1.3 cm and lower margin 1.6 cm.

First page

The type of the paper should be indicated on the first page on the right side in Slovenian and English language following by title of the paper and authors. Full names of authors are used (first name and surname). Each name of the author should have been added an index, which is put immediately after the author(s), and contains address of the institution and academic degree of the author, in the language of the paper. The address of the institution in which the author works is indicated. If the research was realised elsewhere, the author should name the headquarters of the institution. E-mail is optional.

Under the address of the authors some space for dates of arrival and acceptance for publishing should be left. A comprehensive and explicit abstract up to 250 words follows indicating the objective and methods of work, results, discussion and conclusions. Key words follow the abstract.

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References

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