ACTA AGRICULTURAE SLOVENICA

105•1
2015

Biotehniška fakulteta Univerze v Ljubljani
Biotechnical Faculty University of Ljubljana

Acta agriculturae Slovenica • ISSN 1581-9175 • 105 – 1 • Ljubljana, marec 2015

Acta agriculturae Slovenica
Volume / Letnik **105** · Number / Številka **1** · **2015**

VSEBINA / CONTENTS

Članki / Articles

Krajši prispevki/Short Communications

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.01
Agrovoc descriptors: wines, red wines, volatile compounds, provenance, indigenous organisms, aromatic compounds, esters, smell, extraction

Agris category code: f60, q04

Volatile profile of wine Teran PTP

Helena BAŠA ČESNIK[1], Dejan BAVČAR[1], Klemen LISJAK[1]*

Received November 19, 2014; accepted December 18, 2014.
Delo je prispelo 19. novembra 2014, sprejeto 18. decembra 2014.

ABSTRACT

Teran PTP is a protected wine with a recognized traditional denomination produced from a grapevine variety 'Refošk' in winegrowing district Kras in Slovenia (European Union, 2009; Pravilnik, 2008). The aromatic profile of 82 Teran PTP wines produced in years 2011, 2012 and 2013 was monitored. In total the content of 16 volatile compounds was determined. The volatile compounds from wine were extracted following the liquid-liquid extraction and determined with a GC-MS method. The odour activity values and relative odour contributions were calculated for each volatile compound identified. Among sensorial important volatiles the highest odour activity values were determined for ethyl octanoate, ethyl hexanoate, isoamyl acetate and ethyl butyrate. Other research papers also showed, that all red wines investigated except one contained ethyl octanoate, ethyl hexanoate, isoamyl acetate and ethyl butyrate above sensory thresholds.

Key words: Teran PTP, wine, volatile compounds, esters, aroma, GC/MS

IZVLEČEK

AROMATIČNE SPOJINE VINA TERAN PTP

Vino Teran PTP je zaščiteno vino s priznanim tradicionalnim poimenovanjem, ki ga pridelujejo iz grozdja sorte 'Refošk' v vinorodnem okolišu Kras v Sloveniji (European Union, 2009; Pravilnik, 2008). Aromatični profil vina Teran PTP smo spremljali tri leta v 82 vzorcih iz letnikov 2011, 2012 in 2013. Določevali smo vsebnost 16 hlapnih spojin. Za določitev hlapnih spojin smo uporabili ekstrakcijo tekoče-tekoče, kateri je sledila določitev z GC-MS. Za vseh 16 spojin smo izračunali aktivne vonjalne vrednosti in njihove relativne prispevke. Med senzorično pomembnimi hlapnimi spojinami smo največje aktivne vonjalne vrednosti določili za etil oktanoat, etil heksanoat, izoamil acetat in etil butirat. Drugi raziskovalni članki so tudi pokazali, da so vsa preiskovana rdeča vina z izjemo enega vsebovala etil oktanoat, etil heksanoat, isoamil acetat in etil butirat nad pragom zaznave.

Ključne besede: Teran PTP, vino, hlapne spojine, estri, aroma, GC/MS

1 INTRODUCTION

Teran PTP (Recognised Traditional Denomination) is a typical wine from the Kras winegrowing district of the Primorska wine growing region in Slovenia, where it is usually called just »Teran«. The production of this unique red wine has been known since the first century AD (Vodopivec, 1999). Teran PTP is a wine produced from a variety 'Refošk' and known for its deep reddish-violet colour, caused by an abundance of anthocyanins and its medium tannin content (Vanzo et al., 2012). Due to its favorable phenolic compounds (anthocyanins), it is known for having positive health effects and good nutritional value for consumers (Fornasario et al., 2012). Taste of wine teran is both astringent and sour, derived from its high phenolic content and high total acidity. The unique feature of this wine is its fruity odour, reminiscent of raspberries, strawberries and cherries. Fruity odour of Teran PTP is considered most pronounced in the first year after vinification (Vodopivec, 1999).

The volatile fraction of wine determines to a great extent its aroma, which is one of the most

[1] Agricultural Institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia, PhD,* corresponding author: klemen.lisjak@kis.si

important characteristics influencing wine quality and consumer preferences (García-Carpintero et al., 2012b). However, the wine volatile fraction is extremely complex, where more than 1000 aromatic compounds have been identified, originating from different chemical groups, mostly higher alcohols, aldehydes, ethyl esters of fatty acids, fatty acids, ketones, monoterpenes and volatile phenols (Andujar-Ortiz et al., 2009). A content of listed aromatic compounds in wines range from a few ng l^{-1} to hundreds of mg l^{-1} (Andujar-Ortiz et al., 2009). The presence, abundance and various combinations of volatile compounds can be greatly affected by viticultural practices (variety, cultivation, grape thinning etc.), environmental conditions (climate, soil) and oenological measures (fermentation, yeasts, post-fermentation treatments etc.) (Ribéreau Gayon et al., 2006; Welke et al., 2014).

Not all volatile compounds present in wine contribute to aroma (Welke et al., 2014). The influence of volatile compounds on the final aroma depends on their content and the specific perception threshold of each compound. The threshold of olfactory perception is defined as the lowest content capable of producing an olfactory sensation detected by the human nose, and that can be identified by at least 50 % of the judges on a sensory evaluation panel (Welke et al., 2014).

In our research, esters were of particular interest as they are usually responsible for the typical aroma of most not-aromatic varieties of wine (Etievant 1993; Ferreira et al, 1995). One factor that supports the use of this approach is the typical fruity smell of Teran PTP wines, which is correlated to abundant contents of esters. The results of the present study are particularly interesting, since the aromatic profile of Teran PTP wine has never been investigated before and even less over a multi-year period. Previous studies of Teran PTP were mostly concentrated on phenolic compounds and their influence on wine technologies or wine quality (Novak, 2011; Vrščaj Vodošek and Košmerl, 2004).

To determine the volatile compounds in the wine, different analytical techniques were used: discontinuous or continuous liquid-liquid extraction (LLE) (Andujar-Ortiz et al., 2009), solid phase extraction (SPE) (García-Carpintero et al., 2012a; García-Carpintero et al., 2014), solid phase microextraction (SPME) (Revi et al., 2014) and stir bar sorptive extraction (SBSE) (Martínez-Gil et al., 2012; Košmerl and Zlatić et al., 2009). It should be mentioned that the LLE technique is slowly being replaced by more manageable and solvent-free techniques. However, this type of extraction is still a reference technique used for wine aromatic compounds extraction. The main advantages of this technique are its capacity to extract a wide range of compounds of different volatilities (as long as they have an affinity to the solvent), its high repeatability level and the possibility of carrying out simultaneous extractions (Andujar-Ortiz et al., 2009). For determination, gas chromatography (GC) was used, coupled to a flame ionization detector (FID) (Pino and Queris, 2011; Moreno-Pérez et al., 2013) or a mass spectrometer (MS) (Callejón et al., 2009; Pino and Queris, 2011). The mass spectrometer is the most widely used, because it enables unequivocal qualitative and quantitative detection of substances. Quantitative detrermination of the impact of aromatic compounds on the overall aroma of wine can be done by calculating the odor activity value (OAV) and relative odour contribution (ROC). On the other hand, a qualitative evaluation can be done based on the odor descriptors of each component e.g., floral, fruity, green, solvent, plastic, toasted and others (Welke et al., 2014).

The aims of the present work are to present a volatile profile of Teran PTP wine produced in the Primorska winegrowing region (Kras district) in vintages 2011, 2012 and 2013 and a differentiation of an obtained volatile profile from other red wines. We focused our research on the different groups of volatile compounds formed during alcoholic fermentation together with C6 compounds deriving from the grapes. Some wine physico-chemical characteristics were also determined for all samples and presented in the article.

2 MATERIALS AND METHODS

2.1 Samples

The Teran PTP wine samples were collected from the winegrowing district Kras wine producers directly from stainless steel tanks and/or wooden barrels. During a three-year monitoring period, 82 wines were sampled from different producers; 39 samples from the 2011 vintage, 22 samples from the 2012 vintage and 21 samples from the 2013 vintage. The wines were sampled 9 months after fermentation, after the completion of malolactic fermentation and before bottling. Analyses of the volatile compounds and standard wine parameters were performed one month after sampling at the Central Laboratories of the Agricultural Institute of Slovenia.

2.2 Materials used for determining the volatile compounds

The standard volatile compounds, with the highest available purity (minimum of 98 %), were obtained from Merck, Sigma Aldrich, Fluka and SAFC. Stock solutions of each of the standard volatile compounds in pure dichloromethane were prepared with contents ranging from 1.8 to 2.5 g l^{-1}. From the stock solutions, one mixed solution of all the minor volatile compounds was prepared. The final standards were prepared with proper dilutions from this flask. Internal standard 4-nonanol (0.12 g dissolved in 100 ml dichloromethane) was added using a 0.05 ml Hamilton syringe to 10 ml of dichloromethane standard solution and mixed.

2.3 Extraction and determination of volatile compounds

For the extraction of minor volatile compounds (μg l^{-1}), discontinuous liquid-liquid extraction (LLE) with dichloromethane (Sigma-Aldrich) was used. The wine (100 ml) was then transferred into a 250 ml Erlenmeyer flask. To this, 23 μg of 4-nonanol was added as an internal standard, using a 0.05 ml Hamilton syringe from the corresponding ethanol solution. Dichloromethane (40 ml) was added and the mixture was stirred at 350 rpm for 20 min. Then the mixture was centrifuged (8500 g, 10 min) and the organic phase was recovered. The aqueous phase was re-extracted twice, using the same method. Finally, the organic phases were combined and dried over sodium sulphate. They were concentrated to a final volume of 1 ml with a rotary evaporator and nitrogen gas flow prior to GC–MS analysis. This method is described in detail in other articles (Bavčar et al., 2011a; Bavčar et al., 2011b; Bavčar and Baša Česnik, 2011).

2.4 Calculation of odour activity value and relative odour contribution

The OAV is a quantitative approach for determining the impact that volatile compounds have on the aroma of wine. The OAV is obtained from the ratio between the content of an individual compound and its perception threshold. A volatile compound contributes to aroma when its content in wine is above the perception threshold. Therefore, only compounds with an OAV>1 can be perceived. (Pino and Queris, 2011; Welke et al., 2014). Another quantitative factor is the relative odor contribution (ROC), which is the percentage of the impact of a particular aroma compound. It is the ratio of the OAV percentage of each individual compound and the sum of the OAV of compounds that showed OAV>1 (Welke et al., 2014).

2.5 Statistical analysis

Data were collected and edited using Excel (Microsoft Office Professional Plus 2010) and analysis of variance (one-way ANOVA) was performed on physico-chemical characteristics and aromatic compounds data using Statgraphics® Centurion XVI statistical software package (StatPoint Technologies).

3 RESULTS AND DISCUSSION

Over a three-year period, we also monitored the basic parameters of Teran PTP wine. The results of the wine physico-chemical characteristics were obtained by adhering to standard EEC methods (European Union, 1990). These results are presented in Table 1. Most of statistically different physico-chemical characteristics were found in wines from 2011 vintage. But differences between the 2011, 2012 and 2013 vintages are not extensive and most probably caused only by different climatic conditions (Ribéreau Gayon et al., 2006; Vodopivec, 1999). We can confirm that Teran PTP wines contain moderate levels of alcohol (12 vol %), are high in total dry extract and acidity levels, and have surprisingly low contents of both free and total sulphur dioxide in correlation with regulation's demands (Pravilnik, 2004).

Table 1: Average physico-chemical characteristics of Teran PTP wines for the 2011, 2012 and 2013 vintages

Preglednica 1: Povprečne fizikalno-kemijske značilnosti vin Teran PTP letnikov 2011, 2012 in 2013

	Vintage 2011	Vintage 2012	Vintage 2013
	n=39	n=22	n=21
Alcohol (vol. %)	12.01±0.60 A	11.95±0.58 A	12.06±0.46 A
Extract (g l^{-1})	30.0±2.4 B	27.3±1.7 A	27.1±2.6 A
Total acidity (g l^{-1} as tartaric)	7.5±0.7 A	8.0±0.8 B	7.5±0.8 AB
Volatile acids (g l^{-1}as acetic)	0.62±0.17 B	0.45±0.11 A	0.73±0.13 C
Free SO_2 (mg l^{-1})	13±3 B	12±1 A	12±4 A
Total SO_2 (mg l^{-1})	43±6 B	40±9 B	35±7 A
pH	3.37±13 B	3.26±0.12 A	3.33±0.14 AB
Relative density	0.9958±0.0001 B	0.9948±0.0007 A	0.9946±0.0008 A
Lactic acid (g l^{-1})	2.1±0.4 B	1.5±0.4 A	2.5±0.6 C
Reducing sugar (g l^{-1})	2.5±0.7 B	1.2±0.3 A	1.3±0.7 A

n = number of samples
all data present mean value ± standard deviation
significant differences between vintages are indicated A, B, C at $p \leq 0.05$

The focus of our research was to determine the presence of certain volatile compounds in Teran PTP wines from the 2011, 2012 and 2013 vintages. The results are presented in Table 2. We found that Teran PTP wine contains high amounts of 1-hexanol (the mean value of the three vintages was 1292 µg l^{-1}) and γ-butyrolactone (the mean value of the three vintages was 12920 µg l^{-1}). We also found significant amounts of 2-phenyl-ethyl-acetate (the mean value of the three vintages was 49 µg l^{-1}), isoamyl acetate, benzaldehyde, benzyl alcohol, *cis*-3-hexen-1-ol, ethyl butyrate, ethyl decanoate, ethyl dodecanoate, ethyl hexadecanoate, ethyl hexanoate and ethyl octanoate.

Table 2: Contents (µg l^{-1}) of volatile compounds in Teran PTP wines from years 2011, 2012 and 2013 and their threshold values according to ((a) Li et al., 2008; (b) Duarte et al., 2010; (c) García-Carpintero et al., 2012a; (d) Rocha et al., 2004, 2005, (e) Sánchez-Palomo et al., 2012; (f) García-Carpintero et al., 2014; (g) Welke et al., 2014)

Preglednica 2: Vsebnosti (µg l^{-1}) hlapnih spojin v vinih Teran PTP letnikov 2011, 2012 in 2013 in njihovi pragovi zaznave kot navedeno v ((a) Li et al., 2008; (b) Duarte et al., 2010; (c) García-Carpintero et al., 2012a; (d) Rocha et al., 2004, 2005, (e) Sánchez-Palomo et al., 2012; (f) García-Carpintero et al., 2014; (g) Welke et al., 2014)

	Vintage 2011		Vintage 2012		Vintage 2013		Sensory treshold
	min - max	average±std	min - max	average±std	min - max	average±std	
ALDEHYDES							
n-Hexaldehide (Capronaldehide)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Benzaldehyde	n.d. - 91	8±18 A	<LOQ - 10	4±3 A	<LOQ - 75	16±20 B	350 (c, e, f)
Benzyl alcohol	42 - 799	164±152 A	67 - 474	163±90 A	42 - 842	285±210 B	200000 (c)
C6 COMPOUNDS							
1-Hexanol	310 - 2538	1205±454 A	632 - 2218	1227±462 AB	598 - 3008	1522±672 B	8000 (a, b, c, e, f)
Cis-3-hexen-1-ol	15 - 125	50±24 A	18 - 190	55±35 A	23 - 278	103±67 B	400 (c, e, f)
ESTERS							
2-Phenyl-ethyl-acetate	13 - 108	51±24 B	15 - 72	35±16 A	41-90	62±14 C	250 (b, c, e, f)
Ethyl butanoate (Ethyl butyrate)	30 - 301	116±52 B	57 - 104	80±14 A	37 - 245	108±44 B	20 (a, b, c, e, f)
Ethyl decanoate (Ethyl caprate)	29 - 237	84±45 B	23 - 84	56±16 A	73 - 291	184±57 C	200 (a, b, c, e, f)
Ethyl dodecanoate (Ethyl laurate)	n.d. - 46	29±25 A	n.d. - 13	8±4 A	n.d. - 56	40±14 A	3500 (f)
Ethyl hexadecanoate (Ethyl palamitate)	<LOQ - 717	84±141 A	1 - 152	26±42 A	2 - 751	92±163 A	1500 (a, f)
Ethyl hexanoate (Ethyl caproate)	81 - 304	186±49 B	84 - 211	157±36 A	132 - 300	191±43 B	14 (a, b, c, e, f)
Ethyl octanoate (Ethyl caprylate)	105 - 376	216±59 B	88 - 259	170±41 A	118 - 253	187±41 A	5 (a, b, c, e, f)
Hexyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1500 (a, c, e, f)
Isoamyl acetate	163 - 803	393±143 B	163 - 563	264±82 A	232 - 609	406±98 B	30 (c, e, f)
LACTONES							
γ-Butyrolactone	6840 - 18907	14036±3408 B	4444 - 12999	9423±2449 A	8401 - 19879	14512±2983 B	5000 (d)
KETONES							
β-Ionone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.5 (b)

n.d. - not detected
<LOQ - below limit of quantification
significant differences between vintages are indicated A, B, C at $p \leq 0.05$

A comparison between the three vintages shows that the 2012 vintage has a lower content of 2-phenyl-ethyl-acetate, isoamyl acetate, ethyl butyrate, ethyl decanoate, ethyl hexanoate and γ-butyrolactone than the 2011 and 2013 vintages. The lowest volatile contents were mostly found in wines from vintage 2012.

OAV and ROC values were calculated to estimate the sensory contribution of the aromatic compounds to the overall aroma of the wine. According to these calculations the most important volatile compound for Teran PTP wines was ethyl octanoate which reminiscent of sweet red cherry fruity. The second most abundant compound was isoamyl acetate reminiscent of banana, fruity and sweet smell and ethyl hexanoate with fruity, apple and strawberry odour. Important were also ethyl butyrate, with fruity odour and γ-butyrolactone described as sweet, toast and caramel. These results are presented in Table 3.

Table 3: OAV and ROC (%) values and their standard deviations for volatile compounds significant for the aroma of Teran PTP wines from the 2011, 2012 and 2013 vintages

Preglednica 3: OAV in ROC (%) vrednosti in njihovi standardni odkloni za hlapne spojine, ki so signifikantne za vonj vin Teran PTP letnikov 2011, 2012 in 2013

	OAV			ROC (%)		
	2011	2012	2013	2011	2012	2013
ALDEHYDES						
n-Hexaldehide (Capronaldehide)	0	0	0	/	/	/
Benzaldehyde	0.02 ± 0.05	0.012 ± 0.008	0.05 ± 0.06	/	/	/
Benzyl alcohol	0.0008 ± 0.0008	0.0008 ± 0.0005	0.001 ± 0.001	/	/	/
C6 COMPOUNDS						
1-Hexanol	0.15 ± 0.06	0.15 ± 0.06	0.19 ± 0.08	/	/	/
Cis-3-hexen-1-ol	0.13 ± 0.06	0.14 ± 0.09	0.26 ± 0.17	/	/	/
ESTERS						
2-Phenyl-ethyl-acetate	0.20 ± 0.09	0.14 ± 0.06	0.25 ± 0.06	/	/	/
Ethyl butyrate (Ethyl butanoate)	5.8 ± 2.6	4.0 ± 0.7	5.4 ± 2.2	7.4 ± 0.1	6.7 ± 0.1	7.4 ± 0.1
Ethyl decanoate (Ethyl caprate)	0.4 ± 0.2	0.28 ± 0.08	0.9 ± 0.3	/	/	/
Ethyl dodecanoate (Ethyl laurate)	0.008 ±0.007	0.002 ± 0.001	0.011 ± 0.004	/	/	/
Ethyl hexadecanoate (Ethyl palamitate)	0.06 ± 0.09	0.02 ± 0.03	0.06 ± 0.11	/	/	/
Ethyl hexanoate (Ethyl caproate)	13.3 ± 3.5	11.2 ± 2.5	13.7 ± 3.1	17.0 ± 0.1	18.8 ± 0.2	18.8 ± 0.2
Ethyl octanoate (Ethyl caprylate)	43.1 ± 11.7	33.9 ± 8.2	37.3 ± 8.3	55.2 ± 0.4	56.7 ± 0.6	51.2 ± 0.5
Hexyl acetate	0	0	0	/	/	/
Isoamyl acetate	13.1 ± 4.8	8.8 ± 2.7	13.5 ± 3.3	16.8 ± 0.2	14.7 ± 0.2	18.6 ± 0.2
LACTONES						
γ-Butyrolactone	2.8 ± 0.7	1.9 ± 0.5	2.9 ± 0.6	3.59 ± 0.02	3.15 ± 0.04	3.99 ±0.04
KETONES						
β-Ionone	0	0	0	/	/	/

Many of the analyzed volatile compounds in Teran PTP are below OAV. Despite this, they could express a synergistic effect with a positive character to the wine aroma. It is known, that the OAV does not provide a definitive answer to the impact that different compounds can have on the overall aroma of a wine (Benkwitz et al., 2012). The content of some compounds may significantly differ between samples, but this has little or no impact on the sensory properties of these wines. This may be due to the masking and/or enhancing effects of other volatile and nonvolatile components within the wine, found also at various content-levels (Benkwitz et al., 2012).

Although it is difficult to compare volatile compounds of Teran PTP with publish data as each author used specific method, samples were analyzed at different aging time and wines were vinificated with different technologies, we have compared volatile compounds of some other varieties with volatiles of Teran PTP (Table 4).

Table 4: Comparison of volatile compound contents in Teran PTP wines (average of three sequential vintages and their standard deviations) with the volatile compound values of a selection of Spanish red wines (µg l^{-1}) ((a) García-Carpintero et al., 2012b (average of four consecutive vintages); (b) Moreno-Pérez et al., 2013 (6 months after fermentation); (c) García-Carpintero et al., 2014 (control wine of oak treatment), New Zealand Pinot Noirs from Central Ottago (average of three regions) ((d) Imre et al., 2012) and French red wines with their standard deviations ((e) Antalick et al., 2014)

Preglednica 4: Primerjava vsebnosti hlapnih spojin v vinih Teran PTP (povprečje treh zaporednih letnikov in njihovi standardni odkloni) z vrednostmi hlapnih spojin določenih v izbranih španskih rdečih vinih (µg l^{-1}) ((a) García-Carpintero et al., 2012b (povprečje štirih zaporednih letnikov); (b) Moreno-Pérez et al., 2013 (6 mesecev po fermentaciji); (c) García-Carpintero et al., 2014 (kontrolno vino po tretiranju s hrastom), New Zealand Pinot Noirs iz Central Ottago (povprečje treh območij) ((d) Imre et al., 2012) in francoskih rdečih vin z njihovimi standardnimi odkloni ((e) Antalick et al., 2014)

	Teran PTP	Cabernet Sauvignon (b)	Syrah (b)	Monastrell (b)	Bobal (c)	Moravia Dulce (a)	Rojal (a)	Tortosí (a)	Pinot noir (d)	young red wines (e)	aged red wines (e)	Beaujolais Noveau red wines (e)
ALDEHYDES												
n-Hexaldehide (Capronaldehide)	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Benzaldehyde	10±17	n.a.	n.a.	n.a.	7	27	3	9	n.a.	n.a.	n.a.	n.a.
Benzyl alcohol	195±164	n.a.	n.a.	n.a.	688	234	278	438	n.a.	n.a.	n.a.	n.a.
C6 COMPOUNDS												
1-Hexanol	1292±531	1020	1500	1481	1375	1835	1938	1547	583	n.a.	n.a.	n.a.
Cis-3-hexen-1-ol	65±47	33	102	9	81	146	178	35	39	n.a.	n.a.	n.a.
ESTERS												
2-Phenyl-ethyl-acetate	49±22	56	442	115	297	960	80	70	12	58±19	37±16	311±195
Ethyl butanoate (Ethyl butyrate)	104±45	n.a.	n.a.	n.a.	85	60	60	90	31	177±42	147±32	367±95
Ethyl decanoate (Ethyl caprate)	102±65	n.a.	n.a.	n.a.	125	70	70	70	20	108±45	53±33	364±120
Ethyl dodecanoate (Ethyl laurate)	22±19	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	3±3	0.6±0.7	13±10
Ethyl hexadecanoate (Ethyl palamitate)	70±131	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ethyl hexanoate (Ethyl caproate)	179±46	189	93	10	631	360	380	420	43	387±94	326±72	793±158
Ethyl octanoate (Ethyl caprylate)	196±53	99	25	n.d.	428	330	420	430	66	479±106	365±95	1001±221
Hexyl acetate	n.d.	5	15	14	9	3670	2670	4350	10	2±2	2±1	23±15
Isoamyl acetate	362±132	511	644	57	1076	240	240	580	177	507±142	324±98	3190±1788
LACTONES												
γ-Butyrolactone	12920±3712	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
KETONES												
β-Ionone	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.2	n.a.	n.a.	n.a.

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

Comparing our samples with other red wines shows that Teran PTP did not contain hexyl acetate, which is responsible for green and floral aromas, and was found in other red wines (García-Carpintero et al., 2012b; Moreno-Pérez et al., 2013; García-Carpintero et al., 2014). This was also possible because of its typical decomposition in months following alcoholic fermentation as our samples were analyzed at least 9 months after its completion (Ramey and Ough, 1980). A further comparison with other articles shows that 1-hexanol, a compound with intensive herbal, woody aroma is present in all red wines, including Teran PTP.

Antalick et al. (2014) showed that young (one year old) red wines from Bordeaux, Loire valley and the Rhône's southern region (n=19) contain substantial amounts of ethyl butyrate (mean content 177 µg l^{-1}), ethyl hexanoate (mean content 387 µg l^{-1}), ethyl octanoate (mean content 479 µg l^{-1}), ethyl decanoate (mean content 108 µg l^{-1}) and isoamyl acetate (mean content 507 µg l^{-1}). Substantial amounts of these esters were also found in Teran PTP wines. They are of additional interest, as they are known to give a positive, synergistic effect on the wine odour (Ferreira et al., 1998).

The Teran PTP contained isoamyl acetate at slightly higher contents than the three Spanish varieties from the Jumilla Controlled Appellation – 'Moravia Dulce' and 'Rojal' (García-Carpintero et al., 2012b), higher contents than the 'Monastrell' (Moreno-Pérez et al., 2013) and Pinot noir (Imre et al., 2012), but lower contents than the 'Cabernet Sauvignon' and 'Syrah' (Moreno-Pérez et al., 2013), 'Bobal' (García-Carpintero et al., 2014), 'Tortosí' (García-Carpintero et al., 2012b), the young French red wines (Antalick et al., 2014) and the 'Beaujolais Noveau' red wines (Antalick et al., 2014). The Teran PTP contained isoamyl acetate at approximately the same content as the aged French red wines (Antalick et al., 2014).

García-Carpintero et al. (2014) studied the volatile profile of 'Bobal' wine from the La Macha region (Spain) and the influence of oak chips added during vinification. Other authors have shown that the addition of oak chips before the start of alcohol fermentation increases the amount of ethyl esters from straight chain fatty acids. In comparison with Teran PTP wine, which is traditionally vinificated without oak chips, the Bobal wines showed higher amounts of isoamil acetate, ethyl hexanoate and ethyl octanoate. Teran PTP on the other hand, contained higher contents of ethyl butyrate than 'Bobal' (García-Carpintero et al., 2014), 'Moravia Dulce', 'Rojal' and 'Tortosí' (García-Carpintero et al., 2012b).

During four consecutive vintages (2006-2009) García-Carpintero et al., (2012b), analyzed the volatile profile of three red minor varieties: 'Moravia Dulce', 'Rojal' and 'Tortosí', grown in the La Mancha region (Spain). All three varieties showed a higher content of C6 compounds, ethyl hexanoate and ethyl octanoate. On the other hand, comparing the volatile profile of Teran PTP with the 'Pinot Noir' from Central Otago (New Zealand) (Imre et al., 2012), the Teran PTP wines showed a higher content of both esters and C6 compounds.

Cerdan et al. (2004) showed that ethyl butyrate decreased during the aging of 'Merlot' and 'Cabernet Sauvignon' in American oak barrels. After 18 month of aging in oak barrels its content was lower compared to the three-year aging average of Teran PTP. The ethyl hexanoate content in Teran PTP was higher than in the 'Syrah', 'Monastrell' (Moreno-Pérez et al., 2013) and 'Pinot Noir' (Imre et al., 2012) wines. Its content was approximately the same as in the 'Cabernet Sauvignon' (Moreno-Pérez et al., 2013) and lower than the young French red wines (n=22) (Antalick et al., 2014), aged French red wines (n=61) and the 'Beaujolais Nouveau' red wines (n=19) (Antalick et al., 2014). As reported by Cerdan et al. (2004), the content of ethyl hexanoate did not alter during the first six months of aging in oak barrels, but subsequently it increased until it reached a maximum at 12 months of aging. After one year, the contents of this compound diminished. On the other hand, Antalick et al. (2014) showed that contents of ethyl esters of fatty acids (EEFA) decrease with the age of wines only when the EEFA have the longest carbon chain, such as ethyl decanoate and dodecanoate. Ethyl butyrate, hexanoate and octanoate contents were, for the most part, unaltered by the age of wines.

Teran PTP contained lower contents of ethyl octanoate than the 'Bobal' (García-Carpintero et al., 2014), 'Moravia Dulce', 'Rojal' and 'Tortosí' wines (García-Carpintero et al., 2012b), the young French red wines, the aged French red wines and the 'Beaujolais Noveau' red wines (Antalick et al., 2014). But it contained higher contents than the 'Cabernet Sauvignon', 'Syrah' (Moreno-Pérez et al., 2013) and 'Pinot Noir' (Imre et al., 2012). Cerdan et al. (2004) showed that ethyl octanoate increased during the first six months of aging in oak barrels. Then it remained practically constant until it decreased due to hydrolysis. The same authors have also shown that changes in ethyl esters during the aging of 'Merlot' and 'Cabernet Sauvignon' in oak barrels were not related to the alcohol contents and pH values of the wine.

4 CONCLUSIONS

According to the results of our study, esters are a very influential aromatic group in Teran PTP wines and do contribute to its typical fruity aroma. The aromatic profile of Teran PTP wines was most affected by ethyl octanoate, which reminiscent of a fruity sweet red-cherry. The second most important ester was ethyl hexanoate, with its fruity apple and strawberry odour. The third was isoamyl acetate, with a fruity sweet banana odour and was followed ethyl butyrate with fruity smell. A comparison of our results with red wines from other varieties showed some differences in ester contents. However, like for other wines esters were found to be one of the most important volatile compounds in Teran PTP.

5 ACKNOWLEGEMENTS

The authors would like to thank the Teran PTP producers for providing samples and those who contributed to the work: Mrs. Mateja Fortuna and other co-workers at the Central Laboratories of the Agricultural Institute of Slovenia. For financial support, we would like to express our thanks to the Agrotur/Karst agrotourism project, which was implemented as part of the Cross-Border Cooperation Programme Italy-Slovenia 2007-2013 – funded by the European Regional Development Fund and national funds. More information about the Agrotur project is available at http://www.agrotur.si/en/

6 REFERENCES

Andujar-Ortiz I., Moreno-Arribas M. V., Martín-Álvarez P. J., Pozo-Bayón M. A. 2009. Analytical performance of three commonly used extraction methods for the gas chromatography-mass spectrometry analysis of wine volatile compounds. Journal of Chromatography A, 1216: 7351-7357, DOI: 10.1016/j.chroma.2009.08.055

Antalick G., Perello M. C., de Revel G. 2014. Esters in wines: New Insight through the establishment of a database of French wines. American Journal of Enology and Viticulture, article in press, DOI: 10.5344/ajev.2014.13133

Bavčar D., Baša Česnik H.,Čuš F., Košmerl T. 2011a. The influence of skin contact during alcoholic fermentation on the aroma composition of Ribolla Gialla and Malvasia Istriana *Vitis vinifera* (L.) grape wines. International Journal of Food Science and Technology, 46: 1801-1808, DOI: 10.1111/j.1365-2621.2011.02679.x

Bavčar D., Baša Česnik H., Čuš F., Vanzo A., Gašperlin L., Košmerl T. 2011b. Impact of Alternative Skin Contact Procedures on the Aroma Composition of White Wine. South African Journal of Enology and Viticulture, 32: 190-203

Bavčar D., Baša Česnik H. 2011. Validation of the method for the determination of some wine volatile compounds. Acta agriculturae Slovenica, 97: 285-293, DOI: 10.2478/v10014-011-0023-7

Benkwitz F., Nicolau L., Lund C., Beresford M., Wohlers M., Kilmartin P. A. 2012. Evaluation of key odorants in Sauvignon Blanc wines using three different methodologies. Journal of Agricultural and Food Chemistry, 60: 6293 – 6302, DOI: 10.1021/jf300914n

Callejón R. M., Tesfaye W., Torija M. J., Mas A., Troncoso A. M., Morales M. L. 2009. Volatile compounds in red wine vinegars obtained by submerged and surface acetification in different woods. Food Chemistry, 113: 1252-1259, DOI: 10.1016/j.foodchem.2008.08.027

Cerdán T. G., Goñi D. T., Azpilicueta C. A. 2004. Accumulation of volatile compounds during ageing of two red wines with different composition. Journal of Food Engineering, 65: 349-356, DOI: 10.1016/j.jfoodeng.2004.01.032

Duarte W. F., Dias D. R., Oliveria J. M., Vilanova M., Teixeria J. A., Almeida e Silva J. B., Schwan R. F. 2010. Raspberry (Rubus idaeus L.) wine: Yeast selection, sensory evaluation and instrumental analysis of volatile and other compounds. Food Research International, 43: 2303-2314, DOI: 10.1016/j.foodres.2010.08.003

Etievant P.X. 1993. Wine. In: Volatile compounds in food and beverages. Maarse H. (ed.). New York, Marcel Dekker: 483-545

European Union: Commission Regulation (EEC) No. 2676/90 determining Community methods for the analysis of wines

European Union: Information from European Union institutions and bodiescommission, List of quality wines produced in specified regions, 2009/C 187/01, Official Journal from 8.8.2009

Ferreira V., Fernardez P., Pena C., Escudero A., Cacho J.F. 1995. Investigation of the role played by fermentation esters in the aroma of young Spanish wines by multivariate analysis. Journal of the Science of Food and Agriculture, 67, 3: 381-392; DOI: 10.1002/jsfa.2740670316

Ferreira V., Lopez R., Escudero A., Cacho J. F. 1998. The aroma of Grenache red wine: Hierarchy and nature of its main odorants. Journal of the Science of Food and Agriculture, 77, 2: 259-267; DOI: 10.1002/(SICI)1097-0010(199806)77:2<259::AID-JSFA36>3.0.CO;2-Q

Fornasario S., Tramer F., Žiberna L., Passamonti S. 2012. Biološka uporabnost in aktivnost pigmentov grozdja pri živalih: implikacije za zdravje ljudi, Simpozij AGROTUR, Ljubljana, 28. November 2012, pp. 7-13

García-Carpintero E. G., Gómez Gallego M. A., Sánchez-Palomo E., González Viñas M. A. 2012a. Impact of alternative technique to ageing using oak chips in alcoholic or in malolactic fermentation on volatile and sensory composition of red wines. Food Chemistry, 134: 851-863, DOI: 10.1016/j.foodchem.2012.02.194

García-Carpintero E.G., Sánchez-Palomo E., Gómez Gallego M. A., González-Viñas M. A. 2012b. Free and bound volatile compounds as markers of aromatic typicalness of Moravia Dulce, Rojal and Tortosí red wines. Food Chemistry, 131: 90-98, DOI: 10.1016/j.foodchem.2011.08.035

García-Carpintero E. G., Sánchez-Palomo E., Oliveria González Viñas M. A. 2014. Volatile composition of Bobal red wines subjected to alcoholic/malolactic fermentation with oak chips. Food Science and Technology, 55: 586-594, DOI: 10.1016/j.lwt.2013.10.024

Imre S. P., Kilmartin P. A., Rutan T., Mauk J. L., Nicolau L. 2012. Influence of soil geochemistry on the chemical and aroma profiles of Pinot noir wines. Journal of Food, Agriculture and Environment, 10: 280-288

Li H., Tao Y. S., Wang H., Zhang L. 2008. Impact odorants of Chardonnay dry white wine from Changli County (China). European Food Research and Technology, 227: 287-292, DOI: 10.1007/s00217-007-0722-9

Košmerl T., Zlatić E. 2009. Determination of 2-aminoacetophenone in wines using the stir bar sorptive extraction method coupled with GC-MS and GC-NPD. Mitteilungen Klosterneuburg, 59: 121-126

Martínez-Gil A. M., Garde-Cerdán T., Zalacain A., Pardo-García A. I., Rosario Salinas M. 2012. Applications of an oak extract on Petit Verdot grapevines. Influence on grape and wine volatile compounds. Food Chemistry, 132: 1836-1845, DOI: 10.1016/j.foodchem.2011.12.016

Moreno-Pérez A., Vila-López R., Fernández-Fernández J. I., Martínez-Cutillas A., Gil-Muñoz R. 2013. Influence of cold pre-fermentation treatments on the major volatile compounds of three wine varieties. Food Chemistry, 139: 770-776,DOI: 10.1016/j.foodchem.2013.01.052

Novak S. 2011. The influence of oenological tannins on quality of wine Teran PTP. Graduathion thesis, Biotehnical Faculty, Ljubljana 2011, 65 str.

Pino J. A., Queris O. 2011. Analysis of volatile compounds of mango wine. Food Chemistry, 125: 1141-1146, DOI: 10.1016/j.foodchem.2010.09.056

Pravilnik o vinu z oznako priznanega tradicionalnega poimenovanja – teran, Uradni list Republike Slovenije 16/2008 z dne 15.2.2008, str 1166

Ramey D., Ough C.S. 1980. Volatile ester hydrolysis or formation during storage of model solutions and wines. Journal of Agricultural and Food Chemistry, 28, 5:28-934; DOI: 10.1021/jf60231a021

Revi M., Badeka A., Kontakos S., Kontominas M.G. 2014. Effect of packaging material on enological parameters and volatile compounds of dry white wine. Food Chemistry, 152: 331-339, DOI: 10.1016/j.foodchem.2013.11.136

Ribéreau Gayon, P., Dubourdieu, D., Doneche, B., Lonvaud, A. 2006 (2nd ed). Handbook of Enology. Volume 1. The Microbiology of Wine and Vinifications. John Wiley & Sons, Chichester

Rocha S.M., Coutinho P., Delgadillo I., Cardoso A.D., Coimbra M. A. 2005. Effect of enzymatic aroma release on the volatile compounds of white wines presenting different aroma potentials. Journal of the Science of Food and Agriculture, 85, 2: 199-205; DOI: 10.1002/jsfa.1937

Rocha S.M., Rodrigues F., Coutinho P., Delgadillo I., Cardoso A.D., Coimbra M. A. 2004. Volatile composition of Baga red wine. Assessment of the identification of the would-be impact odorants. Analytica Chimica Acta, 513, 1: 257-262; DOI: 10.1016/j.aca.2003.10.009

Sánchez-Palomo E., Gómez García-Carpintero E., Gómez Gallego M. A., González Viñas M. A. 2012. Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications. The Aroma of Rojal Red Wines from La ManchaRegion – Determination of Key Odorants. Salih B., Çelikbiçak Ö. (eds.). Published online, InTech: 147-170, DOI: 10.5772/2517

Vanzo A., Šuklje K., Jenko M., Čuš F., Bavčar D., Lisjak K. 2012. Polifenolni potencial terana. Bioaktivne spojine Terana, Simpozij AGROTUR, Ljubljana, 28. November 2012, pp. 29-50

Vodopivec M. 1999. Kraški teran, Ljubljana, ČZP Kmečki glas: 162 str.

Vrščaj Vodošek T., Košmerl T. 2004. Določanje fenolnih spojin in barvnih parametrov v vinih refošk in teran različnega geografskega porekla. V: Jubilejni 10.slovenski kemijski dnevi 2004, Maribor, 23. in 24. september 2004. Maribor 1-12

Welke J. E., Zanus M., Lazzarotto M., Alcaraz Zini C. 2014. Quantitative analysis of headspace volatile compounds using comprehensive two-dimensional gas chromatography and their contribution to the aroma of Chardonnay wine. Food Research International, 59: 85-99, DOI: 10.1016/j.foodres.2014.02.002

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.02
Agrovoc descriptors: allium, antioxidants, bulbs, antibacterial properties, antimicrobial properties, antimicrobials, phenolic compounds, phenolic content, extracts, indigenous organisms

Agris category code: f60, q04

Variation in antioxidant, and antibacterial activities and total phenolic content of the bulbs of mooseer (*Allium hirtifolium* Boiss.)

Abdollah GHASEMI PIRBALOUTI[1,2], Yazdan AHMADZADEH[1], Fatemeh MALEKPOOR[1]

Received April 24, 2014; accepted December 04, 2014.
Delo je prispelo 24. aprila 2014, sprejeto 04. decembra 2014.

ABSTRACT

Allium hirtifolium Boiss. (mooseer) belonging to the family Alliaceae, is an endemic species of Iran which grows wild in the Zagros Mountains range, western and southwestern Iran. The bulb of *A. hirtifolium* has been used as a flavouring agent, especially dairy foods and pickles by the indigenous people, southwestern Iran. In this study, the bulbs of various populations of the plant were collected from the alpine regions in Chaharmahal va Bakhtiari province, Iran. The total phenolic content of the ethanol extract was determined by Folin–Ciocalteu method, the antioxidant activity was evaluated by measuring 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the antibacterial activity of the extracts against four bacteria, including *Bacillus cereus*, *Listeria monocytogenes*, *Proteus vulgaris*, and *Salmonella typhimurium* was determined by serial dilution assay. Results indicated that the total phenolic content in the ethanol extracts of different populations of *A. hirtifolium* ranged between 34 to 44 mg gallic acid/g extract. In addition, the extracts of *A. hirtifolium* indicated moderate–to–good inhibitory activities (MICs = 0.062 to 0.250 mg/ml) against four bacteria, especially against *B. cereus*. The antioxidant activity of the bulbs of *A. hirtifolium* indicated the extract acted as an effective DPPH scavenger, but were not as effective as the BHT control. This finding suggests that the bulbs of *A. hirtifolium* may be considered as a natural source of antioxidants and antimicrobial agents.

Key words: Alliaceae, biological activity; endemic herbs; mooseer

IZVLEČEK

SPREMENLJIVOST ANTIOKSIDACIJSKEGA IN ANTIBAKTERIJSKEGA DELOVANJA CELOKUPNIH FENOLNIH IZVLEČKOV IZ ČEBULIC PERZIJSKE ŠALOTKE (*Allium hirtifolium* Boiss.)

Perzijska šalotka (*Allium hirtifolium* Boiss. (mooseer), *Allium stipitatum* Regel) spada v družino lukovk (Alliaceae), je endemična vrsta Irana, ki raste samoniklo v zahodnem in jugozahodnem delu države na območju gorovja Zagros. Prebivalci jugozahodnega Irana jo uporablja kot začimbo v mlečnih izdelkih in vlaganju zelenjave. V tej raziskavi so bile analizirane čebulice različnih populacij, nabrane v alpinskih predelih province Chaharmahal va Bakhtiari. Celokupna vsebnost fenolov je bila določena v etanolnem izvlečku po metodi Folin–Ciocalteu, antioksidativno delovanje je bilo ovrednoteno in izmerjeno z 1,1-difenil-2-pikrilhidrazilom (DPPH), antibakterijsko delovanje izvlečkov je bilo določeno proti štirim vrstam bakterij, *Bacillus cereus*, *Listeria monocytogenes*, *Proteus vulgaris*, in *Salmonella typhimurium* s serijskim razredčitvenim testom. Rezultati so pokazali, da so etanolni izvlečki celokupnih fenolov iz čebulic različnih populacij te vrste vsebovali od 34 do 44 mg galične kisline na g izvlečka. Izvlečki so pokazali zmerno do dobro inhibitorno aktivnost (MICs = 0.062 do 0.250 mg/ml) proti omenjenim štirim vrstam bakterij, še posebej proti vrsti *B. cereus*. Antioksidativno delovanje izvlečkov čebulic je pokazalo, da so izvlečki delovali kot učinkoviti lovilci DPPH, vendar so bili manj učinkoviti kot BHT v kontroli. Izsledki kažejo, da so lahko čebulice perzijske šalotke (*A. hirtifolium*) dober naravni vir antioksidantov in antimikrobnih snovi.

Ključne besede: *A. hirtifolium,* Alliaceae, biološka aktivnost, antioksidant, antimikrobna snov

[1] Department of Medicinal Plants, Faculty of Agriculture and Food Science, Shahrekord Branch, Islamic Azad University, Shahrekord, 88148 (PO. Box: 166), Iran. E-mail: ghasemi@iaushk.ac.ir or aghasemipir@psis.umass.edu

[2] Medicinal Plants Program, Stockbridge School of Agriculture, College of Natural Sciences, Massachusetts University, Amherst, 01003, MA, USA

1 INTRODUCTION

Plant extracts are rich sources of natural antioxidant and antibacterial compounds. Phenolic compounds present in spice plants as dietary sources possess bioactive properties protecting cellular systems against oxidative stress (Ghasemi Pirbalouti et al., 2013a). Recently, interest in finding naturally occurring antioxidants to replace synthetic antioxidants in foods and medicines has increased considerably, primarily due to the possible carcinogenicity of the synthetic antioxidants (Velioglu et al., 1998).

The genus of *Allium* L. is the largest and important representative genus of the Alliaceae family comprises 700 species; each with different tastes, forms and colors; nonetheless, they are close in biochemical, phytochemical, and nutraceutical properties (Tepe et al., 2005). *Allium* species are revered to possess antibacterial, antifungal, antiviral, antiprotozoal, and anthelmintic activities (Ariga and Seki, 2006; Benkeblia, 2005) and they contain the powerful antioxidants, sulphur and other numerous phenolic compounds which have aroused great interests for food industries. The *Allium* species have been used for a long time as a medicinal for the prevention and treatment of certain diseases such as diabetes, arthritis, colds and flu, stress, fever, coughs, headache, hemorrhoids, asthma, arteriosclerosis, cancer, respiratory, gastrointestinal, rheumatic, and inflammatory disorders (Najjaa, et al., 2009; Kojuri et al., 2007; Amin, 1991). Biological and medical functions of *Allium* species are due to their sulphur compounds, such as S-alk (en) yl-L'Cysteine sulfoxides (Fritsch and Keusgen, 2006), however, presence of phenolic compounds are also beneficial for human health (Corzo-Martinez et al., 2007).

Mooseer (*Allium hirtifolium* Boiss.), is an endemic plant of Iran which wild grows in the alpine regions in Zagros Mountains range from Northwestern to Southwestern of Iran with the climate of very to moderate cold (Ghahreman, 1984; Rechinger, 1984). *A. hirtifolium* is a nutritive plant with special taste which its dried bulb slices are used as an additive to yogurt and also pickling mixtures, rice, meat, sauces and salads. The bulbs of *A. hirtifolium* have been used as a flavouring agent, especially dairy foods and pickles by the indigenous people, southwestern Iran (Ghasemi Pirbalouti, 2009). In Iranian folk medicine, mooseer has been successfully used for treating rheumatic and inflammatory disorders. In addition, different medicinal properties such as antitrichomonas, antiproliferative, and immunomodulatory activities have also been reported for the bulbs of mooseer (Mozaffarian, 2008; Ghodrati Azadi et al., 2008; Jafarian et al., 2003; Amin, 1991). Results of previous studies (Ashrafi et al., 2004; Ismail et al., 2013) indicated that the aqueous and methanol extracts of mooseer bulbs have antimicrobial properties.

Mooseer (*Allium hirtifolium* Boiss.), is an endemic plant of Iran which wild grows in the alpine regions in Zagros Mountains range from Northwestern to Southwestern of Iran with the climate of very to moderate cold (Ghahreman, 1984; Rechinger, 1984). *A. hirtifolium* is a nutritive plant with special taste which its dried bulb slices are used as an additive to yogurt and also pickling mixtures, rice, meat, sauces and salads. The bulbs of *A. hirtifolium* have been used as a flavouring agent, especially dairy foods and pickles by the indigenous people, southwestern Iran (Ghasemi Pirbalouti, 2009). In Iranian folk medicine, mooseer has been successfully used for treating rheumatic and inflammatory disorders. In addition, different medicinal properties such as antitrichomonas, antiproliferative, and immunomodulatory activities have also been reported for the bulbs of mooseer (Mozaffarian, 2008; Ghodrati Azadi et al., 2008; Jafarian et al., 2003; Amin, 1991). Results of previous studies (Ashrafi et al., 2004; Ismail et al., 2013) indicated that the aqueous and methanol extracts of mooseer bulbs have antimicrobial properties.

To our knowledge, there are no published reports on diversity of total phenolic content, antibacterial and antioxidant activities of various populations of *A. hirtifolium.* The main objective of this study was to evaluate content of phenolic compounds, antioxidants and antibacterial activities of the ethanol extracts from the bulbs of various populations of *A. hirtifolium*, and to evaluate them as potential sources of natural antioxidants and antimicrobial.

2 MATERIAL AND METHODS

2.1 Plant material

The samples of the bulb of *A. hirtifolium* collected from wild populations of the plants growing in various alpine regions of southwestern Iran were used in this study. In total, three replicate samples of 30 plants were gathered from three natural habitats at the early flowering between April 30th to May 20th 2012. The slope and elevation information were obtained from the Digital Elevation Model (DEM) using two well-known GIS software packages ILWIS (3.0 Academic). This array was geo-referenced using a metric UTM coordinate system and the geometric correction were carried out in the GIS ILWIS (Table 1). Soil physical and chemical characteristics, including pH, electrical conductivity (EC), organic carbon (OC%), and soil texture were determined (Table 1). Climatic data of the locations were determined using data collected by the nearest meteorology stations (Table 1). Plant identity was confirmed by Prof. V. Mozaffarian, and a representative voucher specimen (No. 1265) was been placed in the Herbarium of Research Center of Natural Resources of Chaharmahal va Bakhtiari province, Shahrekord, Iran.

2.2 Extract preparation

Immediately following collection, the leaves of *A. hirtifolium* from each plant sample were separated and bagged independently. The bulbs were cleaned with tap water and cut into small slices by using a kitchen mixer. The tissue samples were subsequently air-dried in a shaded room at 30 ± 5 °C. A 100 g sample was extracted with 250 ml ethanol (96%, Merck, Darmstadt, Germany) at 45 °C for 8 h followed by a Soxhlet apparatus. The ethanol was subsequently removed under reduced pressure on a rotary evaporator (Model Zirbus 302 W, Italy) at 40 °C. The extracts were filtered using a Whatman No. 2. The extract samples were stored in universal bottles and refrigerated at 4 °C prior to use.

Table 1. Geographical and climate of natural habitats of *Allium hirtifolium*

Region	Altitude (m)	Latitude (UTM)	Longitude (UTM)	P* (mm)	T (°C)	pH	E.C. (dS/m)	O.C (%)	Sand (%)	Silt (%)	Clay (%)
Samsami	2742	0435278	3565206	779.9	12.6	6.85	0.528	1.931	26	36	38
Khaki	2487	0448579	3587078	327.3	10.6	7.5	0.442	1.541	20	42	38
Dasht-e-Laleh	2336	0428599	3599942	1025.1	9.7	7.23	0.348	1.117	32	32	36

* P: Annual precipitation (mm), T: Average temperature (°C), E.C.: Electrical conductivity (dS/m), O.C.: Organic carbon (%).

Meteorological information was obtained from weather stations located within the study area and the surrounding zone; each value in the mean of 10 to 15 year data.

Soil characteristics are based on average of samples taken from three farms in each region.

2.3 Determination of total phenolic content (TPC)

The total amount of phenolic compounds in each extract was determined using the Folin–Ciocalteu method following procedure of Singleton and Rossi (1965) with some modifications. Briefly, 0.5 ml of the sample was mixed with 2.5 ml of Folin- Ciocalteu's (Sigma–Aldrich Co., Steineheim, Germany) phenol reagent for 5 min at 37 °C, 2 ml of saturated Na_2CO_3 (7.5%) (Merck Co., Darmstadt, Germany) was added, and the mixture was brought to 10 ml with the addition of deionized, distilled water. The mixture was maintained at room temperature in the dark for 120 min and then the absorbance was measured at 765 nm against a reagent blank using a Perkin–Elmer Lambda UV/Vis spectrophotometer. Gallic acid (Merck Co., Darmstadt, Germany) was used as the reference standard and the total phenolic content was expressed as mg of gallic acid equivalents per gram of each extract on dry basis (mg GAE/g extract).

2.4 Antioxidant test

The DPPH radical scavenging activity of the ethanol extract was determined using the method proposed by Hung et al. (2005). The extracts

(100 µL) at concentrations of 8, 16, 32, 62.5, 125, 250, and 500 µg/ml were mixed with 3.9 mL an equal volume of 0.2 mM ethanol solution of DPPH (Sigma–Aldrich Co., Steineheim, Germany). The disappearance of the DPPH after 30 min of incubation at room temperature was determined using a Perkin–Elmer Lambda UV/Vis spectrophotometer at 515 nm against a blank, i.e. without DPPH. Ethanol was used to zero the spectrophotometer and the absorbance of the DPPH radical without antioxidant and measure daily served as the control. The amount of sample necessary to decrease the absorbance of DPPH by 50 % (IC_{50}) was calculated graphically and the percentage inhibition was determined according to the equation:

$$\% \text{ inhibition} = \left[\frac{AC(0) - AA(t)}{AC(0)}\right] * 100$$

where AC_0 is the absorbance of the control at t = 0 min and AA_t is the absorbance of the antioxidant at t = 30 min. The food preservative butylated hydroxytoluene (BHT) was used as positive control. All measurements were replicated three times.

2.5 Antibacterial test

Antibacterial activity of the extracts were tested using clinical isolates of four bacteria strains, the Gram-positive bacteria (*Bacillus cereus* and *Listeria monocytogenes*) and the Gram-negative bacteria (*Proteus vulgaris* and *Salmonella typhimurium*). The bacteria, originally obtained from chicken meat samples, were provided by the Food Microbiology Laboratory, Veterinary Medicine Faculty, (I.A.U.) Iran and had been positively identified using PCR-RFLP along with conventional morphological and biochemical tests. The population of each bacterial strain was increased by culturing in an overnight Mueller Hinton broth (MHB) at 37 °C. To quantify the antibacterial activity of the extracts, bacteria populations were prepared for testing by adjusting each population to 1.0 McFarland standards (1.0×10^7 CFU/mL), using a spectrophotometer (Perkin–Elmer Lambda UV/Vis, USA). Minimum inhibitory concentrations (MIC) were determined using the broth-serial dilution method following standardized methods (CLSI, 2012). The extracts and the antimicrobial agents (ciprofloxacin, and flumequine) were each dissolved in 5 % dimethyl sulfoxide (DMSO) and then diluted to the highest test concentration (500 µg/mL). Subsequent test concentrations were made in a series of two-fold dilutions to develop concentration levels of 8 to 500 µg/ml in sterile, 10 ml test tubes containing MHB. A population of bacteria was subsequently added to each tube containing an essential oil or antimicrobial agent and then incubated at 37 °C for 48 h. After the incubation period, the absorbance of each incubated solution was measured at 630 nm using a spectrophotometer (Perkin–Elmer Lambda UV/Vis, USA) as a measure of bacterial growth to indicate MIC values. The minimum bactericidal concentration (MBC) of each essential oil was determined according to the MIC values by transferring 5 µL from MIC tubes to agar plates and incubating at 37 °C for 48 h. The MBC was recorded as the minimum concentration of extract in which no viable bacterial growth was observed. All experimental tests were replicated three different times.

2.6 Statistical analysis

Data were analyzed by one-way analysis of variance with three replications using the SPSS 19.0 statistical software. Means were compared with Duncan test at $p \leq 0.05$ level.

3 RESULTS AND DISCUSSION

3.1 Extraction yield

The color of the ethanol extract from the bulbs of *A. hirtifolium* was light yellow. Statistical analysis indicated that there was significant difference ($p \leq 0.05$) among various populations for extract yield (Table 2). The highest extract yield was obtained from the Samsami population with 14.6% w/w on dry weight basis (Table 2). The lowest value of extract yield was obtained from the bulbs of *A. hirtifolium* collected in Koohrang population with 8.2% w/w on dry weight basis (Table 2). An earlier study by Jafarian et al. (2003) reported the

hydroalcohol extract yield from the bulbs of *A. hirtifolium* collected from Khansar (Isfahan), Iran was 34% using percolation method. In addition, results of a study by Kazemi et al. (2010) indicated the hydroalcohol extract yield from *A. hirtifolium* bulbs was 51.9 g extract obtained from 100 g powder by polyphenolic fraction method. A comparison of our results with the previous reports suggests differences in the extract yield of the plant material could be attributed to extraction methods.

Table 2: Extract yield, antioxidant activity, and total phenolic content of the ethanol extracts from the bulbs of *Allium hirtifolium*

Species	Part used	Populations	Extract yield (% w/w)	Total phenolic (mg GAE /g extract)	IC_{50} (mg/g)
A. hirtifolium	Bulb	Samsami	14.58 ± 3.94 a	38.11 ± 5.06 bc	3.09 ± 0.65 c
A. hirtifolium	Bulb	Khaki	11.12 ± 0.75 b	34.50 ± 4.12 c	2.51 ± 0.61 bc
A. hirtifolium	Bulb	Dasht-e-Laleh	8.17 ± 1.36 bc	44.28 ± 6.58 a	1.90 ± 0.31 b
BHT	-	-	-	-	0.21 ± 0.03 a
ANOVA			$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.01$

†Values in column having similar letter are not statistically different at $p \leq 0.05$

3.2 Total phenolic contents

In present study, total phenolic content in each extract was determined spectrometrically according to the Folin–Ciocalteu method and calculated as gallic acid equivalent (GAE). A significant difference ($p \leq 0.05$) for total phenolic content was measured among the extracts. The maximum total phenolic content was obtained from the extract of the Dasht-e-Laleh population with 44.28 ± 6.58 mg GAE/g extract (Table 2). Results of an earlier study by Ghahremani-majd et al. (2012) indicated that the total phenolic content in the methanol extracts from the bulbs of *A. hirtifolium* populations ranged from 8.4 to 0.5 mg GAE/g sample. Results of a study by Parakesh et al. (2007) indicted that total phenolic contents in the extracts from four (red, violet, white and green) varieties of *Allium cepa* varied from 4.6 to 74.1 mg/g GAE. Within the vegetable family, the composition and quantity of the phenolic are vary significantly according to different intrinsic and extrinsic factors, such as plant genetics and cultivar, soil and growing conditions, maturity state and harvest conditions (Jaffery et al., 2003).

3.3 Antioxidant test

Antioxidant properties are very important in counteracting the deleterious role of free radicals in food and biological systems. In our study, the antioxidant activity of the extract from the various populations of *A. hirtifolium* was expressed as IC_{50} with values from 1.90 to 3.09 mg/ml that indicating the extracts act as moderate to good DPPH scavenger (Table 2). Significant difference ($p < 0.01$) in IC_{50} values were found for the extracts and control (BHT). The extract from the Dasht-e-Laleh population with the highest total phenolic content showed the highest antioxidant activity. Ghahremani-majd et al. (2012) have observed a linear response between total phenolic and antioxidant capacity of the extracts from *A. hirtifolium* bulbs in FRAP, ABTS, and DPPH assays. The antioxidant activity of *Alliums* species was reported by numerous investigators (Velioglu et al., 1998).

3.4 Antibacterial test

The antibacterial activity of the extract from the various populations of *A.hirtifolium* was tested against the four pathogenic bacteria (*Bacillus cereus, Listeria monocytogenes*, *Proteus vulgaris*, and *Salmonella typhimurium*) by using the serial-dilution method. Extracts demonstrated relatively inhibitory activities against the pathogenic bacteria tested, the MICs and MBCs of the tested samples are presented in Table 3. Results of present study indicated that the different bacteria species

demonstrated different levels of sensitivity to the extracts. The MICs of the extracts were within concentration ranges from 0.062 to 0.25 mg/ml, and the respective MBCs were from 0.125 to > 0.50 mg/ml. Generally, the ethanol extracts from the bulbs of *A. hirtifolium* indicated moderate to good inhibitory activities against four bacteria. The highest antibacterial activity was obtained from the extracts of the bulbs of the Dasht-e-Laleh and Samsami populations against *Listeria monocytogenes* and *Bacillus cereus*, respectively. Similarly, results obtained from the measurements of MICs in a study by Ghahremani-majd et al. (2012) showed that *B. subtilis* was the most sensitive microorganism tested to the extracts from the bulbs of *A. hirtifolium* with the lowest MIC values from 1.87 to 15 mg/ml. In addition, they reported the methanol extract from the bulbs of the Isfahan mooseer population had the highest antibacterial and antifungal activities against six bacteria and two fungi. Probably, in present study the phenolic compounds are responsible of the antibacterial activity of the extracts from the bulbs of *A. hirtifolium*. In other study (Amin and Kapadnis, 2005), the extract of bulbs of *A. hirtifolium* had the high antimicrobial activity against wide range of pathogenic and nonpathogenic bacteria and fungi with the MIC values from 0.001 to 0.010 mg/ml .

Table 3: Antibacterial activity (MICs and MBCs) of the ethanol extracts from the bulbs of *Allium hirtifolium* against four bacteria

Species / Antibiotics	Part used	populations	*Bacillus cereus*		*Listeria monocytogenes*		*Proteus vulgaris*		*Salmonella typhimurium*	
			MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
A. hirtifolium	Bulb	Samsami	**62.5**	**125**	125	500	125	500	125	250
A. hirtifolium	Bulb	Khaki	125	250	250	500	125	250	125	250
A. hirtifolium	Bulb	Dasht-e-Laleh	62.5	250	125	250	125	250	125	250
Ciprofloxacin	-	-	**32.2**	**125**	**32.2**	**62.5**	**62.5**	**125**	**62.5**	**125**
Ampicillin	-	-	62.5	125	62.5	125	125	250	125	250

The mechanisms by which plant extracts can inhibit microorganisms vary (Ahmad and Beg, 2001; Rodriguez et al., 2009; Thormar, 2011). Phenolic compounds can act at two different levels: the cell membrane and cell wall of the microorganisms (Taguri et al., 2006). They can interact with the membrane proteins of bacteria by means of hydrogen bonding through their hydroxyl groups which can result in changes in membrane permeability and cause cell destruction (Ghasemi Pirbalouti et al., 2013b).

4 CONCLUSION

The present study is apparently the first report of quantitative total phenol profile, antioxidant and antibacterial activities of the ethanol extracts from the bulbs of *A. hirtifolium* collected from southwestern Iran. The results of current study demonstrated that the ethanol extract from some populations of *A. hirtifolium* with the maximum total phenolic content had the highest antioxidant activity by the DPPH assay. Total phenolic compounds present in the plant are responsible for its effective free radical scavenging, antioxidant and antimicrobial activities. In total, significant antioxidant and antibacterial activities of the extract of the studied herb provide a scientific validation for the traditional use of the plant as an accessible source of natural antioxidants and antimicrobial with consequent health benefits.

5 ACKNOWLEDGMENT

Authors would like to acknowledge the Deputy of Research and Technology of Shahrekord Branch, I.A.U., Iran for supporting this study.

6 REFERENCES

Ahmad, I., Beg, A.Z. 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethnopharmacology, 74: 113–123; DOI: 10.1016/S0378-8741(00)00335-4

Amin, G.R. 1991. Popular medicinal plants of Iran, Tehran: Iranian Ministry of Health publication, p. 37.

Ariga, T., Seki, T. 2006. Antithrombotic and anticancer effects of garlic-derived sulfur compounds: a review, BioFactors, 26: 93–103; DOI: 10.1002/biof.5520260201

Ashrafi, F., Akhavan Sepahi, A., Kazemzadeh, A. 2004. Effect of aqueous extract of shallot (*Allium ascalonicum*) on inhibition of growth of *Pseudomonas aeroginosa*. Iranian Journal of Pharmaceutical Research, 2: 71–71.

Benkeblia, N. 2005. Free-radical scavenging capacity and antioxidant properties of some selected onions (*Allium cepa* L.) and garlic (*Allium sativum* L.) extracts. Brazilian Archives of Biology and Technology, 48: 753–759; DOI: 10.1590/S1516-89132005000600011

CLSI (Clinical and Laboratory Standards Institute). 2012. Performance standards for antimicrobial disks susceptibility tests: approved standards - Eleventh edition. M02-A11, CLSI, USA.

Corzo-Martinez, M., Corzo, N., Villamiel, M. 2007. Biological properties of onion and garlic. Trends in Food Science Technology, 18: 609–625; DOI: 10.1016/j.tifs.2007.07.011

Fritsch, R.M., Keusgen, M. 2006. Occurrence and taxonomic significance of cysteine sulphoxides in the genus *Allium* L. (Alliaceae). Phytochemistry, 67: 1127–1135; DOI: 10.1016/j.phytochem.2006.03.006

Ghahremani-majd, H., Dashti, F., Dastan, D., Mumivand, H., Hadian, J., Esna-Ashari, M. 2012. Antioxidant and antimicrobial activities of Iranian mooseer (*Allium hirtifolium* Boiss) populations. Horticulture, Environment, and Biotechnology, 53: 116–122; DOI: 10.1007/s13580-012-0131-2

Ghasemi Pirbalouti, A. 2009. Medicinal plants used in Chaharmahal and Bakhtyari districts, Iran. Herba Polonica, 55: 69–75.

Ghasemi Pirbalouti, A., Setayesh, M., Siahpoosh, A., Mashayekhi, H. 2013a. Antioxidant activity, total phenolic and flavonoids contents of three herbs used as condiments and additives in pickles products. Herba Polonica, 59: 51–62.

Ghasemi Pirbalouti, A., Fatahi-Vanani, M., Craker, L., Shirmardi, H. 2013b. Chemical composition and bioactivity of essential oils of *Hypericum helianthemoides*. *Hypericum perforatum* and *Hypericum scabrum*. Pharmaceutical Biology, 52(2): 175-181; DOI: 10.3109/13880209.2013.821663

Ghahreman, A., 1984. Color Atlas of Iranian Plants. Institute of Forestries and Grasslands, Botany Division, No. 5, 512 pp.

Ghodrati Azadi, H., Ghaffari, S.M., Riazi, G.H., Ahmadian, S., Vahedi, F. 2008. Antiproliferative activity of chloroformic extract of Persian Shallot, *Allium hirtifolium*, on tumor cell lines. Cytotechnology, 56: 179–185; DOI: 10.1007/s10616-008-9145-0

Hung, D., Ou, B., Prior, R.L. 2005. The chemistry behind antioxidant capacity assay. J. Journal of Agricultural and Food Chemistry, 53: 1841–1856; DOI: 10.1021/jf030723c

Jafarian, A., Ghannadi, A., Elyasi, A. 2003. The effects of *Allium hirtifolium* Boiss on cell-mediated immune response in mice. Iranian Journal of Pharmaceutical Research, 2: 51–55.

Jaffery, E.H., Brown, A.F., Kurilich, A.C., Keek, A.S., Matusheski, N., Klein, B.P. 2003. Variation in content of bioactive components in broccoli. Journal of Food Composition and Analysis, 16: 323–330; DOI: 10.1016/S0889-1575(03)00045-0

Ismail, S., Jalilian, F. A., Talebpour, A. H., Zargar, M., Shameli, K., Sekawi, Z., Jahanshiri, F. 2013. Chemical composition and antibacterial and cytotoxic activities of *Allium hirtifolium* boiss. BioMed Research International, DOI: 10.1155/2013/ 696835.

Kazemi, S., Asgary, S., Moshtaghian, J., Rafieian, M., Adelnia, A., Shamsi, F. 2010. Liver-protective effects of hydroalcoholic extract of *Allium hirtifolium* boiss. In rats with alloxan-induced diabetes mellitus. ARYA Atheroscler, 6: 11–15.

Kojuri, J., Vosoughi, A. R., Akrami, M. 2007. Effects of *Anethum graveolens* and garlic on lipid profile in hyperlipidemic patients. Lipids Health Disease, 6: 1- 5; DOI: 10.1186/1476-511X-6-5

Mozaffarian, V. 2008. A pictorial dictionary of medicinal plants. Latin–English–French–Germany–Persian/Complied, Farahang Moaser, Tehran, pp. 522.

Najjaa, H., Ammar, E., Neffati, M. 2009. Antimicrobial activities of protenic extracts of *Allium roseum* L., a wild edible species in North Africa. Journal of Food Agriculture and Environment, 7: 150–154.

Prakash, D., Singh, B. N., Upadhyay, G. 2007. Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). Food Chemistry 102(4): 1389–1393; DOI: 10.1016/j.foodchem.2006.06.063

Rechinger, K.H. 1984. *Flora Iranica*, Alliaceae, vol. 76. Akademische Druck, Univ. Verlagsanstalt Graz, Austria, 85 pp.

Rodriguez, H., Curiel, J.A., Landete, J.M., De las Rivas, B., De Felipe, F.L., Gomez- Cordoves, C., Mancheno, J.M., Munoz, R. 2009. Food phenolics and lactic acid bacteria. International Journal of Food Microbiology, 132: 79–90; DOI: 10.1016/j.ijfoodmicro.2009.03.025

Singleton, V.L., Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. American Society for Enology and Viticulture, 16: 144–158.

Taguri, T., Tanaka, T., Kouno, I. 2006. Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. Biological & Pharmaceutical Bulletin, 29: 2226–2235; DOI: 10.1248/bpb.29.2226

Tepe, B., Sokmen, M., Akpulat, H.A., Sokmen, A. 2005. In vitro antioxidant activities of the methanol extracts of five *Allium* species from Turkey. Food Chemistry, 92: 89–92; DOI: 10.1016/j.foodchem.2004.07.016

Thormar H. 2011. Lipids and essential oils as antimicrobial agents. John Wiley & Sons, Ltd, Chichester, UK. DOI: 10.1002/9780470976623.

Velioglu, Y. S., Mazza, G., Gao, L., Oomah, B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. Journal of Agricultural and Food Chemistry, 46: 4113–4117; DOI: 10.1021/jf9801973

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.03
Agrovoc descriptors: hyoscyamus niger, alcaloids, antioxidants, particle size, dimensions, crop yield, enzymes, biochemistry

Agris category code: f60

Activating antioxidant enzymes, hyoscyamine and scopolamine biosynthesis of *Hyoscyamus niger* L. plants with nano-sized titanium dioxide and bulk application

Mansour GHORBANPOUR[1,*], Mehrnaz HATAMI[1], Mahmoud HATAMI[2]

Received August 27, 2014; accepted October 13, 2014.
Delo je prispelo 27. avgusta 2014, sprejeto 13. oktobra 2014.

ABSTRACT

Application of nanotechnology is now widely distributed overall the life, especially in agricultural systems. This study intended to indicate the impacts of nano-sized titanium dioxide particles (NT) and bulk (BT) on antioxidant enzymes activities including superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT), and variations of two major tropane alkaloids such as hyoscyamine (HYO) and scopolamine (SCO) in *Hyoscyamus niger* L. Plants were treated with different concentrations of NT and BT (0, 20, 40 and 80 mg l^{-1}). Alkaloids extracted were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis. Results showed that SOD activity increased with increasing titanium dioxide concentration in both nano-particles and bulk treated plants. However, the highest and the lowest POX activity were observed in plants exposed to NT at 40 mg l^{-1} and control, respectively. Generally, all tested enzymes activities were higher in NT treated plants that those of BT except CAT activity at 80 mg l^{-1}. The highest alkaloids content values, HYO: 0.286 g kg^{-1} and SCO: 0.126 g kg^{-1}, were achieved in plants treated with NT at 80 and 20 mg l^{-1}, respectively. The maximum and minimum plant biomass and subsequently total alkaloids yield were obtained in plants exposed to NT at 40 mg l^{-1} and controls, respectively. Our results suggest that NT in appropriate level (40 mg l^{-1}) may act as an elicitor for biochemical responses and tropane alkaloids biosynthesis in *H. niger* plants.

Key words: black henbane, tropane alkaloids, antioxidant enzymes, nano-anatase TiO_2

IZVLEČEK

AKTIVIRANJE AKTIVNOSTI ANTIOKSIDACIJSKIH ENCIMOV, BIOSINTEZE HIOSCIAMINA IN SKOPOLAMINA PRI ČRNEM ZOBNIKU (*Hyoscyamus niger* L.) Z NANO IN CELOKUPNIMI DELCI TITANOVEGA DIOKSIDA

Uporaba nanotehnologije je v svetu danes zelo razširjena v znanostih o življenju, še posebej v kmetijstvu. V raziskavi je bil preučevan vpliv nano delcev (NT) in celukopnih delcev (BT) titanovega dioksida na antioksidacijsko aktivnost encimov kot so superoksid dismutaza (SOD), peroksidaza (POX) in katalaza (CAT) in vpliv tega obravnavanja na variabilnost vsebnosti dveh glavnih tropanskih alkaloidov, hiosciamina (HYO) in skopolamina (SCO) v črnem zobniku (*Hyoscyamus niger* L.). Rastline so bile tretirane z naslednjimi koncentracijami NT in BT delcev: 0, 20, 40 and 80 mg l^{-1}. Ekstrahirani alkaloidi so bili analizirani in določeni s plinsko kromatografijo (GC) in plinsko kromatografijo povezano z masno spektroskopijo (GC-MS). Rezultati so pokazali, da se je aktivnost SOD povečala pri tretmajih z NT in BT delci z naraščanjem njihove koncentracije. Aktivnost POX pa je bila največja pri rastlinah izpostavljenih NT delcem pri 40 mg l^{-1} in najmanjša pri kontroli. Nasplošno so bile aktivnosti vseh testiranih encimov večje pri rastlinah tretiranih z NT delci kot pri tretmaju z BT delci, razen aktivnosti CAT pri tretmaju z 80 mg l^{-1}. Največji vsebnosti alkaloidov, HYO: 0.286 g kg^{-1} in SCO: 0.126 g kg^{-1}, sta bili doseženi pri rastlinah tretiranih z NT delci pri koncentracijah 80 in 20 mg l^{-1}. Največja biomasa in največji pridelek alkaloidov sta bila dosežena pri rastlinah tretiranih z NT pri 40 mg l^{-1} in najmanjša pri kontroli. Rezultati kažejo, da NT delci v primernih koncentracijah (40 mg l^{-1}) delujejo kot elicitorji za biokemične odzive in biosintezo tropanskih alkaloidov pri črnem zobniku.

Ključne besede: črni zobnik, tropanski aklaloidi, antioksidacijski encimi, nano-delci TiO_2

[1] Department of Medicinal Plants, Faculty of Agriculture and Natural Resources, Arak University, Arak, Iran; * corresponding author: email: m_ghorbanpour@yahoo.com

[2] Iran Technical and Vocational Training Organization

1 INTRODUCTION

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing compounds found in about 20% of plant species. Solanaceous plants are regarded as rich sources of alkaloids, namely the pharmaceutical by interesting tropane derivatives. Tropane alkaloids, especially hyoscyamine (HYO) and scopolamine (SCO) are widely used in medicine for their mydriatic, antispasmodic, anticholinergic, analgesic and sedative properties (Zehra *et al.,* 1998). SCO, which is the 6,7-epoxide of HYO, is the most valued of the two tropane alkaloids (due to fewer side effects on nervous system), its worldwide demand being 10 times higher than that for HYO and its racemic form, atropine (Hashimoto *et al.,* 1993). The synthetic production of these alkaloids is more expensive than their extraction from plant materials and they are, therefore, currently industrially extracted from various solanaceous plants belonging to the genera *Atropa*, *Duboisia*, *Datura, Scopolia* and *Hyoscyamus*.

Black henbane (*Hyoscyamus niger* L.) has a very long history of use as a medicinal plant. A cosmopolitan, strong-scented annual or biennial herb, which all it's parts (root, leaf, and seed) contain tropane alkaloids such as HYO and SCO (Cuneyt *et al.,* 2004). These metabolites are synthesized in roots and then transported to the aerial parts of the plant (Oksman, 1987).

It has been exhibited that signal molecules are very potential elicitors for induction of plant secondary metabolites. Recent years, the applications of signal components as elicitors have evolved an effective strategy for the production of target secondary metabolites in plant cell cultures. However, it is still uncommon for commercial application. It therefore, application of elicitors in vivo is an easy and direct channel to promote the yield of plant secondary metabolites at the whole plant scale. Nanonmaterials could act as signal compounds to make metabolic and physiological responses but the underlying mechanisms are not fully understood (Hatami and Ghorbanpour, 2014).

The development of nanotechnology on physiology and biochemistry has expanded the application area of nanomaterials in different fields due to their unique characteristics (Scrinis and Lyons, 2007). Also, this technology could open up new approaches in plant sciences and in agricultural researches. In recent years, many scientists have studied the effects of nanomaterials on seed germination and plant growth with the aim to promote its use for agricultural productions. Most of these studies are focused on the potential toxicity of nanoparticles on higher plants and positive, negative or inconsequential effects were presented. Most recently it was revealed that the use of appropriate concentration of nano-TiO_2 increased the seed germination parameters and early growth of some medicinal and aromatic plants (Hatami and Ghorbanpour 2014). According to Lu *et al.,* (2002) treatment of soybean (*Glycine max*) plants with a mixture of nano Sio_2 and Tio_2 increase nitrate reductase activity, stimulate its antioxidant system, and accelerate germination and growth. It is reported that silver nanoparticle treatment of *Brassica juncea* seedlings induced the activities of specific antioxidant enzymes (Priyadarshini *et al.,* 2012). However, the mechanism of these nanoparticles has not been completely established yet. Also, in the field of medicinal and aromatic plants, the use of nanomaterials is relatively new and needs more researches. However, some studies have reported negative effects of TiO_2 nanoparticles (NT) on higher plants that varied between plant tissues, growth stages, plant species, applied concentrations, and specific properties of nanoparticles (Castiglione *et al.,* 2011). Thus, the exploration of their extensive application in agriculture and plant science is still in debate (Kurepa *et al.,* 2010). Therefore, the present study was carried out to elucidate the potential effects of nanosized TiO_2 (NT) and bulk (BT) application on antioxidant enzymes including SOD, POX and CAT activity and elicitation of two main tropane alkaloids such as HYO and SCO on *Hyoscyamus niger* L. plants.

2 MATERIALS AND METHODS

2.1 Transmission electron microscopy (TEM) image of nano-dioxide titanium

Nano-sized TiO_2 were provided from the Iranian Nanomaterials Pioneers Company, NANOSANY (Mashhad, IRAN). The size of the TiO_2 nanoparticles was estimated to be 10-15 nm in diameter. A transmission electron microscopy (TEM) image of the TiO_2 particles is also provided (Fig. 1). The crystal properties of TiO_2 nanoparticles were examined by X-ray diffraction (XRD), which showed that used TiO_2 nanoparticles were all present in the anatase form (Fig. 2).

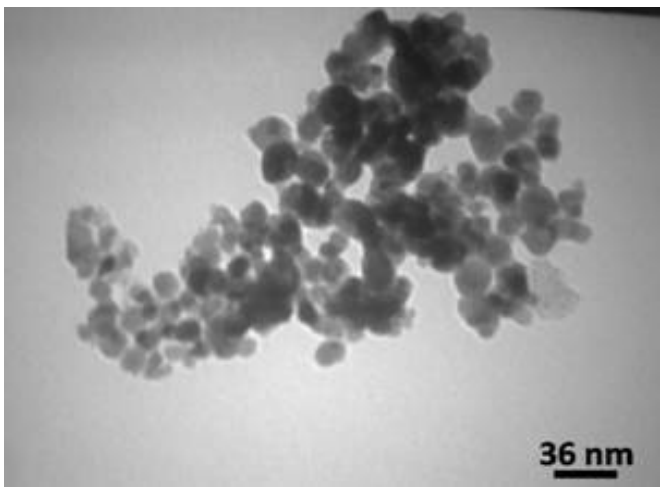


Figure 1: Transmission electron microscopy (TEM) image of TiO_2 nanoparticles. Distribution of particles size was estimated to be 10-15 nm, scale bar = 36 nm

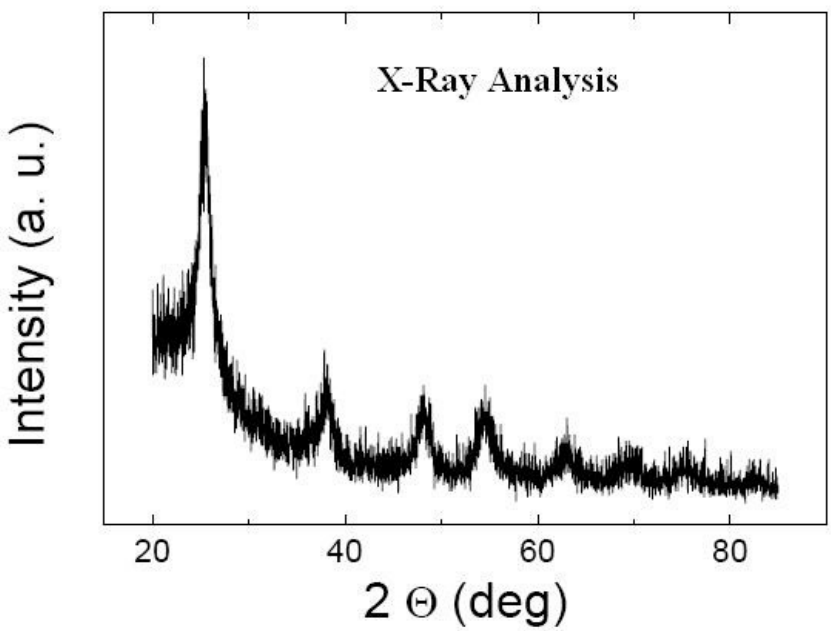


Figure 2: XRD (X-ray diffraction) pattern of Titanium oxide (TiO_2). Nanopowder (TiO_2 anatase)- size: 10-15 nm- purity: 99%- surface area: 200-240 m^2 g^{-1}- pH: 6-6.5- bulk density: 0.24 g cm^{-3}- true density: 3.9 g cm^{-3}- color: white

2.2 Plant growth conditions and treatments

The experiment was carried out in greenhouse conditions (25 °C day/17 °C night temperature, natural light 16 h light: 8 h dark and 75% relative humidity). Henbane seeds generally have low germination rate under normal laboratory conditions. Therefore, seeds were treated with 250 mg L^{-1} gibberellic acid (GA_3) for 48 h at room temperature (25 ± 0.5 °C) for breaking dormancy and accelerating germination. After that seeds were surface-sterilized in 70% ethanol for 2 min and then in 25% commercial bleach (containing 6% sodium hypochlorite) for 10 min and finally rinsed with sterile distilled water. Subsequently, seeds were placed in petri dishes on two layers of filter paper (Whatman No.1) moistened with 4 ml distilled water. After 3 days, 90% of seeds germinated steadily. After germination, individual, healthy and uniform seedlings (when they had three true leaves) were transplanted into experimental pots (25 cm diameter and 30 cm deep, containing 8 kg soil). The physical and chemical characteristics of employed soil are given in table 1. Deionised water was used to prepare 0, 20, 40 and 80 mg l^{-1} NT and BT solutions. Then, three month-old plants at flowering stage were treated with 50 ml of employed solutions. Both sides of the leaves and stems i.e., whole foliage of the plants were sprayed with equal amounts of 50 ml aqueous solution of NT and BT by hand atomizer. Control plants were only treated with deionised water. The study was set up as completely randomized design with three replicates. All pots were harvested at the end of flowering stage and subsequently plant dry matter was weighted with a precision of 0.0001 g scale and was finely powdered in an electronic blender for enzymes assays and alkaloids extraction.

2.3 Antioxidant enzymes assays

A crude enzyme extract was prepared by homogenizing 0.5 gram of powdered leaf sample in extraction buffer containing 0.5% Triton X-100 and 1% polyvinyl pyrrolidone in 100 mM potassium phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate was centrifuged and the supernatant was used for the following enzyme assays.

2.3.1. Superoxide dismutase (SOD, EC 1.15.1.1)

SOD activity was determined according to Beauchamp and Fridovich (1971). The reaction mixture contained 1.17×10^{-6} mol l^{-1} riboflavin, 0.1 mol l^{-1} methionine, 2×10^{-5} mol l^{-1} KCN and 5.6×10^{-5} mol l^{-1} nitroblue tetrazolium (NBT) salt dissolved in 3 ml of 0.05 mol l^{-1} sodium phosphate buffer (pH 7.8). 3 ml of the reaction medium was added to 1 ml of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Philips 40 W fluorescent tubes in a single row. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity is expressed in U mg^{-1} protein. (U = change in 0.1 absorbance h^{-1} mg^{-1} protein under assay conditions).

2.3.2. Catalase (CAT, EC 1.11.1.6)

CAT activity was assayed according to the method of Chandlee and Scandalios (1984). The assay mixture contained 2.6 ml of 50 mmol l^{-1} potassium phosphate buffer (pH 7.0), 0.4 ml of 15 mmol l^{-1} H_2O_2 and 0.04 ml of enzyme extract. Changes in the absorbance were read at 240 nm. The enzyme activity was expressed in U mg^{-1} protein (U=1mM of H_2O_2 reduction min^{-1} mg^{-1} protein). The enzyme protein was estimated by the method of Bradford (1976).

2.3.3. Peroxidase (POX, EC 1.11.1.7)

POX activity was determined by the method of Kumar and Khan (1982). Assay mixture of POX contained 2 ml of 0.1 mol l^{-1} phosphate buffer (pH 6.8), 1 ml of 0.01mol.L^{-1} pyrogallol, 1 ml of 0.005 mol l^{-1} H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 2.5 mol l^{-1} H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 mol l^{-1} H_2SO_4 at zero time. The activity was expressed in U mg−1 protein. One U is defined as the change in the absorbance 0.1 min^{-1} mg^{-1} protein.

2.4 Alkaloid extraction

Leaf samples were air dried, grinded into fine powder and sieved with laboratory mesh (size 30, mesh opening 545 μm). A subsample of one gram

from each samples was added to appropriate volume of $CHCl_3$: MeOH: NH_4OH 25%, (15:5: 1), sonicated for 20 min and then kept at water bath (40 ºC) for one hour. Subsequent sample preparation and alkaloids extraction were based essentially on the method described by Kamada *et al.,* (1986).

2.4.1. Alkaloid analysis and quantification

Alkaloids extracted were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis. GC analysis was performed using a GC system equipped with a flame ionization detector (FID) and HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). Injector and detector temperatures were set at 220 and 290 °C, respectively. The column temperature was initially kept at 50 °C for 5 min, then gradually increased to 300 ºC at a rate of 3 ºC/min and maintained for 3 min. The flow rate of gas He was 0.8 ml/min. Then, 1 µL of extract was directly injected into the gas chromatograph. Each extraction was replicated three times and the compound percentages are the means of the three replicates. GC-MS analysis was carried out on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, USA) fitted with a fused silica HP-5MS capillary column (30m×0.25mm×0.25µm). Oven temperature was programmed from 50 ºC to 285 ºC at 3 ºC/min, and helium was used as carrier gas (0.8 mL/min), Mass spectra were obtained in an Agilent 5973 system operating in electron impact mode (EIMS) at 70 eV, coupled to an GC system. The identification of alkaloids was based on the comparison of their GC retention time and mass spectra (MS) data with their standards substances (HYO. HCl and SCO. HBr, Merck). The total tropane alkaloids (HYO + SCO) yield was quantified by both alkaloid content and dry weight; Total alkaloid yield (g $plant^{-1}$) = Alkaloid content (g kg^{-1}) × Plant dry weight (g $plant^{-1}$).

2.5 Statistical analysis

The data were subjected to ANOVA based on a completely randomized design (CRD) with three replications and were analyzed by SAS and MSTAT-C program, and probabilities of significance were used to test for significance among treatments and interactions, and the Duncan's multiple range test ($p \leq 0.05$) was used to compare means. Values obtained were expressed as mean ± SD (standard deviation) from three replications (n=3) of each treatment.

3 RESULTS

3.1 Plant biomass and antioxidant enzymes status

Analysis of variance showed that the most measured traits of this study have been significantly ($P \leq 0.05$) affected by NT levels. Mean comparison of data revealed that increasing titanium dioxide concentration especially at nano-sized (10-15 nm) up to 40 mg l^{-1} significantly improved the plant dry weight to 42% compared to the unexposed control plants (table 2). However, there were no significant differences among BT levels on plant biomass production. The maximum and minimum plant biomass, 7.53 and 3.24 g $plant^{-1}$, were obtained in NT treated plants at 40 and 80 mg l^{-1}, respectively. There were noticeably differences in antioxidant enzymes activities among the employed treatments. SOD activity increased with NT and BT levels, and TiO_2 application played a significant role in adjusting the enzyme activity (Fig 3 and 4). SOD activity increased with increasing TiO_2 concentration in both nano-sized and bulk treatments. On the other hand, the highest SOD activity was observed at the highest NT and BT supply. CAT activity increased with NT application up to 20 mg l^{-1} and then decreased compared to other NT level, whereas, BT at all concentrations enhanced the CAT activities (Fig 3).

Table 1: The physical and chemical characteristics of soil used in current experiment

Characteristic	Quantity	Characteristic	Quantity
Soil texture	Sandy loam	CEC (Cmol(c)kg^{-1})	11.23
Clay(%)	14.32	total nitrogen(%)	0.051
Silt(%)	16	available phosphate ($mgkg^{-1}$)	9.12
Sand(%)	69.68	available potassium ($mgkg^{-1}$)	175
pH	7.0	Fe ($mgkg^{-1}$) *	8.4
EC (dS/m)	1.04	Mn ($mgkg^{-1}$) *	10.15
$CaCO_3$%	5.82	Cu ($mgkg^{-1}$) *	0.84
OC%	0.81	Zn ($mgkg^{-1}$) *	0.52
SP%	28.2		

* DTPA-Extractable

Table 2: Mean values for plant biomass, major tropane alkaloids including hyoscyamine (HYO) and scopolamine (SCO) content and yield (mean ± S.D., n=3) in *H. niger* plants treated with different nano-sized TiO_2 (NT) and bulk (BT) levels

Treatment ($mg\ l^{-1}$)	Biomass ($g\ plant^{-1}$)	Alkaloid content ($g\ kg^{-1}$)		Alkaloid yield ($g\ plant^{-1}$)		Total alkaloids yield ($g\ plant^{-1}$)
		HYO	SCO	HYO	SCO	
Control	4.32±0.14^{c}	0.168±0.006^{e}	0.084±0.013^{ef}	0.725±0.014^{f}	0.362±0.021^{f}	1.087±0.025^{g}
NT 20	5.81±0.22^{b}	0.216±0.004^{c}	0.126±0.004^{a}	1.254±0.012^{b}	0.732±0.012^{b}	1.986±0.022^{b}
NT 40	7.53±0.16^{a}	0.252±0.005^{b}	0.114±0.005^{b}	1.897±0.014^{a}	0.858±0.014^{a}	2.755±0.014^{a}
NT 80	3.24±0.15^{d}	0.286±0.003^{a}	0.114±0.003^{b}	0.926±0.016^{c}	0.434±0.011^{e}	1.360±0.012^{d}
BT 20	4.35±0.18^{c}	0.176±0.005^{d}	0.108±0.002^{c}	0.765±0.011^{e}	0.469±0.013^{d}	1.234±0.021^{e}
BT 40	5.91±0.21^{b}	0.142±0.008^{f}	0.098±0.009^{de}	0.839±0.021^{d}	0.579±0.015^{c}	1.418±0.022^{c}
BT 80	4.41±0.19^{c}	0.161±0.009^{e}	0.105±0.003^{cd}	0.710±0.019^{f}	0.463±0.021^{d}	1.173±0.027^{f}
LSD	0.17	0.008	0.005	0.016	0.012	0.023

Means in each column with similar letters are not significantly ($p \leq 0.05$) different through the Duncan's multiple range test

The maximum CAT activity was observed in BT at 80 mg l^{-1} treatment. With regard to the effects of NT and BT on adjusting CAT activity, low NT and high BT application significantly increased CAT activity up to 50% compared to untreated control plants. However, POX activity significantly increased under employed NT up to 40 mg l^{-1}, and then decreased with NT concentration (Fig 3). Application of the high NT and BT concentrations significantly decreased POX activity; however, the final value was not lower than that of control in NT treated plants. However, the highest and the lowest POX activity were observed in plants exposed to NT at 40 mg l^{-1} and control, respectively. Generally, all tested enzymes activities were higher in NT treated plants that those of BT except CAT activity at 80 mg l^{-1}.

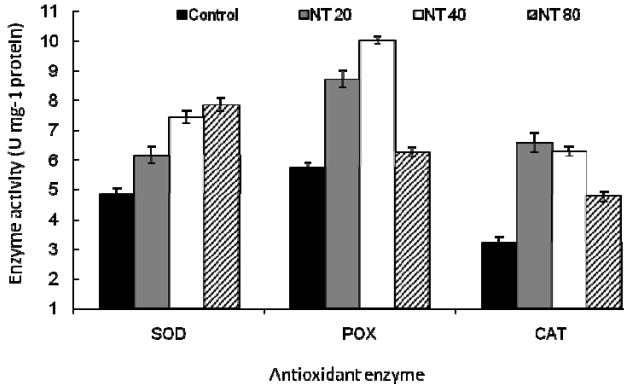


Figure 3: Influence of Nano-sized titanium dioxide (NT) concentrations (0, 20, 40 and 80 mg l^{-1}) on superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) activities in *H. niger* plants. Values are given as mean ± S.D., (n=3)

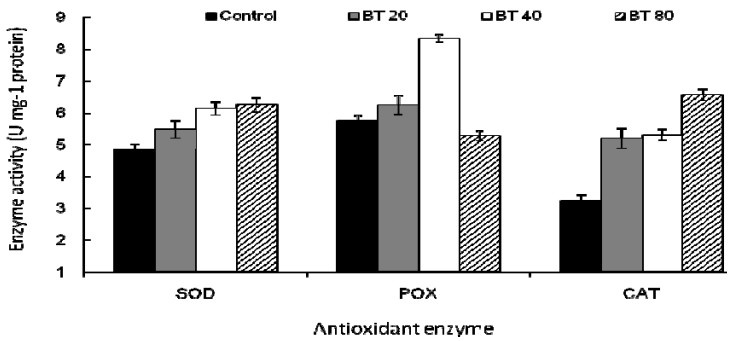


Figure 4: Influence of bulk titanium dioxide (BT) concentrations (0, 20, 40 and 80 mg l^{-1}) on superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) activities in *H. niger* plants. Values are given as mean ± S.D., (n=3)

3.2. Alkaloids biosynthesis

The results demonstrated that the applied treatments affected the shoot HYO and SCO content of black henbane plants (table 2). At high NT concentration (80 mg l^{-1}), the highest HYO content (0.286 g kg^{-1}) was obtained. By contrast, low NT concentration, 20 mg l^{-1}, resulted in high SCO content (126 g kg^{-1}). The lowest content of HYO (142 g kg^{-1}) and SCO (0.087 g kg^{-1}) were observed in plants treated with BT at 40 mg $l^{-1}$and control groups, respectively. The yield of both HYO and SCO in black henbane plants was increased with NT application at 40 mg l^{-1} as presented in table 2. However, the minimum HYO (0.710 g $plant^{-1}$) and SCO (0362 g $plant^{-1}$) yield were recorded with application of the highest BT dose and unexposed control plants, respectively. The largest total alkaloids (HYO+ SCO) yield (2.755 g $plant^{-1}$) was achieved in medium NT application (40 mg l^{-1}) mainly because of high dry weight under this situation in comparison with the other treatments (table 2).

4 DISCUSSION

Nowadays, nanoparticles happen to interest, mostly because of their possible use in varied technologies. They can be defined as objects ranging in size from 1-100 nm that because of their size may differ in the properties from the bulk materials. This can result from the high surface to volume ratio that increases their reactivity, the ability to penetrate cell membranes and possible biochemical activity. Application of nanotechnology is now widely distributed overall

the life, especially in agricultural systems. Nanoparticles because of their physicochemical characteristics e.g., large surface area to volume ratio, ability to engineer electron exchange and highly surface reactive capabilities, are among the potentially candidates for modulating the redox status and changing the growth, performance and quality of plants (Mukherjee and Mahapatra, 2009).

The ninth most abundant element and the second most abundant transition metal in the earth's crust is titanium element (about 6.32 ppm). Metal oxide nanoparticles, represented by titanium dioxide (TiO_2), is of great technological importance in the field of heterogeneous catalysis for catalytic support of a wide variety of metals (Biener *et al.,* 2005). The most important effects of TiO_2 compounds on plants are enhancement of the yield of various crops (~10-20%); an improvement of some essential element contents in plant tissues; an increase in the peroxidase, catalase, and nitrate reductase activities in plant tissues; and an enhancement of the chlorophyll content in paprika (*Capsicum anuum* L.) and green alga (*Chlorella pyrenoidosa*) (Hruby *et al.,* 2002). In our current work, different responses of the examined traits to various nano-sized TiO_2 dosages could be due to the following principal factors that previously reported by many researchers: concentration of nanoparticles, particle size and specific surface area, physicochemical properties of nanoparticles, plant species, plant age/life cycle stage, growth media conditions, nanoparticles stability, and dilution agent.

In our current experiment, NT treated *H. niger* plants at proper concentration caused higher biomass production than that of bulk and the control untreated plants. Whereas, at the highest NT concentration caused no positive impacts, when compared to the control, indicating the potential toxicity of NT particles with this adverse effect. Yin *et al.,* (2011) mentioned that increasing nanosilver concentration caused a decrease in plant root growth, which indicate an increase in phytotoxicity of nano particles. Also, in our present research activity of antioxidant enzymes, SOD, POX and CAT were affected differently under various employed NT and BT treatments. In both NT and BT treated plants, there was an increase in activity of SOD and POX at certain concentrations, however, a significant decrease was observed for CAT activity at the highest NT concentration when compared to the other NT and BT treatments.

According to Priyadarshini *et al.,* (2012) Nano-silver particles decreased H_2O_2 production and increased the efficiency of redox reactions. And also they reported that higher concentration of nano-silver enhanced the activity of H_2O_2 metabolizing enzymes.

It is well known that SOD is an enzyme that catalyzes the conversion of the O^{-2} to O_2 and H_2O_2 (Hafis *et al.,* 2011). Enhanced SOD activity of leaves under employed treatments may be interpreted as a direct response to augmented O^{-2} formation. It is previously suggested that the overexpression of SOD, if this is accompanied by increment of H_2O_2 scavenging mechanisms like POX and CAT, has been considered as a strategy to cope with oxidative damage (Kohler et al., 2006). Our results also indicated significant role of NT, particularly application of moderate levels (40 $mg.L^{-1}$), provides a protective mechanism by increasing the activity of defense enzymes. Similar result was reported by Krishnaraj *et al.,* (2012) that high activity of CAT and POX were recorded from leaf samples of plants subjected to nanosilver treatment, implying less ROS formation, resulting in less toxicity to the plants. They also reported that CAT and POX are enzymes that plays major role in ensuring protection against oxidative damage in plants exposed to nanosilver particles treatments. Lei *et al.,* (2008) stated that nanoparticles (TiO_2) declined oxidative damage in spinach chloroplast by increasing APX, SOD, POX, and CAT activity. It is suggested that combined reduction of APX, SOD and CAT activity resulted in high generation of intercellular ROS concentrations, which may be directly or indirectly be involved in the lipid peroxidation, senescence and cell death of plant (Debasis *et al.,* 2007).

In our research, the decrease in antioxidant enzymes activity observed in control plants may be directly attributed to lower secondary metabolites, HYO and SCO, biosynthesis. The content of alkaloids in plants could be increased through genetic and or environmental manipulations. However, not much information is available on the

effect of nano-sized material impacts on the content of tropane alkaloids in *H. niger* plants. Here, HYO was found as a main alkaloid in the aerial parts of black henbane plants.

5 CONCLUSION

Our results suggest that nano-sized titanium dioxide particles in proper levels may act as elicitor for physiological and biochemical responses and tropane alkaloids biosynthesis pathway in *H. niger* plants. In addition, low NT concentration showed enhancement on the production of plant HYO and SCO yield.

6 REFERENCES

Beauchamp, C., Fridovich, I. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem. 44: 276-287; DOI: 10.1016/0003-2697(71)90370-8

Biener, J., Farfan-Arribas, E., Biener, M., Friend, C.M., Madix, R.J. 2005. Synthesis of TiO_2 nanoparticles on the Au (111) surface. J. Chem. Phys. 123:0947051–6; DOI: 10.1063/1.1999607

Bradford , M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Annu. Rev. Bioch. 72: 248-254; DOI: 10.1016/0003-2697(76)90527-3

Castiglione, M.R., Giorgetti, L., Geri, C., Cremonini, R. 2011. The effects of nano-TiO2 on seed germination, development and mitosis of root tip cells of *Vicia narbonensis* L. and *Zea mays* L. J. Nanopart. Res. 13: 2443–2449; DOI: 10.1007/s11051-010-0135-8

Chandlee, J.M., Scandalios, J.G. 1984. Analysis of variants affecting the catalase development program in maize scutellum. Theor. Appl. Genet. 69: 71–77; DOI: 10.1007/BF00262543

Cuneyt, C., Kudret, K., Birsen, S. 2004. Physical and Physiological Dormancy in Black Henbane (*Hyoscyamus niger* L.) seeds. Journal of Plant Biology. 47: 391-395; DOI: 10.1007/BF03030556

Debasis, C., Chatterjee, J., Datta, S.K. 2007. Oxidative stress and antioxidant activity as the basis of senescence in chrysanthemum florets. Plant Growth Regul. 53: 107-115; DOI: 10.1007/s10725-007-9208-9

Flores, H.E., Vivanco, J.M., Loyola-Vargas, V.M. 1999. 'Radicle' biochemistry: the biology of root-specific metabolism. Trends Plant Sci. 4: 220–226; DOI: 10.1016/S1360-1385(99)01411-9

Hafis, C., Romero-Puertas, M.C., Rio, L.A., Abdelly, C., Sandalio, L.M. 2011. Antioxidative response of Hordeum maritimum L. to potassium deficiency. Acta Physiol Plant. 33: 193–202; DOI: 10.1007/s11738-010-0537-3

Hashimoto, T., Yun, D.J., Yamada, Y. 1993. Production of tropane alkaloids in genetically engineered root cultures. Phytochemistry. 32: 713–718; DOI: 10.1016/S0031-9422(00)95159-8

Hatami, M., Ghorbanpour, M., Defense enzymes activity and biochemical variations of *Pelargonium zonale* in response to nanosilver particles and dark storage. Turkish Journal of Biology, 2014, vol. 38, pp. 130-139; DOI: 10.3906/biy-1304-64

Hatami, M., Ghorbanpour, M., Salehiarjomand, H. 2014. Nano-anatase TiO_2 modulates the germination behavior and seedling vigority of the five commercially important medicinal and aromatic plants. Journal of Biological and Environmental Sciences. 8(22): 53-59

Hruby, M., Cigler, P., Kuzel, S. 2002. Contribution to understanding the mechanism of titanium action in plant. J Plant Nutr. 25: 577–598; DOI: 10.1081/PLN-120003383

Kamada, H., Okamura, N., Satake, M., Harada, H., Shimomura, K. 1986. Alkaloid production by hairy root cultures in *Atropa belladonna*. Plant Cell Rep. 5: 239- 242; DOI: 10.1007/BF00269811

Krishnaraj, C., Jagan, E.G., Ramachandran, R., Abirami, S.M., Mohan, N., Kalaichelvan, P.T. 2012. Effect of biologically synthesized silver nanoparticles on *Bacopa monnieri* (Linn.) Wettst. plant growth metabolism. Process Biochem. 47: 651-658; DOI: 10.1016/j.procbio.2012.01.006

Kumar, K.B., Khan, P.A. 1982. Peroxidase and polyphenol oxidase in excised ragi (*Eleusine coracana* cv. PR 202) leaves during senescence. Ind. J. Exp. Bot. 20: 412-416.

Kurepa, J., Paunesku, T., Vogt, S., Arora, H., Rabatic, B.M., Lu, J.J., Wanzer, M.B., Woloschak, G.E.,

Smalle, J.A. 2010. Uptake and distribution of ultrasmall anatase TiO2 alizarin red S nanoconjugates in *Arabidopsis thaliana*. Nano Lett. 10: 2296–2302; DOI: 10.1021/nl903518f

Lei, Z., Mingyu, S., Xiao, W., Chao, L., Chunxiang, Q., Liang, C., Hao, H., Xiao-qing, L., Fashui, H. 2008. Antioxidant stress is promoted by nano-anatase in spinach chloroplasts under UV-B radiation. Biol Trace Elem Res. 121: 69–79; DOI: 10.1007/s12011-007-8028-0

Lu, C.M., Zhang, C.Y., Wen, J.Q., Wu, G.R. 2002. Research on the effect of nanometer materials on germination and growth enhancement of *Glycine max* and its mechanism. Soybean Sci. 21: 68–171.

Mukherjee, M., Mahapatra, A. 2009. Effect of coinage metal nanoparticles and zwitterionic surfactant on reduction of $[Co(NH_3)5Cl](NO3)_2$ by iron. Colloid Surface. 350: 1-7; DOI: 10.1016/j.colsurfa.2009.08.021

Oksman, K. 1987. Scopolamine and Hyoscyamine Production by Plants and Cell Cultures of *Hyoscymus muticus*. PhD thesis, University of Helsinki, Helsinki, Finland,

Priyadarshini, S., Deepesh, B., Zaidi, M.G.H., Pardha-saradhi, P., Khanna, P.K., Arora, S. 2012. Silver Nanoparticle-Mediated Enhancement in Growth and Antioxidant Status of *Brassica juncea*. Appl Biochem Biotech. 167: 2225- 2233; DOI: 10.1007/s12010-012-9759-8

Scrinis, G., Lyons, K. 2007. The Emerging Nano-Corporate Paradigm: Nanotechnology and the Transformation of Nature, Food and Agri-Food Systems. Int J Soc Agric Food. 15: 22–44.

Yin, L., Cheng, Y., Espinasse, B., Colman, B.P., Auffan, M., Wiesner, M., Rose, J., Liu, J., Bernhardt, E.S. 2011. More than the ions: the effects of silver nanoparticles on *Lolium multiflorum*. Environ Sci Technol. 45: 2360-7; DOI: 10.1021/es103995x

Zayed, R., Wink, M. 2004. Induction of tropane alkaloid formation in transformed root cultures of *Brugmansia suaveolens* (Solanaceae). Z Naturforsch. 59: 863–867.

Zehra, M., Banerjee, S., Naqvi, A.A., Kumar, S. 1998. Variation in Growth and Tropane Alkaloid Production Capability of the Hairy Roots of *Hyoscyamus albus*, *H. muticus* and their Somatic Hybrid. Plant Science. 136: 93-99; DOI: 10.1016/S0168-9452(98)00091-0

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.04

Agrovoc descriptors: chamomilla recutita, flowers, fertilizer application, foliar application, iron, zinc, essential oils, soil types, greenhouses

Agris category code: f04, f62

Effects of soil and foliar applications of iron and zinc on flowering and essential oil of chamomile at greenhouse conditions

Yousef NASIRI[1,*] and Nosratollah NAJAFI[2]

Received September 25, 2013; accepted October 28, 2014.
Delo je prispelo 25. septembra 2013, sprejeto 28. oktobra 2014.

ABSTRACT

In order to study the effects of soil and foliar applications of iron (Fe) and zinc (Zn) on flowering, flower yield and essential oil production of German chamomile a pot experiment was conducted under greenhouse conditions at the Faculty of Agriculture, University of Tabriz, Iran in 2012. The experiment was arranged as completely randomized design with 12 treatments and three replications. Treatments were as follow: T_1: control – without Fe or Zn fertilizers, T_2: 30 mg $FeSO_4.7H_2O$ kg^{-1} dry soil, T_3: 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil, T_4: 30 mg $FeSO_4.7H_2O$ + 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil, T_5: foliar spraying of $FeSO_4.7H_2O$ (3.5 g L^{-1}), T_6: foliar spraying of $FeSO_4.7H_2O$ (7.0 g L^{-1}), T_7: foliar spraying of $ZnSO_4.7H_2O$ (2.5 g L^{-1}), T_8: foliar spraying of $ZnSO_4.7H_2O$ (5.0 g L^{-1}), T_9: T_5+T_7, T_{10}: T_5+T_8, T_{11}: T_6+T_7, T_{12}: T_6+T_8. The foliar spraying was done two times during the growing period. The results revealed that the flower number, flower yield, essential oil content and essential oil yield were significantly increased by soil and foliar applications of Fe + Zn, compared with the control (untreated). The highest flower number (477 $plant^{-1}$), flower yield (11.6 g pot^{-1}), essential oil content (0.88 %) and essential oil yield (119 mg pot^{-1}) were recorded for the soil application of Fe + Zn (T_4) by 58, 68, 21.4 and 105 % increment compared to the control, respectively. Foliar application of Fe + Zn (T_{12}) was placed at the next rank; however this treatment had no significant difference with the soil application of Fe + Zn (T_4). Other treatments did not show significant differences with the control. Generally, the results showed that soil or foliar application of Fe + Zn can be effective on increase or improve of quantity and quality of chamomile yield. Moreover, use of foliar application as a low cost method especially in areas with alkaline or calcareous soils can be recommended.

Key words: Application methods, Essential oil, Iron, *Matricaria chamomilla*, Zinc

IZVLEČEK

UČINKI TALNEGA IN FOLIARNEGA DODAJANJA ŽELEZA IN CINKA NA CVETENJE IN VSEBNOST ETERIČNIH OLJ PRAVE KAMILICE (*Chamomilla recutita* (L.) Rauschert), GOJENE V RASTLINJAKU

Lončni poskus gojenja prave kamilice (*Chamomilla recutita* (L.) Rauschert) je bil izveden v rastlinjaku z namenom ugotavljanja talnega in foliarnega dodajanja železa (Fe) in cinka (Zn) na njeno cvetenje, pridelek cvetov in produkcijo eteričnih olj na Faculty of Agriculture, University of Tabriz, Iran, leta 2012. Poskus je bil izveden kot popoln naključni poskus z 12obravnavanji in tremi ponovitvami. Obravnavanja so bila: T_1: kontrola – brez gnojenja s Fe ali Zn, T_2: 30 mg $FeSO_4.7H_2O$ kg^{-1} suhih tal, T_3: 22 mg $ZnSO_4.7H_2O$ kg^{-1} suhih tal, T_4: 30 mg $FeSO_4.7H_2O$ + 22 mg $ZnSO_4.7H_2O$ kg^{-1} suhih tal, T_5: škropljenje listov z $FeSO_4.7H_2O$ (3.5 g L^{-1}), T_6: škropljenje listov z $FeSO_4.7H_2O$ (7.0 g L^{-1}), T_7: škropljenje listov s $ZnSO_4.7H_2O$ (2.5 g L^{-1}), T_8: škroplenje listov z $ZnSO_4.7H_2O$ (5.0 g L^{-1}), T_9: T_5+T_7, T_{10}: T_5+T_8, T_{11}: T_6+T_7, T_{12}: T_6+T_8. Škropljenje listov je bilo opravljeno dvakrat v rastni dobi. Rezultati so pokazali, da je talno in foliarno gnojenje z Fe + Zn značilno povečalo število cvetov, pridelek cvetov, vsebnost in pridelek eteričnih olj v primerjavi s kontrolo. Največje število cvetov (477 na rastlino), največji pridelek cvetov (11.6 g na lonec), največja vsebnost eteričnih olj (0.88 %) in največji pridelek eteričnih olj (119 mg na lonec) so bili izmerjeni pri talnem dodajanju Fe + Zn (T_4), povečanje je bilo za 58, 68, 21.4 in 105 % glede na kontrolno obravnavanje. Učinek foliarnega dodajanja Fe + Zn (T_{12}) je bil takoj za talnim dodajanjem Fe + Zn (T_4), vendar se od njega ni značilno razlikoval. Druga obravnavanja niso dala značilnih odstopanj od kontrole. V splošnem so rezultati pokazali, da lahko tako talno kot foliarno dodajanje Fe + Zn učinkovito poveča ali izboljša količino in kvaliteto pridelka prave kamilice. Uporabo foliarnega dodajanja bi kot poceni način gnojenja še posebej priporočali na območjih , kjer so tla bazična ali apnenčasta.

Ključne besede: metode gnojenja, železo, cink, *Matricaria chamomilla*, eterična olja

[1] Assistant Professor, Agronomy and Plant Breeding Department, Faculty of Agriculture, University of Maragheh, 5518183111, Maragheh, Iran; Corresponding author E-mail: ysf_nasiri@maragheh.ac.ir

[2] Associate Professor, Soil Science Department, Faculty of Agriculture, University of Tabriz, 5166616471, Tabriz, Iran; n-najafi@tabrizu.ac.ir

1 INTRODUCTION

Today medicinal plants are one of the resources of drugs for treatment of many diseases. *Matricaria chamomilla* is an annual plant belonging to the Asteraceae family. It is widely used and well-documented medicinal plants in the world. It is included in the pharmacopoeia of 26 countries (Hendaway and Khalid, 2011). Chamomile has many pharmacological properties. It is a traditional treatment for numerous disorders, including sleep disorders, digestion/intestinal conditions, skin infections/inflammation (including eczema), wound healing, infantile colic, teething pains, and diaper rash. It has been also reported that chamomile has moderate antioxidant and antimicrobial activities (Simpson, 2001; McKay and Blumberg, 2006).

In order to obtain high quality and yield of crop, nutrients must be sufficient in growing environment of plant. Micronutrients as iron (Fe) and zinc (Zn) are the trace elements that play essential role in plant growth and increasing crop yields. Moreover, they improve plant nutrition and increase soil productivity (Marschner, 1995). Many crops respond to foliar and soil applications of micronutrients in terms of growth and crop yields. It is widely reported that foliar application of micronutrients at active growth stages will improve plant growth and consequently yield and quality in various crops (Kalidasu et al., 2008).

Iron is a cofactor for a large number of enzymes that catalyze several biochemical processes within the plant (Brittenham, 1994; Marschner, 1995). It plays a vital role in the chlorophyll formation, thylakoid synthesis and chloroplast development and also functions in the respiratory enzymes. Moreover, iron serves in the transportation of energy in the plant (Miller et al., 1995).

Zinc is known to have an important role either as a metal component of enzymes or as a functional, structural or regulatory cofactor of many enzymes. Zinc also has many essential roles in the plant growth and development including production of biomass, chlorophyll production, pollen function, fertilization, metabolism of RNA, proteins and the DNA formation (Marschner, 1995; Pandey et al., 2006; Cakmak, 2008). It is also, required for the synthesis of tryptophan, a precursor of IAA (Indole-3-Acetic Acid) which acts as a growth promoting substance (Marschner, 1995; Miller et al., 1995).

Generally plants obtain their nutrients requirements from the soil, but they are capable to absorb nutrients through the leaves. Foliar plant nutrition is one of the techniques that farmers use for plant nutrition since 1950s, when they were learned that foliar fertilization was effective and economic (Ebrahimian et al., 2010). Foliar fertilization is extensively used as a practice to accurate the nutritional deficiencies in plants caused by inappropriate deliver of nutrients to roots (Silberbush and Ling, 2002). The most important use of foliar sprays has been in the application of micronutrients (Havlin et al., 2004).

Micronutrients are added to foliar fertilizers, in order to compensate their deficiencies especially in arid and semi-arid regions with calcareous soils (Nasiri et al., 2010). Many recent researches have shown that a small amount of nutrients as Zn, Fe and Mn, applied by foliar spraying increases significantly the yield of crops (Said-Al Ahl and Mahmoud, 2009 & 2010; Nasiri et al., 2010; Zehtab-Salmasi et al., 2008 & 2012; Saedh et al., 2009). Nasiri et al. (2010) reported that flower yield, essential oil percentage and essential oil yield of chamomile were increased by foliar application of Fe and Zn compared with the control at farm conditions. Also Said-Al Ahl and Mahmoud (2010) reported that the highest plant height, branches per plant, fresh and dry biomass and essential oil yield of basil plants were obtained by foliar application of Zn and/or Fe in normal soil. The highest seed yield, oil yield, oil percentage, thousand seed weight and protein percentage of sunflower were obtained from the soil and foliar applications of Fe + Zn (Ebrahimian et al., 2010). Foliar spraying of Zn (100 mg L^{-1}) in blue sage enhanced the length of peduncle and main inflorescence, number of inflorescence and florets, and fresh and dry weight of inflorescences/plant (Abd El-Aziz and Balbaa, 2007). Application of micronutrients increased fresh and dry mater, leaf area of plant, bush and leaf essential oil percentage and essential oil yield of peppermint (Zehtab-salmasi et al., 2008).

Although the importance of micronutrients (such as Fe and Zn) on the growth and production of herbs in many research presented; however, there is little information about effectiveness of application methods of Zn and Fe on the growth and development of chamomile. In our previous research we investigated the effects of only foliar application of Fe and Zn on German chamomile at field conditions (Nasiri et al., 2010). Therefore, the purpose of present investigation was to study the effects of Fe and Zn application methods (Foliar spraying and Soil application) and different concentrations of them on flowering, yield and essential oil content of German chamomile.

2 MATERIALS AND METHODS

Chamomile plants (*Matricaria chamomilla*) were grown in a sandy loam alkaline soil in the greenhouse of the Faculty of Agriculture, University of Tabriz, Iran in 2012. The seeds obtained from Hungary were sown in plastic pots (30 cm diameter) filled with 6 kg of dry soil which according to Table 1 was deficient in Fe and Zn (Hazelton and Murphy, 2007). Physicochemical characteristics of the soil used in the study were measured by methods of Gee and Bauder (1986) and Sparks et al. (1996). Each pot was supplied with 450 mg NH_4NO_3, 44 mg $Ca(H_2PO_4)_2.H_2O$, 150 mg K_2SO_4, 8 mg $CuSO_4.5H_2O$, 85 mg $MgCl_2.6H_2O$ and 20 mg $MnSO_4.4H_2O$ per kg of dry soil according to the soil testing.

Table 1. Physicochemical characteristics of the soil used as potting media.

pH	7.81
EC_e (dS m^{-1})	0.71
Organic carbon content (%)	0.11
Calcium carbonate equivalent (%)	Negligible
Sand (%)	70
Silt (%)	18
Clay (%)	12
Texture	Sandy loam
Total N (%)	0.08
Available-P (mg kg^{-1})	5.7
Available-K (mg kg^{-1})	250
Available-Mg (mg kg^{-1})	99.1
Available-Ca (mg kg^{-1})	1149
Available-Fe (mg kg^{-1})	1.8
Available-Mn (mg kg^{-1})	1.1
Available-Zn (mg kg^{-1})	0.42
Available-Cu (mg kg^{-1})	1.3

EC_e= Electrical conductivity of saturated soil paste extract

Before seed planting, the above mentioned nutrients were dissolved in enough water and then were mixed with the pot soil. After the emergence of the seedlings four plants were kept per pot.

Treatments in the experiment were as follow: T_1: control, T_2: 30 mg $FeSO_4.7H_2O$ kg^{-1} dry soil, T_3: 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil, T_4: 30 mg $FeSO_4.7H_2O$ + 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil, T_5: foliar spraying of $FeSO_4.7H_2O$ (3.5 g L^{-1}), T_6: foliar spraying of $FeSO_4.7H_2O$ (7.0 g L^{-1}), T_7: foliar spraying of $ZnSO_4.7H_2O$ (2.5 g L^{-1}), T_8: foliar spraying of $ZnSO_4.7H_2O$ (5.0 g L^{-1}), T_9: T_5+T_7, T_{10}: T_5+T_8, T_{11}: T_6+T_7, T_{12}: T_6+T_8. These 12 treatments were arranged in a completely randomized design (CRD) with three replicates. The volume of the spraying solution was maintained just to cover completely the plant foliage till drip. The plants were sprayed twice at stem elongation and flowering stages.

The plant flowers were harvested eight times in 4-5 days intervals. After each harvest flowers were

dried in a shady place and were kept in a convenient location for essential oil extraction. The data recorded were: number of flowers in plant, dry weights of flowers in each pot as flower yield, essential oil percentage, and essential oil yield. Five grams dry flowers were hydro-distilled in a modified Clevenger apparatus in 1000 mL round bottomed flask with 500 mL distilled water for 4 h (Hoelz and Demuth, 1975; Letchamo, 1993). Essential oil yield was determined by multiplying essential oil percentage × average of dry weights of flowers per pot.

The results were statistically analyzed using MSTATC software. The graphs were plotted using Excel software and the Duncan's Multiple Range Test at 5 % level was used to compare the means of treatments.

3 RESULTS AND DISCUSSION

3.1 Flower number

The results in Table 2 show that flower number was significantly ($p < 0.05$) affected by different micronutrient fertilizer treatments. Means comparison indicated that the highest number of flowers (average 462 per plant) was noticed with the soil application of Fe + Zn (30 mg $FeSO_4.7H_2O$ and 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil) and foliar spraying of Fe + Zn (7.0 g $FeSO_4.7H_2O$ L^{-1} and 5.0 g $ZnSO_4.7H_2O$ L^{-1}). These treatments increased flower number by 58 and 48 % compared with the control, respectively. Although soil application of Fe and Zn increased flower number 6 % compared to the foliar spraying, but there was no significant difference between these two methods of fertilizer application.

Table 2: Effects of iron and zinc on flower, flower yield and essential oil of chamomile

Treatments	Flower number/plant*	Flower yield (g/pot)**	Essential oil content (%) **	Essential oil yield (mg/pot)***
T_1: Control– without Fe or Zn fertilizer	301±9.81^{c}	6.9±0.28^{c}	0.84±0.024^{b}	58±2.43^{c}
T_2: Fe (30 mg $FeSO_4.7H_2O$ kg^{-1} dry soil)	349±42.2^{bc}	8.19±1.25^{c}	0.87±0.034^{b}	72±13.51^{bc}
T_3: Zn (22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil)	354±14.16^{bc}	8.14±0.52^{c}	0.88±0.031^{b}	71±6.2^{a}
T_4: Fe + Zn (T_2+ T_3)	477±38.4^{a}	11.6±0.56^{a}	1.02±0.026^{a}	119±8.5^{bc}
T_5: Fe foliar spraying (3.5 g $FeSO_4.7H_2O$ L^{-1})	371±20.42^{abc}	8.17±0.44^{c}	0.86±0.029^{b}	70±4.02^{bc}
T_6: Fe foliar spraying (7.0 g $FeSO_4.7H_2O$ L^{-1})	382±23.58^{abc}	8.44±0.54^{bc}	0.85±0.027^{b}	72±6.61^{bc}
T_7: Zn foliar spraying (2.5 g $ZnSO_4.7H_2O$ L^{-1})	329±25.35^{c}	7.87±0.45^{c}	0.86±0.033^{b}	68±5.95^{bc}
T_8: Zn foliar spraying (5.0 g $ZnSO_4.7H_2O$ L^{-1})	339±6.62^{bc}	7.3±0.13^{c}	0.85±0.025^{b}	62±2.74^{bc}
T_9: T_5 + T_7	363±14.61^{bc}	8.32±0.20^{bc}	0.92±0.026^{ab}	76±0.41^{bc}
T_{10}: T_5 + T_8	385±32.06^{abc}	8.01±0.40^{c}	0.93±0.024^{ab}	74±4.04^{bc}
T_{11}: T_6 + T_7	333±42.89^{c}	8.46±0.85^{bc}	0.93±0.026^{ab}	78±6.1^{b}
T_{12}: T_6+ T_8	447±28.91^{ab}	10.53±0.47^{ab}	1.01±0.019^{a}	106±3.86^{a}
F Test	*	**	**	***

Value represents mean ± standard error of three replicates.
F Test: *** $p<0.001$; ** $p<0.01$; *: $p<0.05$.
Means followed by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at 5 % level.

3.2 Flower yield

Data presented in Table 2 show that soil application of 30 mg $FeSO_4.7H_2O$ + 22 mg $ZnSO_4.7H_2O$ per kg of dry soil) or foliar application of 7.0 g $FeSO_4.7H_2O$ + 5.0 g $ZnSO_4.7H_2O$ L^{-1} significantly ($p < 0.01$) increased flower yield in pot. The increments on flower yield were by 68.1 and 52.6 % respectively for the Fe + Zn soil application (T_4) and Fe + Zn foliar application (T_{12}) compared to the control. The lowest dry flower yield (6.9 g pot^{-1}) was recorded in control treatment. Increment of flower yield might be due to the increased number of flowers per plant as a result of positive effects of iron and zinc application that mentioned in the previous section. Increment of flowers number is directly responsible for higher flower yield in chamomile.

3.3 Essential oil content

The response of essential oil (EO) content (%) of chamomile to soil or foliar application with Fe and Zn is available in Table 2. EO % was significantly ($p < 0.01$) increased as a result of soil and foliar applications of Fe + Zn (T_4 and T_{12} treatments). The increments were 21.4 and 20.2 %, respectively compared to the control plant. Although other treatments also increased this parameter compared to the control in chamomile plants, but these increments were not significant. Chamomile essential oil changes affected by the different treatments of this study are shown in Figure 1.

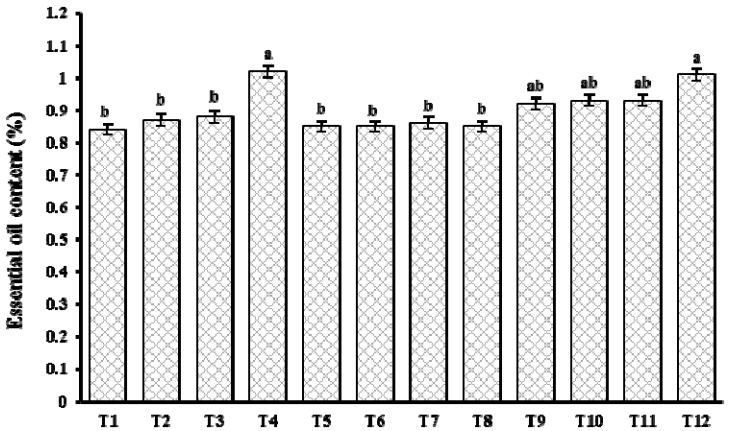


Figure 1: Mean comparison of essential oil content of chamomile in different treatments of iron and zinc application. The same letters in columns indicate no significant difference according to Duncan's Multiple Range Test at 5 % level. Error bars represent standard errors (n=3).
T_1: Control, T_2: 30 mg $FeSO_4.7H_2O$ kg^{-1} dry soil, T_3: 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil, T_4: 30 mg $FeSO_4.7H_2O$ + 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil, T_5: foliar spraying of $FeSO_4.7H_2O$ (3.5 g L^{-1}), T_6: foliar spraying of $FeSO_4.7H_2O$ (7.0 g L^{-1}), T_7: foliar spraying of $ZnSO_4.7H_2O$ (2.5 g L^{-1}), T_8: foliar spraying of $ZnSO_4.7H_2O$ (5.0 g L^{-1}), T_9: T_5+T_7, T_{10}: T_5+T_8, T_{11}: T_6+T_7 and T_{12}: T_6+T_8.

3.4 Essential oil yield

The obtained results in Table 2 show significant differences ($p < 0.001$) were manifested in the plant essential oil yield (EOY) of chamomile due to Fe and Zn application treatments. The highest values of this parameter were obtained from the Fe + Zn soil application (T_4) (119 mg pot^{-1}), Fe + Zn foliar application (T_{12}) (106 mg pot^{-1}), and T_{11} (78 mg pot^{-1}) that were 105, 85 and 34.4 % greater than the control (58 mg pot^{-1}), respectively. Although EO yield was increased by other treatments but these increments were not significant compared to the control (Figure 2).

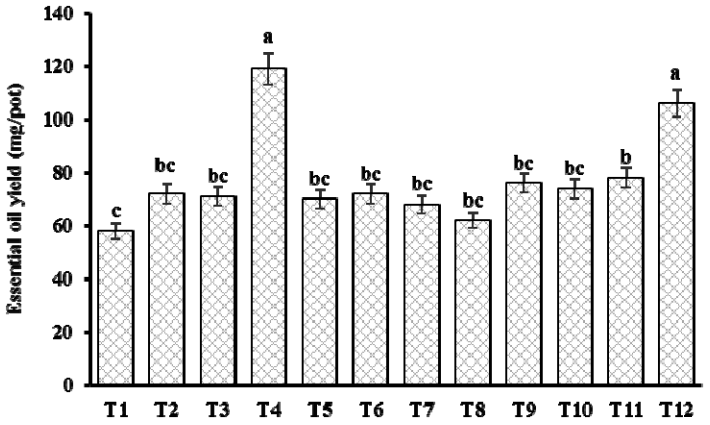


Figure 2: Mean comparison of essential oil yield of chamomile in different treatments of iron and zinc application. The same letters in columns indicate no significant difference according to Duncan's Multiple Range Test at 5 % level. Error bars represent standard errors (n=3).
T_1: Control, T_2: 30 mg $FeSO_4.7H_2O$ kg^{-1} dry soil, T_3: 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil, T_4: 30 mg $FeSO_4.7H_2O$ + 22 mg $ZnSO_4.7H_2O$ kg^{-1} dry soil, T_5: foliar spraying of $FeSO_4.7H_2O$ (3.5 g L^{-1}), T_6: foliar spraying of $FeSO_4.7H_2O$ (7.0 g L^{-1}), T_7: foliar spraying of $ZnSO_4.7H_2O$ (2.5 g L^{-1}), T_8: foliar spraying of $ZnSO_4.7H_2O$ (5.0 g L^{-1}), T_9: T_5+T_7, T_{10}: T_5+T_8, T_{11}: T_6+T_7 and T_{12}: T_6+T_8.

The results of this experiment show that flower number and flower yield affected by soil or foliar application of Fe and Zn treatments**.** These results were in consonance with the findings of Abd El-Aziz and Balbaa (2007) on blue sage, Kaldiasu et al. (2008) on coriander and Ravi et al. (2008) on safflower. They reported the beneficial effects of iron and zinc on flower production of different plants. This beneficial effect of Zn and Fe can be attributed to the role of Zn in the synthesis of IAA, photosynthesis and nitrogen metabolism and the role of iron in the chlorophyll synthesis and nitrogen fixation (Marschner, 1995; Miller et al, 1995).

On the other hand, increment of flower yield might be due to the increased number of flowers per plant as a result of positive effects of iron and zinc application that mentioned in the previous section. Increment of flowers number is directly responsible for higher flower yield in chamomile. These results are in agreement with those obtained by Grejtovský et al. (2006) and Nasiri et al. (2010) on chamomile, Said-Al Ahl and Omer (2009) on coriander and Said-Al Ahl and Mahmoud (2010) on basil. They stated that soil or foliar application of iron and zinc led to the increment of flowering parameters and plant yield.

In the case of essential oil percent and essential oil yield of chamomile plant, results showed significantly addition in their amount with application of Fe and Zn. The maximum amount of these parameters was observed in Fe + Zn soil and foliar application treatments, respectively.

The increase in essential oil due to zinc and /or iron was also reported in Japanese mint (Misra and Sharma, 1991), cumin (El-Sawi and Mohamed, 2002), peppermint (Akhtar et al., 2009) and sweet basil (Said-Al Ahl and Mahmoud, 2010) and chamomile (Nasiri et al., 2010).

Previous studies indicated that biosynthesis of secondary metabolites is not only controlled genetically but also affected intensely by ecological effects (Naghdi-Badi et al., 2004; Said-Al Ahl and Mahmoud, 2010). Plant nutrition as an environmental variable affects essential oil of medicinal plants. CO_2 and glucose are precursors of monoterpene biosynthesis. Carbohydrates are a resource of energy and reducing power for terpenoid synthesis. CO_2 fixation, content of primary metabolites and sucrose metabolism are closely linked with essential oil accumulation (Srivastava et al., 1997). As zinc is involved in photosynthesis and carbohydrate metabolism and CO_2 and glucose are the most likely sources of carbon utilized in terpenoid biosynthesis, the role

of Zn in influencing of essential oil accumulation seems particularly important. Moreover, iron has important functions in plant metabolism, such as activating catalase enzymes associated with superoxide dismutase, as well as in photorespiration and the glycolate pathway (Marschner, 1995).

Increase of EOY previously reported by Nasiri et al. (2010) in chamomile at field conditions and Said-Al Ahl and Mahmoud (2010) in sweet basil. They found that combined application of Fe + Zn gave the highest values of essential oil yield under normal soil conditions. This increment seems may be due to the raise of flower yield and essential oil percentage as a result of positive effects of Fe and Zn application. Since the EOY is directly associated with the flower yield and EO %, so any increase in these two traits led to the increase of essential oil yield.

Micronutrients such as Fe, Cu, Mn and Zn are essential for growth and development of the living plants. As they are found in the most redox reactions and are fundamental for cellular processes and in proteins and enzymes for structural and catalytic enzyme activities (Hall and Williams, 2003). These nutrients are known to be required for all higher plants and shortage of them in culture media causes deficiency symptoms and reducing plant growth (Marschner, 1995).

Fe and Zn act as metal components of various enzymes and also are associated with saccharide metabolism, photosynthesis, and protein synthesis. Iron has important functions in plant metabolism, such as activating catalase enzymes associated with superoxide dismutase, as well as in photorespiration, the glycolate pathway and chlorophyll content. Zinc is an essential micronutrient for synthesis of IAA, cell division and the maintenance of membrane structure and function. Zn deficiency reduces plant growth, pollen viability, flowering, number of fruits and seed production (Sharma *et al*., 1990; Marschner, 1995). Therefore, sufficient amount of these nutrients in the plant is necessary for normal growth and obtain a satisfactory product.

Many studies have reported that micronutrients such as Fe, Mn and Zn have important roles in plant growth and yield of aromatic and medicinal plants (Abd El- Wahab, 2008). Since, the soil application of micronutrients fertilizers in the cultivation may not meet the crop requirement for growth and nutrient use, thus the alternative effective approach is to apply these micronutrients as a foliar application (Saedh et al., 2009).

The positive influence of application of micronutrients on crop growth may be due to the improved ability of the crop to absorb nutrients, photosynthesis and better sink-source relationship as these play vital role in various biochemical processes (Kalidasu, et al, 2008).

Fe and Zn are absorbed by plant root and shoot as Fe^{2+}, Fe^{3+} and Zn^{2+}, respectively. The mobility and remobilization of these micronutrients in plants are low. The Fe and Zn concentrations in the soil solution are very low. The availability and solubility of Fe and Zn in soils were dependent on pH, organic matter content, texture, redox potential, moisture content, calcium carbonate equivalent percent, interactions with other elements, climate conditions and plant factors. The availability and solubility of Fe and Zn decrease with increased soil pH. So, the Fe and Zn deficiencies in plants can be observed in alkaline calcareous soils. At alkaline pH, Fe and Zn fertilizers used in soils precipitate as insoluble $ZnCO_3$, $FeCO_3$, $ZnFe_2O_4$ and $ZnSiO_4$. Fe and Zn adsorption on the surface of $CaCO_3$, clay minerals and Al/Fe oxides could also reduce the availability and solubility of these nutrients. As a result the effectiveness of these fertilizers is low when applied to soils (Marschner, 1995; Towfighi and Najafi, 2001; Havlin et al., 2004). So, when problems of soil fixation of these nutrients exist, foliar spraying constitutes an effective means of fertilizer application. Foliar fertilization needs lower amounts of fertilizers and provides for more rapid utilization of nutrients and permits the correction of observed deficiencies in less time than would be required by soil treatments (Havlin et al., 2004).

4 CONCLUSIONS

In this study it was found that Fe and Zn had beneficial effect on yield and essential oil production of chamomile plant. The obtained results also showed that the application of these two elements in combination had more positive and significantly effects on yield and essential oil of chamomile compared to the their individual applications (Tab. 2). Although there was no significant effect between two methods of fertilizer application in any of the studied parameters, however, soil application of iron and zinc was slightly more effective than use of them by foliar application, but this difference was not significant. With this interpretation, since the foliar application is low-costly technique of feeding plants by applying liquid fertilizer directly to their leaves (Baloch et al., 2008; Yassen et al., 2010), so the use of this method to compensate of micronutrients deficiency like iron and zinc and to improve of chamomile performance especially in arid and semi-arid regions with calcareous soils would be justified.

5 REFERENCES

Abd-El-Aziz N.G., Balbaa L.K. 2007. Influence of Tyrosine and Zinc on Growth, Flowering and Chemical Constituents of *Salvia farinacea* Plants. Journal of Applied Sciences Research, 3(11): 1479-1489.

Abd-El-Wahab M.A. 2008. Effect of some trace elements on growth, yield and chemical constituents of *Trachyspermum ammi* L. (AJOWAN) plants under Sinai conditions. Research Journal of Agricultural and Biology Sciences, 4(6): 717-724.

Akhtar N., Abdul-MatinSarker M., Akhter H., Katrun-Nada M. 2009. Effect of planting time and micronutrient as zinc chloride on the growth, yield and oil content of *Mentha pipeta*. Bangladesh Journal of Scientific and Industrial Research, 44: 125-130; DOI: 10.3329/bjsir.v44i1.2721

Brittenham G.M. 1994. New advances in iron metabolism, iron deficiency and iron overload. Current Opinion in Hematology, 1: 549-556.

Baloch Q.B., Chachar Q.I., Tareen M.N. 2008. Effect of foliar application of macro and micro nutrients on production of green chilies (*Capsicum annuum* L.). Journal of Agricultural Technology, 4: 177- 184.

Cakmak I. 2008. Enrichment of cereal grains with zinc: Agronomic or genetic bio-fortification. Plant Soil, 302:1–17; DOI: 10.1007/s11104-007-9466-3

Ebrahimian E., Bybordi A., Pasban-eslam B., 2010. Efficiency of zinc and iron application methods on sunflower. Journal of Food, Agriculture & Environment, 8 (3&4): 783-789.

El-Sawi S.A., Mohamed M.A. 2002. Cumin herb as a new source of essential oils and its response to foliar spray with some micro-elements, Food Chemistry, 77: 75-80; DOI: 10.1016/S0308-8146(01)00326-0

Gee, G.W., Bauder, J.W.C., 1986. Methods of soil analysis, Part1, Physical and mineralogical methods (2end Ed.). Am. Soc. Agron., USA.

Grejtovsky A., Markusova K., Eliasova A. 2006. The response of chamomile (*Matricaria chamomilla* L.) plants to soil zinc supply. Plant, Soil and Environment, 52: 1-7.

Hall J.L., Williams L.E. 2003. Transition metal transporters in plants. Journal of Experimental Botany, 54 (393): 2601-2613; DOI: 10.1093/jxb/erg303

Havlin J.L., Beaton J.D., Tisdale S.L., Nelson W.L. 2004. Soil fertility and fertilizers: an introduction to nutrient management. 7th Edition, Prentice Hall, USA.

Hazelton P.A., Murphy, B.W. 2007. Interpreting soil test results: what do all the numbers mean? CSIRO Publishing, Collingwood, Australia.

Hendaway S.F., Khalid K.A. 2011. Effect of chemical and organic fertilizers on yield and essential oil of chamomile flower heads. Medicinal and Aromatic Plant Science and Biotechnology, 5 (1): 43-48.

Hoelz J., Demuth G. 1975. Influence of ecological factors on the composition of essential oil and flavonoids in *Matricaria chamomilla* of different origin. Planta Medica, 27: 46-52.

Kalidasu G., Sarada C., Yellamanda-Reddy T. 2008. Influence of micronutrients on growth and yield of coriander (*Coriandrum sativum*) in rainfed Vertisols. Journal of Spieces and Aromatic Crops, 17 (2): 187-189.

Letchamo W. 1993. Nitrogen application effects on yield and content of active substances in chamomile genotypes. In Janick J. and Simon, E. (eds) New Crops. Willey. New York.

Marschner H. 1995. Mineral nutrient of higher plants. Edition 2, Academic Press Limited. Harcourt Brace and Company Publishers, London.

McKay D.L., Blumberg J.B. 2006. A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.). Phytotherapy Research, 20: 519-530; DOI: 10.1002/ptr.1900

Miller G.W., Huang I.J., Welkie G.W., Pushmik J.C. 1995. Function of iron in plants with special emphasis on chloroplasts and photosynthetic activity. In: Abadia J. (ed.) Iron nutrition in soils and plants. Kluwer Academic Publishers, Dordrecht; DOI: 10.1007/978-94-011-0503-3_4

Misra A., Sharma S. 1991. Critical Zn concentration for essential oil yield and menthol concentration of Japanese mint. Fertilizer Research, 29: 261-265; DOI: 10.1007/BF01052394

Naghdi-Badi H., Yazdani D., Mohammad A.S., Nazari F. 2004. Effects of spacing and harvesting on herbage yield and quality/quantity of oil in thyme, *Thymus vulgaris* L. Industrial Crops and Products, 19: 231–236; DOI: 10.1016/j.indcrop.2003.10.005

Nasiri Y., Zehtab-Salmasi S., Nasrullahzadeh S., Najafi N., Ghassemi-Golezani K. 2010. Effects of foliar application of micronutrients (Fe and Zn) on flower yield and essential oil of chamomile (*Matricaria chamomilla* L.). Journal of Medicinal Plants Research, 4: 1733-1737.

Pandey N., Pathak G.C., Sharma C.P. 2006. Zinc is critically required for pollen function and fertilization in lentil. Journal of trace element and medical biology, 20: 89–96; DOI: 10.1016/j.jtemb.2005.09.006

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

Saedh S.E., EL-Abady M.I., EL-Ghomary A.M., Farouk S. 2009. Influence of micronutrients foliar application and nitrogen fertilization on wheat yield and quality of grain and seed. Journal of Biology Sciences, 9(8): 851-858; DOI: 10.3923/jbs.2009.851.858

Said-Al-Ahl H.A.H., Omer E.A. 2009. Effect of spraying with zinc and / or iron on growth and chemical composition of coriander (*Coriandrum sativum* L.) harvested at three stages of development. Journal of Medicinal Food Plants, 1: 30-46.

Said-Al-Ahl H.A.H., Mahmoud A. 2010. Effect of zinc and / or iron foliar application on growth and essential oil of sweet basil (*Ocimum basilicum* L.) under salt stress. Ozean Journal of Applied Science, 3: 97-111.

Sharma P.N., Chatterjee C., Agarwala S.C., Sharma C.P. 1990. Zinc deficiency and pollen fertility in maize (*Zea mays*). Plant and Soil, 124 (2): 221-225; DOI: 10.1007/BF00009263

Silberbush M., Ling F. 2002. Response of maize to foliar vs. soil application of nitrogen-phosphorus-potassium fertilizers. Journal of Plant Nutrition, 25(11): 2333-2342; DOI: 10.1081/PLN-120014698

Simpson B.B. 2001. Herbal remedies Economic botany plants in our world Pp. 39-43 Edition 3, McGraw-Hill, Boston Burr Ridge, IL Dubuque, IA Madison, Wl, New York.

Sparks, D.L., Page, A.L., Helmke, P.A., Loeppert, R.H., Soltanpour, P.N., Tabatabaei, M.A., Johnson, C.T., Sumner, M.E., 1996. Methods of Soil Analysis. Part 3, Chemical Methods. Soil Science Society of American Book Ser. 5. SSSA, Madison, WI, USA.

Srivastava N.K., Misra A., Sharma S. 1997. Effect of zinc deficiency on net photosynthetic rate, 14C Partition, and oil accumulation in leaves of peppermint. Photosynthetica, 33: 71-79; DOI: 10.1023/A:1022127305883

Towfighi H. and N. Najafi. 2001. Changes in recovery and availability of native and applied zinc in waterlogged and non- waterlogged conditions in paddy soils of north of Iran. pp. 382-384. In: Proceedings of the 7th Iranian Soil Science Congress, 26-29 August, Shahrekord University, Shahrekord, Iran.

Yassen A., Abou El-Nour E.A.A., Shedeed S. 2010. Response of wheat to foliar spray with urea and micronutrients. Journal of American Science, 6: 14-22.

Zehtab-Salmasi S., Heidari F., Alyari H. 2008. Effects of microelements and plant density on biomass and essential oil production of peppermint (*Mentha piperita* L.). Plant Science Research, 1: 24-26.

Zehtab-Salmasi S., Saeideh B., Ghassemi-Golezani K. 2012. Effects of foliar application of Fe and Zn on seed yield and mucilage content of psyllium at different Stages of maturity. Pp.63-65. In: International Conference on Environment, Agriculture and Food Sciences (ICEAFS), August 11-12, Phuket, Thailand.

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.05
Agrovoc descriptors: pistacia vera, antioxidants, enzymes, oxidation, damage, stress, freezing, fertilizer application, foliar application, leaf area, membranes, moisture content

Agris category code: f62

Exogenous silicon leads to increased antioxidant capacity in freezing-stressed pistachio leaves

Ghader HABIBI[1]

Received November 24, 2014; accepted Janury 19, 2015.
Delo je prispelo 24. novembra 2014, sprejeto 19. januarja 2015.

ABSTRACT

Freezing stress limits photosynthesis and growth of plants. This may be attributed to the enhancement of freezing-associated oxidative damage. In this study, we followed precisely changes in the extent of lipid peroxidation and oxidative damage in leaves of pistachio (*Pistacia vera* 'Ahmadaghaii') plants exposed to foliar-applied silicon (Si) under freezing stress. The foliar-applied Si decreased significantly damaging effects of cold on relative water content (RWC), accompanied by an increase in shoot fresh mass (SFM). In addition, pre-Si treatment caused a significant reduction of the leaf area lost by freezing. There was a remarkable increase in phenylalanine ammonia-lyase (PAL) activity during recovery. Since leaf phenolic content was not affected by supplementary Si, the possibility that exogenously applied Si directly influences the activity of PAL seems thin. In the present work, freezing stress caused great membrane damage, as assessed by lipid peroxidation, but Si application significantly reduced the membrane damage because of an efficient scavenging by superoxide dismutase (SOD) and peroxidase (POD). Under freezing, despite the increasing POD activity, Si-supplied plants accumulated the highest levels of hydrogen peroxide (H_2O_2) may act as a signal for recovery ability from freezing injury. A positive correlation was found between the concentration of malondialdehyde (MDA) and the percentage of necrotic leaf area. This study suggests that the possible mechanisms for Si enhanced freezing resistance may be attributed to the higher antioxidant defense activity and lower lipid peroxidation through leaf water retention, in addition to its role as a mere physical barrier.

Key words: antioxidant enzymes, cold stress, Evans dye, hydrogen peroxide, phenylalanine ammonia-lyase, *Pistacia vera*, malondialdehyde, relative water content

IZVLEČEK

TRETIRANJE LISTOV PISTACIJE (*Pistacia vera* 'Ahmadaghaii') S SILICIJEM POVEČA NJIHOVO ANTIOKSIDATIVNO SPOSOBNOST V MRAZNEM STRESU

Mrazni stres omejuje fotosintezo in rast rastlin, kar lahko pripišemo povečanju oksidativnih poškodb zaradi zmrzovanja. V raziskavi sta bili spremljani peroksidacija lipidov in oksidativne poškodbe listov pistacije (*Pistacia vera* 'Ahmadaghaii') izpostavljenih mraznem stresu in foliarnem tretmaju s silicijem (Si). Foliarna uporaba silicija je značilno zmanjšala učinke mraza na ravni relativne vsebnosti vode (RWC), kar je povzročilo povečanje sveže mase poganjkov (SFM). Dodatno je predtretiranje s Si povzročilo značilno zmanjšanje izgube listne površine zaradi zmrzovana. Med okrevanjem po mraznem stresu je bila opazno povečana aktivnost fenilalanin amonik-liaze (PAL). Zaradi nespremenjene vsebnosti fenolov v listih po aplikaciji Si je maloverjetno, da bi foliarno dodani Si neposredno vplival na aktivnost PAL. Mrazni stres je povzročil velike poškodbe membran, ki so bile ocenjene s peroksidacijo lipidov, a jih je uporaba Si značilno zmanjšala zaradi učinkovitega antioksidativnega delovanja superoksid dismutaze (SOD) in peroksidaze (POD). Kljub povečanju aktivnosti POD v razmerah zmrzovanja, so s Si-obravnavane rastline kopičile največje količine vodikovega peroksida (H_2O_2), ki je lahko deloval kot signal za sposobnost okrevanja po poškodbah zaradi zmrzovanja. Ugotovljena je bila pozitivna korelacija med koncentracijo malondialdehida (MDA) in odstotkom nekrotične listne površine. Raziskava kaže, da je možen mehanizen preko katerega Si povečuje odpornost na zmrzovanje večja antioksidativna obramba in manjša peroksidacija lipidov, ki se odraža v večjem zadrževanju vode poleg delovanja Si kot čisto fizikalne prepreke.

Ključne besede: antioksidativni encimi, mrazni stres, Evansovo modrilo-T-1824 ,vodikov peroksid, phenilalanin amoniak-liaza, *Pistacia vera*, malondialdehid, relativna vsebnost vode

[1] Department of Biology, Payame Noor University, I. R. of Iran; Email: gader.habibi@gmail.com

1 INTRODUCTION

Low temperatures severely reduce photosynthetic capacity and growth of plants that may be due to increased production of reactive oxygen species (ROS). Accumulation of ROS is capable of inducing damage to almost all cellular macromolecules including DNA, proteins and carbohydrates (Ding *et al*., 2010; Miller *et al*., 2010). The plant cells respond to elevation in ROS levels by increasing the expression and activity of ROS-scavenging enzymes in order to maintain redox homeostasis (Apel and Hirt, 2004; Miller *et al*., 2010). In addition, some plants of tropical and subtropical regions, exhibit sensitivity to cold stress and usually lack the ability for cold acclimation (Zhu *et al*., 2007; Takahashi *et al*., 2013).

Silicon (Si) has been proved to be beneficial for the healthy growth and development of many plant species, particularly from the Gramineae family (Marschner, 1995; Broadley *et al*., 2011). Si application to crops has been reported to enhance their tolerance of multiple stresses (Ma, 2004; Guntzer, 2011), including pests and pathogens (Garbuzov *et al.*, 2011; Dallagnol *et al.*, 2012), metal toxicity (Rizwan *et al.*, 2012; Habibi, 2014*a*), salt and water stress (Hattori *et al.*, 2005; Liu *et al*., 2014). The mechanisms underlying silicon's capacity to increase stress resistance are still poorly understood. It has been reported that Si causes an improvement of water use efficiency and stimulation of antioxidative defense system in winter wheat (Liang *et al*., 2008), *Paspalum vaginatum* (He *et al*., 2010) and cucumber leaves (Liu *et al.* 2009). Increase in production of antioxidants and decline of ROS generation mediated by Si causes reduction of photo-oxidative damage, maintenance of chloroplast membranes integrity and thus enhancement of plants stress tolerance (Liang *et al*., 2008; Waraich *et al*., 2011).

One of the major problems arising in some pistachio cultivation areas includes different levels of injuries caused by lower temperatures in early spring. Because of the fact that the yield of pistachio was reduced due to frost damage, the understanding of the physiological and biochemical mechanisms improving freezing tolerance of this species is very important.

We hypothesize that the possible mechanisms for Si enhanced freezing stress may be attributed to the higher antioxidant defense activity and lower membrane oxidative damage through better water retention in leaf tissues. To test this hypothesis, we examined the effect of Si on the growth parameters, leaf water retention, and the enzymatic and non-enzymatic antioxidants and the membrane lipid peroxidation of freezing-stressed pistachio plants.

2 MATERIALS AND METHODS

2.1 Plant growth and treatments:

Seeds of pistachio (*Pistacia vera* 'Ahmadaghaii') were sown in top of the cylindrical plastic pots. Pots were 14 cm in diameter and 105 cm in depth, filled with 15 kg sandy loam soil (pH 7.6, EC 1.32 dS m^{-1}, field capacity (FC) 23 %, organic carbon (OC) 1.09 %). After emergence, the seedlings were thinned to one plant per pot and irrigated with distilled water every 5 days to maintain at 90 % field capacity. plants were grown in a growth chamber located near the city of Miandoab, NW Iran (46º6' E and 36º46' N) with day/night temperature of 25 ºC/18 ºC, relative humidity of 45–55 % and daily photon flux density (PFD) of about 1100–1200 µmol m^{-2} s^{-1} throughout the experimental period. Seven weeks after sowing, half of the plants were sprayed with 10 mM Si (as K_2SiO_3, pH adjusted to 5.8 with phosphoric acid). A drop of Tween 20 (0.05 %, v/v) as surfactant was added to 500 ml of the spray solutions. Five days after the treatment, half of the control (untreated with Si) and half of the Si-treated plants were placed to a controlled environment chamber under a 12 h (1±1 °C) light (at 300 µmol m^{-2} s^{-1} photosynthetic photon flux)/12 h (–2±1 °C) dark cycle at 85 % relative humidity for 2 days. After the freezing treatment, all plants were returned to normal conditions as described above, to allow leaves to recover from freezing stress. Samples were taken 2, 48 and 96 h after recovery after cold

treatment. Each measurement was done independently and experiments were repeated at least three times.

2.2 Analysis of growth parameters:

Leaves and roots were separated and washed with distilled water, blotted dry on filter paper and after determination of fresh mass (FM) they were dried for 48 h at 70 °C for determination of dry mass (DM). Relative water content (RWC) was measured and calculated according to Lara *et al*. (2003).

2.3 Assay of enzymes activity and related metabolites:

Activities of antioxidant enzymes were determined according to the methods described elsewhere (Habibi 2014*b*). For the determination of superoxide dismutase (SOD, EC 1.15.1.1) activity, enzyme was extracted in 25 mM HEPES pH 7.8 with 0.1 mM EDTA and the supernatant was added to the reaction mixture contained 0.1 mM EDTA, 50 mM Na_2CO_3 pH 10.2, 13 mM methionine, 63 µM nitroblue tetrazolium chloride (NBT), 13 µM riboflavin. One unit of SOD was defined as the amount of enzyme which produced a 50 % inhibition of NBT reduction under assay conditions. For the determination of catalase (CAT, EC 1.11.1.6) activity, samples were homogenized with 50 mM phosphate buffer pH 7.0 and assayed spectrophotometrically by following the degradation of H_2O_2 at 240 nm. Reaction medium contained 50 mM phosphate buffer pH 7 and 10 mM H_2O_2. Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test at 470 nm. The enzyme was extracted by 10 mM phosphate buffer pH 7.0 and assayed in a solution contained 10 mM phosphate buffer, 5 mM H_2O_2 and 4 mM guaiacol. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed by following reduction in absorbance at 290 nm as ascorbate was oxidized according to the method of Boominathan and Doran (2002). The reaction mixture contained 50 mM phosphate buffer pH 7, 0.2 mM EDTA, 0.5 mM ascorbic acid and 50 µg bovine serum albumin (BSA). Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid (Sigma) at 532 nm. MDA levels were calculated from a 1,1,3,3-tetraethoxypropane (Sigma) standard curve. The hydrogen peroxide (H_2O_2) contents in the leaves were assayed according to the method of Velikova et al. (2000). Leaves were homogenized in ice bath with 0.1% (w/v) TCA. The extract was centrifuged at 12,000 × g for 15 min, after which to 0.5 ml of the supernatant was added 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI, the reaction was improved for 1 h in the dark and measured spectrophotometrically at 390 nm. The content of H_2O_2 was given on a standard curve.

The total soluble proteins were measured according to the Bradford protein assay (Bradford, 1976).

To assay for PAL activity, leaf samples were ground in 50 mM sodium phosphate buffer (pH 7.0) containing 2 % (w/v) polyvinylpolypyrrolidon (PVPP), 2 mM EDTA, 18 mM β-mercaptoetanol and 0.1 % (v/v) Triton X-100. After centrifugation (15000 g for 15 min at 4 °C), PAL was assayed in the supernatant by measuring the formation of cinnamic acid at 290 nm according to modified method of Zucker (1965). Enzyme extracts were incubated at 30 °C for 60 min with 5 mM L-phenylalanine in 60 mM sodium borate buffer (pH 8.8). One unit (U) of PAL activity was defined as the amount of the enzyme that produced 1 nmol cinnamic acid per h. Total phenolic content was determined using the Folin-Ciocalteau method as modified by Velioglu *et al*. (1998). Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh weight.

2.4 Measurement of cell death:

Cell death was measured according to the method described by Schützendübel *et al*. (2001). After cold and Si treatments, leaf tips were inserted in Evans blue solution (0.025 % (w/v) Evans blue in water) for 30 min, followed by washing with water for 15 min. The trapped Evans blue was released from the leaves by homogenizing leaf tips in 1.6 mL of 50 % (v/v) MeOH and 1 % (w/v) SDS, and then centrifuged for 15 min. The optical density of the supernatant was determined at 600 nm and expressed on the basis of fresh mass. The percentage of necrotic area was calculated by measuring separately green and necrotic leaf area according to Irigoyen *et al*. (1996).

Leaves were prepared for determination of Si (Jaiswal 2004) using Inductively-Coupled Plasma-Atomic Emission Spectrometry (*ICP-AES, INTEGRA XL2, GBC,* Australia).

2.5 Statistical analysis:

Experiment was undertaken in complete randomized block design with 4 pots as 4 independent replications. Statistical analyses were carried out using Sigma stat (3.5) with Tukey test ($p < 0.05$). Correlation analysis using Spearman Rank Order Correlation in Sigma Stat (3.5) were conducted to determine the relationship between the measurement metabolites and the percentage of necrotic area.

3 RESULTS

As shown in Table 1, freezing alone significantly reduced the shoot fresh weight of pistachio plants. Addition of 10 mM Si under cold conditions significantly increased the shoot fresh weight of plants as compared with freezing alone. No significant changes of SDM, RFM and RDM were found by foliar application of Si under both freezing and normal temperatures. Cold alone decreased relative water content (RWC) by 13 % after treatment for 96 h recovery, but foliar-applied Si decreased significantly damaging effects of cold on RWC. Freezing alone increased necrotic leaf area by 7.6 % after treatment for 96 h recovery, but the increase was only 3.4 % when Si was applied. Si content was elevated by foliar application of Si, but it was not affected by freezing stress during all treatment periods.

Table 1: Effect of Si supplementation on the shoot fresh mass (SFM), shoot dry mass (SDM), root fresh mass (RFM), root dry mass (RDM), relative water content (RWC), necrotic leaf area, and the content of Si in pistachio plants after 96 h recovery after freezing treatment. Data of each row indicated by the same letters are not significantly different ($p < 0.05$). Data are the mean ± SD ($n = 4$)

Parameters	Control		96 h Rec	
	−Si	+Si	−Si	+Si
SFM (g plant^{-1})	10.4±2.21 ^{a}	10.5±1.86 ^{a}	7.00±1.11^{b}	8.91±1.03 ^{a}
SDM (g plant^{-1})	0.91±0.16 ^{a}	0.84±0.08 ^{a}	0.77±0.10 ^{a}	0.86±0.06 ^{a}
RFM (g plant^{-1})	3.73±0.40 ^{a}	4.16±0.69 ^{a}	3.56±0.22 ^{a}	3.81±0.43 ^{a}
RDM (g plant^{-1})	1.07±0.36 ^{a}	0.98±0.21 ^{a}	0.87±0.27 ^{a}	0.92±0.13 ^{a}
RWC (%)	70±3.2 ^{a}	73±1.8 ^{a}	57±3.0 ^{b}	69±2.4 ^{a}
Necrotic leaf area (%)	00.0±00.0 ^{c}	00.0±00.0 ^{c}	7.60±1.10 ^{a}	3.40±0.52 ^{b}
Leaf Si (mg g^{-1} DM)	0.79±0.22 ^{b}	2.16±0.85 ^{a}	0.86±0.33 ^{b}	2.48±0.52 ^{a}

Freezing treatment dramatically increased the PAL activity. Compared with freezing treatment alone, the PAL activity was not affected after 2, 46 and 96 h recovery, by supplementary Si following the freezing treatment (Fig. 1). Similarly, the leaf phenolic content was not influenced by supplementary Si. The percentage of Evans dye uptake increased under freezing stress in the non-Si-treated plants. In Si-supplemented plants, however, uptake of Evans dye did not rise upon cold exposure during the experimental period.

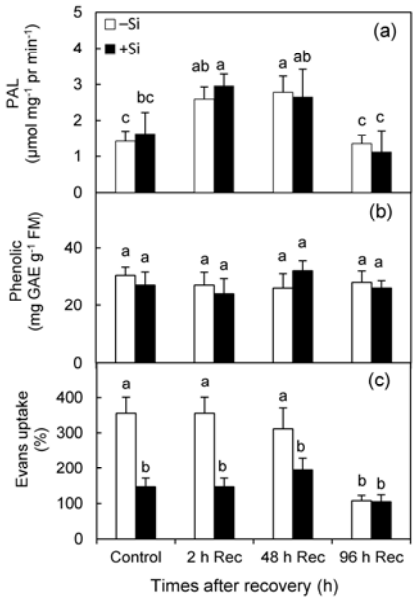


Figure 1: Changes in specific activity of phenylalanine ammonia lyase (PAL) (a), the concentration of total phenolics (b) and the percentage of uptake of Evans dye (c) in pistachio plants grown with (+Si) or without Si (-Si) supplementation after 2, 46 and 96 h recovery after freezing treatment. Bars indicated with the same letter are not significantly different ($p < 0.05$). Data are the mean ± SD ($n = 4$).

Freezing caused a significant increase in the activities of all analyzed antioxidant enzymes with the exception of APX. As shown in Fig. 2, the activities of antioxidant enzymes increased under freezing stress while Si application caused further increase being significant for SOD and POD activities. Freezing stress induced membrane damage as was indicated by higher MDA concentration (Fig. 3). However, the addition of Si to the freezing treatment significantly decreased MDA content compared with the corresponding freezing-treatment with no Si added. Cold stress caused a significant accumulation of hydrogen peroxide, and the continuation of the recovery time with or without Si application caused a further accumulation of H_2O_2. A positive correlation was found between the concentration of MDA and the percentage of necrotic leaf area ($r = 0.96$, $p < 0.01$ in cold treatment; $r = 0.76$, $p < 0.01$ in cold+Si treatment, Fig. 4). There was not a significant correlation between the concentration of H_2O_2 and the percentage of necrotic leaf area.

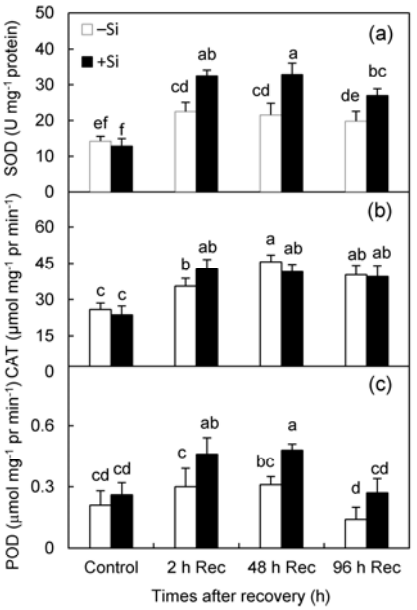


Figure 2: Effect of foliar-applied Si on (a) the specific activity of superoxide dismutase (SOD), (b) catalase (CAT) and (c) peroxidase (POD) in pistachio after 2, 46 and 96 h recovery after freezing treatment. Bars indicated with the same letter are not significantly different ($p < 0.05$). Data are the mean ± SD ($n = 4$).

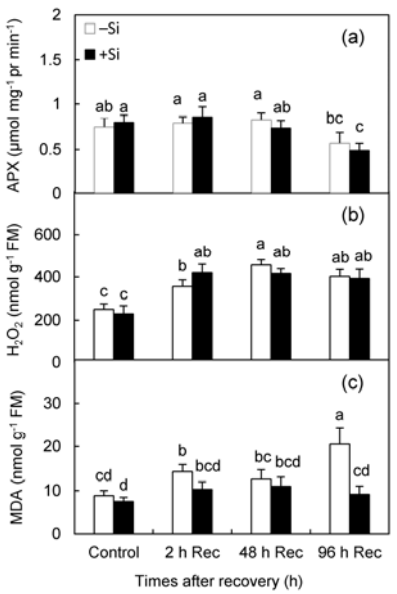


Figure 3: Effect of foliar-applied Si on (a) the specific activity of ascorbate peroxidase (APX), (b) the concentration of hydrogen peroxide (H_2O_2) and (c) malondialdehyde (MDA) in pistachio after 2, 46 and 96 h recovery after freezing treatment. Bars indicated with the same letter are not significantly different ($p < 0.05$). Data are the mean ± SD ($n = 4$).

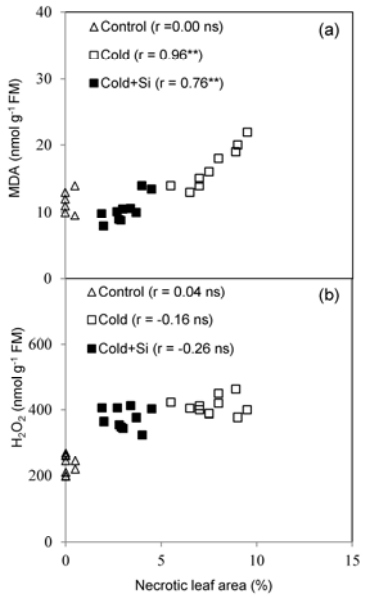


Figure 4: Correlations between the concentration of (a) malondialdehyde (MDA) and the percentage of necrotic leaf area and (b) between the concentration of hydrogen peroxide (H_2O_2) and the percentage of necrotic leaf area in pistachio plants grown with or without Si supplementation after 2, 46 and 96 h recovery after freezing treatment: ns, *, and **: non-significant, significant at the 5 % and 1 % levels of probability, respectively.

4 CONCLUSION

Freezing-sensitive plants exposed to low temperature often show signs of water stress due to decreased root hydraulic conductance, leading to associated decreases in leaf water and turgor potential, followed by a reduction of growth (Waśkiewicz *et al*., 2014). In this study, we have shown that the foliar-applied Si decreased significantly damaging effects of cold on RWC, accompanied by an increase in SFM. The main mechanism for such roles of Si in maintaining higher water content in leaf tissues is hypothesized to be the reduced transpirational water loss via reduction of both cuticular and stomatal transpiration, and through improvement of water uptake via increased volume and weight of roots (Cooke and Leishman, 2011; Sonobe *et al*., 2011). Leaf necrosis is considered as a typical external sign of freezing injury in freezing-sensitive plants. In this work, pre-Si treatment caused a significant reduction of the leaf area lost by freezing.

It has been demonstrated that cold stress induces the activity of PAL, which is the prime intermediary in the biosynthesis of phenolics, and is considered by most authors to be one of the main lines of cell acclimation against stress in plants (Levine *et al*., 1994). In the present study, there was a remarkable increase in PAL activity during recovery. These results are consistent with other authors who consider PAL to be one of the prime elements of cell acclimation against thermal stress in plants (Levine *et al*., 1994; Bharti and Khurana, 1997). The results obtained by Hossain *et al*. (2007) with oat leaves have demonstrated that Si reduces the activity of PAL. In the present experiment, leaf phenolic content was not affected by supplementary Si. Therefore, the possibility that exogenously applied Si directly influences the activity of PAL seems thin.

Earlier experiments support the positive correlation between higher activities of antioxidant enzymes and freezing tolerance (Zhang *et al*., 2011; Kishimoto *et al*., 2014). The magnitude of oxidative damage is usually measured by MDA (an end product of membrane lipid peroxidation), and Evans dye absorption, two markers for the ROS-mediated cell membrane damage (Liu *et al*., 2009). In the present work, freezing stress caused great membrane damage, as assessed by lipid

peroxidation, but Si application significantly reduced the membrane damage because of an efficient scavenging by SOD and POD (Fig. 2). There is data supporting that Si increases the activity of antioxidant enzymes such as POD and SOD (Liu *et al*., 2009), which in turn protect plants against ROS generation and lipid peroxidation. Recently, we have reported that the stability of plasma membranes in leaves of drought-stressed pistachio were mediated by addition of Si (Habibi and Hajiboland, 2013), which is associated with Si-enhanced antioxidant defense capacity in drought-stressed plants (Waraich *et al*., 2011). Thus, it is clear that Si can enhance antioxidant defense activity in plants under drought and freezing stress, resulting in decreased membrane oxidative damage, improved stability of cell membranes and enhanced stress tolerance.

In this research, despite the increasing POD activity, Si-supplied plants accumulated the higher levels of H_2O_2 after freezing treatment. The increase in the concentration of H_2O_2 may act as a signal for recovery ability from freezing injury. The significant correlation between the concentration of MDA and the percentage of necrotic leaf area confirmed the idea that, even if active oxygen formation was increased, the defense mechanisms had sufficient capacity or could be induced, with the result that damage was not apparent. The results indicated that the application of Si could prevent lipid peroxidation of stressed pistachio plants obviously because of higher POD and SOD activities.

In conclusion, Si-supplemented cold-stressed plants exhibited better protection from oxidative damage, and this ability was associated with the higher CAT and SOD activities and the lower level of lipid peroxidation. These data indicate that an application of Si can be used to promote the induction of the antioxidant system in plants, thereby improving stress resistance. One of the major problems arising in some pistachio cultivation areas includes different levels of injuries caused by lower temperatures in early spring. Our results suggest that improvement of plant tolerance to cold stress by Si supplementation is achieved by activation of antioxidant defense capacity. In addition, results demonstrated that the possibility that exogenously applied Si directly influences the activity of PAL seems thin. However further research is needed to solve the relation between the phenolic content and cold tolerance in pistachio plants upon Si supplementation.

5 REFERENCES

Apel K., Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology, 55: 373-399; DOI: 10.1146/annurev.arplant.55.031903.141701

Bharti A.K., Khurana, J.P. 1997. Mutant of *Arabidopsis* as tools to understand the regulation of phenylpropanoids pathway and UVB protection mechanism. Journal of Photochemistry and Photobiology, 65: 765-776; DOI: 10.1111/j.1751-1097.1997.tb01923.x

Boominathan R., Doran P.M. 2002. Ni induced oxidative stress in roots of the Ni hyperaccumlator, *Alyssum bertoloni*. New phytologist, 156: 202-205; DOI: 10.1046/j.1469-8137.2002.00506.x

Bradford M.M. 1967. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254; DOI: 10.1016/0003-2697(76)90527-3

Broadley M., Brown P., Cakmak I., Ma J.F., Rengel Z., Zhao F.P. 2011. Beneficial elements. In: Marschner's Mineral Nutrition of Higher Plants. Marschner P. (eds.). Academic Press, UK, London: 249-269.

Cooke J., Leishman M.R. 2011. Is plant ecology more siliceous than we realise? Trends in Plant Science, 16: 61-68; DOI: 10.1016/j.tplants.2010.10.003

Dallagnol L.J., Rodrigues F.A., Tanaka F.A.O., Amorim L., Camargo L.E.A. 2012. Effect of potassium silicate on epidemic components of powdery mildew on melon. Plant Pathology, 61: 323-330; DOI: 10.1111/j.1365-3059.2011.02518.x

Ding M., Hou P., Shen X. 2010. Salt-induced expression of genes related to Na^+/K^+ and ROS homeostasis in leaves of salt-resistant and salt-sensitive poplar species. Plant Molecular Biology, 73: 251-269; DOI: 10.1007/s11103-010-9612-9

Garbuzov M., Reidinger S., Hartley S.E. 2011. Interactive effects of plant-available soil silicon and

herbivory on competition between two grass species. Annals of Botany, 108: 1355-1363; DOI: 10.1093/aob/mcr230

Guntzer F., Keller C., Meunier J.D. 2011. Benefits of plant silicon for crops: a review. Agronomy for Sustainable Development, 32: 201-213; DOI: 10.1007/s13593-011-0039-8

Habibi G., Hajiboland R. 2013. Alleviation of drought stress by silicon supplementation in pistachio (*Pistacia vera* L.) plants. Folia Horticulturae, 25: 21-29; DOI: 10.2478/fhort-2013-0003

Habibi G. 2014*a*. Role of trace elements in alleviating environmental stress. In: Emerging Technologies and Management of Crop Stress Tolerance Biological Techniques. Ahmad P., Rasool S. (eds.). Elsevier, USA: 313-331; DOI: 10.1016/B978-0-12-800876-8.00014-X

Habibi G. 2014*b*. Silicon supplementation improves drought tolerance in canola plants. Russian Journal of Plant Physiology, 61, 6: 784-791; DOI: 10.1134/S1021443714060077

Hattori T., Inanaga S., Araki H., An P., Morita S., Luxova M., Lux A. 2005. Application of silicon enhanced drought tolerance in *Sorghum bicolor*. Physiologia Plantarum, 123: 459-466; DOI: 10.1111/j.1399-3054.2005.00481.x

He Y., Xiao H., Wang H., Chen Y., Yu M. 2010. Effect of silicon on freezing-induced changes of solutes, antioxidants, and membrane stability in seashore *paspalum turfgrass*. Acta Physiologiae Plantarum, 32: 487-494; DOI: 10.1007/s11738-009-0425-x

Hossain M.T., Soga K., Wakabayashi K., Kamisaka S., Fujii S., Yamamoto R., Hoson T. 2007. Modification of chemical properties of cell walls by silicon and its role in regulation of the cell wall extensibility in oat leaves. Journal of Plant Physiology, 164, 4: 385-393; DOI: 10.1016/j.jplph.2006.02.003

Irigoyen J.J., Juan J.P.D., Diaz M.S. 1996. Drought enhances freezing tolerance in a freezing-sensitive maize (*Zea mays*). New Phytologist, 134: 53-59; DOI: 10.1111/j.1469-8137.1996.tb01145.x

Jaiswal P.C. 2004. Soil, Plant and Water Analysis, (eds.). New Delhi, Kalyani Publishers.

Kishimoto T., Sekozawa Y., Yamazaki H., Murakawa H., Kuchitsu K., Ishikawa M. 2014. Seasonal changes in ice nucleation activity in blue berry stems and effects of cold treatments in vitro. Environmental and Experimental Botany, 106: 13-23; DOI: 10.1016/j.envexpbot.2014.02.010

Lara M.V., Disante K.B., Podesta F.E., Andreo C., Drincovich M.F. 2003. Induction of a crassulacean acid like metabolism in the C_4 succulent plant, *Portulaca oleracea* L.: physiological and morphological changes are accompanied by specific modifications in phosphoenolpyruvate carboxylase. Photosynthesis Research, 77: 241-254; DOI: 10.1023/A:1025834120499

Levine A., Tenhaken R., Dixon R., Lamb C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583-593; DOI: 10.1016/0092-8674(94)90544-4

Liang Y., Zhuc J., Li Z., Chua G., Dingc Y., Zhangc J., Sun W. 2008. Role of silicon in enhancing resistance to freezing stress in two contrasting winter wheat cultivars. Environmental and Experimental Botany, 64: 286-294; DOI: 10.1016/j.envexpbot.2008.06.005

Liu P., Yin L., Deng X., Wang S., Tanaka K., Zhang S. 2014. Aquaporin-mediated increase in root hydraulic conductance is involved in silicon-induced improved root water uptake under osmotic stress in *Sorghum bicolor* L. Journal of Experimental Botany, 65: 4747-4756; DOI: 10.1093/jxb/eru220

Liu J., Lin S., Xu P., Wang X., Bai J. 2009. Effects of exogenous silicon on the activities of antioxidant enzymes and lipid peroxidation in chilling-stressed cucumber leaves. Agricultural Sciences in China, 8, 9: 1075-1086; DOI: 10.1016/S1671-2927(08)60315-6

Ma J.F. 2004. Role of silicon in enhancing the resistance of plants to biotic and abiotic stresses. Soil Science and Plant Nutrition, 50: 11-18; DOI: 10.1080/00380768.2004.10408447

Marschner, H. 1995. Mineral nutrition of higher plants. Acedemic Press, London, UK.

Miller G., Suzuki N., Ciftci-Yilmaz S., Mittler R. 2010. Reactive oxygen species homeostasis and signaling during drought and salinity stresses. Plant, Cell and Environment, 33: 453-467; DOI: 10.1111/j.1365-3040.2009.02041.x

Rizwan M., Meunier J.D., Miche H., Keller C. 2012. Effect of silicon on reducing cadmium toxicity in durum wheat (*Triticum turgidum* L. cv. Claudio W.) grown in a soil with aged contamination. Journal of Hazardous Materials, 209: 326-334; DOI: 10.1016/j.jhazmat.2012.01.033

Schützendübel A., Schwanz P., Teichmann T., Gross K., Langenfeld-Heyser R., Godbold D.L. 2001. Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiationins cotspine roots. Plant Physiology, 127: 887-898; DOI: 10.1104/pp.010318

Sonobe K., Hattori T., An P., Tsuji W., Eneji A.E., Kobayashi S., Kawamura Y., Tanaka K., Inanaga S. 2011. Effect of silicon application on sorghum root responses to water stress. Journal of Plant Nutrition, 34: 71-82; DOI: 10.1080/01904167.2011.531360

Takahashi D., Li B., Nakayama T., Kawamura Y., Uemura M. 2013. Plant plasma membrane proteomics for improving cold tolerance. Frontiers in Plant Science**,** 17: 14-90.

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

Velioglu Y.S., Mazza G., Gao L., Oomah B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. Journal of Agricultural and Food Chemistry, 46: 4113-4117; DOI: 10.1021/jf9801973

Waraich E.A., Amad R., Ashraf M.Y., Ahmad M. 2011. Improving agricultural water use efficiency by nutrient management. Acta agriculturae Scandinavica, 61: 291-304.

Waśkiewicz A., Beszterda M., Goliński P. 2014. Nonenzymatic antioxidants in plants. In: Oxidative damage to plants: Antioxidant networks and signaling. Ahmad P. (eds.). Elsevier, USA, 201-234; DOI: 10.1016/B978-0-12-799963-0.00007-1

Zhang Q., Zhang J.Z., Chow W.S., Sun L.L., Chen J.W., Chen Y.J., Peng C.L. 2011. The influence of low temperature on photosynthesis and antioxidant enzymes in sensitive banana and tolerant plantain (*Musa* sp.) cultivars. Photosynthetica, 49: 201-208; DOI: 10.1007/s11099-011-0012-4

Zhu J., Dong C.H., Zhu J.K. 2007. Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Current Opinion in Plant Biology, 10: 290-295; DOI: 10.1016/j.pbi.2007.04.010

Zucker M. 1965. Induction of phenylalanine deaminase by light, its relation to chlorogenic acid synthesis in potato tuber tissue. Physiologia Plantarum, 40: 779-784; DOI: 10.1104/pp.40.5.779

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.06
Agrovoc descriptors: cynara scolymus, globe artichokes, organic agriculture, biological production, cash crops, green manures, nitrogen, crop yield, soil fertility, biomass, crop residues, organic matter

Agris category code: f01, f04

Artichoke (*Cynara scolymus* L.) as cash-cover crop in an organic vegetable system

Anna LENZI[1], Ada BALDI[2], Romano TESI[3]

Received October 12, 2014; accepted Janury 12, 2015.
Delo je prispelo 12. oktobra 2014, sprejeto 12. januarja 2015.

ABSTRACT

In organic vegetable systems green manure crops play an important role as a nitrogen source, but they cover the soil for several months without producing a direct income. Globe artichoke (*Cynara scolymus* L.) provides both heads to be harvested and particularly abundant plant residues to be possibly incorporated into the soil, so it may play a double role of cash and cover crop. This paper describes an on-farm study in which seed-propagated artichoke, cultivated as an annual crop, preceded zucchini squash and lettuce cultivated in sequence within a vegetable organic system. Artichoke produced about 7 t ha^{-1} of saleable heads and left, after harvest, 50.3 t ha^{-1} of fresh biomass usable as green manure. Zucchini squash and lettuce following artichoke showed a significant increase in yield when artichoke residues were incorporated into the soil. Furthermore, a residual positive effect of green manure on soil fertility was detected after lettuce harvest.

Key words: organic vegetables, globe artichoke, cover crop, cash crop, green manure, nitrogen

IZVLEČEK

ARTIČOKA (*Cynara scolymus* L.) KOT PRODAJNO ZANIMIVA VRTNINA V EKOLOŠKI PRIDELAVI ZELENJAVE

Zeleno gnojenje ima pomebno vlogo pri ekološki pridelavi zelenjave kot vir dušika, a je problem v tem, da rastline namenjene temu zasedajo prostor v kolobarju več mesecev brez neposrednega dohodka. Artičoka (*Cynara scolymus* L.) daje oboje, tržni pridelek v obliki koškov in veliko organskih ostankov, ki se lahko zaorjejo kot zeleno gnojilo, torej igra dvojno vlogo kot tržna in pokrovna kultura. Prispevek opisuje vzorčno raziskavo kmetije, na kateri pridelujejo iz semen vzgojeno artičok kot enoletno kulturo v ekološkem kolobarju pridelovanja zelenjave za bučkami in vrtno solato. Artičoka je dala okrog 7 t ha^{-1} tržnih koškov in po pobiranju pustila na polju 50.3 t ha^{-1} sveže biomase, ki je uporabna kot podor. Bučke in vrtna solata, ki so v kolobarju sledile artičoki, so dale značilno večji pridelek, kadar so se ostanki artičoke vdelali v tla. Pozitivni učinek zelenega gnojenja na rodovitnost tal je bil ugotovljen še po pobiranju pridelka vrtne solate.

Ključne besede: ekološko pridelana zelenjava, artičoka, pokrovna kultura, tržna kultura, zeleno gnojenje, dušik

1 INTRODUCTION

Organically produced food shows increasing interest throughout the world in relation to concerns about food safety, human health, animal welfare, and environmental safeguard (Yiridoe et al., 2005). In fact, organic farming systems may prevent the occurrence of soil degradation, environmental pollution, biodiversity decline, and food contamination that may possibly originate from conventional agricultural practices like recurrent tillage, monoculture, and intensive administration of agrochemicals (Raviv, 2010).

Most consumers associate organic at first with vegetables and fruit (Padel and Foster, 2005), that

[1] Department of Agrifood Production and Environmental Sciences (DISPAA), University of Florence, Piazzale delle Cascine, 18, 50144 Florence, Italy; corresponding author: anna.lenzi@unifi.it

are also the most frequently purchased organic foods (Werner and Alvensleben, 1984; Hay, 1989; Jolly et al., 1989; Davies et al., 1995; Dimitri and Greene, 2000; O'Donovan and McCarthy, 2002). Probably, that is because there is a strong association between heavy agrochemical use and fruit and, especially, vegetable production, and consequently organic produce consumption is seen as an important mean to reduce exposure to chemicals in the diet (Padel and Foster, 2005).

In Italy, the cultivation of organic vegetables covers an estimated area of 23,405 ha (EC DG, 2013), including both certified-organic and in-conversion areas according to European Union (EU) legislation [Council Regulations (EC) No 834/2007 and No 889/2008 as amended]. Such extent, representing 2.1 % of total Italian area occupied by organic crops, is by far the largest one to be devoted to organic vegetables within the EU, where organic vegetable cultivation covers an overall area of 110,955 ha (EC DG, 2013).

In organic vegetable systems, soil fertility management is a crucial and costly cultural practice, and nitrogen is often the most limiting nutrient to efficient and profitable production (Gaskell and Smith, 2007). Possible sources of nitrogen for organic farmers include fixed nitrogen from legumes and organic matter of different, on-farm or off-farm, origins. When animal husbandry is not included in the organic farming system, which is often the case of vegetable farms, green manure crops (cover crops grown for their nutrient value) play an important role in managing nitrogen without or with reduced use of external inputs (Shennan, 1992; Burket et al., 1997; Thönnissen et al., 2000a; Thorup-Kristensen et al., 2001; Lenzi et al., 2009).

The most commonly used cover crops are legume, cereal, and brassica species, depending on farmer goals and circumstances (Snapp et al., 2005). In fact, cover crops are not only a source of nitrogen, but they provide additional potential benefits, like improved soil structure, erosion control, recycling of nutrients other than nitrogen, increased soil biological activity, and pest and weed suppression (Clark, 2012). On the other hand, adopting cover crops may potentially reduce farm income if cover crops interfere with other attractive crops (Snapp et al., 2005). Thus, time or market constraints and the need to intensively farm high value land may limit their use (Cabilovski et al., 2011).

Globe artichoke (*Cynara scolymus* L.) is a horticultural crop cultivated all over the world, but especially widespread in the Mediterranean basin, where its large immature inflorescences (heads) are an important component of the diet. The species is usually vegetatively propagated, but seed-propagated cultivars are also available (Calabrese et al., 2000, 2004, and 2005; Tesi and Lenzi, 2005a). In Italy, where artichoke cultivation covers an area of about 50,000 ha (INEA, 2013), this species is used as both multi-year and annual crop depending on cultivation area and propagation method. Spring production in northern and central regions derives from multi-year crops that may last up to 6-8 years; for autumn-to-spring production in south regions, annual or biennial cycles are adopted, and seed-propagated artichoke is always an annual crop (Tesi, 2010).

Head production may vary depending on cultivar, plant density, and length of the crop cycle, but, in any case, it represents a small part of the total biomass production by the plants (Lattanzio et al., 2009). A research recently conducted on seventeen Italian artichoke genotypes revealed an average aboveground dry biomass yield of 9.7 t ha^{-1} (Ciancolini et al., 2013).

Since artichoke provides both heads to be harvested and particularly abundant plant residues to be possibly incorporated into the soil, it may play a double role of cash and cover crop. Aiming to find some preliminary evidence to this hypothesis, an on-farm study was conducted in which seed propagated artichoke preceded zucchini squash and lettuce cultivated in sequence within a vegetable organic system.

2 MATERIALS AND METHODS

The experiment was carried out in an organic farm (Bonamici Organic Farm) located in San Martino Ulmiano, Pisa, Italy, along the Tyrrhenian coast of central Italy (lat. 43°46', long. 10°24'). Three

crops (seed-propagated artichoke, whose plant residues were removed from the field or incorporated into the soil at the end of the crop cycle, zucchini squash, and lettuce) were organically cultivated in sequence over a 20-month period, with the timing shown in Figure 1.

Plants were cultivated under a high tunnel, 4 m wide and 50 m long, covered with a Long Life Polyethylene film, a type of protection, generally not equipped with heating systems, commonly used in this area for the cultivation of winter vegetables. Monthly average maximum and minimum temperatures during the trial and main soil physical and chemical properties are shown in Figure 1 and Table 1, respectively.

Table 1: Soil characterization of the experimental field (0-30 cm soil depth)

Parameter	Units	
pH		6.89
Organic matter	g kg^{-1}	12
Total N*	g N kg^{-1}	1.45
Available P**	mg P_2O_5 kg^{-1}	120
Exchangeable K	mg K_2O kg^{-1}	185
Sand	%	44
Silt	%	44
Clay	%	12

*Kjeldahl method, ** Olsen method

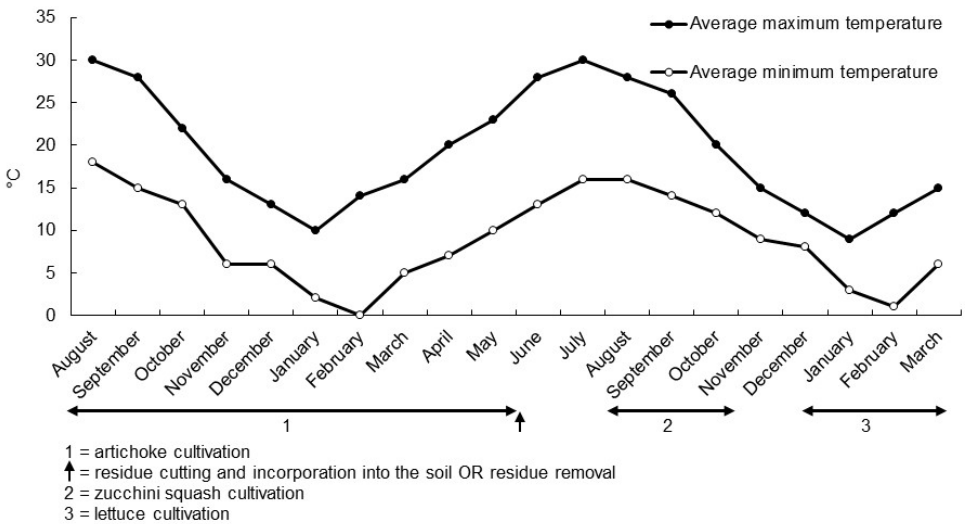


Figure 1: Monthly temperatures in the course of the trial and timing of crop succession

Before artichoke planting, soil was fertilized with 100 g m^{-2} of Guanito (Italpollina, Rivoli Veronese, Italy), a pelleted fertilizer with 6 % total N, 15 % P_2O_5, 55 % organic matter, obtained from guano. No other fertilizer was provided to soil for the whole experiment.

As seed-propagated artichoke, a local selection of the cultivar 'Terom' named 'T3' (Tesi and Lenzi, 2005a) was used. 'T3' plantlets with 3-4 leaves were transplanted in the first week of August in three rows 1.3 m apart with a spacing of 1 m along the rows, resulting in a density of 0.77 plants per m^2. Plants were drip irrigated as needed by means of perforated hoses positioned along each row, and hand-weeding was done twice, at the end of October and of February, respectively. Artichoke heads were harvested from mid-March to end of May.

After artichoke harvest was completed, the experimental area was divided into four plots, each composed of 36 plants (three rows of 12 plants each), in two of which the plants, still green, were cut up by a mulcher for stalks and immediately incorporated into the soil by a spading machine. At the same time, both aerial and underground plant biomass was removed from the other two plots. This plant material was used to estimate the amount of biomass incorporated into the soil and its supply in N, P, and K. With this aim, after weighing the fresh biomass, three samples of both aerial and underground part were oven-dried at 75 °C until constant weight, and dry biomass was analyzed for N, P, and K content.

Zucchini squash (cultivar 'Mora Pisana') followed artichoke crop. 'Mora Pisana' was chosen being a particularly appreciated cultivar on the local market, although characterized by poor yield. At the end of July, after soil was harrowed by a rotary harrow, plantlets of zucchini squash with 3-4 leaves were transplanted into the experimental area in three rows 1.3 m apart with a spacing of 1 m along the rows (plant density of 0.77 plants per m^2). Thus, plants were subjected to two soil treatments (with or without artichoke green manure) repeated twice (two plots per treatment, each plot composed of 36 plants). Zucchini fruits were harvested with attached flowers from September to October excluding, in each plot, the first and the last plant of each row, and their number and weight were recorded.

After the removal of zucchini residue, soil was harrowed again and arranged in beds in order to be prepared for lettuce crop. Plantlets of butterhead lettuce (cultivar 'Cambria') with 4-5 leaves were transplanted into the beds in mid-December in three rows per bed with a 0.3 x 0.3 m spacing within a bed, and 0.5 m between the beds, resulting in a density of about 7.1 plants per m^2. In March, when lettuce heads reached a satisfactory firmness, 30 plants per plot were collected and weighed for yield assessment.

Before zucchini transplant and after lettuce harvest, soil samples were collected at 10-20 cm soil depth and analyzed for pH, electrical conductivity (EC), organic C, total N (Kjeldahl method), available P (Olsen method), and exchangeable K.

Zucchini and lettuce production data and soil data were subjected to analysis of variance (ANOVA) according to a randomized block design with two replicates, and means were compared using the LSD test at $p \leq 0.05$ level of significance.

3 RESULTS AND DISCUSSION

Artichoke produced on average 8.5 heads per plant from mid-March to the end of May (1.5 heads in March, 4 in April and 3 in May), corresponding to 64,450 heads ha^{-1}. Average weight per head was 115 g in March, 110 g in April, and 100 g in May. Although head production per plant, expressed as both number and weight, was consistent with data previously reported for seed-propagated artichoke (Ierna and Mauromicale, 2004; Tesi and Lenzi, 2005a), total yield (7.09 t ha^{-1}) was lower than that observed in the cited studies, where higher plant densities were adopted. Total yield was lower also than the average artichoke yield usually recorded in Italy (about 10 t ha^{-1}) (INEA, 3013), but since in many cases the species is used as a multi-year crop, it must be considered that, in those cases, higher yields are balanced by the costs due to annual agronomic practices like lateral shoot removal at the beginning of a new growing season.

After head harvest, a fresh biomass of 50.3 t ha^{-1} remained usable as green manure (Table 2). The dry biomass yield (10.4 t ha^{-1}, of which 76.6 % represented by aerial parts) was comparable or even higher than that produced by species or their

mixes normally used as cover crops (Snapp et al., 2005).

Nutrient value of green manures especially concerns N (Gaskell and Smith, 2007). Nitrogen supplied to soil by cover crops or plant residues depends on biomass production and biomass N concentration, that vary considerably among and even within species. According to different authors, N accumulation in legume cover crops may range from 28 to 238 kg ha^{-1} (Griffen and Hesterman, 1991; Honeycutt et al., 1995; Ranells and Wagger, 1996; Creamer and Baldwin, 2000; Lenzi et al., 2009), and 23 to 348 kg ha^{-1} N were recorded in different grass cover crops (Honeycutt et al., 1995; Ranells and Wagger, 1996; Creamer and Baldwin, 2000; Braz et al., 2004; Crusciol and Soratto, 2009; Lenzi et al., 2009). Variations in biomass production or N content within the same species may be ascribed to differences in soil fertility and/or climatic conditions (Crusciol and Soratto, 2009; Lenzi et al., 2009). Among vegetable residues, the lowest N amounts have been observed in spinach and radish (about 10 kg ha^{-1}) and the highest in Brussel sprouts (260 kg ha^{-1}); anyway, values over 100 kg ha^{-1} are considered high (Tesi and Lenzi, 2005b). In our study, artichoke accumulated 104.4 kg ha^{-1} N in plant residues, that contained also 50.0 kg ha^{-1} P_2O_5 and 156.8 kg ha^{-1} K_2O (Table 2).

Table 2: Biomass and N, P, and K supply from artichoke plant residues

Plant Residue	Fresh biomass (t ha^{-1})	Dry biomass (t ha^{-1})	N supply (kg N ha^{-1})	P supply (kg P_2O_5 ha^{-1})	K supply (kg K_2O ha^{-1})
Aerial part	43.6	8.0	77.3	38.4	146.7
Roots	6.7	2.4	27.1	11.6	10.1
Total	50.3	10.4	104.4	50.0	156.8

Of course, for nutrients to be released in the soil, green manure must undergo mineralization, whose rate depends on residue quality and quantity, soil moisture and temperature, and specific soil factors such as texture, mineralogy and acidity, biological activity, and already present nutrients (Myers at al., 1994). Part of the released nutrients is temporarily immobilized by soil microbes (Jarvis et al., 1996), or, in the case of N, possibly lost through leaching during irrigation or rainy periods (Gaskell and Smith, 2007). Therefore, estimating and predicting the timing of green manure mineralization as well as the amount and timing of nutrient recovery by the subsequent catch crops is very difficult.

In our study, before zucchini transplant artichoke residues were presumably still undecomposed. In fact, no differences in soil chemical parameters were observed between soil supplied with green manure and soil from which plant residues were removed (Table 3).

Table 3: Effect of artichoke green manure (AGM) on soil chemical parameters

Treatment	pH	EC (dS m^{-1})	Organic C (g kg^{-1})	Total N* (g kg^{-1})	Available P** (mg P_2O_5 kg^{-1})	Exchangeable K (mg K_2O kg^{-1})
before zucchini transplant						
With AGM	7.09 a	0.24 a	12.6 a	1.5 a	211.7 a	187.8 a
Without AGM	7.11 a	0.24 a	11.1 a	1.4 a	199.3 a	155.4 b
after lettuce harvest						
With AGM	7.97 a	0.23 a	11.9 a	1.4 a	194.5 a	180.0 a
Without AGM	8.03 a	0.25 a	11.4 a	1.3 b	175.5 b	137.0 b

Values on the same column followed by different letters are significantly different at $p \leq 0.05$
*Kjeldahl method, ** Olsen method

However, zucchini squash and lettuce crops that followed artichoke seemed to take advantage from green manure, as they showed a significant increase in yield (17 % in squash and 19 % in lettuce) when artichoke residues were incorporated into the soil as compared with when they were removed (Table 4). Squash yield increase was due to the production of a higher number of fruits per plant, while fruit weight was the same with and without green manure (Table 4). With green manure, lettuce produced heavier heads (Table 4). An increasing effect of green manure on lettuce yield was observed also by Thorup-Kristensen (2006) when lettuce followed hairy vetch and winter rye crops. Yield increases in vegetable species following green manure crops were also reported for potato (Honeycutt et al., 1996), tomato (Thönnissen et al., 2000b; Sainju et al., 2001; Lenzi et al., 2009), broccoli (Wyland et al., 1996; Burket et al., 1997), carrot and cabbage (Thorup-Kristensen, 2006). The result depended on the used cover crop, and was mainly ascribed to its nutrient supply capacity.

The nutrient supply capacity of artichoke green manure was not completely depleted by zucchini and lettuce crops. In fact, after lettuce harvest soil supplied with artichoke residues still showed higher amounts in total N, available P, and exchangeable K than soil from which plant residues were removed (Table 3).

Table 4: Production of zucchini squash and lettuce cultivated in sequence after artichoke whose residues were cut and incorporated into the soil (with artichoke green manure = with AGM) or removed from the field (without artichoke green manure = without AGM)

	Zucchini squash				Lettuce	
Treatment	Fruits per plant (n)	Fruit weight (g per fruit)	Fruit weight (kg per plant)	Yield (t ha^{-1})	Head weight (g)	Yield (t ha^{-1})
With AGM	18.2 a	65.6 a	1.2 a	9.2 a	333.2 a	23.7 a
Without AGM	15.3 b	66.8 a	1.0 b	7.9 b	279.7 b	19.9 b

For each crop, values on the same column followed by different letters are significantly different at $p \leq 0.05$

4 CONCLUSIONS

Seed-propagated artichoke produced abundant plant residues, comparable to the biomass obtained by the species most usually employed as cover crops, and accumulating similar nutrient amounts. Unlike cover crops, that cover the soil for several months without producing a direct income, artichoke produced, in a 10-month cycle, about 7 t ha^{-1} of saleable heads. This yield, as well as the residue amount, could be possibly increased by increasing plant density.

Green manure obtained by artichoke plant residues had an increasing effect on yield of succeeding zucchini squash and lettuce. Furthermore, a residual positive effect on soil fertility was still detected after lettuce harvest, when soil supplied with artichoke residues showed higher amounts in total N, available P, and exchangeable K than soil from which plant residues were removed.

Therefore, this study suggests that, in Mediterranean area, seed artichoke can be profitably introduced in organic vegetable systems with a double role of cash and cover crop.

5 ACKNOWLEDGEMENTS

This research was founded by Regione Toscana (Project AGRIBIO). Authors thank all the staff of the Rottaia Experimental Station of Pisa University in San Piero a Grado (Pisa) for their precious collaboration.

6 REFERENCES

Braz A.J.B.P., Silveira P.M., Kliemann H.J., Zimmermann F.P. 2004. Acumulação de nutrients em folhas de milheto e dos capins braquiária e mombaça. Pesquisa Agropecuária Tropical, 34: 83-87.

Burket J.Z., Hemphill D.D., Dick R.P. 1997. Winter cover crops and nitrogen management in sweet corn and broccoli rotations. HortScience, 32: 664-668.

Cabilovski R.R., Manojlovic M.S., Bogdanovic D.M., Rodic V., Bavec M. 2011. Fertilization economy in organic lettuce production. Journal of Sustainable Agriculture, 35: 745-756; DOI: 10.1080/10440046.2011.606491

Calabrese N., De Palma E., Bianco V.V. 2004. Yield and quality of new commercial seed grown artichoke hybrids. Acta Horticulturae, 660: 77-82.

Calabrese N., De Palma E., Bianco V.V. 2005. Yield and quality of seed propagated artichoke hybrid cultivars grown for four years. Acta Horticulturae, 681: 135-142.

Calabrese N., Elia A., Sarli G. 2000. Yield and quality of new artichoke cultivars propagated by seed. Acta Horticulturae, 371: 189-193.

Ciancolini A., Alignan M., Pagnotta M.A., Miquel J., Vilarem G., Crinò P. 2013. Morphological characterization, biomass and pharmaceutical compounds in Italian globe artichoke genotypes. Industrial Crops and Products, 49: 326-333; DOI: 10.1016/j.indcrop.2013.05.015

Clark A. (ed.). 2012. Managing Cover Crops Profitably. Third Edition. Sustainable Agriculture Research and Education (SARE) program. Handbook Series Book 9. University of Maryland, College Park, USA.

Creamer N.G., Baldwin K.R. 2000. An evaluation of summer cover crops for use in vegetable production systems in North Carolina. HortScience, 35: 600-603.

Crusciol C.A.C., Soratto R.P. 2009. Nitrogen supply for cover crops and effects on peanut grown in succession under a no-till system. Agronomy Journal, 101: 41-46; DOI: 10.2134/agronj2008.0054

Davies A., Titterington A.J., Cochrane C. 1995. Who buys organic food? A profile of the purchasers of organic food in Northern Ireland. British Food Journal, 97: 17-23; DOI: 10.1108/00070709510104303

Dimitri C., Greene C. 2000. Recent growth patterns in the U.S. organic foods market. Agriculture Information Bulletin, 777: 1-9.

EC DG for Agriculture and Rural Development. 2013. Facts and figures on organic agriculture in the European Union.

Gaskell M., Smith R. 2007. Nitrogen sources for organic vegetable crops. HortTechnology, 17: 431-441.

Griffen T.S., Hesterman O.B. 1991. Potato response to legume and fertilizer nitrogen sources. Agronomy Journal, 83: 1004-1012; DOI: 10.2134/agronj1991.00021962008300060016x

Hay J. 1989. The consumer's perspective on organic food. Canadian Institute of Food Science Technology Journal, 22: 95-99; DOI: 10.1016/S0315-5463(89)70322-9

Honeycutt C.W., Clapham W.M., Leach S.S. 1995. Influence of crop rotation on selected chemical and physical soil properties in potato cropping systems. American Potato Journal, 72: 721-735;DOI: 10.1007/BF02849157

Honeycutt C.W., Clapham W. M., Leach S.S. 1996. Crop rotation and N fertilization effects on growth, yield, and disease incidence in potato. American Potato Journal, 73: 45-61; DOI: 10.1007/BF02854760

Ierna A., Mauromicale G. 2004. Calendario di produzione, resa e caratteristiche dei capolini di ibridi di carciofo a propagazione gamica. Sementi Elette, 4: 37-42.

INEA 2013. Annuario dell'Agricoltura Italiana 2012, Volume LXVI. INEA, Roma, Italy.

Jarvis S.C., Stockdale E.A., Shepherd M.A., Powlson D.S. 1996. Nitrogen mineralization in temperate agricultural soils: processes and measurement.

Advances in Agronomy, 57:187-237; DOI: 10.1016/S0065-2113(08)60925-6

Jolly D.A., Schutz G.H., Diaz-Knauf K.V., Johal J. 1989. Organic foods: Consumer attitudes and use. Food Technology, 43: 60-65.

Lattanzio V., Kroon P.A., Linsalata V., Cardinali A. 2009. Globe artichoke: A functional food and source of nutraceutical ingredients. Journal of Functional Foods, 1: 131-144; DOI: 10.1016/j.jff.2009.01.002

Lenzi A., Antichi D., Bigongiali F., Mazzoncini M., Migliorini P., Tesi R. 2009. Effect of different cover crops on organic tomato production. Renewable Agriculture and Food System, 24: 92-101; DOI: 10.1017/S1742170508002445

Myers R.J.K., Palm C.A., Cuevas E., Gunatilleke I.U.N., Brossard M. 1994. The synchronization of nutrient mineralization and plant nutrient demand. In P.L. Woomer and M.J. Swift (eds.). The Biological Management of Tropical Soil Fertility. Wiley-Sayce Publication, Chichester, UK. p. 81-116.

O'Donovan P., McCarthy M. 2002. Irish consumer preference for organic meat. British Food Journal, 104: 353-370; DOI: 10.1108/00070700210425778

Padel S., Foster C. 2005. Exploring the gap between attitudes and behavior. Understanding why consumers buy or do not buy organic food. British Food Journal, 107: 606-625; DOI: 10.1108/00070700510611002

Ranells, N.N., Wagger M.G. 1996. Nitrogen release from grass and legume cover crop monocultures and bicultures. Agronomy Journal, 88: 777-882; DOI: 10.2134/agronj1996.00021962008800050015x

Raviv M. 2010. Is organic horticulture sustainable? Chronica Horticulturae, 50:7-14.

Sainju U.M., Singh B.P., Whitehead W.F. 2001. Comparison of the effect of cover crops and nitrogen fertilization on tomato yield, root growth, and soil properties. Scientia Horticulturae, 201-214; DOI: 10.1016/S0304-4238(01)00264-3

Shennan C. 1992. Cover crops, nitrogen cycling and soil properties in semi-irrigated vegetable production systems. HortScience, 27: 749-754.

Snapp S.S., Swinton S.M., Labarta R., Mutch D., Black J.R., Leep R., Nyiraneza J., O'Neil, K. 2005. Evaluating cover crops for benefits, costs and performance within cropping system niches. Agronomy Journal, 97: 322-32.

Tesi R. 2010. Orticoltura Mediterranea Sostenibile. Patron Editore, Bologna, Italy.

Tesi R., Lenzi A. 2005a. Spring production of artichoke propagated by seed. Proceedings of IV IC on Artichoke. Acta Horticultureae, 681: 159-162.

Tesi R., Lenzi A. 2005b. Nutrizione azotata per un'orticoltura sostenibile. Italus Hortus, 12: 57-73.

Thönnissen C., Midmore D.J., Ladha J.K., Olk D.C., Schmidhalter U. 2000a. Legume decomposition and nitrogen release when applied as green manures to tropical vegetable production systems. Agronomy Journal, 92: 253-260; DOI: 10.2134/agronj2000.922253x

Thönnissen C., Midmore D.J., Ladha J.K., Holmer R.J., Schmidhalter U. 2000b. Tomato crop response to short-duration legume green manures in tropical vegetable systems. Agronomy Journal, 92: 245-253; DOI: 10.2134/agronj2000.922245x

Thorup-Kristensen K. 2006. Root growth and nitrogen uptake of carrot, early cabbage, onion and lettuce following a range of green manures. Soil Use and Management, 22: 29-38; DOI: 10.1111/j.1475-2743.2005.00012.x

Thorup-Kristensen K., Magid J., Jensen L.S. 2001. Catch crops and green manures as biological tools in nitrogen management in temperate zones. Advances in Agronomy, 79: 227-302; DOI: 10.1016/S0065-2113(02)79005-6

Werner J., Alvensleben R.V. 1984. Consumer attitudes towards organic food in Germany. Acta Horticulturae, 155: 221-227.

Wyland L.J., Jackson L.E., Chaney W.E., Klonsky K., Koike S.T., Kimple B. 1996. Winter cover crops in a vegetable cropping system: Impacts on nitrate leaching, soil water, crop yield, pests and management costs. Agriculture, Ecosystems and Environment, 59: 1-17; DOI: 10.1016/0167-8809(96)01048-1

Yiridoe E.K., Bonti-Ankomah S., Martin R.C. 2005. Comparison of consumer perceptions and preference toward organic versus conventionally produced foods: A review and update of the literature. Renewable Agriculture and Food Systems, 20: 193-205; DOI: 10.1079/RAF2005113

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.07
Agrovoc descriptors: rivers, alluvial soils, cambisols, soil types, sedimentation, soil classification, soil salinity, site factors, chemicophysical properties

Agris category code: p32

Soil of the lower valley of the Dragonja river (Slovenia)

Tomaž PRUS[1], Nina ZUPANČIČ[2], Helena GRČMAN[1]

Received January 26, 2015; accepted February 25, 2015.
Delo je prispelo 26. januarja 2015, sprejeto 25. februarja 2015.

ABSTRACT

Soil of the lower valley of the river Dragonja developed under specific soil-forming factors. Soil development in the area was influenced by alluvial sediments originating from surrounding hills, mostly of flysch sequence rocks, as a parent material, Sub-Mediterranean climate and the vicinity of the sea. Different soil classification units (Gleysol and Fluvisol) were proposed for that soil in previous researches. The aim of our study was the evaluation of morphological, chemical and mineralogical characteristics of soil, based on detailed soil description and analyses, and to define the appropriate soil classification units. Field examinations revealed that the soil had a stable blocky or subangular structure and did not express substantial hydromorphic forms. Soil pH value was ranging from 6.9 to 7.5. In most locations electroconductivity (ECe) did not exceed 2 ds/m. Base saturation was high (up to 99 %), with a majority of Ca^{2+} ions. Exchangeable sodium percentage (ESP) was ranging from 0.2 to 3.8 %, which is higher compared to other Slovenian soils but does not pose a risk to soil structure. Soil has silty clay loam texture with up to 66 % of silt. Prevailing minerals were quartz, calcite and muscovite/illite. No presence of swelling clay mineral montmorillonite was detected. According to Slovenian soil classification, we classified the examined soil as alluvial soil. According to WRB soil classification, the soil was classified as Cambisol.

Key words: soil classification, soil properties, mineralogical characteristics, salinity

IZVLEČEK

TLA SPODNJEGA DELA DOLINE REKE DRAGONJE (SLOVENIJA)

Tla spodnjega dela doline reke Dragonje so se razvila pod vplivom specifičnih tlotvornih dejavnikov. Rečni sediment iz kamnin flišnega porekla kot matična podlaga, submediteransko podnebje in prisotnost morja so vplivali na razvoj tal. Predhodne raziskave tal na tem območju so tla različno poimenovale; uvrščale so jih bodisi med oglejena bodisi obrečna tla. Namen naše raziskave je bil na osnovi natančnega opisa morfoloških latnosti tal in kemičnih ter mineraloških analiz podati predlog poimenovanja tal. Ugotovili smo, da imajo tla obstojno poliedrično ali oreškasto strukturo in ne izkazujejo intenzivnih hidromorfnih oblik. pH je od 6.9 do 7.5. Na večini vzorčnih mest elektrokonduktivnost nasičenega vzorca tal ne presega 2 ds/m. Zasičenost z bazami je visoka (do 99 %), prevladujejo kalcijevi ioni. Izmenljivi delež Na je od 0.2 to 3.8 %. Tekstura je meljasto glinasto ilovnata, z deležem melja do 66 %. Prevladujoči minerali v tleh so kremen, kalcit in muskovit/illit. Nabrekljivih glinenih mineralov (montmorillonita) nismo ugotovili. Na osnovi slovenske klasifikacije tla uvrščamo med obrečna tla, po WRB klasifikaciji med kambična tla.

Ključne besede: klasifikacija tal, lastnosti tal, mineraloška sestava, slanost

1 INTRODUCTION

Soil of the lower Dragonja river valley formed on alluvial deposits of weathered flysch. The majority of the recent sediments were deposited by the River Dragonja, cutting its riverbed along the contact between flysch rocks and limestone, discharging in the sea near Sečovlje in the form of a minor delta (Pleničar et al., 1973a; 1973b). X-ray diffraction analyses of recent sediment underlying

[1] Biotechnical faculty, p.p.2995, 1001 Ljubljana, helena.grcman@bf.uni-lj.si

[2] Faculty of natural sciences and engineering, Department of Geology, Aškerčeva 12, 1000 Ljubljana

the Sečovlje saltpans showed prevalence of quartz and low Mg-calcite over clay minerals (illite and chlorite group minerals) and minor content of feldspars and dolomite (Ogorelec et al., 1981). The recent sediments of Sečovlje Draga lie over Eocene flysch rocks, where up to 15 cm thick calcarenite beds interchange with marls. Sharp contact between them is at 40 m depth. Sediment pollen analyses showed that Holocene forest vegetation was continuously thermophile, indicating that the entire sediment has been deposited in postglacial times, i.e. less than 10,000 years ago. In the past, the Dragonja River flow has been much more turbulent and able to deposit large amounts of sediment in short periods of time. According to calculations, the average rate of sedimentation was 2.9 cm per year. Such a quantity of sediment material could be explained only by postglacial tectonics, e.g. gradual subsidence of Sečovlje coast, along which the Dragonja River delta has been simultaneously filled up (Ogorelec et al., 1981). Recent climate is Sub-Mediterranean. Average annual temperature and precipitation rate for the period 1971-2000 were 12.8 °C and 931.2 mm, respectively (Slovenian environment agency, 2014).

According to the Soil map of Slovenia 1:25.000, soil of the lower valley of the Dragonja River are characterised as alluvial soil (pedocartographic unit 1086, Figure 1). Earlier studies, which have been made for intended hydromeliorations (Bašić, 1976), reported gleyic properties in the soil, which was thus classified as gley soil. In the study "Soils of the Slovenian Coastal region" (Stepančič et al., 1984) the soil of the investigated area was also classified as gley soil. The renaming of the recognized type to alluvial occurred later, at the time of digitalization and merging of individual soil maps. Revision of the soil map, charting lower valley of Dragonja River (Soil map Buje), was done by Šporar et al. (1994). The researched area is currently in agricultural land use; vineyards and orchards are prevalent. The exception is the northern part, which is abandoned and in the process of overgrowing due to socio-economic factors. Speculations have been raised for this area about lower soil fertility, salinity and extreme hydromorphic soil properties (Rupreht, 2008). Since the Soil map does not refer to any saline soils in Slovenia, we decided to examine the properties of the soil in more detail. The aim of our research was also to propose an appropriate soil name according to Slovenian and WRB soil classification, taking in account morphological, chemical and mineralogical soil properties.

2 MATERIALS AND METHODS

2.1 Field examination

Research area was examined with soil probing (18 locations) from the surface to the depth of 100-120 cm. Four soil profiles were dug to the depth of 80 to 100 cm and described according to the Guidelines for soil description (FAO, 2006). Field examination was done in March and April 2013, March 2014 and January 2015. Locations of the profiles and probings were identified with GPS and are presented in Figure 1. Soil samples were taken from each soil horizon of the profiles for mineralogical and chemical analyses, and from three different depths of soil probes (0-30 cm, 30-60 cm and 80-100 cm) for measurement of electroconductivity.

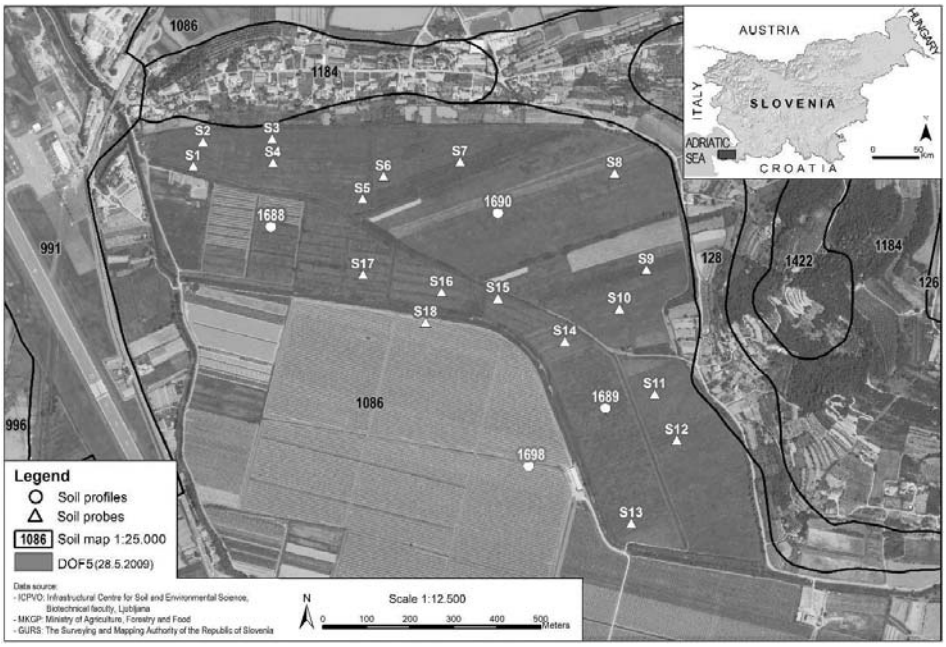


Figure 1: Locations of soil profiles and probes and information of Soil map of Slovenia 1: 25.000. Soils of the research area belong to pedocartographic unit 1086, which consists of two pedosystematic units: 60 % alluvial soil, eutric, deep, on loamy alluvium and 40 % alluvial soil, eutric, deeply gleyed, on loamy alluvium. Soils of surroundings are: pedocartographic unit 128 – Eutric brown soil, on Eocene flysch, colluvial; pedocartographic unit 1184 – Eutric brown soil, on Eocene flysch, calcareous, shallow and pedocartographic unit 991 – Urban area

2.2 Soil analyses

For analysis, soil samples were air-dried and sieved to 2 mm (ISO 11464, 2006). Soil pH was measured in a 1/2.5 (v/v) ratio of soil and 0.01 M $CaCl_2$ suspension (ISO 10390, 2005). Organic matter content was determined by modified Walkley–Black titrations (ISO 14235, 1998), soil texture by the pipette method (ISO 11277, 2009), carbonate content volumetrically after soil reaction with HCl (ISO 10693, 1995), easily extractable P (P_2O_5) and K (K_2O) colorimetrically according to Egner-Riehm-Domingo (ÖNORM L 1087, 1993). Cation exchange capacity (CEC) was determined as a sum of base cations measured after soil extraction with ammonium acetate (pH = 7) and extractable acidity determined with $BaCl_2$ method (Soil Survey laboratory methods manual, 1992). Results are shown in table 1.

Soil salinity was evaluated with tree parameters: electroconductivity of saturated sample (ECe), exchangeable sodium percentage (ESP) and Sodium adsorption ratio (SAR). ECe was calculated from electroconductivity of soil extract (ECw) measured in a 1/5 (v/v) ratio of soil and deionised water (ISO 11265), using factor 9. Sodium adsorption ratio was calculated as a ratio between concentrations of Na^+ versus Ca^{2+} and Mg^{2+} (equation 1) measured in soil water extract (1:5). Exchangeable sodium percentage was calculated as a ratio between Na and cation exchange capacity (equation 2).

$$SAR = \left(\frac{Na^+}{\frac{1}{2}(Ca^{2+} + Mg^{2+})^{1/2}}\right) \quad \text{equation 1}$$

$$ESP = \left(\frac{Na^+}{CEC}\right) 100 \quad \text{equation 2}$$

Qualitative mineral composition of the non-oriented air dried samples was determined by X-ray diffraction (XRD) using a Philips PW 3830/40 diffractometer equipped with CuKα radiation and a graphite monochromator. The X-ray radiation was generated at a voltage of 40 kV and a current of 30 mA. Data were recorded in the range $2^\circ \leq 2\Theta \leq 70^\circ$. Diffractograms were analysed using the PANalytical X'Pert HighScore software.

3 RESULTS AND DISCUSSION

3.1 Soil morphological characteristics

The four examined profiles were very similar in morphological properties. As a result of trench ploughing, a layer with anthric properties (P horizon) formed in the soil. However, due to abandoned land use and the area regrowing with herbs, weeds, grasses and bushes a less than 10 cm thick pale A surface horizon could be distinguished on the top of the anthric matrix in soil profiles 1688, 1689 and 1690. In these three profiles the effect of trench ploughing was visible at depths between 23 and 42 cm. In profile 1698, which is still in agricultural use, two P horizons formed and extended to a depth of 57 cm. Aric P horizons were followed by two layers of clayey sediments with morphological properties showing an initial transformation from alluvial layer to cambic horizon. At the deepest soil layer gleyic properties were recognized by very weakly expressed mottles with reductimorphic colours (IUSS Working Group WRB, 2014). However, soil probing revealed that the mottles have been almost always detected lower than 80 cm. Reductimorphic colour only appeared in profile 1690 at 110 cm as matrix colour GLEY1 6/5GY (greenish gray) according to Munsell, 2013. In all soil profiles well developed, stable, blocky subangular and angular soil structure was clearly observable. No platy or prismatic structure has been recognised even in the lowest examined horizons.

Table 1: Morphological characteristics of soil

Profile	Horizon	Soil depth (cm)	Colour*	Structure	Consistency when moist	Roots	Pedogenetic forms
1688	A	0 - 6	10YR 4/3	blocky-subangular	friable, sticky	many	-
1688	P	6 - 23	10YR 5/3	blocky	friable, sticky	common	-
1688	I(B)	23 - 50	10YR 6/3	blocky	friable, sticky	few	-
1688	II(B)	50 - 80	10YR 6/4	blocky	friable, sticky	very few	few mottles
1689	A	0 - 8	10YR 5/3	blocky-subangular	friable, plastic	common	-
1689	P	8 - 42	10YR 5/4	blocky-subangular	friable	few	-
1689	I(B)	42 - 65	10YR 5/4	blocky	friable	very few	-
1689	II(B)	65 - 80	2,5Y 6/2	blocky	firm	very few	few mottles
1690	A	0 - 8	10YR 4/3	blocky-subangular	friable, sticky	common	
1690	P	8 - 30	10YR 5/3	blocky	friable	few	
1690	I(B)	30 - 65	2,5Y 4/4	blocky	friable, sticky	very few	
1690	II(B)	65 - 80	2,5Y 4/4	blocky	firm, plastic	very few	
1690	III(Go)	80-110+	2,5Y 5/6, GLEY1 6/5GY	blocky	firm, plastic	no	weak mottles
1698	P1	0-29	10YR 4/4	blocky	firm, frable	many	-
1698	P2	29-57	10YR 4.5/4	blocky	firm, friable	common	-
1698	I	57-80	10YR 5/4	blocky	firm, friable	few	-
1698	II	80-100	10YR 5/4	blocky	firm, friable	no	-

*soil colour was identified using Munsell soil colour chart

3.2 Chemical and physical soil characteristics

Analyses of soil samples confirmed soil homogeneity established already by field observation. Texture was silty clay loam with a high proportion of silt (from 57 to 65 % and very low amount of sand, less than 5 %). Soil pH was neutral to slightly alkaline and reached 7.5 at deepest horizons. High pH values were the result of high content of carbonates, which were in the range from 24.1 to 28.9 % and originated from flysch material. The amount of organic matter decreased with soil depth and varied among profiles due to the different land use. In profile 1698, which was located in the vineyard; P horizon contained 2.4 % of soil organic matter. In profiles 1688, 1689 and 1690, which were located in the abandoned orchard or vineyard, soil organic matter in the humus-accumulative horizons ranged from 4.4 to 7.5 %. Higher content of organic matter is a consequence of overgrowth processes, mostly with grasses and herbal plants. All horizons were rich in plant-available potassium as the result of high contents of clay minerals (illite/muscovite) as well as intensive fertilization. Soil from profiles 1688 and 1689 also had high content of plant-available phosphorus due to fertilization in the past. Cation exchange capacity was high; ranging from 38 to 43 $mmol_c$/100 g soil. The high proportion of clay contributes most to the high CEC. Base saturation was very high, almost 99 %. Among cations Ca^{2+} ions were prevalent (from 88 to 99 %).

Table 2: Chemical and physical soil characteristics

Profile	Horizon	Soil depth	Sand	Silt	Clay	Texture	pH	Org. matter	C	N	C/N	P_2O_5	K_2O	Carbonate
		cm	%	%	%			%	%	%		mg/100g		%
1688	A	0 - 6	< 2	60	39	SCL	7.0	7.5	4.3	0.48	9.1	26.0	63.1	24.1
1688	P	6 - 23	< 2	59	40	SCL	7.2	3.7	2.1	0.32	6.6	11.4	41.3	24.9
1688	I(B)	23 - 50	4	57	39	SCL	7.3	2.2	1.3			3.5	28.1	26.1
1688	II(B)	50 - 80	< 2	60	41	SC	7.4	1.5	0.9			2.9	22.3	26.1
1689	A	0 - 8	5	64	31	SCL	6.9	5.1	3.0	0.41	7.4	35.8	60.7	24.1
1689	P	8 - 42	3	64	33	SCL	7.3	2.1	1.2	0.22	5.4	8.0	37.2	28.6
1689	I(B)	42 - 65	2	64	34	SCL	7.4	1.2	0.7			2.8	20.4	27.8
1689	II(Go)	65 - 80	< 2	61	38	SCL	7.4	1.0	0.6			3.0	20.4	27.4
1690	A	0 - 8	< 2	60	39	SCL	7.0	4.6	2.7	0.37	7.3	4.2	35.0	25.7
1690	P	8 - 30	2	59	39	SCL	7.2	2.3	1.3	0.24	5.4	2.7	25.0	25.3
1690	I(B)	30 - 65	< 2	57	42	SC	7.4	1.2	0.7			1.7	22.8	27.4
1690	II(Go)	65 - 80	< 2	58	41	SC	7.4	1.0	0.6			2.4	21.8	27.0
1698	P1	0-29	2	66	32	SCL	7.2	2.4	1.4	0.13	10.8	9.1	28.9	27.3
1698	P2	29-57	3	65	32	SCL	7.2	2.5	1.4	0.12	11.7	6.6	20.8	27.7
1698	I	57-80	< 2	64	36	SCL	7.4	1.3	0.8	0.07	11.4	2.5	15.7	28.9
1698	II	80-100	2	64	34	SCL	7.5	0.9	0.5	0.06	8.3	2.3	14.2	26.2

3.3 Soil salinity

In most locations electro-conductivity of saturated soil samples (ECe) did not exceed 2 ds/m (Table 3); only in location of Profile 1690 and in deeper soil horizons/layers (probe 2 and 3) ECe exceeded 4 ds/m. Higher ECe at soil depth > 80 cm in the locations of probes 2 and 3 could be explained with inflow of the seawater. Salinity parameters in the profile 1690 could not be properly explained; they might be connected to excavation works for a local water supply. Higher ECe in the upper soil layers compared to the deeper soil layers is more likely the result of fertilization than to negative water balance or capillary action. The researched area has a high water table and due to capillary action water can rise through the soil matrix to the surface. However in winter, when precipitation is much higher than evapotranspiration (Table 5), salts move down through the soil profile. We assume that intensive leaching occurred also in the years 2013 and 2014, due to high precipitation rates (Table 5).

Table 3: Parameters of cation exchange capacity and salinity

Profile	Horizon	Soil depth	Ca	Mg	K	Na	H	CEC	Base saturat.	ESP	SAR	ECe
		cm			$mmol_C/100g$				%	%		dS/m
1688	A	0 - 6	36.20	1.53	1.34	0.09	1.55	40.8	96.1	0.22	0.09	1.88
1688	P	6 - 23	37.68	1.32	1.12	0.08	0.75	41.0	98.0	0.20	0.09	1.43
1688	I(B)	23 - 50	37.85	1.26	0.55	1.25	0.70	41.6	98.3	3.00	1.85	3.77
1688	II(B)	50 - 80	38.35	1.56	0.41	0.81	0.75	41.9	98.1	1.93	1.13	2.52
1689	A	0 - 8	34.61	1.18	1.32	0.08	1.35	38.6	96.4	0.21	0.08	1.88
1689	P	8 - 42	37.03	0.95	0.70	0.67	0.50	39.9	98.7	1.68	1.02	2.52
1689	I(B)	42 - 65	36.85	1.11	0.37	0.33	0.45	39.2	98.7	0.84	0.57	1.52
1689	II(Go)	65 - 80	38.98	1.42	0.43	0.45	0.50	41.8	98.8	1.08	0.84	1.97
1690	A	0 - 8	38.35	1.30	0.72	1.67	1.25	43.3	97.0	3.86	3.75	6.12
1690	P	8 - 30	38.86	1.15	0.49	1.50	0.50	42.5	98.8	3.53	2.31	4.22
1690	I(B)	30 - 65	38.43	1.38	0.46	0.72	0.60	41.6	98.6	1.73	1.39	2.42
1690	II(Go)	65 - 80	39.03	1.66	0.45	1.73	0.60	43.5	98.6	3.98	3.24	4.22
1698	P1	0-29	33.04	1.12	0.65	0.07	0.95	35.9	97.2	0.19	0.10	1.17
1698	P2	29-57	33.71	1.12	0.51	0.09	1.05	36.4	97.3	0.25	0.30	1.17
1698	I	57-80	35.99	1.42	0.37	0.11	1.4	39.3	96.4	0.28	0.29	1.08
1698	II	80-100	34.32	1.49	0.32	0.13	0.55	36.8	98.6	0.35	0.61	1.08

Table 4: Electroconductivity of soil samples from soil probing

Soil probe	Altitude	ECe (dS/m)		
	[m a.s.l.]	0 - 30 cm	30 - 60 cm	> 80 cm
S1	1.4	1.35	1.35	2.52
S2	1.2	1.43	2.88	11.78
S3	1.5	0.90	1.35	3.97
S4	1.5	1.35	1.27	1.27
S5	1.9	1.43	1.43	1.35
S6	1.8	1.35	1.27	1.17
S7	1.5	1.35	1.27	2.78
S8	2.1	1.53	1.27	1.17
S9	2.7	1.35	1.27	1.35
S10	2.7	1.35	1.17	2.25
S11	3.5	1.17	1.08	1.17
S12	4.0	1.35	1.08	1.35
S13	3.9	1.17	1.08	1.27
S14	2.6	1.17	1.17	1.08
S15	2.4	1.27	1.17	1.08
S16	2.4	1.27	1.17	1.17
S17	2.2	1.08	1.43	1.08
S18	2.4	1.27	1.35	1.27

Exchangeable sodium percentage (ESP) and sodium adsorption ratio (SAR) were in the range from 0.2 to 3.8 % and from 0.08 to 3.75 %, respectively. ESP values in some soil horizons were higher compared to other soils in Slovenia where the share of sodium ions on adsorption complex is less than 1 % (Prus, 2007). However negative effects on soil structure are less probable;

ESP below 10 % or SAR below 13 % does not pose a risk to soil structure (Brady and Weil, 2002; Rowell, 1994). Additional protection for the soil structure was probably provided by high content of Ca^{2+} ions in the soil.

Table 5: Average monthly temperatures, precipitation, potential evapotranspiration and water balance for the period 1971-2000 and for the years 2012, 2013 and 2014 (Data source: Slovenian environment agency, 2014)

1971-2000													
Month	Jan	Feb	Mar	Apr	May	June	Jul	Aug	Sept	Oct	Nov	Dec	Avg/ Total
Average temp.	4.1	4.5	7.4	11.6	16.4	20.1	22.5	21.7	17.6	13.6	8.4	5.1	12.8
Precipit.	56.3	47.1	61.3	65.3	68.8	85.8	57.6	78.1	123.8	120.5	91.3	75.3	931.2
Evapo-transpir.	30	41	66	90	125	142	163	149	98	64	38	29	1035
Water balance	26.3	6.1	-4.7	-24.7	-56.2	-56.2	-105.4	-70.9	25.8	56.5	53.3	46.3	-103.8
2012													
	Jan	Feb	Mar	Apr	May	June	Jul	Aug	Sept	Oct	Nov	Dec	Avg/ Total
Average temp.	3.5	1.5	9.9	12.8	16.6	22.7	25.5	24.7	19.8	14.9	11.7	5.0	14.0
Precipit.	20.1	20.6	0.1	50.4	117.2	35.1	6.9	36.5	96.5	88.2	145.2	72.9	689.7
Evapo-transpir.	34.7	50.9	88.1	88.5	134.2	161.0	200.3	178.3	103.8	58.4	37.9	25.6	1161.7
Water balance	-14.6	-30.3	-88.0	-38.1	-17.0	-125.9	-193.4	-141.8	-7.3	29.8	107.3	47.3	-472.0
2013													
	Jan	Feb	Mar	Apr	May	June	Jul	Aug	Sept	Oct	Nov	Dec	Avg/ Total
Average temp.	5.6	4.8	7.4	13.2	16.5	20.5	24.3	23.2	18.9	15.3	1.4	6.9	13.2
Precipit.	89.6	99.2	166.2	75.1	118.5	63.8	5.2	53.1	77.8	95.3	190.2	21.1	1055.1
Evapo-transpir.	25.4	40.1	51.6	92.5	112.9	160.9	194.9	179.6	106.4	53.3	43.3	30.4	1091.3
Water balance	64.2	59.1	114.6	-17.4	5.6	-97.1	-189.7	-126.5	-28.6	42	146.9	-9.3	-36.2
2014													
	Jan	Feb	Mar	Apr	May	June	Jul	Aug	Sept	Oct	Nov	Dec	Avg/ Total
Average temp.	9.4	9.8	10.8	13.9	16.2	21.6	21.7	21.5	17.9	15.4	13.0	7.8	14.9
Precipit.	87.6	171.7	47.4	124.1	89	55	264.7	94.5	208.5	115.4	139.3	65.2	1462.4
Evapo-transpir.	24.2	33.2	79.2	81.5	119.8	160.3	134.3	131.4	83	60.4	30.2	28.8	966.3
Water balance	63.4	138.5	-31.8	42.6	-30.8	-105.3	130.4	-36.9	125.5	55	109.1	36.4	496.1

3.4 Mineralogical characteristics of soil

All the soil samples from different profiles and horizons consisted of the same minerals. Prevailing minerals were quartz, calcite, and muscovite/illite (Table 6). Small amount of plagioclases and vermiculite/chlorite group minerals were present in some samples. Muscovite and illite could not be distinguished with certainty due to their similar structure, and vermiculite/chlorite due to their low quantity. A semi-quantitative sample composition estimated by X'Pert HighScore software was controlled and calibrated by measurement of carbonate content (Table 2).

Table 6: Mineralogical characteristics of soil: estimated mineral content in %, minerals presented in traces are marked with *

Profile	Horizon	Quartz	Calcite	Muscovite/Illite	Vermiculite/Chlorite	Plagioclase
1688	P1	40	30	30	*	*
1688	P2	30	30	30	*	10
1688	I	40	20	35	5	
1688	II	45	35	20	*	*
1689	A	45	30	15	*	10
1689	P	40	30	15		15
1689	I	30	35	25		10
1689	II	45	35	15	5	*
1690	A	45	35	17	3	
1690	P	40	35	20	*	5
1690	I	30	40	15	5	10
1690	II	35	35	30		
1698	P1	45	25	20		10
1698	P2	50	30	15		5
1698	I	40	30	15	5	10
1698	II	40	30	20	*	10

Diffractograms of different horizons from the same profile (Figure 2) clearly show that not only mineral composition but also ratios between minerals are similar. The influence of soil depth on mineral composition is minimal. Comparison of samples from the upper soil horizons (P2) of all profiles exhibits the same similarity (Figure 3), which indicates to the same soil forming factors for all the research area. The presence of swelling clay minerals, especially of montmorillonite, a member of the smectite group, was checked by careful examination of the XRD pattern. The presence of swelling clay mineral montmorillonite could not be confirmed in any of the soil samples. There is a possibility that the peak of montmorillonite was overlapped with vermiculite/chlorite, but even in that case the amount of montmorillonite would be small.

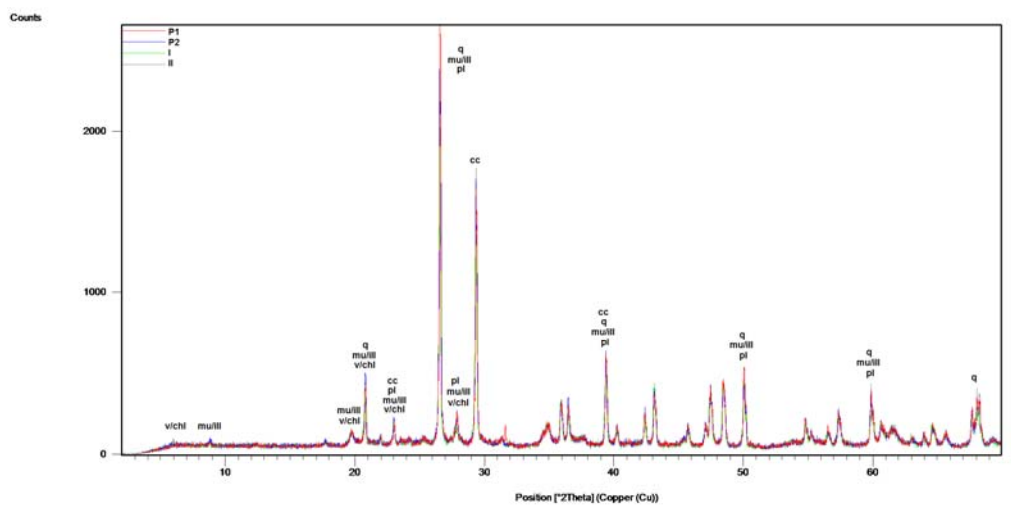


Figure 2: Diffractograms for the profile 1698. Peaks of minerals are labelled with following abbreviations: v/chl – vermiculite/chlorite, mu/ill – muscovite/illite, q – quartz, cc- calcite, pl – plagioclase

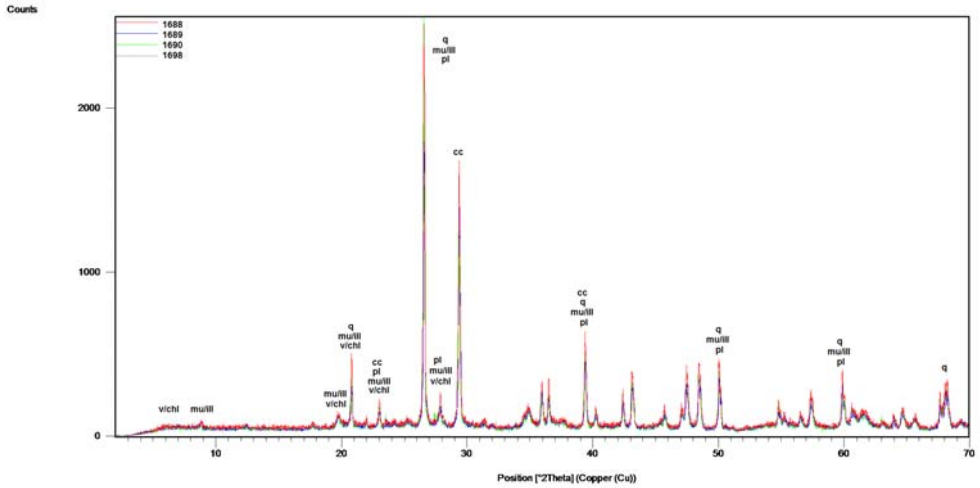


Figure 3: Diffractograms for the P2 horizons of profiles 1688, 1689, 1690 and 1698. Figure 2: Diffractograms for the profile 1698. Peaks of minerals are labelled with following abbreviations: v/chl – vermiculite/chlorite, mu/ill – muscovite/illite, q – quartz, cc – calcite, pl – plagioclase

3.5 Soil classification

Soil was evaluated with the criteria of Slovenian Soil Classification (SSC, Soil map of Slovenia 1:25.000) and World reference base for soil resources (IUSS Working Group WRB, 2014). Three classification units of SSC and reference groups of WRB were discussed: (i) Alluvial soil/Fluvisol, (ii) Gleyic soil/Gleysol (iii) Cambic soil/Cambisol. Both soil classification systems have different criteria as well as different terminology to describe the same soil properties.

The researched soil developed on the fine sediments of the river Dragonja, consequently the first idea was to classify it as Fluvisol. Fluvisols (IUSS Working Group WRB, 2014) have fluvic material ≥ 25 cm thick and starting ≤ 25 cm from the mineral soil surface; or from the lower limit of a plough layer that is ≤ 40 cm thick, to a depth of ≥ 50 cm from the mineral soil surface. Fluvic material refers to fluviatile, marine and lacustrine sediments that receive fresh material or have received it in the past and still show stratification. By the definition of WRB (IUSS Working Group WRB, 2014) stratification is evidenced by a layer that has ≥ 0.2 % soil organic carbon and has a content of soil organic carbon ≥ 25 % (relative) and ≥ 0.2 % (absolute) higher than in the overlying layer.

Alluvial soils by the definition of SSC develop on alluvial material and express either no or weak development of pedogenetic alteration. Soil horizons are difficult to recognise and soil layers are characterised and marked with roman numerals. The researched soil developed on silty clayey fluvic material with more than 0.2 % organic carbon and expresses weak development of pedogenetic alteration, i.e, a well-developed soil structure. We conclude that soil could be classified as alluvial soil according to SSC. However, the criterion of stratification for fluviatile material by WRB (IUSS Working Group WRB, 2014) is not entirely fulfilled as described above and as a consequence Fluvisol is not the appropriate reference group for the subject soil.

Soil materials develop gleyic properties if they are saturated with groundwater (or were saturated in the past, if now drained) for a period that allows reducing conditions to occur. The vicinity of the river Dragonja and fine sediments as the parent material are factors that might lead to development of Gleysols. According to WRB (IUSS Working Group WRB, 2014) Gleysols have a layer ≥ 25 cm thick and starting ≤ 40 cm from the mineral soil surface, that has gleyic properties throughout and reducing conditions in some parts of every sublayer. Gleyic properties comprise of one or both of the following: (i) layer with ≥ 95 % (exposed area) having colours considered to be reductimorphic (Munsell colour hue (moist) of N, 10Y, GY, G, BG, B, PB, or 2.5Y or 5Y with a chroma of ≤ 2, or (ii) a layer with > 5 % (exposed

area) mottles, the colour of which is considered to be oximorphic.

Slovenian soil classification defines Gleysols similarly as defined by WRB (IUSS Working Group WRB, 2014); a hydromorphic surface humus horizon (Aa) must be present at depths less than 50 cm and followed immediately with both gleyic horizons (Go, Gr). Gr must start within 100 cm from the soil surface (Škorić, 1986). The researched soil did not express gleyic horizons starting ≤ 40 cm from the mineral soil surface. In profile 1689, few mottles occur (Go horizon) at 80 cm. Munsell colour hue is mostly 10 YR. Soil from the profile 1690 has Munsell colour hue 2.5Y with the chroma (4/4 or 5/6). Such colour values could be linked to flysch material. In the study "Soils of the Slovenian Coastal region" (Stepančič et al., 1984) reported about several Cambisols developed on flysch material with a Munsell colour hue 2.5Y and chroma >2. Reductimorphic colour GLEY1 6/5GY has been found as matrix colour in the layer at 110 cm soil depth. Generally less intensive expression of gleyic properties in alluvial soils could be explained by the findings of Stepančič (Matičič, 1984), who reported that ground water, due to strong fluctuations, still contains plenty of oxygen and therefore the oxidation and reduction processes in the soil profile are much less pronounced. Nevertheless, due to the morphological properties of the studied soil, it cannot be classified as Gleysol neither according to WRB nor SSC.

Soil profile structure with humus-accumulative or aric topsoil horizon and mineral subsurface is characteristic for cambic soil. Cambisols (IUSS Working Group WRB, 2014) have a cambic horizon starting ≤ 50 cm from the soil surface and having its lower limit ≥ 25 cm from the soil surface. The cambic horizon is a subsurface horizon showing evidence of pedogenetic alteration that ranges from weak to relatively strong. If the underlying layer has the same parent material, the cambic horizon usually shows higher oxide and/or clay contents than this underlying layer and/or evidence of removal of carbonates (at least ≥ 5 % by mass, absolute, fine earth fraction). The pedogenetic alteration of a cambic horizon can also be established by contrast with one of the overlying mineral horizons that are generally richer in organic matter and therefore have a darker and/or less intense colour. In this case, some soil structure development is needed to prove pedogenetic alteration.

Cambic soils, by the SSC definition consist of cambic horizon, which is a mineral soil horizon with well-expressed pedogenetic forms with less than 1 % organic matter. The soil in our research expressed homogeneity in most soil properties (clay content, carbonate content) but the stratification is evident in organic matter content and colour; organic matter content in the upper soil layer is higher. However, almost all soil layers have more than 1 % organic matter. Even though it was difficult to distinguish between alluvial and soil material, soil structure showed evidence of pedogenetic alteration.

Considering all discussed criteria Cambisol is the appropriate reference group for the subject soil according to WRB (IUSS Working Group WRB, 2014).

4 CONCLUSIONS

The researched soil has a silty clay loam texture with a high amount of silt. Soil structure is blocky or subangular with high aggregate stability. High amount of calcium carbonate content contributes to high aggregate stability. Soil has neutral or slightly alkaline pH, among base cations Ca^{2+} ions prevail (up to 99 %). Soil does not express intensive hydromorphic forms; few mottles occur only deeper in the soil profile. Exchangeable sodium percentage is ranging from 0.2 to 3.8 %. In most locations electroconductivity (ECe) does not exceed 2 ds/m; this happens only in some locations and in deeper soil layers, where ECe values exceed 4 ds/m; however in absence of morphological characteristic for salt affected soils (structure, concrete...) the soil could not be characterised as saline soil.

Measured ECe and ESP values in soil from the lower valley of Dragonja are higher compared to other soils in Slovenia. In general, higher precipitation rates in Slovenia favour elluvial-

illuvial processes and development of leached soils. Therefore, soils in the lower valley of the Dragonja River are rare and important for soil diversity in Slovenia.

Prevailing minerals in the soil are quartz, calcite and muscovite/illite. Plagioclase and vermiculite/chlorite were found in small amounts. The presence of swelling clay mineral montmorillonite could not be confirmed in any of the soil samples.

According to WRB soil classification, and based on morphological, chemical and mineralogical analyses, soil of the researched area could be classified as Calcaric Cambisol (aric, siltic). According to the Slovenian national soil classification, two soil types could be determined: (i) alluvial soil, calcaric and (ii) alluvial soil, calcaric, deeply gleyic.

5 ACKNOWLEDGEMENTS

This work was funded by Slovenian Research Agency, Research Programme P4−0085.

6 REFERENCES

Bašić I. 1976. Pedološko-melioraciona studija doline Dragonje i Drnice. Poljoprivredni inštitut Zagreb, Inštitut za pedologiju i poljoprivredne melioracije Zagreb: 60 p.

Brady N.C., Weil R.R. 2002. The Nature and Properties of Soil. 13th edition. Pearson Education Inc: 960 p.

ISO 10390, 2005. Soil Quality-Determination of pH. International Organization for Standardization, Genève, Switzerland.

ISO 10693, 1995. Determination of Carbonate Content. Volumetric Method. International Organization for Standardization, Genève, Switzerland.

ISO 11277, 2009. Determination of Particle Size Distribution in Mineral Soil Material-Method by Sieving and Sedimentation. International Organization for Standardization, Genève, Switzerland.

ISO 11464, 2006. Soil Quality-Pretreatment of Samples for Physico-chemical Analysis. International Organization for Standardization, Genève, Switzerland.

ISO 13878, 1987. Determination of Total Nitrogen Content by Dry Combustion ("Elemental Analysis"). International Organization for Standardization, Genève, Switzerland.

ISO 14235, 1998. Soil Quality- Determination of Organic Carbon by Sulfochromic Oxidation. International Organization for Standardization, Genève, Switzerland.

ISO 11274, 1998. Soil quality — Determination of the water retention characteristic — Laboratory methods. International Organization for Standardization, Genève, Switzerland.

IUSS Working Group WRB. 2014. World Reference Base for Soil Resources 2014.

International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome:181 p.

FAO. 2006. Guidelines for soil description. 4 thedition. Rome: 97 p.

Matičič B. 1984. Izvajanje drenažnih sistemov. UEK v Ljubljani, VDO Biotehniška fakulteta, VTOZD za agronomijo, Katedra za melioracije in urejanje kmetijskega prostora: 358 p.

Munsell soil color charts. 1971 and 2013. Munsel color division. Kollmorgen corporation Baltimore: 7 tabels.

Ogorelec B., Mišič M., Šercelj A., Cimerman F., Faganeli J., Stegnar P. 1981. Sediment sečoveljske soline. Geologija, 24/2: 179–216 p.

ÖNORM L 1087. 1993. Chemische Bodenuntersuchungen Bestimmung von pflanzenverfügbarem Phosphat und Kalium nach der Calcium-Acetat-Lactat (CAL)-Methode, Österreichisches Normungsinstitut: 4p.

Pleničar M., Polšak A., Šikič D. 1973a. Osnovna geološka karta SFRJ Trst 1:100000. Tolmač lista Trst: L 33-88. Beograd. Zvezni geološki zavod Beograd: 68 p.

Pleničar M., Polšak A., Šikić D.. 1973b. Tolmač za list Trst: L 33-88: Socialistična federativna republika Jugoslavija, osnovna geološka karta, 1:100 000: 68 p.

Prus T. Zbijanje in zaslanjevanje tal v Sloveniji. 2007. In: Strategija varovanja tal v Sloveniji (Knapič M. ed): 103-110 p.

Rowell L. D. 1994. Soil science. Methods & Applications. Longman group UK Limited: 350 p.

Rupreht J. 2008. Pedološko poročilo o lastnostih tal na delu Sečoveljske doline: 13 p.

Slovenian environment agency, 2014: http://meteo.arso.gov.si/met/en/climate/ (January, 2015).

Soil map of Slovenia 1:25.000 (1999-2015); Biotechnical Faculty, University of Ljubljana – digital version

Soil Survey laboratory methods manual, 1992. Soil Survey Investigations Report No. 42, Version 2.0.

Stepančič D., Lobnik F., Prus T., Rupreht J. 1984. Tla slovenskega primorja. Biotehniška fakulteta, VTOZD za agronomijo, Katedra za pedologijo, prehrano rastlin in ekologijo: 47 p.

Škorić A. 1986. Postanak, razvoj i sistematika tla. Fakultet poljoprivrednih znanosti Sveučilišta u Zagrebu: 172 p.

Šporar M., Rupreht J., Lobnik F. (Ur.) 1994. Pedološka karta Slovenije 1:25000. PK4612. Buje. Ljubljana, Biotehniška fakulteta, Agronomija, Center za pedologijo in varstvo okolja. 1 zvd. barv.

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.08

Agrovoc descriptors: vicia faba, faba beans, varieties, crop yield, yield factors, seeds, genotypes, indigenous organisms, seed characteristics, plant breeding, selection, genes, environmental factors, heritability, agronomic characters

Agris category code: f03, f62

Genetic variation for seed yield and some of agro-morphological traits in faba bean (*Vicia faba* L.) genotypes

Peyman SHARIFI[1]

Received March 08, 2014; accepted February 23, 2015.
Delo je prispelo 08. marca 2014, sprejeto 23. februarja 2015.

ABSTRACT

An investigation was carried out to select the most successful faba bean genotype(s) and to estimate the heritability for seed yield and some of agro-morphological traits. The results of analysis of variance indicated that the studied genotypes differed significantly for all of the traits. For 100-seed weight, two north's of Iran landraces (G1 and G2) and two improved breeding cultivars containing France (G4) and Barrakat (G10) possessed the heaviest seed weight, 161.33, 139, 119.67 and 166 g, respectively. G1 and G10 presented the highest values for dry seed weight (473.98 and 495.44 g m^{-2}, respectively). G1 and G10 showed significantly higher magnitude values of the other traits. Broad sense heritability (h^2) estimates were generally high to moderate for all of the studied traits. The highest estimates of broad sense heritability was inscribed as 98 % for pod length, dry seed length and dry seed width and 0.95 for hundred seed weight. The estimated broad-sense heritability was 0.80 for dry seed yield per m^2. These results suggested that the environmental factors had a small effect on the inheritance of traits with high heritability. High estimates of heritability indicated that selection based on mean would be successful in improving of these traits. High heritability indicate an additive gene action for the traits, and hence, possible trait improvement through selection. Path coefficient analysis indicated that the traits containing day to harvesting, pod length, hundred seed weight and number of stems per plant play major role in seed yield determination of faba bean. Attention should be paid to these characters for augmentation of seed yield and these traits could be used as selection criteria in faba bean breeding programs. These findings indicate that selection for each or full of the above traits would be accompanied by high yielding ability under such conditions.

Key words: additive gene action, breeding, faba bean, genetic variation, selection

IZVLEČEK

GENETSKA VARIABILNOST PRIDELKA SEMEN IN NEKATERIH AGRONOMSKO-MORFOLOŠKIH LASTNOSTI GENOTIPOV BOBA (*Vicia faba* L.)

Raziskava je bila opravljena za izbor najdonosnejših genotipov boba z namenom oceniti heritabilnost oz. dednostni delež pridelka semen in nekaterih agronomsko-morfoloških lastnosti. Rezultati dobljeni z analizo variance so pokazali, da se vsi obravnavani genotipi značilno razlikujejo v vseh proučevanih lastnostih. Za maso 100-semen sta se izkazali najboljši dve akcesiji iz severnega Irana, (G1 in G2) in dve izboljšani sorti,'France' (G4) 'Barrakat' (G10), katerih masa je znašala, 161.33, 139, 119.67 in 166 g. Genotipa G1 in G10 sta imela največjo maso suhih semen na enoto površine (473.98 in 495.44 g m^{-2}). Ista genotipa sta izkazala tudi večje vrednosti pri drugih analiziranih lastnostih. Ocene dednostnega deleža v širšem smislu (h^2) so bile na splošno velike do zmerne za vse analizirane lastnosti. Največji delež k splošni dednosti so v vrednosti 98 % prispevali dolžina strokov, dolžina in širina suhega zrna in 0.95 masa stotih semen. Ocenjena splošna dednost za pridelek suhih semen na enoto površine je bila 0.80. Ti rezultati so pokazali, da so imeli okoljski dejavniki majhen vpliv na lastnost z velikim dednostnim deležem. Velike ocene dednostnega deleža so pokazale, da je selekcija, ki bi temeljila na povprečnih vrednostih lastnosti primerna za njihovo izboljšanje. Velik dednostni delež lastnosti označuje aditivni učinek genov in možno izboljšanje lastnosti s selekcijo. Analiza združevanja lastnosti je pokazala, da so imele lastnosti, ki so vključevale število dni do žetve, dolžino stroka, maso stotih semen in število stebel na rastlino največjo vlogo pri določanju pridelka semen boba. V žlahniteljskih programih s selekcijo bi bilo potrebno posvečati pozornost tistim lastnostim boba, ki prispevajo k povečanju pridelka semen. Izsledki te raziskave kažejo, da mora biti izbrana in spremljanja vsaka ali vse analizirane lastnosti, ki imajo vpliv na velikost pridelka v danih razmerah.

Ključne besede: aditivno delovanje genov, žlahtnjenje, bob, genetska variabilnost, selekcija

[1] Department of Agronomy and Plant Breeding, Rasht Branch, Islamic Azad University, Rasht, Iran; email: kadose@yahoo.com

1 INTRODUCTION

Faba bean (*Vicia faba* L.), an autogamous plant with partial outcross ranging from 20 to 80 %, is a diploid plant with a relatively few number of large chromosomes (2n = 2x = 12) (Al-Barri and Shtaya, 2013; Basheer et al., 2013; Terzopoulos et al., 2008). It is originated in between the oriental Mediterranean countries and Afghanistan (Cubero, 1974) and most commonly included in the diets of inhabitants of the Middle East, the Mediterranean region, China and Ethiopia, and it can be used as a vegetable, green or dried, fresh or canned (Bond et al., 1985). The species is genetically isolated, tolerating no exchange of genes with any other species including its close relative *Vicia narbonensis* (Hawtin and Hebblethwaite, 1983). Genetic variability of faba bean is quite large. The great part of variability may be due to the presence of intermediate crossing system between autogamy and allogamy (Hanelt and Mettin, 1989).

Yield improvement is a major breeding objective of most crop improvement programs in faba bean. The success of an autogamous plant breeding program depends on the choice of genotypes capable of producing progeny with desired trait combinations. Yield in faba bean, similar to the other crops, is a complex trait and constitute by many of morphological and physiological traits that correlated each other. Plant height, number of stems and pods per plant, biological yield, harvest index, 100-seed weight, days to flowering and maturity are the most important traits in faba bean improvement for increasing seed yield due to direct and indirect correlation with seed yield (Loss and Siddique, 1997)

The genetic improvement of crop desired traits depends on the nature and magnitude of genetic variability and interactions involved in the inheritance of these traits which can be estimated using experimental design techniques. Many researchers studied heritability for seed yield, yield components and the other agro-morphological traits in faba bean. Seed yield is a complex trait that is quantitatively inherited with low heritability value (Bond, 1966). The low heritability and consequent limited genetic advance for yield in response to selection had led many scientists to search for characters which are associated with yield but which are more highly heritable (De Pace, 1979). Toker (2004) grown eight faba bean genotypes at in order to estimate the broad-sense heritability, and the heritability for plant height, number of stems and pods per plant, seed yield, biological yield, 100-seed weight, days to flowering and maturity estimated as 83, 63, 43, 62, 52, 99, 97 and 97 %, respectively. Alghamdi (2007) carried out a research in order to determine the genetic behavior of six faba bean genotypes and the results revealed that the studied genotypes significantly differed for all of the traits including plant height, number of pods per plant, number of seeds per plant, seed weight per plant and seed yield. The highest estimates of broad sense heritability were obtained for flowering date (0.986), number of pods per plant (0.96), number of seeds per plant (0.957) and maturity date (0.905), respectively. Hanna and Hayes (1966) showed low heritability for number of pods per plant (0.24), number of seeds per plant (0.23) and seed weight (0.46), respectively. El-Kadi (1968) indicated that the broad-sense heritability ranged from 6.6 to 52.1 % for seed yield, 48.1 to 65.1 % for number of stems per plant, 42.8 to 63.9 % for number of pods per plant, 51.6 to 62.2 % for plant height and 48.0 to 86.2 % for seed index. El-Kady and Khalil (1979) revealed that broad-sense heritability estimates ranged from 36.2 to 90.6 %, 10.6 to 50.9 % and 27.1 to 62.0 % for seed yield, number of seeds per plant and seed weight per plant, respectively. Abo El-Zahab et al. (1980) studied broad-sense heritability for number of pods per plant, number of seeds per pod, seed weight per plant and seed yield. Their findings indicated that heritability values were 88.4, 99.9, 84.3 and 21.3 % respectively. Bora et al. (1998) stated that the high heritability was followed by high genetic advance for fruiting stems per plant, number of pods per plant and seed yield per plant, indicating the scope for their improvement through selection. Ibrahim (2010) indicated narrow-sense heritability was high for 100-seed weight and low for seed yield per plant. Kalia and Sood (2004) revealed high broad-sense heritability estimates (0.97) along with high genetic advance (126 %) for pod yield.

The present investigation aimed at the agronomic performance of ten faba bean genotypes in order to employ the most successful genotype(s) in a breading program. Heritability *via* variance

components for seed yield and some of agro-morphological traits were also determined on faba bean genotypes in lowland of the north region of Iran.

2 MATERIALS AND METHODS

This study was carried out during 2011 and 2012 in Shanderman, Iran (longitude, 49° 55′ E; latitude, 37° 27′ N; altitude, 71 m above sea level; climate, wet). Experimental material comprised 10 genotypes of faba bean that some of their features is given in Table 1.

2.1 Experimental field area

The sowing of seeds was conducted in 8 December 2011 by hand. Field experiments were conducted in a randomized complete block design with three replications. Each plot consisted of four rows with 6 m long and distance between rows was 50 cm. The seeding rate was 15 plants per m^2. Forty five kilogram nitrogen, phosphorus and potassium per hectare were applied as compose fertilizer including 15 kg ha^{-1} from any of fertilizers (15-15-15) prior to sowing. All recommended agronomic practices were followed to raise good crop.

The following statistical model was adopted for experimental design:

$$Y_{ij} = \mu + G_i + R_j + \varepsilon_{ij}$$

Where,
μ: general mean; R_j: effect of j^{th} block (j = 1, 2, 3); G_i: effect of i^{th} genotype (i = 1, 2 ... 10) and e_{ijk}: experimental Error.

2.2 Estimated characters

Fifteen plants of each plot were harvested by hand and weight of seed and pod per plant were measured before and at physiological maturity stage for all of the genotypes. The characters *via* day to pod initiation (DP), day to harvesting (DH) and plant height (PH) were also calculated before harvesting. The remainder of plants in each plot were harvested by hand at harvest maturity stage and pod length (PL), number of dry seeds per pod (DSP), dry seed length (LS), dry seed width (SW), hundred seed weight (HSW), number of stems per plant (StP), number of pods per plant (PP), number of pods per stem (PSt), number of seeds per stem (SSt) and number of seeds per pod (SP) were measured on ten plants selected randomly from all plots. Dry seed yield per m^2 (SY) and dry seed weight per plant were weighed in maturity harvesting stage.

2.3 Statistical analysis

The analysis of variance was carried out according to Steel and Torrie (1980) in data after collecting and means comparison of any traits in genotypes were performed by the least significant difference (LSD) test.

The genotypic and phenotypic variances (σ_g^2 and σ_p^2) were calculated from the partitioning mean squares expectation (Table 2) as follows:

$$\sigma_g^2 = \frac{MSg - MSe}{r}$$

$$\sigma_p^2 = \sigma_g^2 + \sigma_e^2 / r$$

Where, g and r are number of genotype and replication, respectively; σ_p^2, σ_g^2 and σ_e^2 are components of variance for phenotypic, genotypes and error, respectively. Broad sense heritability (h^2_B) was estimated as: genotypic variance/phenotypic variance (Roy**,** 2000):

$$H^2 = \frac{\sigma_g^2}{\sigma_p^2}$$

Entire analyses were performed with the Statistical Analysis System (SAS) Software and Excel.

GGE (genotype main effect plus genotype-by-environment interaction) biplots are commonly used to analyze two-way data where rows and columns represent different experimental units (e.g. genotypes and traits). The mean values for genotypes across traits are used to form a

symmetrical data matrix from which the first two PC are extracted (Yan and Hunt 2002). All biplots presented in this paper were generated using the software GGE biplot package that runs in a windows environment, an earlier version of which was described in Yan (2001). GGE-biplot was carried out on seven genotypes (g1, g2, g3, g5, g6, g9 and g10) and three remained genotypes (g4, g7 and g8) were excluded from biplot analysis according to their similarity to the other genotypes.

Table 1. Information of some of important traits on studied genotypes

Genotype number	Genotype name	Origin	Breedng status	Seed structure
1	-	North of Iran (Guilan)	Autochthonous landrace	Large
2	-	North of Iran (Mazandaran)	Autochthonous landrace	Large
3	France	France	Improved breeding cultivars	Intermediate
4	Filip3	Syria	Improved breeding cultivars	Small
5	Filip5	Syria	Improved breeding cultivars	Small
6	-	Lorestan (Khorramabad)	Autochthonous landrace	Small
7	-	Lorestan (Borujerd1)	Autochthonous landrace	Small
8	-	Lorestan (Borujerd2)	Autochthonous landrace	Small
9	-	Lorestan (Borujerd3)	Autochthonous landrace	Small
10	Barrakat	Iran/ Gurgaon	Improved breeding cultivars	Large

3 RESULTS AND DISCUSSION

3.1 Analysis of variances

Significance of mean squares due to different sources of variability for studied traits is summarized in Table (2). Results indicated that the studied genotypes differed significantly for all of the traits except plant height, weight of pod per plant before physiological maturity stage, number of stem per plant, number of pod per plant and ratio of pod weight per plant before physiological maturity stage.

3.2 Genotypes' mean performance

The mean performances for different traits of ten faba bean genotypes are given in Table (3). Mean comparison were performed by least significant differences (LSD). Data revealed that the genotype 5 and 8 possessed the earliest pod initiation (140.33 day) and maturity date (167.67 day), respectively. On the other hand, G9 and G1 took 146 and 175.33 days to pod initiation and maturity date, respectively. Respecting to plant height, G10 possessed the tallest plants (88.5 cm) whereas, G2 presented the shortest plants (76.21 cm). With regards to pod length, G10 owned the tallest pods (15.49). On the other side, G6 possessed the shortest ones (5.65). For weight of seed per plant before physiological stage, G2 revealed heaviest seed weight (33.22 g). For ratio of seed length to width, G1 showed the highest ratio (1.51). For number of stems per plant, number of pods per plant and number of pods per stem, G1 recorded 4.46, 12.19 and 2.77, respectively. Results indicated that the G10 possessed the highest values for number of seeds per pod, dry seed length, dry seed width, weight of pod per plant at physiological maturity stage, weight of seed per plant at physiological maturity stage, weight of pod per plant before physiological maturity stage, hundred seed weight, number of seed per plant and

number of seed per stem. For the ratios of seed per pods before and at the physiological stages and ratio of seeds per plant before physiological stage G9 had the highest ratio values 0.59, 0.64 and 0.27, respectively. G2 possessed the highest values of pod per plant ratio before physiological stage. For 100-seed weight, G1, G2, G3 and G10 possessed the heaviest seed weight 161.33, 139, 119.67 and 166 g, respectively. On the other hand, the other genotypes possessed the lowest values. G1 and G10 presented the highest value for dry seed yield, 473.98 and 495.44 g m^{-2}, respectively. On the other hand, G7 exhibited the lowest value for dry seed yield (106.63 g m^{-2}). From the above mentioned results, it could be deduced that G10 followed by G1 indicated the tallest plants and pods, highest number of pods, seeds, seeds weight per plant and dry seed yield (g m^{-2}). These results express that the selection prospects within this genotype to improve the performance through breeding program.

Table 2: Analysis of variance for some of morphological traits in some of faba bean genotypes

SOV	df	DP	DH	PH (cm)	PL (cm)	DSP	LS (cm)	WS (cm)
R	2	0.4ns	24.7*	38.75ns	1.09ns	0.04ns	0.008ns	0.0008ns
G	9	16.03**	18.01*	88.49^{ns}	28.80**	1.82**	0.806**	0.35**
E	18	3.18	5.63	116.86	0.7	0.15	0.02	0.007
CV		1.24	1.39	13.45	10.23	11.44	8.92	7.35

SOV	df	SY (g/m^2)	WPo/Pl(ph) (g/ plant)	WSe/Pl(ph) (g/ plant)	WPo/Pl(bph) (g/ plant)	WSe/Pl(bph) (g/ plant)	HSW (g)	L/W
R	2	5661.45ns	458.88ns	111.73ns	0.033**	0.0068ns	295.43ns	0.0053ns
G	9	63438.15**	8173.14**	1527.88**	0.009^{ns}	0.0106*	7671.87**	0.0152**
E	18	12450.91	932.6	271.28	0.011	0.0039	409.14	0.0029
CV		47.66	41.39	45.20	24.08	36.37	22.52	3.94

SOV	df	StP	PP	PSt	SSt	Se/Po(Ph)	Se/Po(bPh)	Se/Pl(bph)	Po/Pl(bph)
R	2	0.03ns	20.29ns	0.77ns	42.18*	0.0022ns	0.014ns	0.0068ns	0.03ns
G	9	1.23^{ns}	14.68^{ns}	1.25**	28.85*	0.005**	0.068**	0.0106*	0.0089^{ns}
E	18	0.77	10.98	0.39	9.9	0.0011	0.013	0.0039	0.0103
CV		24.98	38.15	24.77	43.6	6.5	27.68	36.37	24.08

SY: Dry Seed Weight per m^2; DP: Day to Pod initiation; DH: Day to Harvesting; PH: Plant Height; PL: Pod Length; LS: Dry Seed Length; WS: Dry Seed Width; DSP: Number of Seed per Plant; WPo/Pl(ph): Weight of Pod per Plant at Physiological maturity Stage; WSe/Pl(ph): Weight of Seed per Plant at Physiological maturity Stage; WPo/Pl(bph): Weight of Pod per Plant before Physiological maturity Stage; WSe/Pl(bph): Weight of Seed per Plant before Physiological maturity Stage; HSW: Hundred Seed Weight; L/W: Ratio of Seed Length to Width; StP: Number of Stem per Plant; PP: Number of Pod per Plant; SP: Number of Seed per Plant; PSt: Number of Pod per Stem; SSt: Number of Seed per Stem; Se/Po(Ph): Ratio of Seed Weight per Pod at Physiological maturity Stage; Se/Po(bPh): Ratio of Seed Weight per Pod before Physiological maturity Stage; Se/Pl(bph): Ratio of Seed Weight per Plant before Physiological maturity Stage; Po/Pl(bph): Ratio of Pod Weight per Plant before Physiological maturity Stage.

ns, not significant

*, significant at the 0.05 probability level

**, significant at the 0.01 probability level

Table 3: Averaged performance for some of morphological traits in faba bean genotypes

Genotype number	DP	DH	PH (cm)	PL	DSP	LS (cm)	WS (cm)
1	142.67	175.33	86.33	8.70	2.40	2.30	1.52
2	140.67	172.33	76.21	9.80	3.22	2.12	1.51
3	141.00	173.33	77.33	10.17	3.46	1.82	1.38
4	144.33	171.33	77.67	5.93	3.12	1.13	0.81
5	140.33	170.00	74.33	8.07	3.46	1.55	1.21
6	144.67	169.67	84.83	5.65	2.76	1.18	0.90
7	147.00	171.00	74.25	6.30	3.37	1.17	0.85
8	144.33	167.67	85.25	5.85	2.81	1.13	0.86
9	146.00	168.00	76.79	5.81	3.43	1.14	0.80
10	142.00	173.33	88.50	15.49	5.30	2.37	1.66
Mean	143.30	171.20	80.15	8.18	3.33	1.59	1.15
SE	0.731	0.774	1.717	0.979	0.246	0.164	0.108
LSD(1%)	4.19	5.57	25.41	1.97	0.89	0.34	0.19

Genotype number	SY (g/ m^2)	WPo/Pl(ph) (g/ plant)	WSe/Pl(ph) (g/ plant)	Wpo/Pl(bph) (g/ plant)	WSe/Pl(bph) (g/ plant)	HSW (g)	L/W
1	315.9867	128.00	64.60	100.89	26.11	161.33	1.51
2	162.2253	92.80	47.37	114.00	33.22	139.00	1.40
3	204.0678	68.97	32.22	51.33	11.44	119.67	1.32
4	107.4393	56.23	30.27	70.78	27.56	48.67	1.39
5	88.15763	61.27	27.83	38.33	14.67	65.33	1.29
6	112.1333	34.83	18.73	33.67	17.89	48.00	1.30
7	71.08951	35.22	19.78	50.83	29.00	52.87	1.38
8	87.13008	33.07	19.13	31.56	17.22	50.00	1.32
9	82.35512	33.48	19.43	25.33	18.56	47.33	1.43
10	330.2933	193.97	85.03	175.89	30.67	166.00	1.42
Mean	156.0878	73.78	36.44	69.26	22.63	89.82	1.38
SE	45.985	16.506	7.136	15.133	2.382	15.995	0.022
LSD(1%)	262.25	71.77	38.71	0.23	0.15	47.54	0.13

Genotype number	StP	PP	SP	PSt	SSt	Se/Po(Ph)	Se/Po(bPh)	Se/Pl(bPh)	Po/Pl(bPh)
1	4.46	12.19	43.11	2.77	9.22	0.50	0.27	0.11	0.41
2	4.31	9.69	43.44	2.19	8.19	0.51	0.34	0.17	0.50
3	3.31	5.18	15.67	1.67	4.48	0.47	0.23	0.10	0.44
4	2.76	10.27	33.11	3.72	11.32	0.54	0.41	0.19	0.48
5	2.64	6.03	12.11	2.35	5.64	0.45	0.39	0.18	0.45
6	3.86	8.67	16.44	2.19	4.08	0.56	0.52	0.18	0.35
7	3.23	10.69	23.39	3.19	6.79	0.57	0.53	0.23	0.42
8	3.17	7.04	17.33	2.18	5.18	0.56	0.56	0.21	0.37
9	3.24	9.58	17.33	3.03	4.36	0.59	0.64	0.27	0.35
10	4.16	7.50	50.78	1.85	12.91	0.44	0.19	0.09	0.47
Mean	3.51	8.68	2.95	2.51	7.22	0.52	0.41	0.17	0.42
SE	0.203	0.700	0.426	0.204	0.980	0.017	0.048	0.018	0.017
LSD(1%)	2.06	7.78	3.12	1.46	7.4	0.08	0.27	0.15	0.24

SE: Standard error; LSD: Least significant differences

3.3 Genetic parameter

Estimates of phenotypic and genotypic variances and broad sense heritability from the partition of mean squares are presented in Table (4). Data indicated that the extent of phenotypic and genotypic variances varied from trait to another.

Broad sense heritability (h^2) estimates were generally high to moderate for all of studied traits. The highest estimates of broad sense heritability was inscribed as 98 % for pod length, dry seed length and dry seed width and 0.95 for hundred seed weight. The estimated broad-sense heritability was 0.80 for dry seed yield per m^2. These results suggested that the environmental factors had a small effect on the inheritance of traits with high heritability. High estimates of heritability indicated that selection based on mean would be successful in improving these traits. The lowest value of h^2 was observed for number of stems and pods per plant with 037 and 0.25, respectively. The estimated traits before maturity stage containing weight of pod per plant at physiological maturity stage, weight of seed per plant at physiological maturity stage and weight of seed per plant before physiological maturity stage had moderate heritability. The moderate value of heritability for number of pods per stem, number of seeds per stem and number of seeds per pod were 0.69, 0.66 and 0.68, respectively. In agreement with the results of this work, Toker (2004) recorded high to moderate heritability for traits containing days to flowering and maturity, 100-seed weight and seed yield and low heritability for number of stems and pods per plant. The results of this research are also similar to the results of Alghamdi (2007), Hanna and Hayes (1966), El-Kadi (1968), El-Kady and Khalil (1979), Bora et al. (1998), Ibrahim (2010) and Kalia and Sood (2004). The magnitude of heritability was affected by the type of genetic material and yield level of environment due to the fact that the plant height, number of stems and pods per plant, dry seed yield, 100-seed weight, days to pod initiation and maturity of plants are created by the effects of genes and environment.

Table 4: Estimated some of genotypic and phenotypic parameters for studied traits in faba bean

Traits	Genotypic variance	Phenotypic variance	Broad-sense Heritability
DP	4.28	5.34	0.80
DH	4.13	6.00	0.69
PL (cm)	9.37	9.60	0.98
DSP	0.56	0.61	0.92
LS (cm)	0.26	0.27	0.98
WS (cm)	0.11	0.12	0.98
SY (g/m^2)	16995.75	21146.05	0.80
WPo/Pl(ph)	2413.51	2724.38	0.89
WSe/Pl(ph)	418.87	509.29	0.82
WSe/Pl(bph)	0.0022	0.0035	0.63
HSW (g)	2420.91	2557.29	0.95
L/W	0.0041	0.0051	0.81
StP	0.15	0.41	0.37
PP	1.23	4.89	0.25
PSt	0.29	0.42	0.69
SSt	6.32	9.62	0.66
SP	1.23	1.82	0.68

3.4 Path coefficient analysis

The results of correlation coefficients between seed yield per m^2 and were positively significant *via* the traits containing DH, PL, HSW, L/W, NStPl, NSeSt and NSePl. The significant negative correlation coefficient was detected between seed yield and days to pod initiation.

The advantage of path analysis is that it permits the partitioning of the correlation coefficient into its components. One component is the path coefficient (or standardized partial regression coefficient) that measures the direct effect of a predictor variable upon its response variable. The other component is the indirect effect(s) of a predictor variable on the response variable through the predictor variables (Dewey and Lu, 1959). The results showed that the coefficient of determination were 54 % (Table 5). It represents the influence of the traits involved in the study on total variability of dry seed yield. Path coefficient analysis indicated that the traits containing day to harvesting (p=0.244), pod length

(p=0.303), hundred seed weight (p=0.206) and number of stems per plant (p=0.216) play major role in seed yield determination of faba bean. This result concur with Alghamdi (2007) detected significant positive correlations between seed yield and each of number of pods per plant, number of seeds per plant, seed weight per plant and biological yield. In confirming with we results, Berhe et al., (1998) indicated the number of seeds per plant and 100-seed weight were the major direct contributors to seed yield per plant. These results also agree with those of Tadesse et al. (2011) and Ulukan et al. (2003) that found out days to maturity, number of pod per plants, seed per pod, thousand seed weight and plant height, pod length, and grain number per pod had high positive direct effect on seed yield per plot.

Table 5: Path coefficients for seed yield components in faba bean. The diagonal under line numbers is direct effects of any trait on seed yield

Traits	DP	DH	PH	PL	HSW	L/W	StP	SSt	SP	Overall effects (r)
DP	-0.019	0.005	0.003	0.011	0.009	-0.003	0.007	0.003	0.008	-0.43
DH	-0.073	0.244	0.059	0.121	0.144	0.099	0.107	0.132	0.115	0.650
PH	-0.006	0.007	0.032	0.009	0.004	0.004	0.016	0.008	0.010	0.330
PL	-0.166	0.151	0.089	0.303	0.229	0.068	0.120	0.149	0.236	0.697
HSW	-0.098	0.122	0.027	0.156	0.206	0.075	0.095	0.080	0.125	0.719
L/W	0.014	0.037	0.013	0.020	0.033	0.092	0.020	0.044	0.028	0.372
NStPl	-0.084	0.095	0.111	0.085	0.099	0.049	0.216	0.062	0.077	0.578
NSeSt	0.006	-0.017	-0.008	-0.015	-0.012	-0.015	-0.009	-0.032	-0.026	0.453
NSePl	0.002	-0.002	0.001	0.004	0.003	0.002	0.002	0.005	0.006	0.581
		R^2=0.54						$\sqrt{1-R^2}$ =0.56		

The symbol of traits is the same as in Table 2

3.5 Biplot analysis

The polygon view of the biplot will be displayed (Figure 1). This view helps identify cultivars with the highest values for one or more traits. The scores of three traits containing PH, NStPl and LW fell in the genotype 10 sector, suggesting that this cultivar had highest or near-highest values for these three traits. Genotype 1 had the highest values for PH, NStPl and L/W. Since the biplot did not explain all the variation, these predictions may not exactly reflect the observed numbers. Nonetheless, cultivars that are among the top with regard to a trait can be identified with confidence. Yan and Kang (2003) demonstrated the numerous utilities of genotype and genotype-by-environment (GGE) biplot in visual analysis of genotype-by-trait data for evaluating cultivars based on traits and comparing cultivars as packages of traits. Sharifi and Safari Motlagh (2011) and Sharifi (2012) were also used biplot techniques for analysis the data obtained from diallel crosses in rice. Mohammadi and Amri (2011) examined the performance of 13 durum (*Triticum turgidum* L. var. durum Desf.) genotypes on the basis of multiple traits by biplot method.

The tester vectors (Figure 2) are the lines that originate from the biplot origin and reach markers of the traits. Since the cosine of the angle between the vectors of any two traits approximates the correlation coefficient between them, this view of the biplot is best for visualizing the interrelationship among traits. Figure 2 suggests close positively associations among PL, L/W, HSW, DH, NStPl and dry seed yield per m^2. Plant height indicated a weak but positive correlation with dry seed yield. GGE biplot were also used for identifying traits that are closely associated with, and therefore can be used in indirect selection for, a target trait (Yan and Kang, 2003). Oladejo et al. (2011) used biplot method to determine the interrelationships among physiological traits of thirty cowpea cultivars and identify suitable traits for indirect selection for improved crop yield.

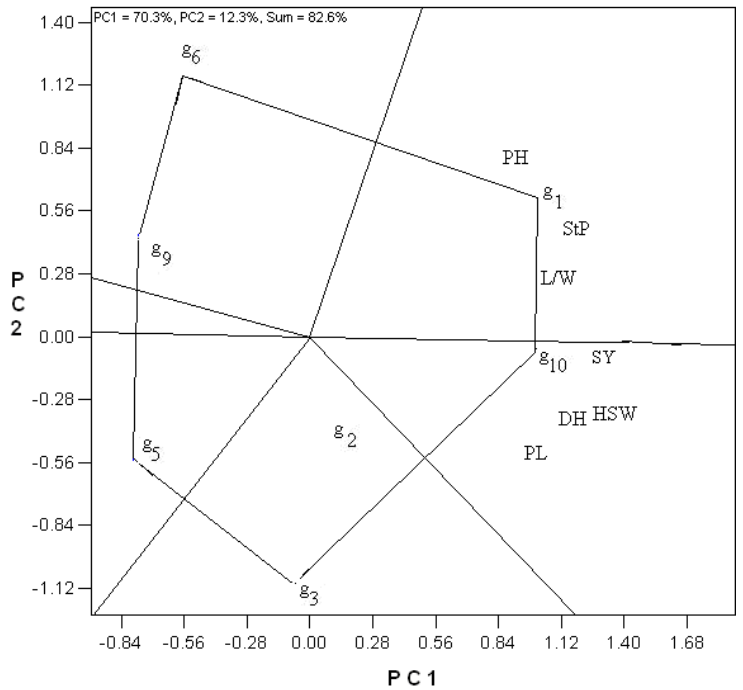


Figure 1: Biplot analysis indicating polygon view for some of traits in faba bean genotypes
g1: Autochthonous landrace (Guilan); g2: Autochthonous landrace (Mazandaran); g3: France; g5: Filip5; g6: Autochthonous landrace (Khorramabad); g9: Autochthonous landrace (Borujerd); g10: Barrakat

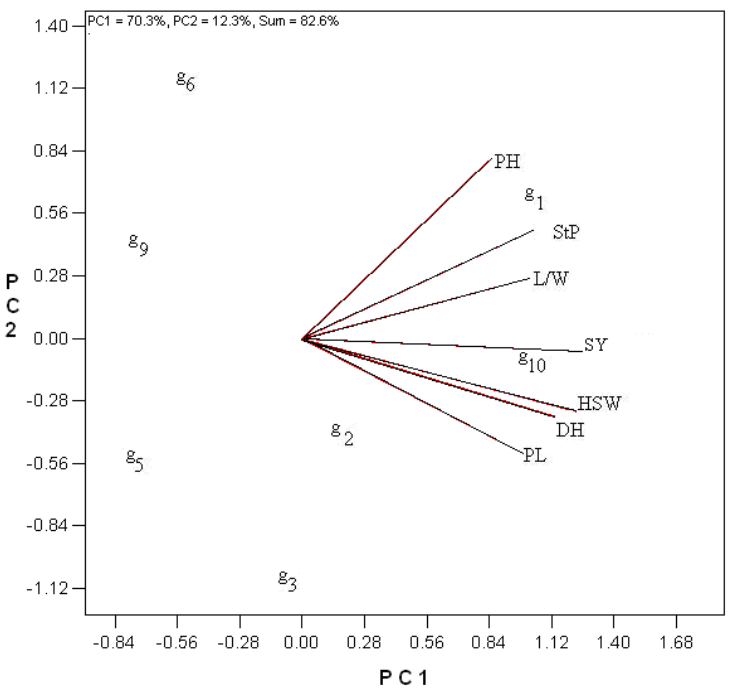


Figure 2: Biplot analysis indicating relationships between some of traits in faba bean genotypes.
g1: Autochthonous landrace (Guilan); g2: Autochthonous landrace (Mazandaran); g3: France; g5: Filip5; g6: Autochthonous landrace (Khorramabad); g9: Autochthonous landrace (Borujerd); g10: Barrakat

4 CONCLUSION

This study indicated that agro-morphological traits were significantly differed in the investigated faba bean genotypes. Among the 10 faba bean genotypes used in this study, G1 and G10 showed significantly higher magnitude values than other genotypes. G1 and G10 are an autochthonous landrace from Guilan, Iran and improved breeding variety, naming Barrakat, respectively. The variability among the faba bean genotypes was expected because of their different origins. Heritability of large number of traits was high and moderate. High estimates of heritability indicated that selection based on mean would be successful in improving these traits. High heritability indicate an additive gene action and, hence, possible trait improvement through selection. Some of genotypes such as G5, G7, G8 and G9 can be crosses with G1 and G10 and used their offspring's for breeding programs such as QTL mapping, diallel analysis and generation mean analysis.

5 ACKNOWLEDGMENTS

This work was the part of first author's research project "Study yield and some of agronomic traits in faba bean (*Vicia faba* L.) genotypes on Guilan and Lorestan province" which supported by "Islamic Azad University, Rasht Branch, Youngh Researchers Club" in Guilan province.

6 REFERENCES

Abo-El-Zahab A.A., Ashor A.M., El-Hadeedy K.H. (1980). Comparative analysis of growth, development and yield of five field bean cultivars (*Vicia faba* L.) Z. Acker-und Pflanzenbau. 149: 1-13.

Alghamdi S.S. (2007). Genetic Behavior of Some Selected Faba Bean Genotypes. African Crop Science Conference Proceedings. 8: 709-714.

Al-Barri T., Shtaya M.J.Y., (2013). Phenotypic characterization of faba bean (Vicia faba L.) landraces grown in Palestine. Journal of Agricultural Science; 5: 110-117.

Basheer-Salimia R., Shtaya M., Awad M., Abdallah J., Hamdan Y. (2013). Genetic diversity of Palestine landraces of faba bean (*Vicia faba*) based on RAPD markers. Genetics and Molecular Research 12 (3): 3314-3323; DOI: 10.4238/2013.September.3.8

Berhe, A., Bejiga, G., Mekonnen, D., (1998). Associations of some characters with seed yield in local varieties of Faba bean. African Crop Science Journal. 6: 197-204.

Bond D.A., Lawes D.A., Hawtin G.C., Saxena M.C., Stephens J.H. (1985). Faba bean (*Vicia faba* L.). In: Summerfield RJ, Roberts EH (Eds) Grain Legume Crops, Collins, London: 199-265.

Bond D.A. (1966). Yield and components of yield in diallel crosses between inbred lines of winter beans (*Vicia faba*). Journal of Agricultural Science of Cambridge. 67: 335-336; DOI: 10.1017/S0021859600017329

Bora G.C., Gupta S.N., Tomer Y.S., Singh S. (1998). Genetic variability, correlation and path analysis in faba bean (*Vicia faba*). Indian Journal of Agricultural Sciences. 68(4): 212-214.

Cubero, J.I. (1974). The evolution of *vicia faba*. Theoretical and Applied Genetics, 45: 47-51; DOI: 10.1007/BF00283475

De Pace C. (1979). Characteristics with significant correlation to seed yield and broad bean population grown in Southern Italy. In: Semi Current research on *Vicia faba* in Western Europe. Ed BABND, GR Scarascia Mugnozza and M.H. Poulsen, Pub. EECEUR, 6244 En., Luxembourg: 144-167.

Dewey D.R., Lu K.H. (1959). A correlation and path coefficient analysis of components of crested wheat grass seed production. Agronomy Journal. 51: 515–518; DOI: 10.2134/agronj1959.00021962005100090002x

El-Kady D.A. (1968). Genotypic and phenotypic variability and their implications in Vicia faba L. breeding. M.Sc. Thesis, Cairo University: 138 p.

El-Kady M.A.K., Khalil S.A. (1979). Behaviour of seed yield components in cross between broad bean cultivars and selection for superior yield. Egyptian Journal of Agronomy. 4: 159-170.

Hanelt P., Metin D. 1989. Biosystematics of the genus *Vicia* L. (*Leguminosae*). Annual review of ecological systems, 20: 199-223.

Hanna A.S., Hayes J.D. (1966). Genetic studies in field beans, Vicia faba L. I. Genetic variability in flower, pod and seed characters in a cross between two inbred lines. Z. Pflanzenzuchtg. 56: 285-294.

Hawtin G.C., Hebblethwaite P.D. (1983). The faba bean (Vicia faba L.).: A basis for improvement, (P.D. Hebblethwaite, ed). Butterworths, London, UK. pp 3-22.

Ibrahim H.M. (2010). Heterosis, Combining Ability and Components of Genetic Variance in Faba Bean (*Vicia faba* L.). Meteorology, Environment and Arid Land Agriculture Environmental Science. 21(1): 35-50.

Kalia P., Sood S.H. (2004). Genetic variation and association analysis for pod yield and other agronomic and quality characters in an Indian Himalayan collection of broad bean (*Vicia faba* L.). SABRAO Journal of Breeding and Genetics. 36(2): 55-61.

Loss S.P., Siddique K.H.M. (1997). Adaptation of faba bean (*Vicia faba* L.) to dryland Mediterranean-type environments. I. Seed yield and yield components. Field Crops Research. 52: 17-28; DOI: 10.1016/S0378-4290(96)03455-7

Mohammadi R., Amri A. (2011). Graphic Analysis of Trait Relations and Genotype Evaluation in Durum Wheat. Journal of Crop Improvement. 25: 680–696.

Oladejo A.S., Akinwale R.O., and Obisesan I.O. (2011). Interrelationships between grain yield and other physiological traits of cowpea cultivars. African Crop Science Journal. 19: 189 – 200.

Roy D. (2000). Plant breeding analysis and exploitation of variation. Alpha Science. 798 p.

Sharifi P. (2012). Graphic analysis of salinity tolerance traits of rice (*Oryza sativa* L.) using biplot method. Cereal Research Communications. 40(3): 416–424; DOI: 10.1556/CRC.40.2012.3.3

Sharifi P., Safari Motlagh M.R. (2011). Biplot analysis of diallel crosses for cold tolerance in rice at the germination stage. Crop and Pasture Science. 62(2): 169–176; DOI: 10.1071/CP10207

Steel R.G.D., Torrie J.H. (1980). Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., New York: 633 p.

Tadesse T., Fikere M., Legesse T., Parven A. (2011). Correlation and path coefficient analysis of yield and its component in faba bean *(Vicia faba L.)* germplasm. International Journal of Biodiversity and Conservation. 3: 376-382.

Terzopoulos P.J., Bebeli P.J. (2008). Genetic diversity analysis of Mediterranean faba bean (*Vicia faba* L.) with ISSR markers. Field Crops Research. 108: 39-44; DOI: 10.1016/j.fcr.2008.02.015

Toker C. (2004). Estimates of broad-sense heritability for seed yield and yield criteria in faba bean (*Vicia faba* L.). Hereditas. 140: 222-225; DOI: 10.1111/j.1601-5223.2004.01780.x

Ulukan H., Mustafa G., Siddik K. (2003). A Path Coefficient Analysis Some Yield and Yield Components in Faba Bean *(Vicia faba L.)* Genotypes. Pakistan Journal of Biological Sciences. 6: 1951-1955; DOI: 10.3923/pjbs.2003.1951.1955

Yan W. (2001). GGE biplot – a Windows application for graphical analysis of multienvironment trial data and other types of two-way data. Agronomy Journal 93: 1111–1118; DOI: 10.2134/agronj2001.9351111x

Yan W., Hunt L.A. (2002). Biplot analysis of diallel data. Crop Science. 42: 21–30; DOI: 10.1201/9781420040371

Yan W., Kang M. (2003). GGE Biplot Analysis: A Graphical Tool for Breeders, Geneticists, and Agronomists. CRC Press, 288 pages.

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.09
Agrovoc descriptors: zea mays, maize, soil salinity, site factors, stress, chemicophysical properties, seeds, germinability, genotypes, varieties, photosynthesis, pigments, carotenoids, chlorophyll

Agris category code: f62

The effect of salt stress on the germination of maize (*Zea mays* L.) seeds and photosynthetic pigments

Sali ALIU[1], Imer RUSINOVCI[1], Shukri FETAHU[1], Bekim GASHI[2], Emilija SIMEONOVSKA[3], Ludvik ROZMAN[4]

Received February 12, 2015; accepted March 09, 2015.
Delo je prispelo 12. februarja 2015, sprejeto 09. marca 2015.

ABSTRACT

The objective of this study was to investigate the effect of salinity stress on seed germination and chlorophyll content in maize. In the study, two maize hybrids were included (Bc 678 and Bc 408) originating from the Bc Institute at Rugvica near Zagreb (Croatia) and two maize populations (LMP-1 and LMP-2) originating from Kosovo. The experiment was conducted in four replicates of 100 seeds, which were germinated on top of double-layered papers, each with 10 ml of salt solution of NaCl and $CaCl_2$ in Petri dishes. Germinated seeds were counted every 24 h for 15 days. The photosynthetic pigments, chlorophylls '*a*' and '*b*' as well as carotenoids were extracted with 80 % acetone. Chlorophyll and carotenoid contents were calculated using absorbance values at 662, 644 and 440 nm. The effects of the NaCl and $CaCl_2$ concentrations accounted for a high proportion of the variance in all analyses. The results showed that both germination percentage and germination index decreased significantly in all cultivars at the highest salt concentrations. The significant differences between different concentrations of salinity were also found in all cultivars for the content of chlorophyll '*a*' and '*b*' and for the content of carotenoids.

Key words: maize, salinity stress, germination, NaCl, $CaCl_2$, chlorophyll, carotenoids

IZVLEČEK

VPLIV SLANOSTNEGA STRESA NA KALIVOST IN FOTOSINTEZNE PIGMENTE KORUZE (*Zea mays* L.)

Namen raziskave je bil proučiti vpliv slanosti tal na kalivost zrnja ter vsebnost kolorofila in karotinoidov pri koruzi. V proučevanje sta bila vključena dva hibrida 'Bc 678' and 'Bc 408', vzgojena na Zavodu za koruzo Inštituta za žlahtnjenje rastlin v Zagrebu ter dve domači populaciji (LMP-1 and LMP-2) s Kosova. Poskus je bil izveden v 4 ponovitvah in sicer za kalivost v petrijevkah po 100 zrn, za vsebnost klorofila in karotinoidov pa v lončkih po 2 rastlini z 1 kg substrata. Vsak genotip je bil, poleg kontrole, tretiran s 4 različnimi koncentracijami (50, 100, 200 in 400 mMol NaCl in $CaCl_2$). Kalivost smo ugotavljali prvih 15 dni vsakih 24 ur. Klorofil '*a*' in '*b*' in karotinoide smo ekstrahirali z 80 % acetonom. Vsebnost klorofila in karotinoidov smo računali s pomočjo absorpcijske vrednosti pri 662, 644 and 440 nm. Pri največji slanosti (400 mMol NaCl in $CaCl_2$) je pri vseh kultivarjih ugotovljen statistično značilno manjši odstotek in indeks kalivosti. Prav tako so pri vseh kultivarjih ugotovljene statistično značilne razlike med različnimi koncentracijami slanosti tudi za vsebnost klorofila '*a*' in '*b*' ter vsebnost karotinoidov.

Ključne besede: koruza, slanost tal, kalivost, NaCl, $CaCl_2$, klorofil, karotinoidi

[1] University of Prishtina, Faculty of Agriculture, Department of Crop Science, Prishtina, Kosovo; e-mail: sali.aliu@uni-pr.edu

[2] University of Prishtina, Faculty of Natural Science, Department of Biology, Prishtina, Kosovo; e-mail: bekim.gashi@uni-pr.edu

[3] The Faculty of Agriculture Science and Food, Skopje, Macedonia

[4] University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia; e-mail: ludvik.rozman@bf.uni-lj.si

1 INTRODUCTION

Salinity stress negatively impacts agricultural yields throughout the world, affecting production, whether for subsistence or economic gain. At present, about 20 % of the world's cultivated land and approximately half of all irrigated land and 2.1 % of the dry agriculture land is affected by salinity (FAO, 2000). Salinization is spreading more rapidly in irrigated lands because of inappropriate management of irrigation and drainage. Moreover, rain, cyclones and wind add NaCl to coastal agricultural lands (FAO, 2008). Maize (*Zea mays* L.) is the important cereal crop, providing basic food and oil for human cosumption, as well as feed for livestock throughout the world, but this crop is normally submissive to salt stress. Maize, a plant with a C4 metabolism, is also classified as moderately sensitive to salinity (Katerji et al., 1994). The rapid increase in the world's population requires an expansion of crop areas to raise food production. In this context, a significant part of agricultural crops is cultivated on low quality soils, which are sometimes affected by salinity (Allen et al., 1983). Different strategies for diminishing the adverse effects of salinity stress on plants are currently in practice. Salinity due to the over-accumulation of NaCl is usually of great concern and is the most damaging factor in arid and semi-arid regions. Saline soils are widespread throughout the world, and their genesis may be natural or accelerated by irrigated agriculture, the intensive use of water resources combined with high evaporation rates and human activity (Lambers, 2003; Arzani, 2008). The osmotic adjustment, i.e. the reduction of cellular osmotic potential by net solute accumulation, has been considered to be an important mechanism of salt and drought tolerance in plants. This reduction in osmotic potential in salt-stressed plants can be a result of inorganic ion (Na^+, Cl^-, and K^+) and compatible organic solute (soluble carbohydrates, amino acids, proline, betaines, etc.) accumulations (Hasegawa et al., 2000). Salinity-induced crop yield reduction takes place due to a number of physiological and biochemical disfunctions in plants grown under salinity stress, which have been listed in a number of comprehensive reviews (Kaya et al., 2013). Salinity is considered to be a major abiotic stress affecting germination, seedling growth, and crop production in arid and semi-arid regions (Yohannes and Abraha, 2013). Moreover, salinity has an adverse effect on seed germination of many crops, by creating an osmotic potential outside the seed, thereby inhibiting the absorption of water, or by the toxic effect of Na^+ and Cl^- (Khajeh-Hosseini et al., 2003). Therefore, salinity is one of the most significant abiotic factors limiting crop productivity (Munns, 1993; Gama et al., 2007). The ability of seeds to germinate at high salt concentrations in the soil is of crucial importance for the survival of many plant species. Although salinity stress mostly reduces the germination percentage and delays the onset of germination, its effects are modified by interactions with other environmental factors, such as temperature and light (Bojović et al., 2010). In saline habitats, satisfactory seed germination takes place after high precipitation, when the soil salinity is reduced (Khan and Rizvi, 1994). Seed priming stimulates many of the metabolic processes involved in the early phases of germination, and it has been observed that seedlings from primed seeds grow more vigorously, and perform better in adverse conditions (Cramer, 2002). It has been shown that soil salinity increases P, Mn, and Zn and decreases K and Fe concentrations in plant tissues (Turan et al., 2010).

The present study was to investigate the response of maize seed germination, the content of chlorophyll '*a*', '*b*' and carotenoid content to different salinity concentration of NaCl and $CaCl_2$.

2 MATERIAL AND METHODS

2.1 Plant material

The plant material that was included in our study was two maize hybrids ('Bc 678' and 'Bc 408') originating from the Maize Dept. of Bc Institute at Rugvica near Zagreb (Croatia) and two maize populations (LMP-1 and LMP-2) originating from Kosovo. The experiment was done in the Department of Crop Science, Laboratory of Plant Breeding, University of Prishtina. The seeds were

disinfected in NaOCl 1% for 60 minutes and then rinsed three times with distilled and sterilized water. Maize seeds were germinated on moistened filter paper. The prepared seeds were placed on the germinator for germination (after addition of 10 ml H_2O) for ten days in temperature 25 °C. Pots were filled with compost (minimum 1 kg/pot) for each cultivar and for each treatment. In total, 32 pots were prepared for salt treatment including NaCl and $CaCl_2$, and a control. During the experiment, solutions with different concentrations for each salt treatment was prepared in the growth period. For two salts (NaCl and $CaCl_2$), the concentrations were 50, 100, 200 and 400 mMol. After 20 days of exposure, the following parameters were determined in different parts of the plants: chlorophyll pigments and concentration, and seed germination. After disinfection, the seeds were divided into nine treatment groups for each salt solution: H_2O (Control), 50, 100, 200, 400 mMol NaCl and 50, 100, 200, 400 mMol $CaCl_2$.

2.2 Soil material

The compost consisted of pH ($CaCl_2$)=5.8; salt concentration (g L^{-1} KCl=0.9; Nitrogen (NH_4+NO_3)=155 mg L^{-1}, $CaCl_2$=120; Phosphorus (P_2O_5) mg L^{-1} CAL=150 and potassium (K_2O) mg L^{-1} CAL=200. The maize seedlings were transfered to compost in 1 kg weight pots in controlled environment cabinets with 12-hour photoperiods and temperatures of 25/19 °C day/night and 75 % relative humidity.

2.3 Seed germination assays

Germination and early seedling growth were compared at 25 °C (optimum temperature) in the dark. The filter papers were moistened with 20 ml distilled water. For all the seeds groups, the experiment was conducted with four replicates of 100 seeds. Seeds were germinated on top of double-layered papers (ISTA, 1996) with 10 ml of each of the salt solutions of NaCl and $CaCl_2$ in 10 cm Petri dishes (4 Petri dishes × 25 seeds = 100 seeds × 4 replications = 400 seeds per treatment). These Petri dishes were placed in sealed plastic bags to avoid moisture loss. Seeds were allowed to germinate at 24±1 ºC and for 16 hours on the light (day) and 8 hours in the dark (night). The germination percentage is an estimate of the viability of seeds. Germinated seeds were counted every 24 hours for 15 days. According to Sharma (2010), seeds were considered to have germinated upon the emergence of radicles (≥ 2 mm).

Full Germination Percent (FGP) was calculating according to equation:

$$FGP = \frac{n}{N} x100$$

where N – is total seed number;
n – number of germinated seeds.

The Mean Germination Time (MGT) was calculated for each lot, using the daily counts, according to the equation (Moradi et al., 2008):

$$MGT = \frac{\sum nD}{\sum n}$$

where n= number of seeds newly germinated at day D,
D – days from the beginning of the germination test,
Σ n –number of all germinated seeds (final germination).

Germination Index (GI) was calculated as described in the Association of Official Seed Analysis (AOSA, 1983) according to the following formula:

$$GI = \left(\frac{G1}{1}\right) + \left(\frac{G2}{2}\right) + \left(\frac{Gx}{x}\right);$$

where GI – is Germination Index,
G1, G2, …. Gx – is germination at 1, 2, ….x day,
1, 2, …. x – day of counting of germinated seeds.

2.4 Pigments analysis

Pigments were extracted by grinding 60–80 mg freshly sampled leaves. At the time of sampling, the plants reached the stage of five leaves; we took the third leaf and put in an 80% acetone/water solution containing $MgCO_3$ (0.5% w/v), at room temperature for 24 hours in the dark. Photosynthetic pigments of all samples were extracted in triplicate to minimize experimental errors. Concentrations of chlorophyll and carotenoid contents were measured by using absorbance recorded at 662, 644 and 440 nm for maximum absorption of chlorophyll '*a*' (*Chl 'a'*), chlorophyll '*b*' (*Chl 'b'*) and carotenoids, respectively. The extinction coefficients were

determined by a UV-Vis spectrophotometer (SECOMAM, Anthelie Advanced 5). Pigment contents were calculated in mg g^{-1} fresh leaf weight (FW) by applying the absorption coefficient equations, described by Lichtenthaler (1986); Aliu et al. (2013 and 2014); Gashi et al. (2013):

Chl 'a' (mg g^{-1} FW) = [9.784 (OD662) – 0.99 (OD644)] × V/FW,
Chl 'b' (mg g^{-1} FW) = [21.426 (OD644) – 4.65 (OD662)] × V/FW,
Carotenoids (mg g^{-1} FW) = [4.695 (OD440) – 0.268 (*Chl a* + *Chl b*)] × V/FW.

Where is:
FW – fresh leaf weight,
OD – optical density,
V – volume of sample.

2.5 Statistical analysis

SPSS version 19 was used for analysis of variance for all parameters and to compare of treatment means with Duncan's Multiple Range Test. Relationships among the traits were estimated with Pearson correlation analysis.

3 RESULTS AND DISCUSSION

Analyses of variance showed a wide range and highly significant effects of NaCl and $CaCl_2$ concentrations on the parameters of seed germination. The effects of the NaCl and $CaCl_2$ concentrations accounted for a high proportion of the variance in all analyses include Full Germination Percent (FGP), Mean Germination Time (MGT) and Germination Index (GI) (Table 1).

The FGP at all maize genotypes ranged from 14 to 100%, depending on treatments. FGP for the hybrid 'Bc 678' was low (16 and 44 %) after treatments with 400 mMol $CaCl_2$ and 400 mMol NaCl, respectively. When comparing these values (16 and 44%) to the any other values (Control, 50, 100 and 200 mMol $CaCl_2$ and mMol NaCl), they were significantly lower. In the case of hybrid 'Bc 408', between different treatments the same differences for FGP are also present, while lower values (20 and 74%) were recorded in treatment with 400 mMol NaCl and $CaCl_2$. In comparison with 'Bc 678', the hybrid 'Bc 408' had the highest (87.8%) average values of FGP. The applied high concentrations with NaCl and $CaCl_2$ for FGP on treatments, as well as 400 mMol in seed Local Maize Populations (LMPs) had negative effects or inhibited the physiological processes. LMP-1 and LMP-2 had significantly lower values of FGP after the treatments of 200 and 400 mMol $CaCl_2$ and NaCl than the control.

In both hybrids, the differences in FGP, in treatments with 50, 100, 200 mMol, both for NaCl and $CaCl_2$ concentrations, resulted in no significant differences, except in the case of the hybrid 'Bc 678' after the treatment with 200 mMol $CaCl_2$. In both populations (LMP-1 and LMP-2) significant differences exist almost among all treatments. Therefore, the populations could be more susceptible to salinity stress than hybrids. However, the seed germination percentage of populations decreased at the highest level of salinity, but the hybrid 'BC 678' expresses significantly higher values of FGP at 50 and 100 mMol $CaCl_2$ and at 50, 100 and 200 mMol NaCl than the control does.

Many authors (Amzallag et al., 1990; Djanaguiraman et al., 2006) found that plants' exposure to low level salinity activates an array of processes leading to an improvement of plant stress tolerance. High salt concentrations negatively affect maize growth. Rahman et al. (2000) reported that maize cultivars were significantly more tolerant to salt stress at germination than at later stages of growth. In order to determine the usefulness of *Tripsacum* in improving salt tolerance in maize, and the effects of NaCl, *in vitro* and *in vivo*, Pesqueira et al., (2006) evaluated an intergeneric hybrid obtained

from crossing *Zea mays* L. with *Tripsacum dactyloides* L.

The different levels of NaCl and $CaCl_2$ concentrations also significantly affected the mean germination time (MGT) and germination index (GI) (Table 1). The significantly greater number of days for MGT at all genotypes were obtained from treatments in 400 mMol NaCl and $CaCl_2$ concentrations; furthermore, the concentration of 400 mMol $CaCl_2$ also resulted in a significantly higher number of days when compared to the same concentration of NaCl.

Similar findings were also obtained for GI. For both parameters, the greatest differences between control and treatments in 400 mMol NaCl and $CaCl_2$ concentrations for maize populations than for hybrids were obtained.

For maize hybrids, the MGT at 400 mMol $CaCl_2$ and NaCl concentration the 6.87 and 6.50 days were obtained, respectively; while for maize populations (LMP) the values 7.94 and 7.88 were obtained. On the basis of these results, we can conclude that the maize populations are more responsive to soil salinity than hybrids. Taiz and Zeiger (2002) concluded that the high concentration of NaCl in the salt solution increases its osmotic potential.

In addition, the high absorption of Na and Cl ions during seed germination can be due to the cell toxicity that finally inhibits or slows the rate of germination and thus decreases the germination percentage. Moreover, the germination indices of all the cultivars decreased with increasing salt stress (Carpici et al., 2009).

The leaf is a very important photosynthetic organ, in which light energy is transformed through the green pigment chlorophyll into the potential energy of asimilates. Our results show that the chlorophyll content concentration was significantly changed under different salinity concentrations (Table 2).

In many cases, the significantly higher content of chlorophyll '*a*' was found at lower salinity concentrations of only NaCl. The significantly higher content of chlorophyll '*a*' than in control was obtained at concentrations of 100 mMol NaCl ('Bc 408' and LPM-1), at concentrations of 50 and 200 mMol NaCl ('Bc 678'), and at concentrations of 50, 100 and 200 mMol NaCl (LPM-2). Significantly lower contents of chlorophyll '*a*' were found at higher concentrations of 100, 200 and 400 mMol $CaCl_2$ ('Bc 678'), 200 and 400 mMol $CaCl_2$ ('Bc 408' and LPM-2) and at 400 mMol $CaCl_2$ (LMP-1).

NaCl was affected on lower chlorophyll '*a*' at all cultivars only at 400 mMol concentrations. Similar results for chlorophyll '*a*' in different treatments of maize were obtained by Daughtry et al. (1999), ranging from 10.4 to 34.6 mg g^{-1}. The content of chlorophyll '*b*' is less variable than chlorophyl '*a*' under different salinity concentrations.

Significantly higher contents of chlorophyll '*b*' were found only at 'Bc 678' (50 mMol $CaCl_2$ and 200 mMol NaCl), at LMP-2 (50, 100 and 200 mMol NaCl); while the lowest chlorophyll '*b*' contents at 200 and 400 mMol $CaCl_2$ ('Bc 678', 'Bc 408' and LMP-2), at 400 mMol $CaCl_2$ (LMP-1) and only at 400 mMol NaCl ('Bc 408', LMP-1 and LMP-2) were found.

In general, the highest salinity concentrations reduced content of both chlorophyll 'a' and '*b*' compared to the control.

The significantly lower content of carotenoides was determined at higher concentrations of treatments, 400 mMol $CaCl_2$, at all cultivars and at 400 mMol NaCl at both LMP (Table 2). The most responsive to salinity stress relating to carotenoides content was LMP-2; at both highest concentrations, it showed the lowest carotenoid content, while at 50, 100 and 200 mMol NaCl concentrations it showed significantly higher carotenoids content than the control.

Table 1: The effect of salinity on seed germination in maize cultivars

Treatment	'Bc 678'			'Bc 408'			LMP-1			LMP-2		
	FGP * (%)	MGT (days)	GI %	FGP (%)	MGT (days)	GI %	FGP (%)	MGT (days)	GI %	FGP (%)	MGT (days)	GI %
Control	96^{b}	4.0^{c}	59.3^{ab}	100^{a}	4.0^{c}	61.7^{a}	100^{a}	4.0^{cd}	61.7^{a}	88^{b}	4.1^{de}	53.4^{b}
50 mM $CaCl_2$	100^{a}	4.0^{c}	61.7^{a}	100^{a}	4.0^{c}	61.7^{a}	100^{a}	4.0^{cd}	61.4^{a}	82^{b}	4.0^{e}	50.5^{b}
100 mM $CaCl_2$	100^{a}	4.0^{c}	61.7^{a}	100^{a}	4.0^{c}	61.5^{a}	98^{a}	4.1^{bc}	59.9^{a}	96^{a}	4.1^{de}	57.9^{a}
200 mM $CaCl_2$	96^{b}	4.1^{c}	57.9^{b}	96^{a}	4.0^{c}	55.8^{b}	68^{b}	4.7^{ab}	35.3^{c}	70^{c}	4.2^{d}	39.5^{d}
400 mM $CaCl_2$	16^{d}	6.9^{a}	3.6^{d}	20^{c}	6.5^{a}	5.2^{d}	28^{c}	7.9^{a}	3.7^{e}	14^{d}	7.9^{a}	2.0^{f}
50 mM NaCl	100^{a}	4.0^{c}	61.7^{a}	100^{a}	4.0^{c}	61.7^{a}	100^{a}	4.0^{cd}	60.5^{a}	86^{b}	4.0^{e}	53.1^{b}
100 mM NaCl	100^{a}	4.0^{c}	61.7^{a}	100^{a}	4.0^{c}	61.7^{a}	94^{a}	4.0^{cd}	57.3^{a}	94^{a}	4.0^{e}	58.0^{a}
200 mM NaCl	100^{a}	4.0^{c}	61.7^{a}	100^{a}	4.0^{c}	61.0^{a}	72^{b}	3.4^{d}	46.1^{b}	88^{b}	4.6^{c}	46.8^{c}
400 mM NaCl	44^{c}	4.6^{b}	23.1^{c}	74^{b}	5.3^{b}	33.6^{c}	20^{c}	5.0^{b}	9.7^{d}	16^{d}	6.2^{b}	5.9^{e}
Average (μ)	83.6	4.4	50.3	87.8	4.4	51.6	75.6	4.6	44.0	70.2	4.8	40.8

*FGP – final germination percentage; MGT – mean germination time; GI – germination index;
* – values within individual columns indicated by at least one equal letter are not significantly different at 0.05 probability level

Table 2: Effect of salinity on photosynthetic pigments content (mg g^{-1} FW) of maize cultivars

Treatment	'Bc 678'					'Bc 408'					LMP-1					LMP-2				
	Chl *a*	Chl *b*	Carot	Total Chl	Ratio *a*/*b*	Chl *a*	Chl *b*	Carot	Total Chl	Ratio *a*/*b*	Chl *a*	Chl *b*	Carot	Total Chl	Ratio *a*/*b*	Chl *a*	Chl *b*	Carot	Total Chl	Ratio *a*/*b*
Control	38.8^{c*}	8.8^{bc}	5.6^{bc}	47.5^{c}	4.4^{bc}	37.3^{b}	10.1^{a}	5.8^{ab}	47.3^{c}	3.8^{a}	39.5^{bc}	10.1^{a}	6.2^{ab}	49.6^{bc}	4.0^{a}	37.9^{b}	8.9^{c}	5.6^{c}	46.7^{b}	4.2^{a}
50 mM $CaCl_2$	44.8^{abc}	10.9^{a}	6.2^{ab}	55.8^{ab}	4.1^{ab}	38.9^{b}	9.5^{abc}	5.7^{ab}	48.4^{c}	4.2^{a}	38.8^{bc}	10.8^{a}	5.2^{b}	49.6^{bc}	3.7^{ab}	41.1^{c}	9.6^{c}	5.9^{bc}	50.7^{b}	4.3^{a}
100 mM $CaCl_2$	31.2^{d}	7.8^{cd}	4.9^{cd}	39.1^{d}	4.0^{cd}	37.3^{b}	9.4^{abc}	5.4^{ab}	46.7^{c}	4.0^{a}	43.8^{b}	10.1^{a}	6.0^{ab}	53.9^{bc}	4.3^{a}	23.4^{d}	5.7^{e}	3.2^{d}	29.1^{d}	4.1^{a}
200 mM $CaCl_2$	27.3^{d}	6.8^{de}	3.9^{d}	34.1^{d}	4.0^{d}	30.6^{c}	7.4^{c}	5.6^{b}	38.0^{d}	4.2^{a}	36.7^{c}	8.6^{a}	5.5^{ab}	45.2^{c}	4.3^{a}	33.0^{c}	7.5^{d}	4.7^{c}	40.5^{c}	4.4^{a}
400 mM $CaCl_2$	20.1^{e}	5.3^{e}	3.8^{d}	25.4^{e}	3.8^{d}	9.7^{d}	3.5^{d}	2.1^{c}	13.2^{e}	2.8^{b}	6.1^{e}	2.2^{b}	1.3^{d}	8.2^{e}	2.8^{b}	10.0^{e}	2.8^{f}	2.2^{d}	12.9^{e}	3.6^{b}
50 mM NaCl	46.2^{ab}	11.6^{a}	7.1^{a}	57.8 ^{ab}	4.0^{a}	40.9^{ab}	9.8^{ab}	5.9^{ab}	50.7^{bc}	4.2^{a}	38.5^{c}	9.2^{a}	5.8^{ab}	47.7^{bc}	4.2^{a}	48.0^{a}	11.3^{b}	7.3^{a}	59.3^{a}	4.3^{a}
100 mM NaCl	41.3^{bc}	9.9^{ab}	6.2^{ab}	51.3^{bc}	4.2^{ab}	44.3^{a}	10.6^{a}	6.4^{a}	55.2^{a}	4.2^{a}	47.0^{a}	11.2^{a}	6.9^{a}	58.2^{a}	4.2^{a}	52.3^{a}	12.6^{a}	8.3^{a}	64.9^{a}	4.2^{a}
200 mM NaCl	48.5^{a}	11.1^{a}	6.6^{ab}	59.6^{a}	4.3^{ab}	42.3^{ab}	10.9^{a}	6.3^{a}	53.1^{ab}	3.9^{a}	39.2^{bc}	9.7^{a}	5.4^{b}	49.0^{bc}	4.1^{a}	49.7^{a}	11.9^{ab}	7.1^{ab}	61.7^{a}	4.2 ^{a}
400 mM NaCl	21.5^{c}	6.2^{ab}	6.1^{ab}	27.7^{d}	3.5^{d}	29.8^{c}	7.7^{bc}	4.9^{ab}	37.5^{d}	3.9^{a}	15.1^{d}	4.2^{b}	2.7^{c}	19.2^{d}	3.6^{ab}	20.0^{d}	5.9^{e}	3.4^{d}	25.1^{d}	3.9^{b}
Average	35.5	8.7	5.6	47.0	4.1	34.5	8.8	5.3	43.3	3.9	33.9	8.4	5.0	42.3	3.9	35.1	8.5	5.3	43.4	4.1

* – values within individual columns indicated by at least one equal letter are not significantly different at 0.05 probability level

In general, the correlation coefficients between all the studied properties for most cultivars were positive and statistically significant, except for the MGT, for which there was a negative and significant correlation (Table 3). Only for hybrid 'Bc 678' were a lower value of correlation coefficients between the ratio of chlorophyll '*a*' and '*b*' and other properties obtained; statistically nonsignificant correlation coefficients were obtained only for MGT and chlorophyll '*a*'. Wue et al. (2008) investigated similar issues and have obtained similar results.

Table 3: The correlation coefficients between investigated traits in maize cultivars

Treatment		FGP	MGT	GI	*Chl 'a'*	*Chl 'b'*	Carotenoids	*Chl (a+b)*
Bc 678	FGP	1						
	MGT	-0.90**	1					
	GI	1**	-0.90**	1				
	Chl 'a'	0.50**	-0.62**	0.51**	1			
	Chl 'b'	0.48*	-0.58**	0.48*	0.95**	1		
	Carot.	0.50**	-0.62**	0.51**	0.99**	0.96**	1	
	Total *Chl*	0.39*	-0.48**	0.40*	0.92**	0.92**	0.93**	1
	Ratio *a*/*b*	0.30	-0.38*	0.30	0.42*	0.13	0.37	0.24
Bc 408	FGP	1						
	MGT	-0.97**	1					
	GI	0.98**	-0.99**	1				
	Chl '*a*'	0.92**	-0.93**	0.92**	1			
	Chl '*b*'	0.86**	-0.89**	0.87**	0.93**	1		
	Carot.	0.91**	-0.93**	0.92**	0.99**	0.95**	1	
	Total Chl	0.85**	-0.87**	0.85**	0.93**	0.98**	0.95**	1
	Ratio *a*/*b*	0.76**	-0.72**	0.73**	0.75**	0.50**	0.71**	0.54**
LMP-1	FGP	1						
	MGT	-0.69**	1					
	GI	0.98**	-0.80**	1				
	Chl '*a*'	0.88**	-0.84**	0.92**	1			
	Chl '*b*'	0.86**	-0.82**	0.90**	0.93**	1		
	Carot.	0.89**	-0.85**	0.92**	0.99**	0.95**	1	
	Total Chl	0.85**	-0.82**	0.88**	0.96**	0.91**	0.96**	1
	Ratio *a*/*b*	0.55**	-0.65**	0.58**	0.72**	0.45*	0.68**	0.69**
LMP-2	FGP	1						
	MGT	-0.91**	1					
	GI	0.99**	-0.93**	1				
	Chl 'a'	0.75**	-0.76**	0.75**	1			
	Chl 'b'	0.74**	-0.73**	0.74**	0.99**	1		
	Carot.	0.75**	-0.75**	0.75**	1**	0.99**	1	
	Total Chl	0.67**	-0.66**	0.67**	0.96**	0.97**	0.97**	1
	Ratio *a*/*b*	0.61**	-0.75**	0.62**	0.60**	0.51**	0.58**	0.49**

* - Correlation is significant at the 0.05 level,
** - Correlation is significant at the 0.01 level.

4 CONCLUSIONS

The study involving NaCl and $CaCl_2$ with different concentrations indicated that maize seed germination and chlorophyll content are sensitive to salt stress. In general, no significant decrease at 50 and 100 mMol salt concentrations for all maize cultivars and all investigated traits were found, while the highest concentrations of 400 mMol NaCl and $CaCl_2$ negatively affected all cultivars and for all properties. In some cases, mainly moderate concentrations (50 or 100 mMol) of NaCl had a positive impact on investigated traits. Hybrids are less sensitive to salinity than populations because they were not genetically improved. Therefore, due to the genetic variability of populations and their responsiveness to salinity, they can serve as a good starting material for breeding of genotypes resistant to salinity stress.

5 ACKNOWLEDGMENT

The authors would like to express their sincere appreciation to Professor Marc Lemmens, University of Natural Resources and Life Science, Institute for Biotechnology, Vienna, Austria for his continuous support and suggestions in editing of this article.

6 REFERENCES

Aliu S., Fetahu Sh., Rozman L., Salillari A. 2008. General and specific combining ability studies for leaf area in some maize inbreeds in agroecological conditions of Kosovo. Acta agriculturae Slovenica, 91, 1: 67-73; DOI: 10.2478/v10014-008-0007-4

Aliu S., Gashi B., Rusinovci I., Fetahu Sh. Vataj R. 2013. Effects of some heavy metals in some morphological and physiological parameters in maize seedlings. American Journal of Biochemistry and Biotechnology, 9, 1: 27-33; DOI: 10.3844/ajbbsp.2013.27.33

Aliu S., Rusinovci I., Gashi B., Kaul H.P., Rozman L., Fetahu Sh. 2014. Genetic diversity for mineral content and photosynthetic pigments in local bean (*Phaseolus vulgaris* L.) populations. Journal of Food, Agriculture & Environment, 12, 2: 635-639

Allen S.G., Dobrenz A.K., Schonhorst M.H. Stoner J.E. 1983. Heritability of NaCl tolerance in germinating alfalfa seeds. Agronomy Journal, 77: 99-101; DOI: 10.2134/agronj1985.00021962007700010023x

Amzallag N., Lerner H.R., Poljakoff Mayber A. 1990. Induction of increased salt tolerance in Sorghum bicolor by NaCl pretreatment. Journal of Experimental Botany, 41: 29–34; DOI: 10.1093/jxb/41.1.29

Arzani A. 2008. Improving salinity tolerance in crop plants: a biotechnological view. Vitro Cell. Dev. Biol. Plant 44: 373-383; DOI: 10.1007/s11627-008-9157-7

Association of Official Seed Analysis (AOSA). 1983. Seed Vigour Testing Handbook. Contribution No. 32 to the handbook on Seed Testing, 18-19

Bojović B., Đelić G.,Topuzović M., Stanković M. 2010. Effects of NaCl on seed germination in some species from families *Brassicaceae* and *Solanaceae*, Kragujevac Journal of Science, 32: 83-87

Carpıcı E.B., Celık N., Bayram G. 2009. Effects of salt stress on germination of some maize (*Zea mays* L.) cultivars. African Journal of Biotechnology, 8, 19: 4918-4922.

Cramer, G.R. 2002. Sodium-calcium interaction under salinity stress, University of Nevada, Reno, NV 89557 USA. Chapter 10: 205-228.

Daughtry C., Walthall L., Kim K., Brown E., McMurtrey E. 1999. Estimating Corn Leaf Chlorophyll Concentration from Leaf and Canopy Reflectance. Remote sens. Environment, 74: 229-239 ; DOI : 0.1016/S0034-4257(00)00113-9

Djanaguiraman M., Sheeba J.A., Shanker A.K., Devi D.D., Bangarusamy U. 2006. Rice can acclimate to lethal level of salinity by pretreatment with sublethal level of salinity through osmotic adjustment. Plant and Soil 284: 363-373; DOI: 10.1007/s11104-006-0043-y

FAO 2000. Global network on integrated soil management for sustainable use of salt-affected soils. Available in:

http://www.fao.org/ag/AGL/agll/spush/intro.htm (28.Jan.2015)

FAO. 2008. Land and Plant Nutrition Management Service. http://www.fao.org/ag/agl/agll/. Accessed on November/ 15/2012.

Gama P.B.S., Inagana S., Tanaka K., Nakazawa R. 2007. Physiological response of common bean (*Phaseolus Vulgaris*. L.) seedlings to salinity stress. African Journal of Biotechnology, 2: 79-88

Gashi B., Babani F., Kongjika E. 2013. Chlorophyll fluorescence imaging of photosynthetic activity and pigment contents of the resurrection plants *Ramonda serbica* and *Ramonda nathaliae* during dehydration and rehydration. Physiology Molocular Biology of Plants, 19, 3: 333-341; DOI: 10.1007/s12298-013-0175-5

Hasegawa P.M, Bressan R.A., Zhu J.K., Bohnert H.J. 2000. Plantcellular and molecular responses to high salinity. Annu. Rev. Plant Physiology, 51: 463-499; DOI: 10.1146/annurev.arplant.51.1.463

Katerji N., Van Hoorn J.W., Hamdy. A., Karam F., Mastroruilli M. 1994. Effect of Salinity on Emergence and on Water Stress and Early Seedling Growth of Sunflower and Maize. Agric. Wat. Mang., 26: 81-91; DOI: 10.1016/0378-3774(94)90026-4

Kaya C., Ashraf M., Murat Dikilitas M,, Atilla L. 2013. Alleviation of salt stress-induced adverse effects on maize plants by exogenous application of indoleacetic acid (IAA) and inorganic nutrients – A field trial. Australian Journal of Crop Science, 7, 2: 249-254

Khajeh-Hosseini M., Powell A.A., Bimgham I.J. 2003. The interaction between salinity stress and seed vigor during germination of soybean seeds. Seed Science Technology, 31: 715-725; DOI: 10.15258/sst.2003.31.3.20

Khan A. M., Rizvi Y. 1994. Effect of salinity, temperature and growth regulators on the germination and early seedling growth of *Atriplex griffthii.* Canadian Journal of Botany, 72, 475-479; DOI: 10.1139/b94-063

Lambers H. 2003. Introduction, dry land salinity: a key environmental issue in Southern Australia. Plant Soil, 257: 5-7; DOI: 10.1023/B:PLSO.0000003909.80658.d8

Lichtenthaler H. 1986. Laser-Induced Chlorophyll Fluorescence of Living Plants, Proceedings of the Remote Sensing Symposium, Band III, ESA Publication Division, Nordwijk, 1571-1579

Moradi D.P., Sharif-Zadeh F., Janmohammadi M. 2008. Influence of priming techniques on seed germination behavior of maize inbred lines (*Zea mays* L.). Journal of Agricultural and Biological Sciences, 3, 3: 22-25

Munns R. 1993. Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. Plant Cell Environment, 16: 15-24; DOI: 10.1111/j.1365-3040.1993.tb00840.x

Pesqueira J., Garcia M.D., Staltari S., Molina M.D.C. 2006. NaCl effects in *Zea mays* L. x *Tripsacum dactyloides* L. hybrid calli and plants. Electronic Journal of Biotechnology, 9, 3: 285-290; DOI: 10.2225/vol9-issue3-fulltext-29

Rahman M., Kayani S.A., Gul S. 2000. Combined Effects of Temperature and Salinity Stress on Corn Sunahry Cv., Pakistan Journal of Biological Sciences, 3, 9: 1459-1463; DOI: 10.3923/pjbs.2000.1459.1463

Sharma R.K., Sharma S. 2010. Effect of storage and cold-stratification on seed physiological aspects of *Bunium persicum*: A threatened medicinal herb of Trans-Himalaya. Int. J. Bot., 6, 2: 151-156; DOI: 10.3923/ijb.2010.151.156

Taiz L., Zeiger E. 2002. Plant Physiology. 3rd Edn., Sunderland, Sinauer Associates, Inc.: 85-87

Turan M., Elkarim H., Taban N., Suleyman Taban S. 2010. Effect of salt stress on growth and ion distribution and accumulation in shoot and root of maize plant. African Journal of Agricultural Research, 5, 7: 584-588

Wue Ch., Niu Zh., Tang Q., Huang W. 2008. Estimating chlorophyll content from hyperspectral vegetation indices: Modeling and validation. Agricultural and forest meteorology, 148: 1230–1241; DOI: 10.1016/j.agrformet.2008.03.005

Yohannes G., Abraha B. 2013. The role of seed priming in improving seed germination and seedling growth of maize (*Zea mays* L.) under salt stress at laboratory conditions. African Journal of Biotechnology, 12, 46: 6484-6490

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.10
Agrovoc descriptors: orchidaceae, endangered species, seeds, plant propagation, germinability, site factors, disinfection, chemicophysical properties, growing media

Agris category code: f02, f03

Successful disinfection protocol for orchid seeds and influence of gelling agent on germination and growth

Tomaž JEVŠNIK[1], Zlata LUTHAR[2]

Received February 27, 2015; accepted March 08, 2015.
Delo je prispelo 27. februarja 2015, sprejeto 08. marca 2015.

ABSTRACT

Artificial propagation of endangered orchid species is one of the most important actions of conservationists often jeopardized by low numbers of acquired seed, its contamination and viability. Disinfection and chemical composition of media are two of the most important factors contributing to better germination in temperate orchid species. The article deals with three world genera (*Epidendrum nocturnum, Prosthechea garciana, Maxillaria rufescens)* and one commercial hybrid (*Zygopetalum*) and describes an effective method of orchid seed disinfection carried out in a centrifuge. Germination percentages of all three genera and one hybrid were between 60 and 90 % from which we concluded that the risk of physical damage to the seeds by centrifugation is not significant. The time needed for disinfected seeds (*E. nocturnum, P. garciana, M. rufescens)* to swell-form protocorms was 10 days shorter compared to undisinfected seeds (*Zygopetalum* hybrid - green capsule method) and some other studies. Adequate wetting and stratification of the seed is very important for successful germination, which resembles processes in natural environment. Additionally, this method solves the problems of collecting and transferring the seeds after disinfection. It is also important that the time needed for disinfection is shorter, which is desirable for some sensitive species. Our study also focuses on importance of gelling agent, namely Gellan gum and agar, since we noticed an obvious superiority of the former in all phases of *in vitro* development.

Key words: orchids, seed, disinfection, wetting, gelling agent, agar, Gellan gum, germination morphological stage

IZVLEČEK

USPEŠNA METODA RAZKUŽEVANJA SEMEN ORHIDEJ IN VPLIV STRJEVALCA NA KALITEV IN RAST

Razmnoževanje ogroženih vrst orhidej je ena od najpomembnejših dejavnosti konservatorjev teh rastlin, ki jih pogosto ogrožajo majhne količine dostopnega semena, okuženost s patogeni in viabilnost. Razkuževanje in kemična sestava gojišča sta najpomembnejša dejavnika, ki vplivata na boljšo kalivost semena orhidej iz območij z zmernim podnebjem. V raziskavo so bili vključeni trije rodovi orhidej iz Srednje in Južne Amerike (*Epidendrum nocturnum, Prosthechea garciana, Maxillaria rufescens)* ter komercialni križanec (*Zygopetalum*). V delu je prikazana uspešna metoda razkuževanja semena orhidej z uporabo centrifuge. Kalivost semen vseh štirih rodovov orhidej je bila med 60 in 90 %, kar potrjuje, da so posledice poškodb zaradi vrtilnega momenta pri centrifugiranju zanemarljive. Čas, ki so ga razkužena semena (*E. nocturnum, P. garciana, M. rufescens*) potrebovala za razvoj protokormov, je bil za 10 dni krajši v primerjavi z nerazkuženim semenom (*Zygopetalum* - zelena semenska glavica) in s primerljivimi študijami. Za uspešno kalitev je pomembna zadostna omočitev semenske ovojnice in stratifikacija semena, s čimer se približamo procesom v naravnem okolju. Olajšano je tudi rokovanje s semenom, ki se zaradi centrifugalne sile sesede na dno mikrocentrifugirke. Zaradi učinkovitosti metode je seme manj časa izpostavljeno razkuževalnemu sredstvu, na katerega so semena nekaterih orhidej občutljiva. Proučevali smo tudi vpliv dveh strjevalcev gojišč in ugotovili, da je v vseh fazah *in vitro* kalitve in razvoja rastlin Gellan gum v primerjavi z agarjem učinkovitejši.

Ključne besede: orhideje, semena, razkuževanje, omočitev, strjevalci gojišč, agar, Gellan gum, kalivost, rast

[1] Ocean Orchids d.o.o., Dobrovnik 297, SI-9223 Dobrovnik, e-mail: tomaz.jevsnik@oceanorchids.si

[2] Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, e-mail: zlata.luthar@bf.uni-lj.si

1 INTRODUCTION

The gateway to massive hybridization and conservation goals was opened when Knudson (1922) described and demonstrated his breakthrough method of non-symbiotic germination of orchid seed. Many technical and technological problems have been solved since, mainly understanding and satisfying the needs of individual genera and species of orchids. The existence of increasingly high number of orchid species around the globe is threatened (Koopowitz, 2001; Johnson et al., 2007). Artificial propagation of endangered species is therefore one of the most important actions of conservationists often jeopardized by low numbers of acquired seeds, their contamination and viability.

Sterilization and chemical composition of media are two of the most important factors contributing to better germination in temperate orchid species (Rasmussen, 1995), with the embryo itself being afforded little protection therefore vulnerable to physical damage (Hicks, 2000). Common technique for disinfection of orchid seeds that has been used by breeding companies, research institutions and gene banks is agitation of disinfection solution containing seeds. Additionally, some authors (Arditti, 1982; Snow, 1985; Hicks, 2000) describe a method called presoak, before adding a disinfectant, in a sugar or honey solution at various time intervals, which improves disinfection efficiency of heavily contaminated seeds and also enhances germination. Sugar or honey solution causes bacteria and fungal spores that are hidden in porous seed surface to germinate, consequently making them vulnerable to disinfection solution. It is important to understand other mechanisms behind this treatment and its influence in relation to the structure of the seed and the process of germination that takes place in natural environment. Orchid seeds are difficult to wet because the outer walls of their testa cells (outer integument) are hard, lignified and covered with a lipid cuticle. As the seeds ripen, testa cells lose moisture and their walls curve inwards. Both the cells of testa and as interior of seeds are filled with air. When seeds that fall into water or drop onto a moist substrate are chilled by cool water or reduced atmospheric temperatures the air inside testa contracts. This creates suction that draws water into seed through micropylar opening (Arditti and Ghani, 2000). Up to this point, presence of water is a crucial factor but after initial swelling of protocorms the fungus (symbiotic method) or constituents of media (asymbiotic method) play the most important role.

This paper describes a reliable protocol for sterilization of orchid seeds regardless of the level of contamination by various pathogens and signifies the importance of adequate wetting of the seed, which might often be the reason for low germination rates. Our studies also focus on importance of gelling agent, Gellan gum and agar, since we noticed an obvious superiority of Gellan gum in all phases of *in vitro* development in three genera and one commercial hybrid.

2 MATERIALS AND METHODS

The seeds of botanical genera *Epidendrum nocturnum* Jacq., *Prosthechea garciana* Garay & Dunst. (syn. *Encyclia garcianum* Carnevali & I. Ramírez) and *Maxillaria rufescens* Lindl. were kindly provided by Dr. Michel Vacherot, France in a form of dry seeds extracted from capsules. Additionally, a capsule of a commercial hybrid of *Zygopetalum* was used as control.

2.1 Disinfection and inoculation of seeds on the medium

Disinfection of seeds was carried out in 1.5 ml microcentrifuge tubes. Approximately 20 mg of isolated seeds were disinfected in 16.6 g/l Na_2 salt of dichloroisocyanuric acid (Sigma) dissolved in sterile distilled water with a drop of wetting agent Tween 20 (Sigma).

Seeds were soaked in disinfection agent for 8 min at room temperature, and then centrifuged for 2 min at 4000 rpm (1900x g) in a Beckman J2-HS centrifuge at 4 °C. A supernatant was removed and washed three times in sterile water using the following procedure: supernatant was removed and seeds were mixed in sterile water for few seconds and sedimented by centrifugation at the same conditions as described. Finally 0.8 ml of sterile water was added to each microcentrifuge tube and after resuspending seeds were inoculated using 1000 µl pipete in Petri dishes (90 x 15 mm) on media. Glass spreader was used to spread the seeds on the medium surface. Seed capsule from *Zygopetalum* was picked few days before dehiscence of the capsule. The whole capsule was disinfected for 15 min. in 16.6 g/l Na_2 salt of dichloroisocyanuric acid including Tween 20 and washed for three times. Seeds were extracted and immediately inoculated.

2.2 Subcultivation and culture conditions

Further subcultivations were carried out in bigger petri dishes (90 x 20 mm) and baby jars (55 x 72 mm). All vessels were sealed with Parafilm and cultured in a growth chamber under 16/8 photoperiod at 25^{+}/- 1 °C and illumination of 40 µmol m^{-2} s^{-1}.

2.3 Medium

The culture medium used for germination and subculturing was composed of Gamborg B5 macro-salts (Gamborg et al., 1976) and full strength MS micro-salts (Murashige and Skoog, 1962). Iron ions were added as 25 mg/l Na_2Fe-EDTA and 25 g/l sucrose were added as carbohydrate source. Media were solidified using either 8 g/l agar (Difco Bacto), mark A or 2.6 g/l Gellan gum (Sigma), mark G. The pH was adjusted to 5.4 prior to autoclaving.

3 RESULTS

3.1 Disinfection

The method used in our study, including preliminary tests, has proven to be 100 % efficient in disinfecting seeds of four epiphytic orchids (Table 1). Washing of seeds in the centrifuge was also efficient, since we observed no abnormalities caused by possible remnants of dichloroisocyanuric acid at germination. Centrifugation forced the seeds to gather at the bottom of microcentrifuge tubes, which enabled transfer to Petri dishes without difficulties, using a pipette.

Table 1: Efficiency of disinfection of three orchid genera and one hybrid

Genotype	Material for disinfection	Disinfection efficiency (%)	Germination (%)
Epidendrum nocturnum	isolated seeds	100	70
Prosthechea garciana	isolated seeds	100	70
Maxillaria rufescens	isolated seeds	100	60
Zygopetalum hybrid	intact capsule	100	90

3.2 Germination

We estimated the germination percentage visually with the use of stereomicroscope. Presented percentages should be considered approximate and are as follows: 70 % for *E. nocturnum* and *P. garciana*, 60 % for *M. rufescens* and above 90 % for *Zygopetalum* hybrid (Figs. 1, 2 and 3). Individual genus needed the same time for germination on medium A as well as on medium G. After 7 days we observed first swollen embryos - protocorms in *E. nocturnum* and *P. garciana*. The actual day of germination is considered to be after 9 days since by that day more than half of the embryos formed protocorms (Fig. 1). *M. rufescens* formed protocorms after 11 days *in vitro*. The longest period needed for germination (21 days)

was observed in *Zygopetalum* hybrid, which was the only seed material that was not disinfected in the centrifuge (Table 2).

Protocorms of *E. nocturnum* and *P. garciana* left the testa after 21 days and covered themselves with rhizoids. *Zygopetalum* protocorms reached this phase after 30 days.

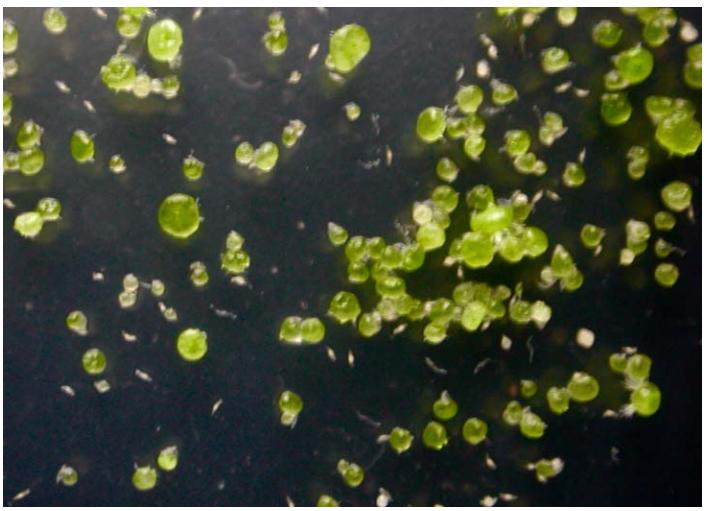


Figure 1: Developing protocorms of *Epidendrum nocturnum* 11 days after inoculation on medium G

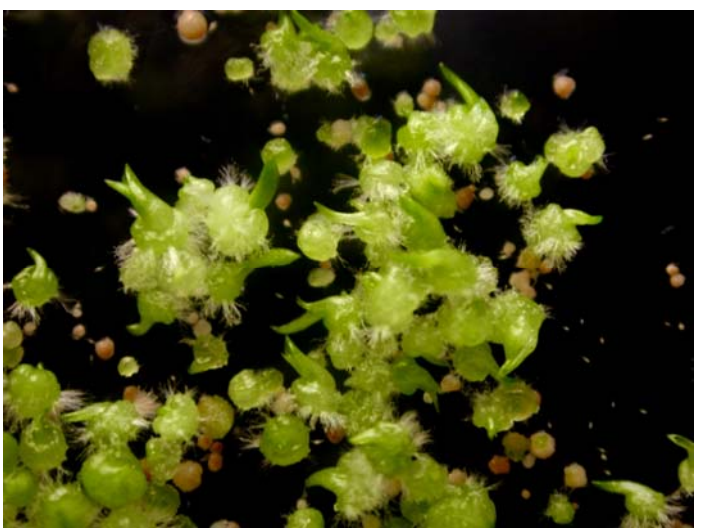


Figure 2: Uneven germination of *Zygopetalum* hybrid as a consequence of green capsule technique used

After initial development of protocorms *M. rufescens* entered stagnation and by the end of the experiment, after 223 days, only few formed structures as small as 1-2 mm that would eventually become first and second leaf. *E. nocturnum* reached the first leaf phase after 35 days, *Zygopetalum* and *P. garciana* reached it after 45 days (Fig. 2). After this period of time obvious differences between plants grown on medium A and G were observed (Table 2 and Figs. 3 and 4).

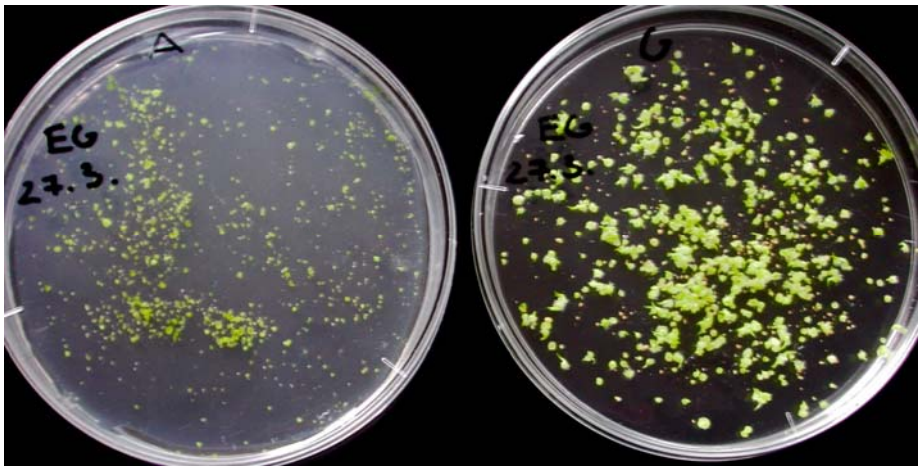


Figure 3: Differences in protocorm development between medium A (left) and medium G (right) 45 days after inoculation *Prosthechea garciana*

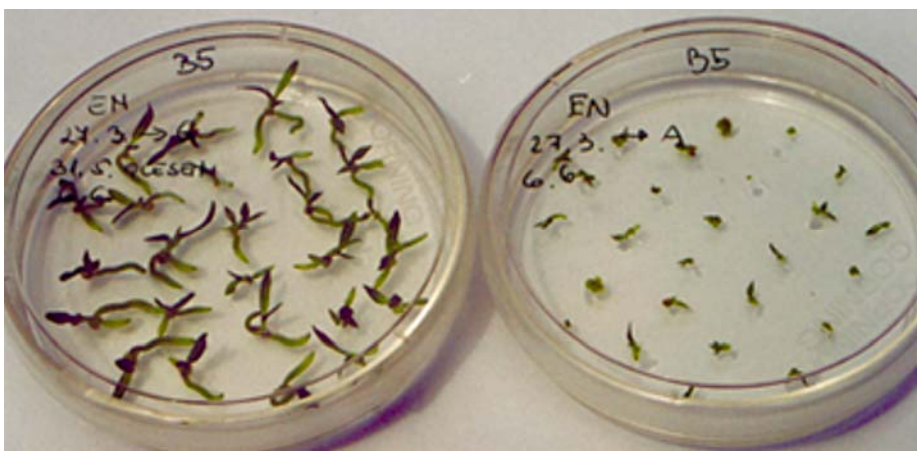


Figure 4: The difference in the growth and development of plants of *Epidendrum nocturnum*, which were at the stage of germination, cultivated on medium containing Gellan gum (left) and agar (right), after the first subcultivation

3.3 Subcultivation

By the time of first subcultivation for *E. nocturnum* and *P. garciana*, 65 days after inoculation, both genera that was cultured on medium G developed 3-4 mm big protocorms with first leaf that was 4 mm long. Protocorms on medium A were smaller (2-3 mm) and were covered with much less rhizoids than those cultured on medium G. Very few of them developed first leaf, which was only 1-2 mm long. We only subcultivated plants germinated on medium G (Table 2, Fig. 4).

Subcultivation for *Zygopetalum* was performed 80 days after inoculation, mainly because of uneven germination on both media (Fig. 2). Half of the seedlings (protocorms with one or more roots) were 4-8 mm big with two leaves 5-8 in length and a short root, the rest were smaller protocorms some still in early germination phases. Protocorms cultured on medium A were on average smaller, less developed and was consequently not subcultivated.

Protocorms of *E. nocturnum* and *P. garciana* became seedlings 11 days after first subcultivation. After second subcultivation in bigger Petri dishes (90 x 20 mm) on media were solidified with 2.6 g/l

Gellan gum, and 70 days *in vitro*, seedlings of *E. nocturnum* and *P. garciana* developed 1-2 leaves (0.5-1.5 cm in length) and 1-2 roots (1-2 cm in length) (Table 2 and Fig. 4). After another subcultivation in baby jars and 172 days *in vitro*, seedlings of *E. nocturnum* and *P. garciana* developed 4-6 leaves (2-4 cm in length) and 2-4 roots (3 cm in length) and were transferred to acclimatization (Table 2).

After another subcultivation in baby jars and 150 days *in vitro*, 50 % of *Zygopetalum* seedlings developed 3-4 leaves (4-5 cm in length), two roots (3 cm in length) and were transferred to acclimatization. Other half of the plants followed gradually, using approximately the same amount of time for growth, if we calculated the time from germination and not their overall *in vitro* presence. All results are presented in table 2.

Table 2: Number of days taken for *in vitro* germination and subsequent growth and development in three orchid genera and one hybrid

Genus/ morphological stages	Medium (number of days after inoculation)	
	A	B
Epidendrum nocturnum		
Germination	9	9
1 leaf initiation	45	35
2 leaf initiation	128	106
3 leaf initiation	/	135
4 leaf initiation	/	160
1 root initiation	106	76
2 root initiation	/	114
Prosthechea garciana		
Germination	9	9
1 leaf initiation	76	45
2 leaf initiation	128	92
3 leaf initiation	/	120
4 leaf initiation	/	158
1 root initiation	115	76
2 root initiation	/	158
***Zygopetalum* hybrid**		
Germination	21	21
1 leaf initiation	65	45
2 leaf initiation	102	80
3 leaf initiation	/	112
4 leaf initiation	/	150
1 root initiation	/	80
2 root initiation	/	112
Maxillaria rufescens		
Germination	11	11
1 leaf initiation	180	180
2 leaf initiation	/	223
3 leaf initiation	/	-
4 leaf initiation	/	-
1 root initiation	/	-
2 root initiation	/	-

Legend: / = Plants were not subcultured; - = Plants did not reach that phase

Table 3: Number and size of the leaves and roots of seedlings after subcultivation

Genotype/ days after inoculation	No. of leaves		Length of leaves		No. of roots		Length of roots	
	A	G	A	G	A	G	A	G
Epidendrum nocturnum								
65	1	1	1-2 mm	4 mm				
172		4-6		2-4 cm		2-4		3 cm
Prosthechea garciana								
65	1	1		4 mm				
172		4-6		2-4 cm		2-4		3 cm
***Zygopetalum* hybrid**								
80	1	2	2-3 mm	5-8 mm		1 or more		3 mm
150		3-4		4-5 cm		2		3 cm

Legend: A – medium with agar; G – medium with Gellan gum

4 DISCUSSION

Various approaches have been reported for orchid seed disinfection. Among them are agitation of disinfection solution with seeds, filtration (vacuum induced passing of the solution through the seeds), gas disinfection and others. They employ different chlorine based gases and solutions (various hypochlorites, Na_2 salt of dichloroisocyanuric acid), Virkon S, hydrogen peroxide, etc. (Hicks, 2000). In addition to conventional disinfection, Hicks (2000) describes a method called presoak in water or any sugar-based solution. This method is effective in invoking fungus vulnerability but it also wets the seed, which is an important aspect of germination process (De Pauw and Remphrey, 1993; Rasmussen, 1995; Arditti, 2000). The latter author describes five important factors enhancing germination of *Cypripedium* sp., one of many hard-to-germinate terrestrial orchids. These are disinfection, cold stratification, soaking, appropriate light regime and chemical constitution of the medium. Method described in this paper successfully satisfies first three factors with the use of centrifuge. We emphasize the stratification at 4 °C, causing micropylar suction of water, which occurs in natural circumstances. Additionally, this method solves the problems of collecting and transferring the seeds after disinfection. It is also important that the time needed for disinfection is shorter, which is desirable for some sensitive species.

For the limited amount of species studied, the storage characteristics of orchid seeds are classified as 'orthodox' in the sense that seed longevity is enhanced by reducing moisture contents (from around 20 %, wet basis to 5 %) and decreasing storage temperatures (from 62 to 0 °C) (Pritchard and Seaton, 1993). When stored dry at 5-8 °C, the time taken for viability to fall to 50 % can be 8-14 years, assuming high initial seed quality (Koopowitz, 2001). At cryogenic temperatures (-196 °C), all metabolic processes and physicochemical changes are arrested which provides a possibility for storing these materials alive in a state of anabiosis for decades (Nikishina, 2001). Thorough wetting and stratification offered by this method might raise the percentage of viable seeds after desiccation and prolonged period of time in seed storage banks. More comprehensive study on the subject is needed to confirm this hypothesis.

Germination percentages of all four genotypes were between 60 and 90 % from which we

concluded that the risk of physical damage to the seeds by rotation moment is not significant. Elevated rotation speeds would probably reduce seed viability, although this was not established in our research.

The time needed for embryos to swell-form protocorms was much lower compared to some studies reporting 20 and up to 160 days needed to reach this phase (Arditti, 1992; Bhattacharjee et al., 1999a; Bhattacharjee et al., 1999b). *Zygopetalum* seeds were not subjected to sterilization procedure, which explains longer time needed for germination compared to *E. nocturnum*, *P. garciana* and *M. rufescens*. This confirms our hypothesis about importance of adequate wetting and stratification of the seeds prior to inoculation on the medium.

After germination phase, constitution of the medium plays the most important role, which can be compared to natural conditions where appropriate fungus invades germinating seeds and their rhizoids. Our experiment showed retarded growth in medium solidified with agar, whereas the same medium with Gellan gum enabled very good development in *E. nocturnum*, *P. garciana* and *Zygopetalum* hybrid. None of the used media, regardless of the gelling agent suited *M. rufescens*. Post experiments showed normal growth of that genus, using commercially available medium (Sigma P-1056) solidified with Gellan gum. Uneven germination of *Zygopetalum* was probably due to green capsule technique using immature seed capsule.

5 REFERENCES

Arditti J. 1982. Orchid seed germination and seedling culture. A manual. In: Orchid Biology: Reviews and Perspectives, Vol. 2. Arditti, J (ed.). New York: Cornell University Press: 243–293

Arditti J. 1992. Fundamentals of Orchid Biology. New York, John Wiley & Sons: 712 p.

Arditti J., Ghani A.K.A. 2000. Numerical and physical properties of orchid seed and their biological implications. Tansley Review, 145: 367-421

Bhattacharjee S., Khan H.A., Reddy P.V. 1999a. *In Vitro* Germination of *Phalaenopsis* Hybrid Seed. Seed Research, 27: 11-13

Bhattacharjee S., Khan H.A., Reddy P.V. 1999b. Effect of Sucrose Levels on *in vitro* Seed Germination of *Phalaenopsis* Hybrid. Journal of Hill Research, 12: 58-60

De Pauw M. A., Remphrey W.R. 1993. *In vitro* germination of three *Cypripedium* species in relation to time of seed collection, media, and cold treatment. Canadian Journal of Botany, 71: 879-885; DOI: 10.1139/b93-100

Gamborg O.L., Murashige T., Thorpe T.A., Vasil I.K. 1976. Plant tissue culture media. In Vitro, 12: 473-478; DOI: 10.1007/BF02796489

Hicks A.J. 2000. Asymbiotic Technique of Orchid Seed Germination. Chandler, The Orchid Seedbank Project: 134 p.

Hinnen M.G.J., Pierik R.L.M., Bronsema F.B.F. 1989. The Influence of Macronutrients and Some Other Factors on Growth of *Phalaenopsis* Hybrid Seedlings *In Vitro*. Scientia Horticulturae, 41: 105-116; DOI: 10.1016/0304-4238(89)90054-X

Johnson T.R., Stewart S.L., Dutra D., Kane M.E., Richardson L. 2007. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae) preliminary evidence for the symbiotic culture advantage. Plant Cell Tiss. Org. Cult., 90: 313-323; DOI: 10.1007/s11240-007-9270-z

Knudson, L. 1922. Non-symbiotic Germination of Orchid seed. Bot. Gaz., 73: 1-25; DOI: 10.1086/332956

Koopowitz H. 2001. Orchids and their Conservation. London, B.T. Batsfort Ltd.: 176 p.

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

Nikishina T.V., Popov A.S., Kolomeitseva G.L., Golovkin B.N. 2001. Effect of cryoconservation of seed germination of rare tropical orchids. Russian Journal of Plant Physiology, 48 (6): 810-815; DOI: 10.1023/A:1012520927743

Pritchard H.W, Seaton P.T. 1993. Orchid seed storage: Historical perspective, curent status and future prospects for long-term conservation. Selbyana, 14: 89-104

Rasmussen H.N. 1995. Terrestrial orchids from seed to mycotrophic plant. Cambridge, Cambridge University press: 444 p; DOI: 10.1017/CBO9780511525452

Snow R. 1985. Improvements in methods for the germination of orchid seed. American Orchid Society Bulletin, 54: 178-181

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.11
Agrovoc descriptors: genotypes, crop yield, statistical data, statistical methods, plant breeding, models

Agris category code: u10, f30

Identification of the most stable genotypes in multi-environment trials by using nonparametric methods

Naser SABAGHNIA[1]

Received November 14, 2014; accepted February 28, 2015.
Delo je prispelo 14. novembra 2014, sprejeto 28. februarja 2015.

ABSTRACT

Genotype performances in multi-environment trials are usually analyzed by different univariate and multivariate parametric models for assessing yield stability and genotype × environment (GE) interaction investigation. One of the alternative strategies can be nonparametric statistics approach which is particularly useful in situations where parametric statistics fail. For an estimation of yield stability of genotypes in various environments two new nonparametric stability statistics ($NS_i^{(1)}$ and $NS_i^{(2)}$) have been used which are based upon the ranks of the genotypes in each environment. These statistics use median as a nonparametric central tendency, and two nonparametric index of statistical dispersion as inter-quartile range and inter-decile range. The $NS_i^{(1)}$ and $NS_i^{(2)}$ nonparametric stability statistics which presented here is similar to the nature and concept of environmental coefficient of variation. Results indicated that the most stable genotype based on the lowest values of these two nonparametric statistics, had the highest mean yield among studied genotypes. Plotting of mean yield versus $NS_i^{(1)}$ and $NS_i^{(2)}$ verified the above results and indicated that the highest mean yielding genotype is identified as the most stable genotype. These nonparametric statistics would be useful for simultaneous selection for mean yield and stability. They can be very helpful in selection for yield stability and determination of favorable genotypes in plant breeding programs.

Key words: adaptation, GE interaction, stability, yield

IZVLEČEK

DOLOČANJE NAJBOLJ STABILNIH GENOTIPOV V RAZLIČNIH OKOLJIH Z NEPARAMETRIČNIMI METODAMI

Za analizo in ocenjevanje stabilnosti pridelka in interakcije genotipov z različnim okoljem se navadno uporabljajo univariatni in multivariatni parametrični modeli. Eden izmed alternativnih pristopov bi lahko bila uporaba neparametričnih modelov, še posebej v primerih kjer je parametrični pristop težko izvedljiv. Za ocenitev stabilnosti pridelka genotipov v različnih okoljih sta bili uporabljeni dve novi neparametrični metodi ($NS_i^{(1)}$ in $NS_i^{(2)}$), ki temeljita na rangih genotipov v danem okolju. Ti metodi uporabljata mediano kot neparametrično osrednjo tendenco in dva neparametrična indeksa porazdelitve kot inter-kvartilno in inter-decilno območje. Predstavljeni neparametrični metodi, $NS_i^{(1)}$ in $NS_i^{(2)}$, sta podobni konceptu koeficienta okoljske spremenljivosti. Rezultati so pokazali, da so imeli najbolj stabilni genotipi, opredeljeni z najmanjšimi vrednostimi $NS_i^{(1)}$ in $NS_i^{(2)}$ največji pridelek med vsemi analiziranimi genotipi. Primerjanje povprečnega pridelka z $NS_i^{(1)}$ in $NS_i^{(2)}$ je potrdila zgoraj navedene rezultate, kar kaže, da so genotipi, ki dajejo največji pridelek tudi najbolj stabilni. Te neparametrične metode bi lahko bile uporabne za hkratno selekcijo povprečnega pridelka in njegove stabilnosti. Lahko bi bile v pomoč pri selekciji primernih genotipov za stabilnost pridelka v žlahtniteljskih programih.

Ključne besede: adaptacija, GE interakcije, stabilnost, pridelek

[1] Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Maragheh, Maragheh, Iran. E-mail sabaghnia@maragheh.ac.ir

1 INTRODUCTION

Multi-environment yield trials are conducted in multiple years and test sites, are central to every plant breeding programs to evaluate and improve different crop plants. These trials are the most common and important experiments in agricultural research and different statistical methods for effective analysis of yield trials have received considerable development and discussion (Karimizadeh and Mohammadi, 2010). Although, only simple statistical method would be needed if genotypes performed similarly in all test environments but, in most cases, genotypes and environments interact such that different rankings often exist for the same set of genotypes tested over a range of test environments (Stoilova and Dechev, 2002; Sabaghnia et al., 2008b). This failure of two or more genotypes to respond similarly to a test environment which is known as genotype × environment (GE) interaction complicates their evaluation with respect to relative performance and usefulness. The GE interaction had an important affect on improvement for better genotypes buffering (Allard and Bradshaw, 1964).

The GE interaction prevents the extrapolation of agronomic evaluations from one environment to another, and so requiring more knowledge of the magnitudes of GE interactions and the various sources of variation in GE interaction. Ignoring the GE interaction is problematic when it is larger than the genotype main effect, which is a common issue in multi-environment yield trials (Gauch, 2006; Arslanoglu and Aytac, 2010; Sabaghnia et al., 2012a). Furthermore, GE interaction complicates genotype recommendations because genotypes must be targeted to specific test sites. In most cases, analysis of variance estimates the existence, significance and large magnitude of GE interaction but cannot explain its importance and so, several statistical strategies had been developed to analysis of the GE interaction pattern.

The question, whether the statistical strategy is sufficiently good to explain the GE interaction, is still discussed. The first strategy which is the classical analysis of variance model for the two-way layout of GE matrix is reviewed by Lin et al. (1986). Also, this strategy involves some linear regression models (Finlay and Wilkinson, 1963; Eberhart and Russell, 1966) for yield stability analysis.

The linear regression model has received much attention in the literature and by including further terms using multivariate statistical procuresses as the second strategy. It has been developed into the additive main effects and multiplicative interaction (AMMI) model (Gauch, 1992) and a thorough review of the theory and applications of this model especially versus genotype plus GE interaction (GGE) biplot model has been given by Gauch (2006) and Gauch et al. (2008). All of the stability methods of both mentioned strategies are parametric. In contrast, there are nonparametric stability statistics as the third strategy which are largely unaffected by data distribution. These stability methods are based on ranks and a special genotype is considered stable if its ranking is constant across environments. Several nonparametric stability statistics have been developed to explain the GE interaction (Huehn, 1979; Kang, 1988; Ketata et al., 1989; Fox et al., 1990; Thennarasu, 1995).

The nonparametric stability statistics separate genotypes based on their similarity of response to a range of test environments. The nonparametric strategy is based on ranks of genotypes and provides an important alternative to the parametric strategies including univariate and multivariate statistics. According to Huehn (1990a) and Huehn (1996), the nonparametric strategy has some advantages over the parametric strategies such as: (i) reduction of the bias caused by outliers, (ii) no assumptions are required about the data distribution, (iii) easy to use and to interpret, (iv) additions or deletions of few genotypes or environments do not cause much variation of estimates, and (v) for many applications such as selection in plant breeding programs and cultivar testing trials, the rank order of genotypes is the most essential information.

The good ability of the nonparametric stability statistics for detecting the most stable genotypes as well as GE interaction investigation have been demonstrated in different crops such as lentil (Sabaghnia et al., 2006; Karimizadeh et al., 2012), chickpea (Ebadi-segherloo et al., 2008) and durum

wheat (Sabaghnia et al., 2012b). The objective of this study was estimation of stability performance of genotypes in different test environments using two nonparametric stability statistics which are based upon the ranks of the genotypes in each environment.

2 MATERIALS AND METHODS

If x_{ij} is denoted as observed mean value of the *i*th genotype in the *j*th environment (*i* = 1, 2, .. ., M; *j* = 1, 2, . . ., N). Then, r*ij* is considered as the rank of genotype *i* in environment *j* which the lowest value is rank 1 and the highest value is rank of K. The concept of yield stability is practicable; a genotype is the most stable over test environments if its ranks are similar over environments, and so maximum stability = equal ranks over all test environments. The two nonparametric stability statistics as $NS_i^{(1)}$ and $NS_i^{(2)}$ which are proposed in this paper are:

$$NS_i^{(1)} = (Q_3 - Q_1) / M_{di}$$

$$NS_i^{(2)} = (D_9 - D_1) / M_{di}$$

In the above nonparametric statistics, $Q_3 - Q_1$ is the inter-quartile range, also called the mid-spread or middle fifty, is a nonparametric index of statistical dispersion, being equal to the difference between the upper and lower quartiles. M_{di} is the median of the genotypes' ranks in the test environments. Also, $D_9 - D_1$ is the inter-decile range is the difference between the first and the ninth deciles. The inter-decile range is another nonparametric index of statistical dispersion of the values in a set of data, similar to the inter-quartile range.

The $NS_i^{(1)}$ and $NS_i^{(2)}$ nonparametric stability statistics which presented here is similar to the nature and concept of environmental coefficient of variation (Francis and Kannenberg, 1978). The important central tendency of ranks is the median and its related measures of dispersion are inter-quartile or inter-decile range. It would be interesting that compare these nonparametric stability statistics with the environmental coefficient of variation (CV). The CV was designed primarily to exploration in investigation on the physiological basis for yield stability (Francis and Kannenberg, 1978), and was found more practical to characterize genotypes on a group basis rather than individually. However, this procedure and its related concept could be used in the plant breeding because it represents a simple and descriptive tool for investigation of genotypes' stability. Considering these benefits of CV concept, using new nonparametric stability statistics ($NS_i^{(1)}$ and $NS_i^{(2)}$) could be useful in GE interaction interpreting and identification of the most stable genotypes especially in nonparametric strategy.

3 RESULTS

The classic dataset of Yates and Cochran (1938) are used in this study and its two-way layout of yield performance for five barley genotypes at six environments yield is shown in Table 1. Also, genotypes mean, the ranks of genotypes in environments and the median of these ranks are given in Table 1. The required statistics for computation of the new nonparametric stability statistics ($NS_i^{(1)}$ and $NS_i^{(2)}$), including the first quartile, the third quartile, the inter-quartile range, the first decile, the third decile and the inter-decile range are shown in Table 2. According to the obtained results, genotype Trebi was the most stable genotype based on the lowest values of these two nonparametric statistics. This genotype had the high mean yield among studied genotypes (Table 1).

Plotting of mean yield versus $NS_i^{(1)}$ (Fig. 1) and $NS_i^{(2)}$ (Fig. 2) verified the above results and indicated that the high mean yielding genotype is identified as the most stable genotype. In other word, these nonparametric statistics would be useful for simultaneous selection for mean yield

and stability. Simultaneous selection for mean yield and stability of performance is an important issue in breeding programs (Yan and Kang, 2003). Kang and Pham (1991) have studied several stability methods for simultaneous selection for yield and stability of performance. Also, Kang (1988) proposed a nonparametric stability statistic (rank-sum) using stability variance of Shukla (1972) and genotype mean rank. According to literature, all nonparametric statistics of Huehn (1979) [Sabaghnia et al. 2006]; nonparametric statistics of Thennarasu's (1995) [Ebadi-Segherloo et al. 2008]; and nonparametric statistics of Ketata et al. (1989) [Dehgahni, 2008] could not be useful for simultaneous selection of mean yield and stability.

Table 1: Two-way layout of genotype × environment cited from the paper of Yates and Cochran (1938)

	E1	E2	E3	E4	E5	E6	Mean
Yield							
Manchuria	161.7	247.0	185.4	218.7	165.3	154.6	188.8
Svansota	187.7	257.5	182.4	183.3	138.9	143.8	182.3
Velvet	200.1	262.9	194.9	220.2	165.8	146.3	198.4
Trebi	196.9	339.2	271.2	266.3	151.2	193.6	236.4
Peatland	182.5	253.8	219.2	200.5	184.4	190.1	205.1
Ranks							Median
Manchuria	1	1	2	3	3	3	2.5
Svansota	3	3	1	1	1	1	1
Velvet	5	4	3	4	4	2	4
Trebi	4	5	5	5	2	5	5
Peatland	2	2	4	2	5	4	3

Table 2: Nonparametric despersive statistics for five genotypes across six environments

Genotypes	Q_1	Q_3	Q_3- Q_1	NS^1	D_1	D_9	D_9- D_1	NS^2
Manchuria	1.0	3.0	2.0	0.8	1	3	2	0.8
Svansota	1.0	3.0	2.0	2.0	1	3	2	2.0
Velvet	2.8	4.3	1.5	0.4	2	5	3	0.8
Trebi	3.5	5.0	1.5	0.3	2	5	3	0.6
Peatland	2.0	4.3	2.3	0.8	2	5	3	1.0

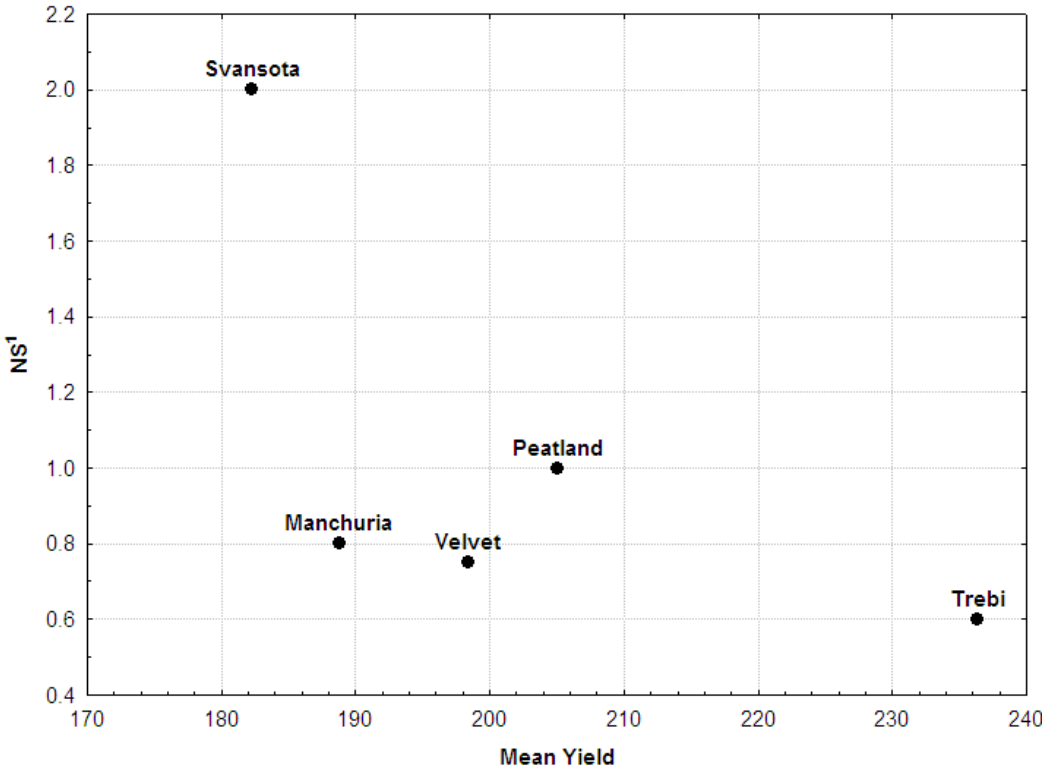


Figure 1: Plot of mean yield versus the first nonparametric stability statistic (NS^1).

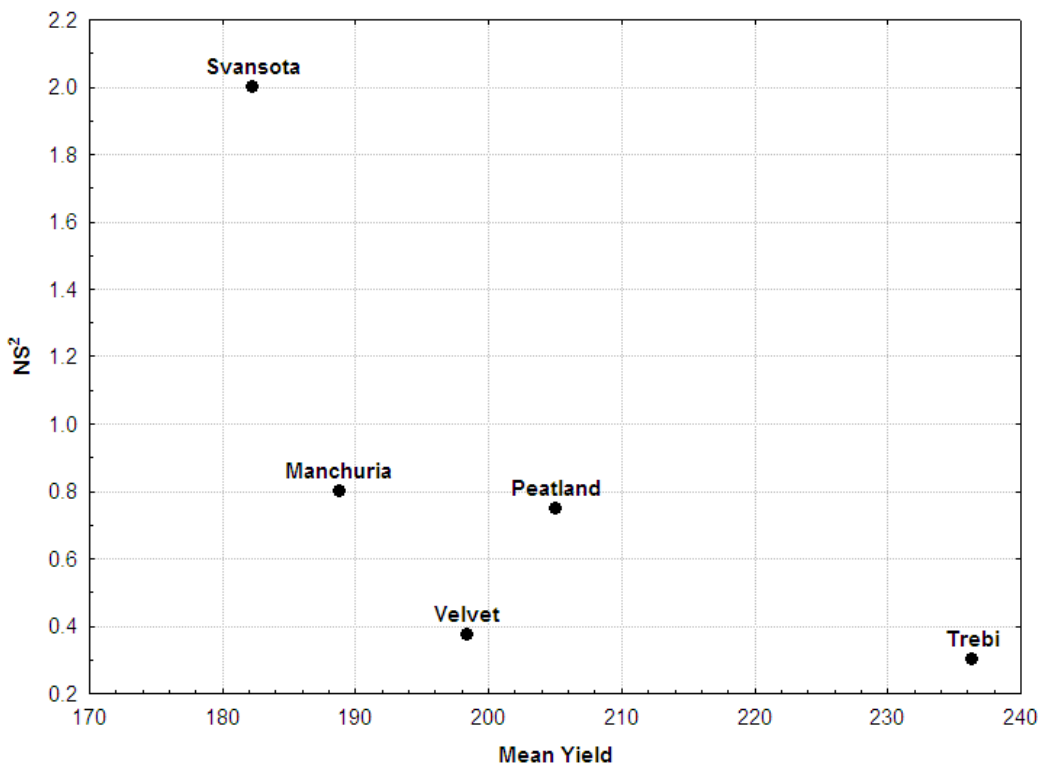


Figure 2: Plot of mean yield versus the second nonparametric stability statistic (NS^2).

4 DISCUSSION

Though several statistical strategies have been proposed for yield stability analysis, they each reflect different aspects of stability nature and maybe no single method can adequately explain genotype performance across test environments. The stability of yield is defined as the ability of a genotype to avoid substantial fluctuations in yield over a range of environmental conditions. The different stability models are broadly classified according to Lin et al. (1986) into there are three types of stability known as Type 1, 2 and 3. Lin and Binns (1991) conclude that stability models of Types 1 and 4 are useful for selection, while those of types 2 and 3 are not useful due to non-heritability. According to Becker and Leon (1988), at least two fundamentally different concepts of stability exist, the static and the dynamic. Both concepts are valuable, but their application depends on the trait considered. It seems that static type of stability is not acceptable for most yield performance breeders, who would prefer a dynamic (agronomic) concept of stability (Becker, 1981; Sabaghnia et al., 2008a). In the agronomic concept of stability, it is not required that the genotypic response to environmental conditions should be equal for all genotypes (Becker and Leon, 1988). For the more important agronomic traits such as grain yield, oil content and etc., the static concept type of stability analysis would not be beneficial for the farmers and is equivalent to type 1 of stability while the dynamic concept of stability is equivalent to type 2 of stability (Lin et al., 1986).

It seems that the new nonparametric stability statistics ($NS_i^{(1)}$ and $NS_i^{(2)}$) have similar nature and concept of environmental CV and so benefits from type 1 of stability while by identification of high mean yield genotype as the most stable genotype benefits from dynamic concept of stability. However, for simultaneous selection of mean yield and stability, it is necessary to use mean yield in the formula of each stability statistic. This concept could be seen in rank-sum (Kang, 1988) as nonparametric stability statistic or desirability index (Hernandez, 1993) as parametric stability statistic. The selection of genotypes for a particular trait depends upon their mean performance and stability statistics. The selected genotypes must have high mean value coupled with stable performance. Most of the nonparametric methods utilized classic stability concept (static or biological concept) for selection of the most favorable genotypes. It seems that there are good potencials in the new introduced nonparametric stability statistics in distinction of favorable genotypes in plant breeding programs. These methods thus provide some flexibility in the hands of plant breeders for simultaneous selection for yield and stability.

There are several statistical models for investigating stability and determination of GE interaction. Each of them reflects different aspects of stability and usually no single approach can fully explain genotype performance across environments. The nonparametric stability statistics seem to be useful alternatives to parametric methods (Huehn 1990b; Yue et al., 1997), although they do not supply information about genotype adaptability. There are several reasons why the use of nonparametric stability statistics could be preferred. These statistics avoid the bias of outliers and no assumptions are required about the distribution of the observations. Furthermore, these methods are easy to use and to interpret; therefore, estimation of stability seems to be an adequate strategy. Many parametric (univariate and multivariate) and nonparametric statistics of stability have been presented and compared in the literature (Lin et al., 1986; Flores et al., 1998; Sabaghnia et al. 2006). For making practical recommendations, it would be necessary to analyse the relationship among these statistics and compare their powers in different stability situations. This topic will be considered in detail in a subsequent paper.

5 REFERENCES

Arslanoglu F., Aytac S. 2010. Determination of stability and genotype × environment interactions of some agronomic properties in the different soybean (*Glycine max*. (L) Merrill) cultivars. Bulgarian Journal of Agricultural Science, 16: 181–195.

Becker, H.C., Leon J. 1988. Stability analysis in plant breeding. Plant Breeding, 101:1–23; DOI: 10.1111/j.1439-0523.1988.tb00261.x

Becker, H.C. 1981. Correlations among some statistical measures of phenotypic stability. Euphytica, 30: 835–840; DOI: 10.1007/BF00038812

Dehghani H., 2008. Estimating yield stability by nonparametric stability analysis in maize (Zea mays L.). Plant Breeding and Seed Science, 58: 61–77.

Ebadi-Segherloo A., Sabaghpour S.H., Dehghani H., Kamrani M. 2008. Non-parametric measures of phenotypic stability in chickpea genotypes (*Cicer arietinum* L.). Euphytica, 2: 221-229; DOI: 10.1007/s10681-007-9552-x

Eberhart S.A. Russell W.A. 1966. Stability parameters for comparing varieties. Crop Science, 6: 36–40; DOI: 10.2135/cropsci1966.0011183X000600010011x

Finlay K.W. Wilkinson G.N. 1963. The analysis of adaptation in a plant breeding program. Australian Journal of Agriculture Research, 14: 742–754; DOI: 10.1071/AR9630742

Flores F., Moreno M.T., Cubero, J.I. 1998. A comparison of univariate and multivariate methods to analyze G × E interaction. Field Crops Research, 56: 271–286; DOI: 10.1016/S0378-4290(97)00095-6

Fox P.N., Skovmand B., Thompson B.K., Braun H.J., Cormier R. 1990. Yield and adaptation of hexaploid spring triticale. Euphytica, 47: 57–64; DOI: 10.1007/BF00040364

Francis T.R. Kannenberg L.W. 1978. Yield stability studies in short-season maize: I. A descriptive method for grouping genotypes. Canadian Journal of Plant Science, 58: 1029–1034; DOI: 10.4141/cjps78-157

Gauch H.G. 1992. Statistical Analysis of Regional Yield Trials: AMMI Analysis of Factorial Designs. Elsevier, Netherlands, Amsterdam.

Gauch H.G. 2006. Statistical analysis of yield trials by AMMI and GGE. Crop Science, 46: 1488–1500; DOI: 10.2135/cropsci2005.07-0193

Gauch, H.G., Piepho H.P., Annicchiaricoc P. 2008. Statistical analysis of yield trials by AMMI and GGE. Further considerations. Crop Science, 48: 866–889; DOI: 10.2135/cropsci2007.09.0513

Hernández C.M., Crossa J., Castillo A. 1993. The area under the function: an index for selecting desirable genotypes. Theoretical Applied Genetics, 87: 409–415; DOI: 10.1007/BF00215085

Huehn M. 1979. Beitrage zur Erfassung der phänotypischen Stabilität. EDV Medizin und Biologie, 10: 112–117.

Huehn M. 1990a. Non-parametric measures of phenotypic stability: Part 1. Theory. Euphytica, 47: 189–194.

Huehn M. 1990b. Non-parametric measures of phenotypic stability: Part 2. Application. Euphytica, 47: 195–201.

Huehn M. 1996. Non-parametric analysis of genotype × environment interactions by ranks. p. 213–228. In M.S. Kang and H.G. Gauch (ed.) Genotype by environment interaction. CRC Press, Boca Raton, F; .DOI: 10.1201/9781420049374.ch9

Kang M.S., Pham H.N. 1991. Simultaneous selection for high yielding and stable crop genotypes. Agronomy Journal, 83: 161–165; DOI: 10.2134/agronj1991.00021962008300010037x

Kang M.S. 1988. A rank–sum method for selecting high-yielding, stable corn genotypes. Cereal Research Communications, 16: 113–115.

Karimizadeh R., Mohammadi M. 2010. AMMI adjustment for rainfed lentil yield trials in Iran. Bulgarian Journal of Agricultural Science, 16: 66–73.

Karimizadeh R., Mohammadi M., Sabaghnia N., Shefazadeh M.K., Pouralhossini J. 2012. Univariate stability analysis methods for determining genotype × environment interaction of durum wheat grain yield. African Journal of Biotechnology, 11: 2563–2573.

Ketata H., Yan S.K., Nachit M. 1989. Relative consistency performance across environments. Int. Symposium on Physiology and Breeding of Winter Cereals for stressed Mediterranean Environments. Montpellier, July 3–6, 1989.

Lin, C.S. Binns M.R. 1991. Genetic properties of four types of stability parameters. Theoretical Applied Genetics, 82: 505–509; DOI: 10.1007/BF00588606

Lin, C.S., Binns M.R., Lefkovitch L.P. 1986. Stability analysis: Where do we stand? Crop Science, 26: 894–900; DOI: 10.2135/cropsci1986.0011183X002600050012x

Sabaghnia N., Dehghani H., Sabaghpour S.H. 2006. Nonparametric methods for interpreting genotype · environment interaction of lentil genotypes. Crop Science, 46: 1100–1106 ; DOI : 10.2135/cropsci2005.06-0122

Sabaghnia N., Dehghani H., Sabaghpour S.H. 2008a. Graphic analysis of genotype by environment interaction for lentil yield in Iran. Agronomy Journal, 100: 760–764; DOI: 10.2134/agronj2006.0282

Sabaghnia N., Karimizadeh R., Mohammadi M. 2012a. Genotype by environment interaction and stability analysis for grain yield of some lentil genotypes. Žemdirbystė=Agriculture 99: 305–312.

Sabaghnia N., Karimizadeh R., Mohammadi M. 2012b. The use of corrected and uncorrected nonparametric stability measurements in durum wheat multi-environmental trials. Spanish Journal of Agricultural Research 10: 722–730; DOI: 10.5424/sjar/2012103-384-11

Sabaghnia N., Sabaghpour S.H., Dehghani H. 2008b. The use of an AMMI model and its parameters to analyse yield stability in multi-environment trials. Journal of Agricultural Science, 146: 571–581; DOI: 10.1017/S0021859608007831

Shukla G.K. 1972. Some aspects of partitioning genotype-environmental components of variability. Heredity 28:237–245; DOI: 10.1038/hdy.1972.87

Stoilova A. Dechev D. 2002. Genotype environment interaction and phenotypic stability of economic traits in cotton lines. Bulgarian Journal of Agricultural Science, 8: 485–491.

Thennarasu K. 1995. On certain non-parametric procedures for studying genotype–environment interactions and yield stability. Ph.D. thesis. P.J. School, IARI, New Delhi, India.

Yan W., Kang M.S. 2003. GGE-biplot analysis:A graphical tool for breeders, geneticists, and agronomists. CRC Press, Boca Raton, FL, USA.

Yates F. Cochran W.G. 1938. The analysis of groups of experiments. Journal of Agricultural Science, 28: 556–580; DOI: 10.1017/S0021859600050978

Yue, G.L., Roozeboom K.L., Schapaugh W.T., Liang G.H. 1997. Evaluation of soybean cultivars using parametric and nonparametric stability estimates. Plant Breeding, 116: 271–275; DOI: 10.1111/j.1439-0523.1997.tb00995.x

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.12

Agrovoc descriptors: grasses, gramineae, dry matter content, crop yield, statistical data, statistical methods, models, simulation, weather data

Agris category code: u10, f01

Calibration of the LINGRA-N model to simulate herbage yield of grass monocultures and permanent grassland in Slovenia

Tjaša POGAČAR[1], Domen IPAVEC[2], Janko VERBIČ[3], Lučka KAJFEŽ-BOGATAJ[4]

Received January 30, 2015; accepted March 09, 2015.
Delo je prispelo 30. januarja 2015, sprejeto 09. marca 2015.

ABSTRACT

In this study, we calibrated the LINGRA-N model using the minimization of *RMSE*, and proceeded to evaluate its performance. We simulated herbage dry matter yield of cock's foot (*Dactylis glomerata* L.) and perennial ryegrass (*Lolium perenne* L.) in Jablje in the period 1998–2013, and multiple-species grassland in Ljubljana (S72) in 1974–1993. The overall performance of LINGRA-N is fair for perennial ryegrass (*RMSE%* < 25%) and good for cock's foot and S72 (*RMSE%* < 15%). The index of agreement (*d*) suggests that LINGRA-N is not calibrated well enough to simulate the interannual herbage yield variability for S72, so the model cannot yet be used for the simulation of multi-species grassland herbage yield. In contrast, the herbage yields of cock's foot and perennial ryegrass in Jablje are simulated correctly (with *d* values 0.84 and 0.78, respectively). One of our further goals is to use the calibrated model on a specific location for the simulation of the herbage yield of grass monocultures under various weather conditions as well as for the simulation of climate change effect on it.

Key words: grassland herbage yield, simulation, LINGRA-N, calibration, evaluation, variability, cock's foot, perennial ryegrass

IZVLEČEK

UMERJANJE MODELA LINGRA-N ZA SIMULACIJO PRIDELKA POSAMEZNIH VRST TRAV IN TRAJNEGA TRAVINJA V SLOVENIJI

V raziskavi smo model LINGRA-N umerjali na podlagi minimizacije kvadratnega korena napake (*RMSE*) ter ocenjevali kakovost umerjenega modela. Uporabili smo podatke o pridelku suhe snovi navadne pasje trave (*Dactylis glomerata* L.) in trpežne ljuljke (*Lolium perenne* L.) v Jabljah v obdobju 1998–2013 ter trajnega travinja v Ljubljani (S72) v obdobju 1974–1993. Glede na *RMSE* se je izkazalo, da je bilo umerjanje primerno (*RMSE%* < 25 %) za trpežno ljuljko in dobro za navadno pasjo travo ter trajno travinje (*RMSE%* < 15 %). Vendar pa je indeks ujemanja (*d*) pokazal, da za S72 model ni dovolj dobro umerjen, da bi sledil medletni variabilnosti pridelka, kar pomeni, da ga v taki obliki ne moremo uporabiti za nadaljnje modeliranje pridelka trajnega travinja. Za navadno pasjo travo (*d* = 0,84) in trpežno ljuljko (*d* = 0,78) so rezultati dobri. Umerjen model bomo zato uporabili za simulacijo pridelka posamezne vrste trave pri različnih vremenskih razmerah in pod vplivom podnebnih sprememb na specifični lokaciji.

Ključne besede: pridelek travne ruše, modeliranje, LINGRA-N, umerjanje, ocena kakovosti, variabilnost, navadna pasja trava, trpežna ljuljka

[1] univ. dipl. meteorol., University of Ljubljana, Biotechnical Faculty, Jamnikarjeva 101, SI-1000 Ljubljana, tjasa.pogacar@bf.uni-lj.si

[2] student, University of Ljubljana, Faculty of Mathematics and Physics, Jadranska 19, SI-1000 Ljubljana, domen.ipavec@gmail.com

[3] univ. dipl. inž. agr., Agricultural Institute of Slovenia, Hacquetova 17, SI-1000 Ljubljana, janko.verbic@kis.si

[4] prof. dr., University of Ljubljana, Biotechnical Faculty, Jamnikarjeva 101, SI-1000 Ljubljana, lucka.kajfez.bogataj@bf.uni-lj.si

This article is based on research undertaken for a doctoral dissertation by Tjaša Pogačar. Supervisor: prof. dr. Lučka Kajfež-Bogataj.

Članek je nastal na osnovi raziskav v okviru doktorske disertacije Tjaše Pogačar. Mentorica: prof. dr. Lučka Kajfež-Bogataj.

1 INTRODUCTION

Grasslands are an important agroecosystem in Europe with essential functions for fodder and ecosystem service supply. Impact assessment modelling of European agriculture and the environment needs to consider grasslands and requires spatially explicit information on grassland distribution and productivity, which is not available (Smit et al., 2008). As grassland budgeting must precede production of the grass, its effectiveness is severely limited by the uncertainty of future herbage supply. This is due to grass growth rates being highly variable both in time, i.e. within and between seasons at one location, and in space, i.e. between locations at any one time (Barrett et al., 2005).

According to SURS (2014), in Slovenia the area of permanent grasslands has changed very little in last 10 years: from 285,410 ha in 2000 to 277,492 ha in 2010 (excluding common grassland: 22,786 ha in 2000 and 8221 ha in 2010). This accounts for the biggest share of utilised agricultural area, 58.5 % (SURS, 2014). On the other hand, the area of sown grassland has been increasing lately to provide enough quality forage (Tehnološka priporočila ... , 2008). Although the area of sown grassland has not changed (7632 ha, compared to 7702 ha previously), the area of grass-clover and clover-grass mixtures increased from 3918 ha in 2000 to 16,675 ha in 2010 (SURS, 2014).

A considerable number of models dealing with various agronomical and ecological aspects of grassland have been developed in the past decades (Herrmann et al., 2005). Process-based models can be used to study the interactions between soil and weather conditions, and management and crop growth, thereby facilitating harvest decisions that require optimization of forage yield and nutritive value (Jego et al., 2013). One of the main advantages of crop model application is the possibility to use the models under various weather and soil conditions and in various environments in various regions of the world (Žalud et al., 2006). We can use data from archives or future scenarios to run the model (Barrett and Laidlaw, 2005). Hence crop simulation models have lately become an essential tool to study climate change impacts and to perform other scenario analyses with the aim of determining crop yield and production security. Furthermore, the awareness of a potential use of models in decision support systems for livestock grazing and forage supply planning has increased (Barrett and Laidlaw, 2005; Barrett et al., 2005).

The most complex model is not necessarily the most appropriate one to simulate grass sward growth and grassland herbage yield, because we may need input data that are not easily available. There are two modelling approaches: a simple static model without a description of process rates, and a dynamic model where state variables change in accordance to fluctuating process rates (Bouman et al., 1996).

However, in Slovenia there is no simulation model in use that would serve to monitor or forecast grass sward growth and grassland herbage yield. Our long-term objective is to develop a tool that is sensitive to climatic variation, soil properties and management practices for simulating and evaluating the growth and herbage yield of sown or permanent grasslands. In this paper we describe the first steps of our work with the LINGRA-N model. The aim of the present optimisation was to obtain a common parameter set that will serve as input for LINGRA-N in order to use it for simulations under varied input climate conditions. We expect the calibrated model to explain the major part of interannual grassland herbage yield variability in Slovenia (on a specific location) and as such to be useful for further simulations. This is also strategically important for the planning of forage supply and the adaptation of Slovene grasslands to various weather conditions.

2 METHODS AND DATA

2.1 The LINGRA-N model

The LINGRA model (Bouman et al., 1996; Schapendonk et al., 1998; Wolf, 2006; Pogačar and Kajfež-Bogataj, 2011) is an intermediate type of model in which both static and dynamic descriptions are used. Only a small number of processes involving the key parameters are simulated dynamically. On the other hand, parameters that have relatively little impact on crop growth, or of which knowledge is scarce, have been treated using the static approach. The simulated key processes are light utilization, leaf formation, leaf elongation, tillering, and carbon partitioning. LINGRA was designed for applications such as forecasting of (regional) grassland herbage yield and quantitative land-use evaluation and to study the effects of climate change on grassland herbage yields (Schapendonk et al., 1998).

Our research is based on a new version of the model, LINGRA-N (Wolf, 2012). It is an extension of LINGRA for forage production under sub-optimal nutrient availability. It can be used for potential, water limited and nitrogen (N) limited growing conditions, but it has not yet been widely used for research. LINGRA-N is largely equal to LINGRA, but the new model structure allows simulations for different grass sward types growing under a large range of soil and weather conditions with different management regimes (Wolf, 2012). For performing land use studies at the regional scale the possibility to do simulations for all these combinations is essential and is made possible by putting all the input data in separate input files (Wolf, 2012). In both models, crop growth after the winter period is initialized when a 10-day moving average of daily air temperatures is higher than the given base temperature. Growth only takes place when the supply (photosynthesis plus reallocation from the reserve pool) exceeds or equals the demand function. Conversely, carbohydrates will be stored in the reserve pool when the photosynthetic supply exceeds the demand. To calculate the grassland herbage yield, Y (g dry matter m^{-2}) can be calculated by multiplying total biomass by dynamic grass specific partitioning factors, *HI*:

$$Y = \int (f_t PAR_t E_t) HI \quad (1)$$

where f_t is the fraction of photosynthetically active radiation (*PAR*) intercepted by the foliage, PAR_t the incoming amount of *PAR* (MJ m^{-2} d^{-1}), and E_t the light utilisation efficiency (g dry matter (MJ PAR)$^{-1}$). Intercepted radiation is calculated from the leaf area index. Light use efficiency is made dependent on air temperature, level of *PAR* and possibly occurring water (Bouman et al., 1996) or nitrogen stress (new for LINGRA-N).

As others (e.g. Merot et al., 2008) we did not take into account the complex processes occurring on the crop during winter. It is assumed that the crop is also optimally protected against pests, diseases and weeds (Bouman et al., 1996). Water and nutrient availability are both subject to change by management but, in contrast with the extensive use of fertilizers, irrigation of agricultural grasslands is not widely practised.

As input for LINGRA-N user has to prepare meteorological, soil and grass crop data. The following daily weather data are used to run the model: minimum and maximum temperatures, irradiation, precipitation, mean wind speed, and early morning vapour pressure. Two output files are produced from each simulation run. One gives the soil and grass crop status at a defined regular interval during the crop growth period. The other gives mainly the total amount of cut grass, the cumulative components of the water balance, the cumulated crop's nitrogen (N) uptake and N losses (Wolf, 2012).

2.2 Calibration and performance evaluation of the model

Most studies on the agronomic performance of grassland are restricted to a few years, which is too short to allow for an analysis of production stability or for an estimation of the probable range of grassland productivity at a given site (Herrmann et al., 2005). Ideally, experiments used for grass model development should include detailed repetitive measurements of crop performance over the growing season, in addition to exact

information about the weather and soil conditions at the experimental site (Persson et al., 2014). The lack of long-term experimental data limits the use of grassland models, as reliable and sufficiently extensive data are essential for their calibration and verification (Trnka et al., 2006). Grassland herbage yield data over at least 10 year period are needed for the LINGRA or LINGRA-N calibration, to indicate both the mean yield level and yield variation (Wolf, 2006).

Parameter estimation and model evaluation are essential phases in every modelling project. Most of the studies are based on a "trial and error" approach whereby different values of the parameters are tested until the simulation fits the data reasonably well (Merot et al., 2008; Shibu et al., 2010). We conducted a simple sensitivity analysis (without interaction taken into account) in order to identify non-influential parameters that can be omitted from the calibration. Values of non-influential parameters were then set as default values in LINGRA-N. Influential parameters were calibrated by minimizing the difference (minimization of *RMSE*, e.g. Jego et al., 2013) between the simulated and measured forage dry matter yield. At first in four groups of parameters changing simultaneously (around 40,000 iterations for each group), secondly in six groups (parameters changing with higher precision) and finally in two more groups, depending on previous results. Together, this made 12 steps of the calibration procedure.

The commonly used correlation measures such as Pearson's correlation coefficient (r) and its square, coefficient of determination (r^2), and tests of statistical significance in general are often inappropriate or misleading when used to compare model predicted and observed variables. Difference measures, however, seem to contain appropriate and insightful information (Willmott, 1982). So we decided to use the root mean square error ($RMSE$) and its relative value in % (*RMSE%*) to evaluate the model performance (e.g. Willmott, 1982; Jego et al., 2013):

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(O_i - P_i)^2}\ , \qquad (2)$$

$$RMSE\% = \frac{RMSE}{\overline{O}} \times 100 \qquad (3)$$

where n is the number of measurements, O_i the measured value, $\overline{O}$ the mean of the measured values and P_i the value simulated by the model. The simulation is considered to be excellent when $RMSE\% \le 10\%$, good when $10\% < RMSE\% \le 20\%$, fair when $20\% < RMSE\% \le 30\%$, and poor when $RMSE\% > 30\%$ (Jamieson et al., 1991 op. cit. Jego et al., 2013).

Also according to Willmott (1982) we should determine how much of $RMSE$ is systematic in nature and what portion is unsystematic. The systematic part can be described by $RMSE_s$ and the unsystematic part takes the form of $RMSE_u$:

$$RMSE_s = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(\hat{P}_i - O_i)^2} \qquad (4)$$

$$RMSE_u = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(P_i - \hat{P}_i)^2} \qquad (5)$$

where $\hat{P}_i$ is derived from the least-squares regression, $\hat{P}_i = a + bO_i$ (a is the intercept and b is the slope).

Moreover, the difference between the simulated and observed dry matter was evaluated by means of Willmott's index of agreement (Willmott, 1982):

$$d = 1 - \frac{\sum_{i=1}^{n}(Pi - Oi)^2}{\sum_{i=1}^{n}(|P_i'| + |O_i'|)^2} \qquad (6)$$

where n, P_i, and O_i are defined as in (2). P_i' and O_i' are $P_i - \overline{O}$ and $O_i - \overline{O}$, respectively. d is intended to be a descriptive measure, and is both a

relative and bounded measure which can be widely applied in order to make a cross-comparison of models (Willmott, 1982).

2.3 Input data

The application of LINGRA-N in Slovenia brought along major challenges regarding input parameters. During our research we came across two extensive collections of grassland herbage yield data.

Firstly, measurements of herbage yield of sown grassland have been performed since 1998 (at some locations even since 1983) on several locations as part of the research of the Agricultural Institute of Slovenia (KIS, 2014). Taking into consideration the available nearby meteorological and soil data we decided to use the experiments in Jablje and Rakičan. Only the results for Jablje are presented in the article. The advantage of the experiments is that the measurements were performed for grass monocultures. We used cock's foot (*Dactylis glomerata* L., DG), as it is said to be more drought resistant (Kapun, 2005), and perennial ryegrass (*Lolium perenne* L., LP), which was often used abroad for the calibration of LINGRA. The experiment was carried out from 1998 to 2013, except in 2005 and 2006 for DG, and except in 2000, 2005 and 2008 for LP.

The herbage yield of sown grass sward is usually considerably lower in the seeding year (development is still in progress, the first mowing is only to remove the weeds) and reaches its optimum in the second (LP) or third (DG) year. Afterwards the herbage yield starts to diminish: faster for LP, which usually disappears from stands in the fourth year. DG still grows even in the sixth year; the herbage yield is decreasing more slowly. Weather conditions can outweigh the development significance for grass growth, but only in extremes like severe drought. Consequently, it would be ideal to have herbage yield data on the grasses sown every year; otherwise this has substantial implications for a general model to predict grass production. On this ground, because of a significant deviation from our expectations, in view of weather conditions and other herbage yield measurements we had to eliminate the years 2004 and 2007 for DG, and 2001 and 2004 for LP. In 2004 DG was in its fifth year and the herbage yield was extremely low. On the other hand, in 2007 the ley was in the second year and the herbage yield was very high. Something similar could be seen with LP, where 2001 was the first year and 2004 the fourth. For other years herbage yields were averaged for leys of various age to minimize the effect of younger and older leys.

Both experiments were carried out in randomized block design in four replicates with a plot size of 6 m^2. There were in average five varieties of cock's foot (from 2 to 8) and nine varieties of perennial ryegrass (from 5 to 14). We calculated the minimum, average and maximum herbage yields for each year from all replicates. Total datasets of averages for 12 years (11 for LP) were divided into two parts, the odd six years for calibration and the even six (five for LP) for validation. Some simplifications were made to arrange the data for the use in the model. Average cutting days were used (Table 2), although not all years of the experiment involved four mowings, but between two and five. Additionally, the fertilization amount of nitrogen (N) is averaged for LP.

Secondly, we studied the whole unpublished paper archive of prof. dr. Mirko Leskošek from the Biotechnical Faculty (BF) at the University of Ljubljana. He had done an exceptional job, performing fertilizing experiments on permanent grassland, starting from the year 1955. Some experiments contain 10 or even 20 years of data. Even though a major part of the data is not useful for us, it would be a serious loss never to use them, so we collected them in a file to be available for further studies. Basic information about the data is presented in Table 1.

Table 1: The base of experiments carried out by prof. dr. Mirko Leskošek, now arranged in a file
Preglednica 1: Preged poskusov prof. dr. Mirka Leskoška, ki smo jih uredili v elektronski obliki

Experiment number	Period	Number of mowings	Variants of fertilization	Location
S2	1955–1966	2	6	Hoče
S5	1955–1969	2	6	Pristava near Mestinje
S6	1955–1960	2	6	Imeno
S7	1955–1970	2	4	Sela near Podčetrtek
S8	1955–1968	2	6	Brestanica
S9	1955–1970	2	4	Rožno near Brestanica
S10	1955–1970	2	4	Arto near Sevnica
S11	1955–1968	2	4	Škofljica
S12	1955–1968	2	6	Blatna Brezovica near Vrhnika
S13	1956–1965	2	7	Predoslje near Kranj
S18	1958–1963	2	9	Litija
S26	1960–1970	2	6	Horjul
S27	1961–1970	1, 2	5	Dragatuš
S34	1963–1968	2	5	Rožno near Brestanica
S35	1963–1968	2	5	Vnanje Gorice
S40	1972–1981	2	6	Rožno near Brestanica
S42	1972–1980	2	6	Gabrovčec near Krka
S44	1969–1973	2	5	Polšnik above Litija
S45	1969–1973	2	5	Nova vas – Bloke
S46	1969–1973	2	6	Lome above Idrija
S47	1969–1973	2	5	Preska above Litija
S48	1969–1973	2	6	Sorica above Škofja Loka
S49	1969–1973	2	5	Cerknica
S50	1969–1973	2	6	Gorjuše above Bohinj
S72	**1974–1993**	**2, 3, 4**	**11**	**Ljubljana BF**
S77	1975–1980	5	11	Bašelj
S78	1975–1978	4	9	Letenice
S79	1975–1979	3	11	Ljubljana BF
S80	1981–1988	3	7	Ljubljana BF
S87	1984–1989	3	8	Ljubljana BF

This article presents the results for the experiment S72 in Ljubljana. It was carried out on multi-species permanent grassland of the laboratory field of the Biotechnical Faculty in Ljubljana from 1974 to 1993. The meadow is situated in the Pre-Alpine area at an altitude of about 300 m. Despite a relatively large amount of rainfall the habitat is quite dry, which is also reflected in the grassland plant community, which can be found on the unfertilized *Bromo-Plantaginetum mediae* (Žitek, 1991). The experiment was carried out on 64 parcels with an area of 14 m^2 each in 16 variants of fertilization and three cutting regimes (two, three or four mowings). The data are already averaged for each fertilization and cutting variant. We used the data from the following treatment combination: three mowings and the fertilization application of 180 kg N ha^{-1}, 120 kg P_2O_5 ha^{-1} and 165 kg K_2O ha^{-1}. Again, total dataset was divided into two parts, the odd 10 years for calibration and the even 10 for validation. Management practices such as the date and rate of N fertilization as well as cutting dates (Table 2) were used to create the management file.

Table 2: Average cutting days for four mowings in Jablje (DG and LP) and for three mowings in Ljubljana (S72) used at the beginning of calibration and N fertilization rates for all experiments

Preglednica 2: Povprečni datumi za štiri košnje v Jabljah (DG in LP) in tri v Ljubljani (S72) ter stopnja gnojenja z dušikom za vse poskuse

Experiment	Average cutting days 1^{st}-2^{nd}-3^{rd}-4^{th} mowing (Julian day)	Fertilization rate 1^{st}-2^{nd}-3^{rd} (kg N ha^{-1})
DG	136-187-242-287	60-50-46
LP	135-176-237-280	60-50-46
S72	145-206-267	60-60-60

For all three experiments day 91 was set as the date of the first fertilization; it is a simplified marker of the beginning of the vegetation period. The first days after the first and second mowing were set as the dates of the second and third fertilization. At the beginning, crop specific parameters were set as default. For more common parameters (16) the calibration range was determined from the literature, and for the others (11) as a 30 % range around the default value. In the paper the statistic criteria (averages, standard deviations, the root mean square error, its systematic and unsystematic part, the index of agreement) are calculated for the whole datasets.

We acquired the meteorological data from the Slovenian Environment Agency (ARSO, 2014). Minimum and maximum air temperatures (°C), and precipitation (mm) are measured, mean wind speed (m s^{-1}) is averaged, whereas irradiation (kJ m^{-2}) and early morning vapour pressure (kPa) are calculated. Jablje and Ljubljana both have the moderate continental climate of the central Slovenia. For Jablje we can use the nearest meteorological station Airport Jože Pučnik Ljubljana (Brnik) and for Ljubljana the station Ljubljana Bežigrad (Ljubljana). This of course brings some uncertainty to the modelling results, because the data from Brnik are not representative for Jablje in every meteorological situation. Particularly in the summer time, the water balance can vary due to local convective events that do not occur at both locations. The missing values for less than five days were interpolated as part of model calculations (this is not possible for precipitation, but no precipitation data were missing). More data were missing for early morning vapour pressure in Brnik and were replaced with the data for Ljubljana.

In Brnik, in the period 1981–2010 the average summer (June to August) maximum air temperature was 25.0 °C and the annual 14.7 °C. For the minimum air temperature the summer average was 12.4 °C and the annual 4.0 °C. The average annual precipitation was 1363 mm. Temperature conditions in Ljubljana are warmer. Temperatures in the reference period (1961–1990) are closer to the ones in the period 1981–2010 in Brnik. The average summer maximum air temperature was 25.0 °C and the average annual maximum air temperature 14.8 °C in the reference period. In the period 1981–2010 these average air temperatures were higher by 1.2 °C and 0.8 °C, respectively. The average summer minimum air temperature was 13.4 °C and the annual 5.5 °C in the reference period. In the second period those averages were higher by 1.4 °C and 1.1 °C, respectively. The average annual precipitation was practically the same as in Brnik, 1393 mm in the reference period and 31 mm less in the second period.

The soil type in Jablje is pseudogley-gley, deep and moderate, the texture is silty clay. Soils are described in the proceedings of the conference about IOSDV experiments (Tajnšek, 2003). The basic soil data for the experiment in Ljubljana are from the Centre of soil and environmental science (CPVO, 2014). The experiment had similar conditions than the ones in Jablje: pseudogley on gravel, the texture is silty clay. Soils are medium deep and brown. Soil input parameters (Table 3) were derived from the basic soil data using the SPAW model, version 6.02.75, developed by Saxton (Saxton and Rawls, 2006).

Table 3: Soil data for Jablje (DG and LP) and Ljubljana (S72)
Preglednica 3: Podatki o tleh za Jablje (DG in LP) in Ljubljano (S72)

Experiment	Soil moisture content at field capacity ($cm^3\ cm^{-3}$)	Soil moisture content at wilting point ($cm^3\ cm^{-3}$)	Soil moisture content at saturation ($cm^3\ cm^{-3}$)	Maximal percolation rate to deeper soil layers ($cm\ d^{-1}$)
DG and LP	0.36	0.14	0.50	30
S72	0.29	0.13	0.49	39

The initial soil water content was set to field capacity, which is representative of soil water status when simulations start during winter or at the beginning of spring (Schapendonk et al., 1998; Lazzarotto et al., 2009; Jego et al., 2013).

3 RESULTS AND DISCUSSION

After conducting the sensitivity analysis, we eliminated 10 non-influential parameters and continued the calibration with 27 parameters. From the much simpler LINGRA model evaluation and sensitivity analysis conducted by Bouman et al. (1996), only four influential parameters were selected for calibration: minimum threshold temperature for photosynthesis, threshold temperature after which photosynthesis reaches a maximum value, leaf area index after cutting, and maximum light use efficiency. Besides those the most important in the LINGRA-N model are the fraction of precipitation lost by surface runoff, the initial number of tillers, dates of mowing, the total mineral soil N available at the start of the growth period, the fraction of total biomass to roots under stressed conditions and the recovery fractions of fertiliser N applications.

In the calibration using the data for Jablje, 16 out of 27 default parameters were changed for cock's foot (DG), and 22 for perennial ryegrass (LP). For S72 (the experiment in Ljubljana) there were 19 such parameters. For DG and LP the meteorological, soil and management data are the same (except the mowing dates), so differently calibrated parameters only mean variation in their crop characteristics. The difference between the predicted herbage yields for both grass species can be seen in Figure 1. The interannual variability due to weather conditions is similar, but in general the herbage yields are lower for LP. The simulations are for instance the same for the year 2009, but on the other hand the difference between the observed values for the year 2009 is 3000 kg DM ha^{-1}, and it is unfortunately practically impossible to determine the reason.

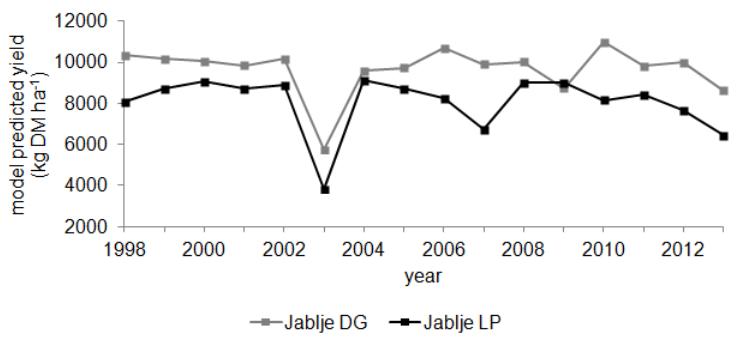


Figure 1: Model predicted herbage yield after the calibration for cock's foot (Jablje DG) and perennial ryegrass (Jablje LP) in Jablje
Slika 1: Z umerjenim modelom izračunan pridelek za navadno pasjo travo (Jablje DG) in trpežno ljuljko (Jablje LP) v Jabljah

As Figure 2 demonstrates, the dynamics of herbage yield are quite well fitted for DG. As an illustration we can additionally see some intermediate steps in the calibration procedure. Throughout the period, the final model predicted herbage yield is firmly inside the minimum-maximum frame of observed values. The simulation is excellent for the year 2003, when extreme drought reduced the herbage yield to a considerable extent. The simulation is also very good for the years 2001, 2011, 2012, and 2013.

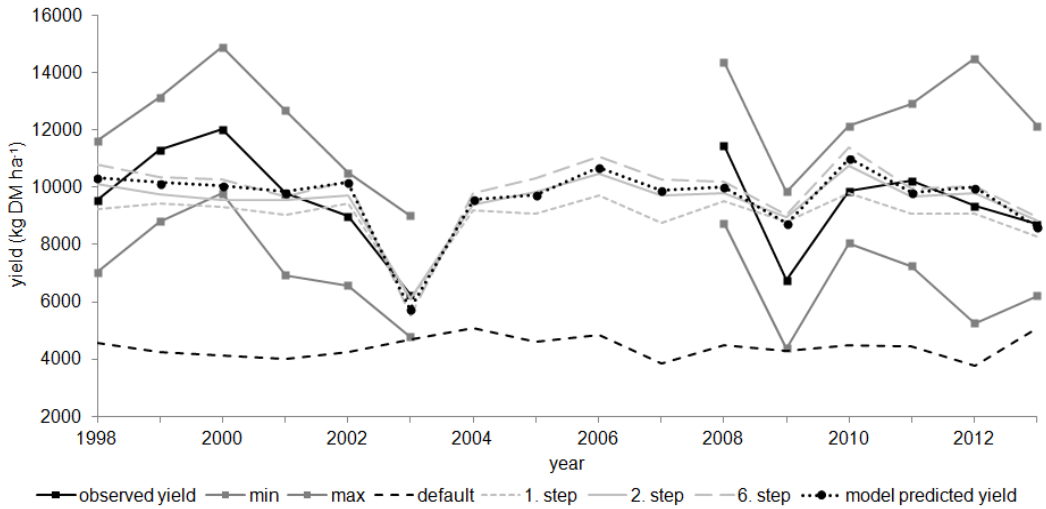


Figure 2: The herbage yield of cock's foot in Jablje: average (observed yield), minimum (min) and maximum (max) values of observed herbage yield, the default output of the model without calibration (default), 1st, 2nd, and 6th step herbage yield results of the calibration procedure, and model predicted herbage yield at the end of calibration

Slika 2: Pridelek navadne pasje trave v Jabljah: povprečne (observed yield), najmanjše (min) in največje (max) izmerjene vrednosti pridelka, modelska simulacija pridelka pred umerjanjem modela (default), rezultat simulacije 1., 2. in 6. koraka pri umerjanju modela in simulacija pridelka z umerjenim modelom

As regards the other years we have to keep in mind that there can be bigger differences because of the aging of the grass sward. In the year 2000 the ley was in the second year, so the observed herbage yield of cock's foot is much higher than predicted. A similar situation was seen in the year 2008. In contrast, the year 2009 was the ley's fourth year, grass sward growth slowed down, weeds started to overgrow it. Thus the observed herbage yield is much lower than predicted.

For LP, the predicted herbage yields are very close to observed ones in the years 2002, 2003, 2009, 2011 (figure not presented). The fit altogether is not as good as for DG, but it exceeds the maximum observed herbage yield only a little in 1999. The reason could be the same as with DG; the ley was in the third year, which is past the optimum for LP, and due to weeds there were also only two mowings. Furthermore, the observed herbage yields in the years 2007 and 2010 were quite low (so the simulation is too high). A comment for both years could be that sometimes already after its second overwintering LP stops to grow well and becomes very sensitive to high summer temperatures and soil moisture deficit.

Additionally, on the scatter plots (Figure 3) we can clearly see the relationship between the observed and predicted values for both grass species in Jablje. The plots alone indicate that the model is more variable in the case of LP.

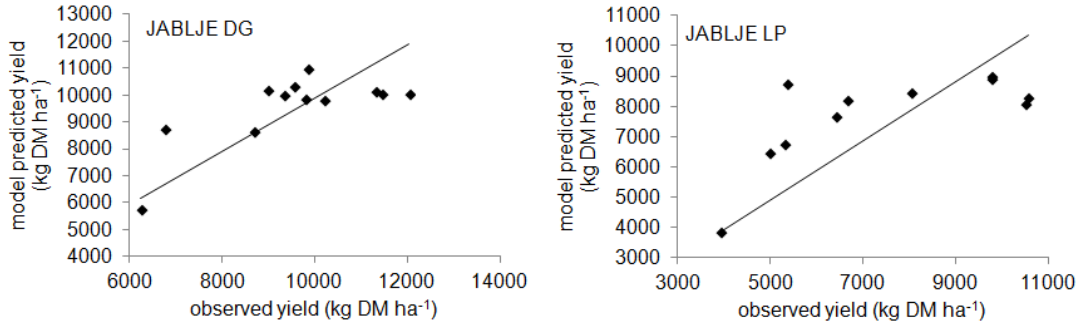


Figure 3: Scatterplots of the observed versus model predicted herbage yield for two grass species on the same location (Jablje). Left: cock's foot (DG), right: perennial ryegrass (LP)

Slika 3: Razsevna diagrama (izmerjeni vs. simulirani pridelek) za obe vrsti trave na isti lokaciji, v Jabljah. Levo: navadna pasja trava (DG), desno: trpežna ljuljka (LP)

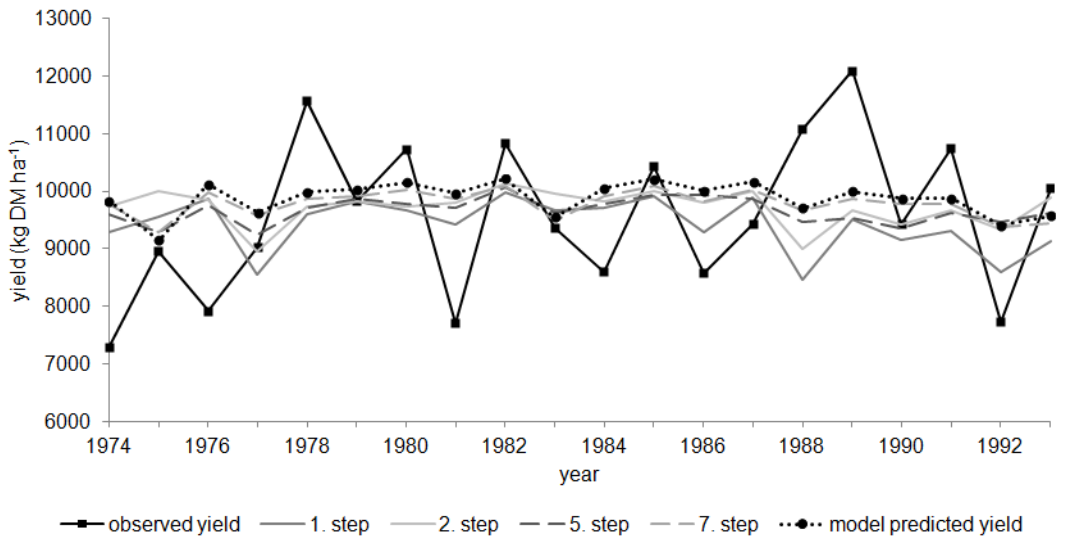


Figure 4: The herbage yield of multi-species grassland in the experiment S72 in Ljubljana: observed herbage yield, 1st, 2nd, 5th and 7th step herbage yield results of the calibration procedure, and model predicted herbage yield at the end of calibration

Slika 4: Pridelek poskusa S72 na travniku v Ljubljani: izmerjene vrednosti pridelka (observed yield), rezultat simulacije 1., 2., 5. in 7. koraka pri umerjanju modela in simulacija pridelka z umerjenim modelom

Even though calibration results for Jablje are good, we have a problem with S72 in Ljubljana (Figure 4). We may say the trouble is in the multi-species grassland. If there are already quite big differences between DG and LP (Figure 1), the difficulty caused by multi-species grass is only greater. So it seems as if the model can only be fully calibrated if we have a monoculture.

However, Figure 4 demonstrates that in this case the model may be able to reproduce the overall herbage yield level (the production potential) of the site, whereas the representation of interannual herbage yield variability is inaccurate. This can only have limited benefits in modelling in general, e.g. for overall grassland herbage yield levels on many locations in Slovenia or even in one part of Europe.

An examination of averages indicates that for each experiment the average predicted herbage yield ($\overline{P}$)overestimates the corresponding average observed herbage yield ($\overline{O}$) (Table 4). In contrast,

according to Schapendonk et al. (1998) LINGRA grassland herbage yield simulations underestimated observations on most of 35 locations around Europe. Nevertheless, the differences are not big: 308 kg DM ha^{-1} between the average predicted and average observed herbage yield for S72, 251 kg DM ha^{-1} for LP and only 23 kg DM ha^{-1} for DG. On the other hand for each experiment the standard deviation of the observed herbage yields (σ_O) is higher than the standard deviation of predictions (σ_P) (Table 4). The biggest problem is in the standard deviation of predictions for S72, which is only 289 kg DM ha^{-1} and does not correspond to the variability of the observed herbage yield.

Table 4: Number of measurements (n), the average observed herbage yield ($\overline{O}$) and its standard deviation (σ_O), the average predicted herbage yield ($\overline{P}$) and its standard deviation (σ_P) for the performance evaluation of LINGRA-N for three study experiments (DG and LP in Jablje, S72 in Ljubljana)

Preglednica 4: Število meritev (n), povprečni izmerjeni pridelek ($\overline{O}$) in standardni odklon (σ_O) ter povprečni simulirani pridelek ($\overline{P}$) in standardni odklon (σ_P) za oceno kakovosti modela LINGRA-N pri treh obravnavanih poskusih (DG in LP v Jabljah, S72 v Ljubljani)

Experiment	n	$\overline{O}$ (kg DM ha^{-1})	σ_O (kg DM ha^{-1})	$\overline{P}$ (kg DM ha^{-1})	σ_P (kg DM ha^{-1})
DG	12	9525	1742	9548	1359
LP	11	7398	2439	7649	1512
S72	20	9569	1368	9877	289

The most interesting result of the statistical analysis (Table 5) is that with respect to *RMSE*, no meaningful distinction can be made between the goodness of fit of model predictions for DG and S72. The overall performance of LINGRA-N is fair for LP (*RMSE%* = 23 %) and good for DG (*RMSE%* = 12 %) and S72 (*RMSE%* = 14 %). For example, in Scandinavia *RMSE%* for timothy was 43 % after the calibration (Van Oijen et al., 2005). However, good evaluation is needed that includes several statistical criteria. Regarding the systematic and unsystematic errors we can say that only for S72 the systematic error prevails over the unsystematic one. The index of agreement for S72 (d = 0.37) suggests that LINGRA-N is not calibrated well enough to simulate a multi-species grassland herbage yield, so for now we will not be able to use it for this purpose. Meanwhile the index of agreement is much higher for DG and LP, 0.84 and 0.78, respectively. For illustration, Persson *et al*. (2014) outlined the difference between leys of various age. For the first ley year were *RMSE%* 31 % and *d* 0.36, while for the second ley year both were much better, *RMSE%* 22 % and *d* 0.98. Considering both *RMSE* and *d,* we can be satisfied with the calibration of LINGRA-N for DG and a little less for LP.

Table 5: Statistical criteria: the root mean square error (*RMSE*), its relative value in % (*RMSE%*), its systematic ($RMSE_s$) and unsystematic ($RMSE_u$) part and the index of agreement (d) for additional performance evaluation of LINGRA-N for three study experiments (DG and LP in Jablje, S72 in Ljubljana)

Preglednica 5: Statistični kriteriji: kvadratni koren napake (*RMSE*), njegova relativna vrednost v % (*RMSE%*), njegov sistematični ($RMSE_s$) in nesistematični ($RMSE_u$) del ter indeks ujemanja (d) za nadaljnjo oceno kakovosti modela LINGRA-N pri treh obravnavanih poskusih (DG in LP v Jabljah, S72 v Ljubljani)

Experiment	*RMSE%* (%)	*RMSE* (kg DM ha^{-1})	$RMSE_s$ (kg DM ha^{-1})	$RMSE_u$ (kg DM ha^{-1})	d
DG	12	1134	711	882	0.84
LP	23	1709	1353	1043	0.78
S72	14	1329	1199	249	0.37

4 CONCLUSIONS

We have to concur with Barrett et al. (2005) that as grass sward growth is determined by the interaction of many environmental and management factors, forecasting grass sward growth rates is particularly difficult. Unfortunately we do not have datasets consisting of multiple years of observations from sequential measurements of grassland herbage yield, together with e.g. leaf area, tiller density, reserve carbohydrates, leaf appearance rate, leaf elongation rate, or specific leaf area, possibly during spring growth and first summer regrowth, in swards of one to two years of age (likewise e.g. Persson et al., 2014). That would improve calibration by dividing it in several stages. Considering the simplifications we made, we can conclude that the herbage yield of cock's foot and perennial ryegrass in Jablje is correctly simulated by the calibrated LINGRA-N. Two sets of parameters were defined: one for DG and one for LP. The best results are for DG in Jablje, with *RMSE%* = 12 % and with the index of agreement *d* = 0.84. The results for S72 in Ljubljana are far from optimal. As the interannual variability is not simulated well, we cannot use the model for further analyses in S72.

Calibration was the first step in preparing a tool to simulate grass sward growth and grassland herbage yield on specific locations in Slovenia. The degree of complexity of LINGRA-N construction is appropriate for its intended application, rather than for process understanding. Our further goal is to use the calibrated model for the simulation of the herbage yield of grass monocultures under various weather conditions as well as for the simulation of climate change effect on it. However, further parameterization and validation would be required for locations where the model will be operated using well-monitored swards under realistic parameters.

5 REFERENCES

ARSO – climatological data. 2014. Slovenian Environment Agency (http://www.arso.gov.si/): database output

Barrett P.D., Laidlaw A.S. 2005. Grass growth modelling: to increase understanding and aid decisionmaking on-farm. V: Utilization of grazed grass in temperate animal systems. Murphy J.J. (ed.). Wageningen, Wageningen Academic Publishers: 79-88

Barrett P.D., Laidlaw A.S., Mayne C.S. 2005. GrazeGro: a European herbage growth model to predict pasture production in perennial ryegrass swards for decision support. European Journal of Agronomy, 23: 37-56; DOI: 10.1016/j.eja.2004.09.006

Bouman B.A.M., Schapendonk A.H.C.M., Stol W., Van Kraalingen D.W.G. 1996. Description of the growth model LINGRA as implemented in CGMS. Quantitative Approaches in Systems Analysis, 7: 11-56

CPVO – soil data. 2014. Centre of soil and environmental science, Biotechnical Faculty, University of Ljubljana (http://web.bf.uni-lj.si/cpvo/): database output

Herrmann A., Michael K., Kornher A. 2005. Performance of grassland under different cutting regimes as affected by sward composition, nitrogen input, soil conditions and weather – a simulation study. European Journal of Agronomy, 22: 141-158; DOI: 10.1016/j.eja.2004.02.002

Jego G., Belanger G., Tremblay G.F., Jing Q., Baron V.S. 2013. Calibration and performance evaluation of the STICS crop model for simulating timothy growth and nutritive value. Field Crops Research, 151: 65-77; DOI: 10.1016/j.fcr.2013.07.003

Kapun S. 2005. Pridelovanje pasje trave. Naše travinje 1, 1: 10-11

KIS – herbage yield data of grass monocultures. 2014. Agricultural Institute of Slovenia (http://www.kis.si/): database output

Lazzarotto P., Calanca P., Fuhrer J. 2009. Dynamics of grass-clover mixtures – An analysis of the response to management with the PROductive GRASsland Simulator (PROGRASS). Ecological Modelling, 220: 703-724; DOI: 10.1016/j.ecolmodel.2008.11.023

Merot A., Bergez J.E., Wallach D., Duru M. 2008. Adaptation of a functional model of grassland to simulate the behaviour of irrigated grasslands under a Mediterranean climate: The Crau case. European Journal of Agronomy, 29: 163-174; DOI: 10.1016/j.eja.2008.05.006

Persson T., Höglind M., Gustavsson A.M., Halling M., Jauhiainen L. et al. 2014. Evaluation of the LINGRA timothy model under Nordic conditions. Field Crops Research, 161: 87-97; DOI: 10.1016/j.fcr.2014.02.012

Pogačar T., Kajfež-Bogataj L. 2011. LINGRA: model za simulacijo rasti in pridelka travne ruše. Acta agriculturae Slovenica, 97, 3: 319-327

Saxton K.E., Rawls W.J. 2006. Soil water characteristic estimates by texture and organic matter for hydrologic solutions. Soil Science Society of America Journal, 70: 1569-1578 ; DOI: 10.2136/sssaj2005.0117

Schapendonk A.H.C.M., Stol W., Van Kraalingen D.W.G., Bouman B.A.M. 1998. LINGRA, a sink/source model to simulate grassland productivity in Europe. European Journal of Agronomy, 9: 87-100; DOI: 10.1016/S1161-0301(98)00027-6

Shibu M.E., Leffelaar P.A., Van Keulen H., Aggarwal P.K. 2010. LINTUL3, a simulation model for nitrogen-limited situations: Application to rice. European Journal of Agronomy, 32: 255-271; DOI: 10.1016/j.eja.2010.01.003

Smit H.J., Metzger M.J., Ewert F. 2008. Spatial distribution of grassland productivity and land use in Europe. Agricultural Systems, 98: 208-219; DOI: 10.1016/j.agsy.2008.07.004

SURS – grassland data. 2014. Statistical office of the Republic of Slovenia. http://pxweb.stat.si/pxweb/Database/Okolje/Okolje.asp (17. 12. 2014)

Tajnšek A. 2003. Deset let trajnih poskusov IOSDV v Sloveniji, Jable in Rakičan 1993–2003. In: Namen in cilj trajnih poljskih poskusov IOSDV Jable in IOSDV Rakičan. Žalec, 12. 12. 2003. Tajnšek A., Čeh Brežnik B., Kocjan Ačko D. (eds.). Proceedings of the conference, Slovensko agronomsko društvo: 7-24

Tehnološka priporočila za zmanjšanje občutljivosti kmetijske pridelave na sušo: poljedelstvo, travništvo, zelenjadarstvo in hmeljarstvo. 2008. Ljubljana, Ministry of Agriculture, Forestry and Food: 44 p. http://www.arsktrp.gov.si/fileadmin/arsktrp.gov.si/pageuploads/Aktualno/Aktualno/2013/Tehnoloska_priporocila_za_zmanjsanje_obcutljivosti_na_suso.pdf (18. 11. 2013)

Trnka M., Eitzinger J., Gruszczynsk G., Buchgraber K., Resch R., Schaumberger A. 2006. A simple statistical model for predicting herbage production from permanent grassland. Grass and Forage S cience, 61: 253-271; DOI: 10.1111/j.1365-2494.2006.00530.x

Van Oijen M., Höglind M., Hanslin H.M., Caldwell N. 2005. Process-based modeling of Timothy regrowth. Agronomy Journal, 97: 1295-1303; DOI: 10.1111/j.1365-2494.2006.00530.x

Willmott C.J. 1982. Some comments on the evaluation of model performance. Bulletin of the American Meteorological Society, 63: 1309-1313; DOI: 10.1175/1520-0477(1982)063<1309:SCOTEO>2.0.CO;2

Wolf J. 2006. Grassland data from PASK study and testing of LINGRA in CGMS. ASEMARS Project report no. 2. Wageningen, Alterra: 38 p.

Wolf J. 2012. LINGRA-N: Simple generic model for simulation of grass growth under potential, water limited and nitrogen limited conditions. User guide for LINGRA-N. Wageningen, Wageningen University (available on agreement): 65 p.

Žalud Z., Trnka M., Ruget F., Hlavinka P., Eitzinger J., Schaumberger A. 2006. Evaluation of crop model STICS in the conditions of the Czech Republic and Austria. In: Proceedings of the conference Bioclimatology and water in the land (poster), Strecno, 11.-14. 9. 2006: 7 p. http://www.cbks.cz/sbornikStrecno06/prispevky/PosterII_clanky/P2-16.pdf (11. 11. 2013)

Žitek D. 1991. Dinamika razvoja pridelka travne ruše pri različnem gnojenju v enem letu. Bachelor thesis. Ljubljana, University of Ljubljana, Biotechnical Faculty, Department of Agronomy: 128 p.

COBISS Code 1.02

DOI: 10.14720/aas.2015.105.1.13
Agrovoc descriptors: leguminosae, legumes, varieties, crop yield, drought stress, proteins, drought resistance

Agris category code: f62

Preučevanje odziva na sušni stres pri metuljnicah (Fabaceae) s proteomiko

Tanja ZADRAŽNIK[1], Jelka ŠUŠTAR-VOZLIČ[2]

Received March 02, 2015; accepted March 16, 2015.
Delo je prispelo 02. marca 2015, sprejeto 16. marca 2015.

IZVLEČEK

Sušni stres predstavlja resno grožnjo pri pridelovanju kmetijskih rastlin, saj povzroča slabšo rast in razvoj rastlin ter posledično vpliva na količino in kakovost pridelka. Odziv rastlin na stres se kaže v spremembah metabolizma celice, ki med drugim vključuje spremenjeno izražanje proteinov. Učinkovito analizo proteinov, udeleženih pri odgovoru rastlin na stresne razmere, omogoča proteomika. Raziskave izražanja proteinov pri metuljnicah pri odzivu na sušo igrajo veliko vlogo, saj so metuljnice zelo pomembne v prehrani ljudi in živali ter tudi pogosto izpostavljene suši. Rezultati proteomskih raziskav pri izbranih metuljnicah omogočajo boljše razumevanje molekularnih mehanizmov odgovora rastlin na sušni stres, prispevajo k razvoju markerjev, povezanih z odgovorom rastlin na sušo ter so uporabni pri vzgoji novih, na sušo tolerantnih sort metuljnic.

Ključne besede: metuljnice, stres, suša, proteomika, proteini

ABSTRACT

PROTEOMIC STUDIES OF DROUGHT STRESS RESPONSE IN FABACEAE

Drought stress is a serious threat to crop production that influences plant growth and development and subsequently causes reduced quantity and quality of the yield. Plant stress induces changes in cell metabolism, which includes differential expression of proteins. Proteomics offer a powerful approach to analyse proteins involved in drought stress response of plants. Analyses of changes in protein abundance of legumes under drought stress are very important, as legumes play an important role in human and animal diet and are often exposed to drought. The presented results of proteomic studies of selected legumes enable better understanding of molecular mechanisms of drought stress response. The study of drought stress response of plants with proteomic approach may contribute to the development of potential drought-response markers and to the development of drought-tolerant cultivars of different legume crop species.

Key words: legumes, drought stress, proteomics, proteins

1 UVOD

Rastline so pogosto izpostavljene neugodnim vplivom iz okolja, ki vplivajo na njihovo rast, razvoj in pridelek. Poleg biotskega stresa, ki ga povzročajo bolezni in škodljivci, k zmanjšanju količine in kakovosti pridelka kmetijskih rastlin v veliki meri prispeva tudi abiotski stres. Med vrstami abiotskega stresa je najbolj razširjen sušni stres, ki se pojavlja v povezavi z manjšo količino padavin, njihovo neenakomerno razporeditvijo in pogosto tudi s povišanimi temperaturami. Sušni stres vpliva na pridelovanje kmetijsko pomembnih rastlinskih vrst, tudi na metuljnice.

Metuljnice po količini pridelka zavzemajo tretjo mesto svetovne pridelave kmetijskih rastlin (Popelka in sod., 2004). Vrste med njimi, kot so vrtni grah (*Pisum sativum* L.), bob (*Vicia faba* L.), fižol (*Phaseolus vulgaris* L.), soja (*Glycine max* (L.) Merr.), leča (*Lens culinaris* Medik.), čičerika (*Cicer arietinum* L.) in arašidi (*Arachis hypogaea* L.) zavzemajo pomembno mesto v prehrani ljudi

[1] Dr., Oddelek za poljedelstvo, vrtnarstvo, genetiko in žlahtnjenje; Kmetijski inštitut Slovenije, Hacquetova 17, 1000 Ljubljana; E-mail: tanja.zadraznik@kis.si

[2] Izr. prof. dr., prav tam

po svetu, predvsem zaradi svoje visoke hranilne vrednosti, vsebnosti proteinov, dietnih vlaken in mineralov. Pomembne so tudi krmne metuljnice, kot sta soja in lucerna (*Medicago sativa* L.), ki predstavljajo bogat vir proteinov pri krmi živali. Soja pa je pomembna tudi za pridobivanje olja. V Sloveniji se največ pridelujeta fižol in grah, v manjši meri še soja, bob, leča in čičerika (Kocjan Ačko in sod., 2005). Po obsegu pridelovanja pa je fižol daleč najpomembnejša metuljnica v Sloveniji (Popis vrtnarstva, 2000).

Metuljnice so zelo pomembne tudi zaradi sposobnosti vezave dušika iz ozračja s pomočjo simbiotskih bakterij na koreninah (Muneer in sod., 2012). Po njihovem spravilu ostanejo v tleh rastlinski ostanki, ki so bogat vir dušika za posevke, ki jim sledijo. Najpomembnejše so simbiotske bakterije iz rodu *Rhizobium* in njemu sorodnih rodov, ki živijo v simbiozi z rastlinami in na njihovih koreninah tvorijo posebne gomoljčke - nodule, kjer poteka transformacija atmosferskega dušika v organsko obliko. Učinkovitost vezave dušika iz ozračja in sposobnost uporabe organske oblike dušika pri rastlini je v veliki meri odvisna od abiotskih dejavnikov, med katerimi je tudi sušni stres.

Kombinacija sušnega stresa in visokih temperatur povzroča 18-28 % izgube pridelka zrnatih metuljnic (CGIAR Research program on grain legume, 2012). Po mnenju klimatologov bodo spremembe podnebja, ki jih bodo spremljale manjše količine padavin in povišane temperature, v prihodnje še bolj izrazite kot v zadnjih desetletjih (Kajfež-Bogataj, 2005). Z razvojem tolerantnih sort lahko prispevamo k prilagoditvi kmetijskih rastlin na podnebne spremembe, zato je toleranca na sušo postala cilj številnih žlahtniteljskih programov kmetijsko pomembnih rastlinskih vrst, med njimi tudi metuljnic (Miklas in sod., 2006). Za izbor in vzgojo sort, tolerantnih na sušo, je potrebno poznati fiziološke, morfološke in biokemijske lastnosti, ki določene sorte opredeljujejo kot tolerantne (Bray, 1993). Toleranca na sušo je genetsko kompleksna lastnost, zato je za razvoj tolerantnih sort potrebno dobro poznavanje mehanizmov tolerance in odziva na sušni stres.

Pri odzivu rastlin na sušne razmere se sprožijo celično in tkivno specifični fiziološki in molekularni mehanizmi, ki vključujejo izražanje specifičnih genov in spremembe v vsebnosti določenih proteinov, ki igrajo pomembno vlogo med stresom (Ramachandra Reddy in sod., 2004). Razvoj novih metod je v zadnjem desetletju omogočil velik napredek pri identifikaciji genov in proteinov vključenih v odgovor na stres. Med t.i. metode »omike« uvrščamo poleg genomike, transkriptomike, metabolomike tudi proteomiko. Te metode omogočajo analizo kompleksnih celičnih procesov, medsebojnih vplivov med biološkimi komponentami in razumevanje kompleksnih procesov. Pristopi »omik« omogočajo sistemsko analizo in povezavo med spremembami v genomu, transkriptomu, proteomu, metabolomu z odzivi rastlin na sušni stres in omogočajo pridobitev podatkov/informacij, ki so pomembni za pridobivanje in razvoj sort tolerantnih na stres (Gupta in sod., 2013).

Proteomika obravnava celokupni nabor proteinov v celici, delu celice, tkivu ali organizmu, obsega pa metode, ki omogočajo določitev in identifikacijo velikega števila proteinov, prisotnih v določenih razmerah in v določenem času. Določeno število kodirajočih genov lahko določa veliko večje število proteinov, predvsem zaradi različnih posttranslacijskih sprememb. Proteom tako prikaže realno sliko aktivnosti v celici, saj so prav proteini nosilci funkcij v celici. V prispevku je podan pregled proteomskih raziskav odziva na sušo pri izbranih metuljnicah ter splošni vpogled v strategije preučevanja odziva na sušni stres pri rastlinah s tehnikami proteomike.

2 PROTEOMIKA IN SUŠNI STRES PRI METULJNICAH

Kljub hitremu napredku tehnik in metod s področja proteomike, so do sedaj identificirali samo majhen delež celičnega proteoma. To je uspelo samo pri najbolj preučevanih organizmih, kot so človek, vinska mušica (*Drosophila melanogaster* Meigen), navadni repnjakovec (*Arabidopsis thaliana* (L.) Heynh.) in riž (*Oryza sativa* L.) (Jorrín-Novo in sod., 2009). Vendar celo pri naštetih organizmih ostaja funkcija velikega števila proteinov nepojasnjena. Določen protein ima lahko v

različnih tkivih in celo v isti celici povsem različno funkcijo. Funkcija proteinov je v določeni meri odvisna od lokalizacije proteinov znotraj celice in od njihove dejanske aktivnosti. Znotraj celice so proteini glede na njihove funkcije porazdeljeni neenakomerno, saj je število proteinov, ki so vključeni v uravnavanje celične homeostaze ter v presnovo in strukturo celice, mnogo večje, kot število tistih, ki sodelujejo v procesih signaliziranja. Zato sta določitev in kvantifikacija proteinov, ki v celicah niso prisotni v velikem številu, mnogo težji od določitve proteinov, katerih vsebnosti so večje (Zhang in sod., 2009; Corthals in Rose, 2007).

Objavljene so številne raziskave sušnega stresa na proteomski ravni pri različnih rastlinskih vrstah, kot so riž (Salekdeh in sod., 2002a in 2002b), sladkorna pesa (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* Döll.) (Hajheidari in sod., 2005), koruza (*Zea mays* L.) (Vincent in sod., 2005), pšenica (*Triticum durum* Desf.) (Caruso in sod., 2009) ter druge. V nadaljevanju je predstavljen pregled odziva na sušni stres pri različnih metuljnicah na področju proteomike, kratek povzetek pa je podan v preglednici 1.

2.1 Proteomske analize odziva na sušni stres pri trnati meteljki

Med metuljnicami se je trnata meteljka (*Medicago truncatula* Gaertn.) uveljavila kot modelni organizem zlasti pri študijah simbiotskih interakcij med rastlino in mikrobi, kot tudi na drugih področjih rastlinske biologije (Lei in sod., 2007). Obsežno proteomsko študijo o analizi proteoma trnate meteljke v normalnih rastnih razmerah so opravili Watson in sod. (2003). Identificirali so proteine, ki so jih izolirali iz listov, stebel, korenin, semen, cvetov in celičnih suspenzij. Pri listih, steblih in cvetovih je bilo največje število proteinov povezanih z energijsko presnovo, pri suspenziji celic z energijsko presnovo in skladiščenjem, pri koreninah z obrambnimi procesi ter pri semenih s skladiščenjem. Larrainzar in sod. (2007) so preučevali vpliv simbiotskih interakcij med noduli trnate meteljke in bakterijami *Sinorhizobium meliloti* v povezavi s sušnim stresom. Ločena izolacija rastlinskih in bakterijskih proteinskih frakcij je omogočila neodvisno analizo odziva na stres obeh simbiotskih udeležencev. Največ proteinov, ki so jih identificirali v nodulih rastlin, je bilo udeleženih v presnovi aminokislin ter pri sintezi ali razgradnji proteinov. Ugotovili so, da imajo pomembno vlogo tudi proteini, udeleženi pri obrambnih reakcijah proti stresu, v signalizaciji in pri procesih presnove. Izpostavili so protein saharoza sintazo, ki je vključen v odgovor na sušni stres. Poleg omenjene raziskave so Larrainzar in sod. (2009) objavili raziskavo o povezavi med presnovo ogljika in dušika v nodulih ter simbiotsko fiksacijo dušika pri rastlinah trnate meteljke v sušnem stresu. Na osnovi rezultatov predvidevajo, da je inhibicija simbiotske fiksacije dušika pri rastlinah v sušnem stresu povezana z oslabljenim metabolizmom bakterij in sposobnostjo fiksiranja N_2.

2.2 Proteomske analize odziva na sušni stres pri soji

Proteomske raziskave soje v sušnem stresu so delali tako na koreninah, kot tudi na nadzemnih zelenih delih. Yamaguchi in sod. (2010) so ugotovili, da so proteini, ki so jih identificirali na koreninah rastlin v sušnem stresu, v glavnem udeleženi v mehanizmu tolerance na sušni stres. Proteine so povezali z vlogo pri kontroli mehanizma reaktivnih kisikovih zvrsti (ROS), biosintezi izoflavonoidov, kontroli celične smrti in kontroli razgradnje proteinov. Proteomsko analizo korenin pri soji v sušnem stresu so opravili tudi Alam in sod. (2010). Prisotnost proteina feritina in dehidrina so zasledili samo pri vzorcih v sušnem stresu, čeprav so identificirali še 26 proteinov z različnimi celičnimi funkcijami, kot so presnova ogljikovih hidratov in dušika, transdukcija signalov, obramba celic in programirana celična smrt. Mohammadi in sod. (2012) so analizirali spremembe pri mladih kalečih rastlinah soje podvrženih suši ali osmotskemu stresu, vzpostavljenim s polietilen glikolom. Analizirali so proteine iz listov, hipokotila in korenin. Korenine so bile izmed vseh treh analiziranih tkiv organ z največjim številom proteinov, katerih izražanje se je spremenilo v stresnih razmerah. Pri listih so se v stresnih razmerah povečale vsebnosti proteinov, povezanih z energijsko presnovo, vsebnost proteinov, povezanih s sintezo proteinov pa se je zmanjšala. Ugotovili so, da se je v vseh treh organih rastlin v sušnem stresu zmanjšala vsebnost metionin sintaze. Na tej osnovi ter na osnovi rezultatov izražanja mRNA pri rastlinah v stresu zaradi suše, slanosti in vročine so sklepali, da je izražanje metionin sintaze povezano z odzivom na sušo. Podobno so tudi Nouri in Komatsu (2010) za

analizo proteinov plazmaleme uporabili mlade kaleče rastline soje v osmotskem stresu induciranem s polietilen glikolom. Poseben poudarek so avtorji namenili proteinu kalneksinu, ki se v osmotskem stresu nalaga v plazmalemi in proteinu H^+-ATPazi. Pri obeh proteinih so opazili povečano izražanje v stresnih razmerah. Hossain in sod. (2013) so objavili obsežen pregled proteomskih raziskav abiotskega stresa pri soji. Podali so prednosti in slabosti različnih proteomskih metodoloških pristopov pri ekstrakciji celotnega proteoma. Opredelili so tudi cilje prihodnjih raziskav proteoma soje na ravni posameznih rastlinskih tkiv oz. celotnih rastlin z namenom, da bi dobili boljši vpogled v mehanizem odziva soje na abiotski stres. Ugotovili so, da bi bilo potrebno nameniti več pozornosti preučevanju interakcij protein-protein in protein-ligand ter interdisciplinarnim raziskavam, zlasti v povezavi z metabolomiko, da bi lahko preučili mrežo interakcij med proteini in metaboliti, ki so udeleženi v mehanizem tolerance na abiotski stres. Avtorji so še izpostavili pomembnost preučevanja proteomov posameznih organelov, kar bi pripomoglo do boljšega razumevanja molekularnih mehanizmov celice pri odzivu na neugodne okoljske razmere. Preučevanje odziva soje na različne stresne dejavnike bi bilo zelo zanimivo za nadaljnje proteomske raziskave, saj bi tako dobili globlji in natančnejši vpogled v povezavo signalnih poti ob delovanju različnih abiotskih stresnih dejavnikov.

2.3 Proteomske analize odziva na sušni stres pri čičeriki

Pandey in sod. (2008) so analizirali jedrni proteom čičerike v povezavi s sušo. Med proteini, ki so jih določili, jih je največ vpletenih v gensko transkripcijo in replikacijo, celično signaliziranje ter remodeliranje kromatina. Bhushan in sod. (2007 in 2011) so opravili dve raziskavi o izražanju proteinov pri čičeriki v sušnih razmerah. V prvi so analizirali proteine, ki se različno izražajo v zunajceličnem matriksu rastlin v suši (Bhushan in sod., 2007). Identificirali so 134 proteinov, ki so v glavnem udeleženi v transdukciji signala, modifikacijah celične stene, energijsko presnovo ter celični obrambi. Sklepali so tudi na morebitno vlogo obravnavanih proteinov v toleranci na dehidracijo. V nadaljevanju so analizirali proteom zunajceličnega matriksa čičerike v sušnem stresu pri sorti, občutljivi na sušo, in sorti tolerantni na sušo (Bhushan in sod., 2011). Ugotovili so, da je različno izražanje določenih proteinov odvisno od obravnavanega genotipa. Raziskave jedrnega proteoma čičerike v sušnem stresu so nedavno opravili tudi Subba in sod. (2013). Pri sorti občutljivi na sušo so identificirali 75 proteinov z različno vsebnostjo v suši, ki so jih povezali z različnimi presnovnimi in regulatornimi potmi v celici. Nato so proteom občutljive sorte primerjali s tolerantno sorto, iz česar so sklepali, da je toleranca lahko povezana s spremenjenim izražanjem številnih strukturnih proteinov ter proteinov, ki so povezani s prilagoditvijo na stres, kot so encimi povezani s katabolizmom reaktivnih kisikovih spojin. Najnovejša študija, ki so jo objavili Jaiswal in sod. (2014), obravnava proteomsko analizo membranskih proteinov čičerike v sušnem stresu. Izpostavili so Sad1/UNC-84 protein (CaSUN1), za katerega predvidevajo, da ima pri odzivu na stres vlogo pri signalizaciji odziva na nezvite proteine.

2.4 Proteomske analize odziva na sušni stres pri arašidih

Kottapalli in sod. (2009) so preučevali tri različne genotipe arašidov v sušnem stresu. S proteomsko analizo listov so identificirali proteine, ki so jih glede na funkcije razvrstili v deset skupin. Ugotovili so, da so največje skupine proteinov povezane s fotosintezo, transdukcijo signala in sušnega stresa. Predvidevajo, da so proteini, ki sodelujejo pri ojačanju celične stene, transdukciji signala, energijski presnovi, detoksifikaciji celic in genski regulaciji, udeleženi pri mehanizmu tolerance na sušo pri arašidih. Novejša študija, ki so jo opravili Kottapalli in sod. (2013), pa se osredotoča na proteomsko analizo semen arašidov izpostavljenih suši. Identificirali so 93 proteinov, ki so se različno izražali v suši. Ugotovili so, da ima suša največji vpliv na proteine, udeležene pri glikolizi, presnovi saharoze in škroba ter presnovi maščobnih kislin.

2.5 Proteomske analize odziva na sušni stres pri grahu

Taylor in sod. (2005) so preučevali različne vplive stresa, kot so suša, zmrzal in stres zaradi vpliva herbicidov, na proteom mitohondrijev v listih graha. Mitohondriji predstavljajo ključno mesto pri oksidativnem stresu in celičnem odgovoru na

stresne dejavnike. Ugotovili so, da je prisotnost herbicidov povzročila največji oksidativni stres v mitohondrijih, medtem ko sta zmrzal in suša povzročila stres v milejši obliki. Identificirane proteine so razvrstili v skupine glicin dekarboksilaz in serin hidroksimetiltransferaz, proteine vključene v cikel trikarboksilnih kislin in oksidativno fosforilacijo ter proteine vročinskega šoka. Spremembe v vsebnosti proteinov v nodulih pri grahu v sušnem stresu so preučevali Irar in sod. (2014). Identificirali so 18 proteinov, nekatere med njimi so razvrstili v skupino presnovo flavonoidov, presnovo žvepla in proteine, ki se vežejo na RNA.

2.6 Proteomske analize odziva na sušni stres pri lucerni

Odziv na sušni stres s proteomsko analizo pri lucerni so proučevali Aranjuelo in sod. (2011). Z namenom, da bi preučili odziv fotosintetskega aparata na sušni stres, so analizirali proteom listov. Poleg proteomske analize so opravili meritve fizioloških parametrov za določanje fotosintetske aktivnosti, pa tudi meritve nitrogenazne aktivnosti koreninskih gomoljčkov in analize vsebnosti aminokislin in sladkorjev v listih. Ugotovili so, da je inhibicija fotosinteze povezana z inhibicijo proteina Rubisco. V listih so zasledili zmanjšanje vsebnosti določenih aminokislin, kot sta asparagin in glutaminska kislina ter zmanjšanje vsebnosti proteina Rubisca, kar so povezali z manjšo vsebnostjo dušika, ki je bila posledica zmanjšane nitrogenazne aktivnosti. Sušni stres je vplival tudi na zmanjšanje vsebnosti proteina, ki se veže na Rubisco ter na povečanje izražanja proteaz, ki lahko vplivajo na razgradnjo proteina Rubisco. Zasledili so povečanje aminokisline prolina ter sladkorja pinitola, ki sta udeležena pri osmotski prilagoditvi na sušni stres.

2.7 Proteomika in odziv na sušni stres pri fižolu

V koreninah mungo fižola (*Vigna radiata* (L.) R. Wilczek), izpostavljenega različnim stopnjam sušnega stresa, so s proteomskim pristopom identificirali proteine, ki sodelujejo pri detoksifikaciji ROS, presnovi žvepla, morfogenezi korenin, sintezi proteinov, v energijski presnovi in v celičnem signaliziranju (Sengupta in sod., 2011; Sengupta in Reddy, 2011). Na začetni stopnji sušnega stresa so zaznali zmanjšane vsebnosti proteinov povezanih s citoskeletom, vendar se je njihova vsebnost povečala pri daljši izpostavljenosti stresu. Pri glikoproteinih, kot so lektini, pa so povečano vsebnost zaznali tako pri kratkotrajni kot tudi pri daljši izpostavljenosti stresu, kar nakazuje na pomembno vlogo lektinov pri odzivu na sušo pri metuljnicah. Avtorji predvidevajo, da je povečana vsebnost lektinov povezana z njihovo vlogo pri znotrajcelični regulaciji in poteh signalizacije, kar pripomore k prilagoditvi rastline na sušni stres. V stresu so opazili tudi povečano vsebnost proteinov, povezanih z oksidativnim stresom, kot so Cu/Zn superoksid dismutaza, oksidoreduktaza in aldehid reduktaza (Sengupta in sod., 2011). Yang in sod. (2013) so opravili proteomsko analizo in analizo fosforilacije proteinov (fosfoproteomska analiza) koreninskih vršičkov navadnega fižola v osmotskem stresu induciranem s polietilen glikolom. Pri analizi celokupnih proteinov se je 22 proteinov različno izražalo v razmerah osmotskega stresa, kjer je bilo največ identificiranih proteinov udeleženih v presnovi gljikovih hidratov in aminokislin. Analiza apoplastnih proteinov je pokazala, da je pri petih proteinih prišlo do zmanjšanja vsebnosti pri osmotskem stresu, pri sedmih proteinih pa do povečanja vsebnosti. Pri analizi celokupnih fosfoproteinov so izpostavili protein dehidrin, za katerega predvidevajo, da ima zaščitno vlogo pri osmotskem stresu preko zagotavljanja zaščitne vloge celične stene proti poškodbam in vzdrževanju integritete celične stene.

Na Kmetijskem inštitutu Slovenije smo analizirali odziv na sušo na ravni celokupnih proteinov v listih navadnega fižola pri dveh sortah, 'Tiber' in 'Starozagorski čern', ki se razlikujeta v toleranci na sušni stres (Zadražnik in sod., 2013). Ugotovili smo, da suša najbolj negativno vpliva na vsebnosti proteinov, ki so ključni za fotosintezo, kot so Rubisco, karbonska anhidraza, proteinov vključenih v fotolizo vode ter druge. Pri teh proteinih smo tudi zaznali najbolj izrazite razlike v vsebnosti med tema sortama. Pri sorti 'Starozagorski čern', ki je na sušo bolj občutljiva, se je vsebnost vseh proteinov tega tipa zmanjšala, pri sorti 'Tiber' pa se je vsebnost nekaterih proteinov, kot sta karbonska anhidraza in Rubisco, zmanjšala, pri drugih, kot so proteini vključeni v fotolizo vode, pa povečala. Ugotovili smo tudi, da suša vpliva na vsebnost proteinov, ki so povezani z energijsko presnovo, odzivom na stres, sintezo, proteolizo in zvijanjem proteinov. Na podlagi

rezultatov sklepamo, da lahko določene proteine uporabimo kot markerje v selekcijskem procesu tolerance na sušo pri navadnem fižolu. Za ta namen bi bili najbolj primerni proteini, katerih vsebnost se med sortama razlikuje, to bi bili predvsem proteini vključeni v fotolizo vode.

3 STRATEGIJE PREUČEVANJA SUŠNEGA STRESA S PROTEOMIKO

Proteomske analize odziva na sušni stres so tako pri metuljnicah kot tudi pri ostalih rastlinah usmerjene k primerjalnim analizam vsebnosti proteinov med rastlinami v stresu in kontrolnimi rastlinami, v nekaterih primerih pa vključujejo še proteomsko primerjalno analizo tolerantnih in občutljivih sort na stres (Barkla in sod. 2013). Določitev razlik v vsebnosti proteinov med različnimi vzorci je dokaj zahtevna zaradi velike kompleksnosti proteoma in zahtevne analitike, povezane s preučevanjem proteinov. Rezultati proteomskih analiz v večini primerov ne nudijo celostnega vpogleda odziva rastline na stres na nivoju proteoma (Kosova in sod., 2011). Pri večini predstavljenih raziskav so identificirali samo proteine, kjer so bile vsebnosti največje; pri tem gre večinoma za hidrofilne, nemembranske proteine. Rezultati primerjalnih proteomskih analiz so v veliki meri odvisni od uporabljenih metod.

V proteomiki se največ uporablja metoda dvo-dimenzionalne poliakrilamidne gelske elektroforeze (2D-PAGE). Dobra stran 2D-PAGE je, da se lepo opazijo proteinske izo-oblike in razgradnja proteinov. Uporaba 2D-PAGE pa ima tudi nekaj pomanjkljivosti, ki so povezane s slabo ponovljivostjo izvedbe gelske elektroforeze in problemi pri kvantifikaciji proteinov iz gelov. Za zanesljivo kvantifikacijo je zato potrebno narediti več ponovitev istega vzorca. Za 2D-PAGE se je uveljavilo splošno mnenje, da je identifikacijski proces neobčutljiv in zahteven ter je v veliki meri odvisen od barvanja proteinov ter ostalih tehnik vizualizacije (Weckwerth, 2008). K delni rešitvi teh problemov je pripomogla uvedba diferencialne gelske elektroforeze (DIGE), ki temelji na uporabi fluorescentnih barvil (Timms in Cramer, 2008). Vse bolj se uveljavljajo tudi metode, ki ne temeljijo na uporabi gelov, temveč na ločitvi peptidov oz. proteinov s tekočinsko kromatografijo (LC), ki je sklopljena z masno spektrometrijo (LC/MS) (Swanson in Washburn, 2005). Prednost metod proteomike, ki temeljijo na LC/MS, je možnost analize proteinov v zelo majhnih količinah ter analize hidrofobnih proteinov, kot so membranski proteini (Barkla in sod., 2013).

Raziskave sušnega stresa pri metuljnicah ter ostalih rastlinah se v zadnjem času vse bolj usmerjajo k analizam posameznim celičnih organelov določenih rastlinskih tkiv, ki v glavnem vključujejo analizo proteinov plazemske membrane, apoplasta, tonoplasta, kloroplastov, mitohondrijev ter jeder. Proteomska analiza specifičnih celičnih lokacij in organelov oz. subproteomika je dokaj zahtevna, zaradi težke izolacije subproteomov in njihove kompleksnosti (Barkla in sod., 2013). Poglobljeno znanje in identifikacija subceličnih proteomov sta potrebna za boljše razumevanje odziva rastlin na stres ter tudi za razvoj sort tolerantnih na stres. Prihodnje raziskave se morajo tudi bolj usmeriti v analizo posttranslacijskih modifikacij proteinov ter njihovo spreminjanje pri rastlinah v stresnih razmerah. Veliko število različnih modifikacij, ki pogosto spremenijo fizikalno-kemijske lastnosti proteinov, je samo eden od izzivov proteomike. Proteinske modifikacije, ki otežujejo analizo, so pogosto samo začasno prisotne, časovno in mestno specifične (Reinders in Sickmann, 2007).

Povezava informacij proteomike z ostalimi podatki, pridobljenimi s pomočjo metod »omik«, zlasti z metabolomiko in transkriptomiko, omogoča učinkovito strategijo preučevanja sušnega stresa rastlin ter s tem preučevanje celičnih poti in mehanizmov, ki so potrebni za razumevanje tolerance rastlin na sušni stres (Gupta in sod., 2013).

Preglednica 1: Pregled najpomembnejših raziskav sušnega stres pri metuljnicah na področju proteomike.
Table 1: A summary of drought stress studies using proteomics in major legume species.

Metuljnica	Tkivo/ del celice	Opis stresa	Metoda*	Število identificiranih proteinov s spremenjenimi vsebnostmi v suši	Referenca
trnata meteljka	noduli	10 tednov stare rastline, brez zalivanja za 3 in 6 dni	2D LC-MS/MS	377 proteinov rastlin**	Larrainzar in sod. (2007)
trnata meteljka	noduli	10 tednov stare rastline, brez zalivanja za 3 in 6 dni	LC-MS/MS	141 proteinov rastlin in 169 proteinov bakterij**	Larrainzar in sod. (2009)
soja	korenine	mlade kaleče rastline pri vodnem potencialu -1,6 MPa za 3 dni	2D-MALDI TOF MS/MS	27	Yamaguchi in sod. (2010)
soja	korenine	2 tedna stare rastline, brez zalivanja za 5 dni	2D-MALDI TOF	28	Alam in sod. (2010)
soja	listi, hipokotil, korenine	3 dni stare rastline, 10 % PEG ali brez zalivanja za 4 dni	2D LC-MS/MS	51 proteinov v listih, 49 v hipokotilu, 60 v koreninah	Mohammadi in sod. (2012)
soja	plazmalema	2 dni stare rastline, 10 % PEG za 2 dni	2D LC-MS/MS ter LC-MS/MS	12 proteinov iz gelov ter 86 iz LC-MS/MS	Nouri in sod. (2010)
čičerika	jedro	3 tedne stare rastline, brez zalivanja do 6 dni	2D LC-MS/TOF	147	Pandey in sod. (2008)
čičerika	zunajcelični matriks	3 tedne stare rastline, brez zalivanja za 7 dni	2D LC-MS/TOF	134	Bhushan in sod. (2007)
čičerika	zunajcelični matriks	3 tedne stare rastline, brez zalivanja za 8 dni	2D LC-MS/TOF	81	Bhushan in sod. (2011)
čičerika	jedro	3 tedne stare rastline, brez zalivanja za 6 dni	2D LC-MS/MS	75	Subba in sod. (2013)
čičerika	membrane	3 tedne stare rastline, brez zalivanja za 5 dni	2D MALDI-TOF/TOF	95	Jaiswal in sod. (2014)
arašidi	listi	67 dni stare rastline, brez zalivanja za 7 dni	2D MALDI TOF MS in Q-TOF MS/MS	49	Kottapalli in sod. (2009)
arašidi	semena	2 tedna stare rastline, poseben režim zalivanja, pobirali 110 dni stare rastline v stresu	1D LC-MS/MS	93	Kottapalli in sod. (2013)
grah	noduli	4 tedne stare rastline, brez zalivanja za 7 dni	2D MALDI-TOF/TOF	18	Irar in sod. (2014)
grah	mitohondriji	10 dni stare rastline, brez zalivanja 7 dni	2D Q-TOF	33	Taylor in sod. (2005)
lucerna	listi	91 dni stare rastline, brez zalivanja 7 dni	2D LC-MS/MS	26	Aranjuelo in sod. (2011)
mungo fižol	korenine	30 dni stare rastline, brez zalivanja za 3 in 6 dni	2D MALDI-TOF/TOF	26	Sengupta in sod. (2011)
navadni fižol	listi	5 tednov stare rastline, brez zalivanja za 12 in 17 dni	2D LC-MS/MS	58 proteinov pri tolerantni sorti, 64 pri občutljivi sorti	Zadražnik in sod. (2013)

*2D – dvodimenzionalna elektroforeza; LC-MS/MS – tekočinska kromatografija s tandemsko masno spektrometrijo; MALDI – ionizacija s pomočjo laserske svetlobe; TOF – analizator na čas preleta ionov; Q – kvadrupolni analizator
** število vseh identificiranih proteinov

4 ZAKLJUČKI

Sušni stres spada med abiotske stresne dejavnike, ki vplivajo na slabši pridelek kmetijsko pomembnih rastlin, med katere uvrščamo tudi metuljnice. Odziv metuljnic na sušo še ni podrobno raziskan. Največ proteomskih raziskav sušnega stresa je bilo opravljenih na modelnih rastlinah, kot sta trnata meteljka in soja, ter na nekaterih drugih metuljnicah, kot so čičerika, arašidi, grah, lucerna in fižol. Preučevanje proteinov, povezanih z odzivom na sušo, pomaga k razumevanju molekularnih mehanizmov odziva na sušo, kar je bistveno za razvoj tolerantnih sort, sami rezultati identifikacije proteinov pa so osnova za nadaljnje raziskave odziva na sušo pri metuljnicah, ki imajo pomembno vlogo v prehrani ljudi in krmi živali.

5 VIRI

Alam I., Sharmin S.A., Kim K-H., Yang J.K., Choi M.S., Lee B-H. 2010. Proteome analysis of soybean roots subjected to short-term drought stress. Plant Soil, 333: 491-505; DOI: 10.1007/s11104-010-0365-7

Aranjuelo I., Molero G., Erice G., Avice J.C., Nogués S. 2011. Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.). Journal of Experimental Botany, 62(1):111-123; DOI: 10.1093/jxb/erq249

Barkla B.J., Vera-Estrella R., Pantoja O. 2013. Progress and challenges for abiotic stress proteomics of crop plants. Proteomics, 13, 12-13: 1801-1815

Bhushan D., Jaiswal DK., Ray D., Basu D., Datta A., Chakraborty S., Chakraborty N. 2011. Dehydration-responsive reversible and irreversible changes in the extracellular matrix: comparative proteomics of chickpea genotypes with contrasting tolerance. Journal of Proteome Research, 10, 4: 2027-2046; DOI: 10.1021/pr200010f

Bhushan D., Pandey A., Choudhary M.K., Datta A., Chakraborty S., Chakraborty N. 2007. Comparative proteomics analysis of differentially expressed proteins in chickpea extracellular matrix during dehydration stress. Molecular & Cellular Proteomics, 6, 11: 1868-1884; DOI: 10.1074/mcp.M700015-MCP200

Bray E.A. 1993. Molecular responses to water deficit. Plant Physiology, 103, 4: 1035-1040

Caruso G., Cavaliere C., Foglia P., Gubbiotti R., Samperi R., Laganà A. 2009. Analysis of drought responsive proteins in wheat (*Triticum durum*) by 2D-PAGE and MALDI-TOF mass spectrometry. Plant Science, 177, 6: 570-576; DOI: 10.1016/j.plantsci.2009.08.007

CGIAR Research program on grain legume. 2012. ICRISAT, CIAT, ICARDA, IITA: 236 str. http://www.icrisat.org/crp/CRP3.5_Grain_Legumes_15Aug12.pdf (september, 2014)

Corthals G.L., Rose K. 2007. Quantitation in proteomics. V: Proteome research: concepts, technology and application. Wilkins M.R., Appel R.D., Williams K.L., Hochstrasser D.F. (eds.). 2nd ed. Berlin, Springer: 69-93

Gupta B., Saha J., Sengupta A., Gupta K. 2013. Plant abiotic stress: 'omics' approach. Journal of Plant Biochemistry & Physiology, 1: e108. doi:10.4172/2329-

Hajheidari M., Abdollahian-Noghabi M., Askari H., Heidari M., Sadeghian S.Y., Ober E.S., Salekdeh G.H. 2005. Proteome analysis of sugar beet leaves under drought stress. Proteomics, 5: 950-960; DOI: 10.1002/pmic.200401101

Hossain Z., Khatoon A., Komatsu S. 2013. Soybean proteomics for unraveling abiotic stress response mechanism. Journal of Proteome Research, 12, 11: 4670-4684; DOI: 10.1021/pr400604b

Irar S., González E.M., Arrese-Igor C., Marino D. 2014. A proteomic approach reveals new actors of nodule response to drought in split-root grown pea plants. Physiologia Plantarum, 152, 4: 634-645; DOI: 10.1111/ppl.12214

Jaiswal D.K., Mishra P., Subba P., Rathi D., Chakraborty S., Chakraborty N. 2014. Membrane-associated proteomics of chickpea identifies Sad1/UNC-84 protein (CaSUN1), a novel component of dehydration signaling. Scientific Reports, 4: 4177; DOI: 10.1038/srep04177

Jorrín-Novo J.V., Maldonado A.M., Echevarría-Zomeño S., Valledor L., Castillejo M.A., Curto M., Valero J., Sghaier B., Donoso G., Redondo I. 2009. Plant proteomics update (2007-2008): second-generation proteomic techniques, an appropriate experimental design, and data analysis to fulfill MIAPE standards, increase plant proteome coverage and

expand biological knowledge. Journal of Proteomics, 72, 3: 285-314; DOI: 10.1016/j.jprot.2009.01.026

Kajfež-Bogataj L. 2005. Podnebne spremembe in ranljivost kmetijstva. Acta agriculturae Slovenica, 85, 1: 25-40

Kocjan Ačko D., Tolar Š., Šantavec I. 2005. Stročnice v kolobarju slovenskih ekoloških kmetij. Acta agriculturae Slovenica, 85, 1: 125 – 134

Kosová K., Vítámvás P., Prásil I.T., Renaut J. 2011. Plant proteome changes under abiotic stress-contribution of proteomics studies to understanding plant stress response. Journal of Proteomics, 74: 1301-1322; DOI: 10.1016/j.jprot.2011.02.006

Kottapalli K.R., Rakwal R., Shibato J., Burow G., Tissue D., Burke J., Puppala N., Burow M., Payton P. 2009. Physiology and proteomics of the water-deficit stress response in three contrasting peanut genotypes. Plant, Cell & Environment, 32: 380-407; DOI: 10.1111/j.1365-3040.2009.01933.x

Kottapalli K.R., Zabet-Moghaddam M., Rowland D., Faircloth W., Mirzaei M., Haynes P.A., Payton P. 2013. Shotgun label-free quantitative proteomics of water-deficit-stressed midmature peanut (*Arachis hypogaea* L.) seed. Journal of Proteome Research, 12, 11: 5048-5057; DOI: 10.1021/pr400936d

Larrainzar E., Wienkoop S., Scherling C., Kempa S., Ladrera R., Arrese-Igor C., Weckwerth W., González EM. 2009. Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in *Medicago truncatula* under drought and recovery. Molecular Plant-Microbe Interactions, 22: 1565-1576; DOI: 10.1094/MPMI-22-12-1565

Larrainzar E., Wienkoop S., Weckwerth W., Ladrera R., Arrese-Igor C., González E. 2007. *Medicago truncatula* root nodule proteome analysis reveals differential plant and bacteroid responses to drought stress. Plant Physiology, 144: 1495-1507; DOI: 10.1104/pp.107.101618

Lei Z., Nagaraj S., Watson B., Summer L. 2007. Proteomics of *Medicago truncatula*. V: Plant proteomics. Šamaj J., Thelen J. (eds.). Berlin, Springer: 121-136

Magyar-Tábori K., Mendler-Drienyovszki N., Dobránszki J. 2011. Models and tools for studying drought stress responses in peas. OMICS, 15, 12: 829-838; DOI: 10.1089/omi.2011.0090

Miklas P.N., Kelly J.D., Beebe S.E., Blair M.W. 2006. Common bean breeding for resistance against biotic and abiotic stresses: from classical to MAS breeding. Euphytica, 47: 105-131; DOI: 10.1007/s10681-006-4600-5

Mohammadi P.P., Moieni A., Hiraga S., Komatsu S. 2012. Organ-specific proteomic analysis of drought-stressed soybean seedlings. Journal of Proteomics, 75, 6: 1906-1923; DOI: 10.1016/j.jprot.2011.12.041

Muneer S., Ahmad J., Bashir H., Qureshi M.I. 2012. Proteomics of nitrogen fixing nodules under various environmental stresses. Plant Omics Journal, 5, 2: 167-176

Nouri MZ., Komatsu S. 2010. Comparative analysis of soybean plasma membrane proteins under osmotic stress using gel-based and LC MS/MS-based proteomics approaches. Proteomics, 10, 10: 1930-1945; DOI: 10.1002/pmic.200900632

Pandey A., Chakraborty S., Datta A., Chakraborty N. 2008. Proteomics approach to identify dehydration responsive nuclear proteins from chickpea (*Cicer arietinum* L.). Molecular & Cellular Proteomics, 7: 88-107; DOI: 10.1074/mcp.M700314-MCP200

Popelka J.C.,Nancy Terryn N., Higgins T.J.V. 2004. Gene technology for grain legumes: can it contribute to the food challenge in developing countries? Plant Science, 167, 2: 195-206; DOI: 10.1016/j.plantsci.2004.03.027

Popis vrtnarstva. 2000. Kmetijstvo in ribištvo. Ljubljana, Statistični Urad Republike Slovenije: 56 str. http://www.stat.si/doc/pub/rr-765-01.pdf (september, 2014)

Ramachandra Reddy A., Chaitanya K.V., Vivekanandan M. 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Journal of Plant Physiology, 161: 1189-1202; DOI: 10.1016/j.jplph.2004.01.013

Reddy D.S., Bhatnagar-Mathur P., Vadez V., Sharma K.K. 2012. Grain legumes (soybean, chickpea, and peanut): omics approaches to enhance abiotic stress tolerance. V: Improving crop resistance to abiotic stress, volume 1 & Volume 2, eds N. Tuteja, S. S. Gill, A. F. Tiburcio and R. Tuteja, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, str: 993-1030 doi: 10.1002/9783527632930.ch39; DOI: 10.1002/9783527632930.ch39

Reinders J., Sickmann A. 2007. Modificomics: posttranslational modifications beyond protein phosphorylation and glycosylation. Biomolecular Engineering, 24, 2: 169-177; DOI: 10.1016/j.bioeng.2007.03.002

Salekdeh G.H., Siopongco J., Wade L.J. Ghareyazie B., Bennett J. 2002a. A proteomic approach to analyzing drought- and salt-responiveness in rice.

Field Crops Research, 76: 199-219; DOI: 10.1016/S0378-4290(02)00040-0

Salekdeh G.H., Siopongco J., Wade L.J., Ghareyazie B., Bennett J. 2002b. Proteomic analysis of rice leaves during drought stress and recovery. Proteomics, 2: 1131-1145; DOI: 10.1002/1615-9861(200209)2:9<1131::AID-PROT1131>3.0.CO;2-1

Sengupta D., Kannan M., Reddy AR. 2011. A root proteomics-based insight reveals dynamic regulation of root proteins under progressive drought stress and recovery in *Vigna radiata* (L.) Wilczek. Planta, 233, 6: 1111-1127; DOI: 10.1007/s00425-011-1365-4

Sengupta D., Reddy AR. 2011. Water deficit as a regulatory switch for legume root responses. Plant Signaling & Behavior, 6, 6: 914-917; DOI: 10.4161/psb.6.6.15340

Subba P., Kumar R., Gayali S., Shekhar S., Parveen S., Pandey A., Datta A., Chakraborty S., Chakraborty N. 2013. Characterisation of the nuclear proteome of a dehydration-sensitive cultivar of chickpea and comparative proteomic analysis with a tolerant cultivar. Proteomics, 13, 12-13: 1973-1992

Swanson S.K., Washburn M.P. 2005. The continuing evolution of shotgun proteomics. Drug Discovery Today, 10, 10: 719-725; DOI: 10.1016/S1359-6446(05)03450-1

Taylor N.L., Heazlewood J.L., Day D.A., Millar A.H. 2005. Differential impact of environmental stresses on the pea mitochondrial proteome. Molecular & Cellular Proteomics, 4, 8: 1122-1133; DOI: 10.1074/mcp.M400210-MCP200

Timms J.F., Cramer R. 2008. Difference gel electrophoresis. Proteomics, 8, 23-24: 4886-4897

Vincent D., Lapierre C., Pollet B., Cornic G., Negroni L., Zivy M. 2005. Water deficits affect caffeate O-methyltransferase, lignification, and related enzymes in maize leaves. A proteomic investigation. Plant Physiology, 137: 949-960; DOI: 10.1104/pp.104.050815

Watson B.S., Asirvatham V.S., Wang L., Summer L.W. 2003. Mapping the proteome of barrel medic (*Medicago truncatula*). Plant Physiology, 131: 1104-1123; DOI: 10.1104/pp.102.019034

Weckwerth W. 2008. Integration of metabolomics and proteomics in molecular plant physiology-coping with the complexity by data-dimensionality reduction. Physiologia Plantarum, 132, 2: 176-189; DOI: 10.1111/j.1399-3054.2007.01011.x

Yamaguchi M., Valliyodan B., Zhang J., Lenoble M., Yu O., Rogers E., Nguyen H., Sharp R. 2010. Regulation of growth response to water stress in the soybean primary root. I. Proteomic analysis reveals region-specific regulation of phenylpropanoid metabolismand control of free iron in the elongation zone. Plant, Cell and Environment, 33: 223–243; DOI: 10.1111/j.1365-3040.2009.02073.x

Yang Z.B., Eticha D., Führs H., Heintz D., Ayoub D., Van Dorsselaer A., Schlingmann B., Rao I.M., Braun H.P., Horst W.J. 2013. Proteomic and phosphoproteomic analysis of polyethylene glycol-induced osmotic stress in root tips of common bean (*Phaseolus vulgaris* L.). Journal of Experimental Botany, 64, 18: 5569-5586; DOI: 10.1093/jxb/ert328

Zadražnik T., Hollung K., Egge-Jacobsen W., Meglič V., Šuštar-Vozlič J. 2013. Differential proteomic analysis of drought stress response in leaves of common bean (*Phaseolus vulgaris* L.). Journal of Proteomics, 78: 254-272; DOI: 10.1016/j.jprot.2012.09.021

Zhang D., Ye F., Gao L., Liu X., Zhao X., Che Y., Wang H., Wang L., Wu J., Song D., Liu W., Xu H., Jiang B., Zhang W., Wang J., Lee P. 2009. Proteomics, pathway array and signaling network-based medicine in cancer. Cell Division, 4, 1: 20, doi: 10.1186/1747-1028-4-20: 16 str.

COBISS Code 1.01

DOI: 10.14720/aas.2015.105.1.14
Agrovoc descriptors: malus pumila, tree form, crown, flowering, stems, plant anatomy, crop yield, organic agriculture, biological production, growth control

Agris category code: f50, f62

Ukrepi za zaviranje rasti vrhov jablane 'GALA'

Matjaž BEBER[1]

Received December 11, 2014; accepted February 23, 2015.
Delo je prispelo 11. decembra 2014, sprejeto 23. februarja 2015.

IZVLEČEK

V Sadjarskem centru Maribor smo v časovnem obdobju 2010 – 2013, pri sorti `Gala´, spremljali različne metode umirjanja rasti vrhov jablane: premazovanje prevodnika na višini 2,2 m z 2 % raztopino rastnega regulatorja NAA (Luxan Late – Val) in rastlinske smole, uporaba pripravka Regalis (2-krat), zamenjava vrha s privezovanjem najvišje upognjene veje (zamenjava vrha), odstranjevanje novih poganjkov 28 dni po vrhu cvetenja (mandanje) ter junijska rez vrhov po zaključku primarne rasti. Spremljali smo priraščanje enoletnih poganjkov in pridelek v vrhu (nad 2,2 m). V praksi najbolj pogosto uporabljena ukrepa, junijska rez in mandanje, sta rezultirala z najmočnejšo rastjo v vrhu. Najbolj smo rast umirili z uporabo rastnih regulatorjev (Regalis ter s premazovanjem) in zamenjavo vrha. Največjo rodnost vrhov smo dosegli z uporabo pripravka Regalis, z ukrepom mandanja pa najmanjšo. Ukrep zamenjave vrha je primeren pri ekološkem načinu pridelave, saj brez uporabe rastnih regulatorjev uspešno umiri rast vrhov.

Ključne besede: jablana, `Gala´, vrh krošnje, enoletni poganjki, cvetenje, pridelek, rastni regulatorji, ekološka pridelava

ABSTRACT

MEASURES TO INHIBIT THE GROWTH OF APPLE TREE TOP WITH THE `GALA´ VARIETY

In Fruit Research Center Maribor different methods of less vigorous growth of apple tree top with the variety `Gala´ were conducted during the period from 2010 to 2013: applying a coating of the central leader at a height of 2.2 m with 2 % solution of growth regulator NAA (Luxan Late – Val) and plant resin, the use of the growth retardant Regalis (2 times), of replacing the the top of the tree with the highest appropriate bent branch, removal of new shoots 28 days after flowering (tearing) and the June cut after the completion of the primary growth. Increment of annual shoots and harvest in the top (over 2.2 m) was followed. Most commonly used practices, the June cut and tearing of young shoots resulted in the strongest growth in the top. The vigour of the top of the tree was the best reduced by the use of plant growth regulators (Regalis and NAA top coating) and replacing the top of the tree. The highest yield of the top of the tree was achieved by using Regalis, meanwhile tearing of the young shoots gave the lowest yield. The replacing the top of the tree is suitable measure for organic production, because it successfully reduces the vigour of the tree top without the use of growth regulators.

Key words: apple `Gala´, tree top, shoots, flowering, yield, growth regulators, organic production

1 UVOD

V intenzivnih nasadih jablan je vzdrževanje umirjene rasti vrha težava, ki se s postavljanjem protitočnih mrež v zadnjih letih povečuje, saj vrhovi pogosto zrastejo v mrežo in otežujejo različne tehnološke ukrepe. V vrhu poganjka nastajajo avksini, ki zavirajo rast stranskih poganjkov in povzročajo apikalno dominanco vrha. Količina avksinov v vrhu drevesa je odvisna od gostote sajena. V večji gostoti se tvori več avksinov in rast vrha je izrazitejša (Watanabe in sod., 2006). Avksini, nastali v terminalnem brstu, omejujejo tok vode do lateralnih brstov, kar

[1] mag. kmet., KGZ – zavod Maribor, Sadjarski center Maribor (SCM), Gačnik 77, Pesnica pri Mariboru, e-mail: matjaz.beber@gmail.com

omejuje razraščanje jablane (Wang s sod., 1994). Z odstranitvijo voditeljice, se tvorba avksinov seli v ostale poganjke na vrhu (Bangerth, 2000) in s tem se vzpodbuja razrast vrha. Na intenzivnost rasti vrha vplivajo še drugi dejavniki kot so: tehnološki ukrepi v času rasti, uporaba gnojil, izbira podlage, temperature…

Za umirjanje rasti vrha je potrebno zavreti delovanje avksinov. Vpliv avksinov se zmanjša z autoinhibicijo, ki je naravna pot zaviranja rasti vrhov in s citokinini, ki so antagonisti avksinov in nastajajo v koreninah (Bangerth, 2000). Razmerje med avksini in citokinini je odločujoči dejavnik razvoja poganjkov iz stranskih brstov, rasti drevesa in spreminjanja koncentracije hormonov v koreninah in poganjkih (Tworkoski in Miller, 2007). Autoinhibicija avksinov, kot posledica njihovega nastanka, v vrhu ne deluje vedno. V tem primeru zaviranje delovanja avksinov dosežemo s pomočjo drugih regulatorjev rasti kot so citokinini in giberelini (Greene, 2010). Za manjšo bujnost vrhov se v zadnjem času uporablja pripravek Regalis (proheksadion – kalcij). Pripravek povzroča zmanjšano sintezo giberelinov in posledično zbito rast poganjkov, saj skrajša dolžino internodijev, poveča pa tudi odpornost rastline na ognjevko (Medjdoub in sod., 2005).

Umirjanje rasti vrhov lahko dosežemo tudi z drugim ukrepi. Tako na primer umirjeno rast vrhov dobimo ob velikem ovesku plodov (Baab in Lafer, 2005), saj plodovi predstavljajo glavni ponor asimilatov. Z upogibanjem vej vplivamo na hidravlično prevodnost, vzpodbudimo rast latentnih poganjkov in nastajanje cvetnih brstov (Hann in sod., 2007). Z odstranitvijo vrha nad najvišjo upognjeno vejo in poravnavo upognjene veje kot novi vrh, ohranimo lastnosti upognjene veje. Prav tako bi naj odstranitev pet najvišjih listov pri dolžini poganjka 20 – 25 cm vzpodbudila rast lateralnih brstov s tem se zmanjša rast vrha (Ouellette in Young, 1994). Dencker in Hansen (1994) poročata, da je uporaba amonijevega dušika v fertirigaciji imela učinek na boljši cvetni nastavek, povečala aktivnost korenin in umirila rast vrha. Rast poganjkov je tudi temperaturno pogojena in je pri nižjih temperaturah manjša – večji vpliv vrha (Tromp, 1993).

Po Evropi v intenzivnih nasadih uporabljajo različne gojitvene tehnike za umirjanje rasti vrhov. Sadjarji na Nizozemskem v spomladanskih mesecih premazujejo vrhove z mešanico rastlinske smole in bioregulatorjev rasti. Na Južnem Tirolskem uporablja metodo ravnanja najvišje upognjene veje medtem, ko v drugih pridelovalnih območjih Evrope uporabljajo pripravek Regalis. Pri nas, kljub posameznim poskusom, nismo do sedaj naredili večletnih primerjalnih poskusov v katerih bi primerjali uspešnost različnih načinov umirjanja rasti vrha. Zato je bil cilj študije večletni poskus na sorti 'Gala' s katerim smo skušali dati odgovor na ta pereč problem jablane.

2 MATERIAL IN METODE DELA

Zastavitev poskusa – lokacija:

v Sadjarskem centru Maribor smo v letu 2006 posadili drevesa jablane sorte `Gala Schniga´ (3 x 1 m). Nasad je pokrit s protitočno mrežo in oskrbovan s kapljičnim namakalnim sistemom. V letu 2010 so vrhovi dosegli višino mreže. V tem letu smo pričeli s različnimi ukrepi umirjanja rasti vrhov:

1: Premazovanje: rastlinski smoli (1l Kambisan) smo dodali 2 % naftil ocetne kisline (200 ml pripravka Luxane Late – Val, aktivna snov: naftil ocetna kislina 10 % (NAA), proizvedel: Luxan, Nizozemska). Zmes smo nanesli v 10 cm pasu na deblo v višini 2,2 m od tal. V letu 2010 smo drevesa premazali enkrat (9.apr.) v fenofazi B – C. V letu 2011 postopek ni bil izveden. V letu 2012 je bil premaz izveden dvakrat prvič (13. marec) fenofaza A – B in drugič (13. junij) v zaključku primarne rasti – v nadaljevanju `premaz´;were used for transformation.

2: Uporaba proheksadion kalcija (pripravek Regalis): v letu 2010 smo prvič pripravek uporabili pri 4 razvitem listu v odmerku 1,25 kg Regalisa /ha. Uporabili smo ročno nahrbtno nizkotlačno škropilnico CP3 s šobo porabe 400 l vode/ha. Drugo tretiranje je bilo izvedeno 21 dni kasneje. Pri tem smo tretirali samo vrhove v koncentraciji 0,6 kg Regalisa/ha. Postopek smo v

tesnih letih 2012 in 2013 ponovili – v nadaljevanju `Regalis´;

3: Zamenjava vrha – ukrep, ki se izvaja v pridelovalnem območju Južne Tirolske. Izvedli smo ga samo v letu 2010 (9. apr.). Na drevesih smo zravnali najvišjo izraščajočo vejo, ki je bila v predhodnem letu upognjena (90^{o}). Preostali del vrha smo odrezali - v nadaljevanju `zamenjava vrha´.

4: Z odstranjevanjem novo nastalih poganjkov v vrhu krošnje 20 do 28 dni po vrhu cvetenja skušamo ohraniti zračen in umirjen vrh. Ukrep smo izvajali v vseh tesnih letih v nadaljevanju `mandanje´,

5: Kontrola – junijska rez. V letu 2010 nismo izvajali ukrepov na vrhovih. Rast je bila premočna in vrhovi so poškodovali mrežo, zato je bila v letih 2011, 2012 in 2013 po zaključku primarne rasti (konec junija) izvedena rez vrhov – v nadaljevanju `junijska rez´.

Meritve

v poskus smo imeli vključenih 5 različnih metod, katere so bile izvedene v štirih ponovitvah (5 x 4 dreves). Drevesa smo spremljali v časovnem obdobju 4 let (2010 – 2013). Opravljene so bile meritve:

1: generativnih parametrov: število socvetij, število plodov, količina pridelka (kg) na drevesu in v vrhu;

2: vegetativnih parametrov: obseg debla 20 cm nad cepljenim mestom in meritve rasti vrhov, katere so zajemale meritve sledečih parametrov: enoletni prirast vseh poganjkov v cm, število poganjkov in povprečna dolžina poganjka v cm. Kot vrh smo označili zgornji del drevesa (nad ukrepom), ki je presegal višino 2,2 m od tal.

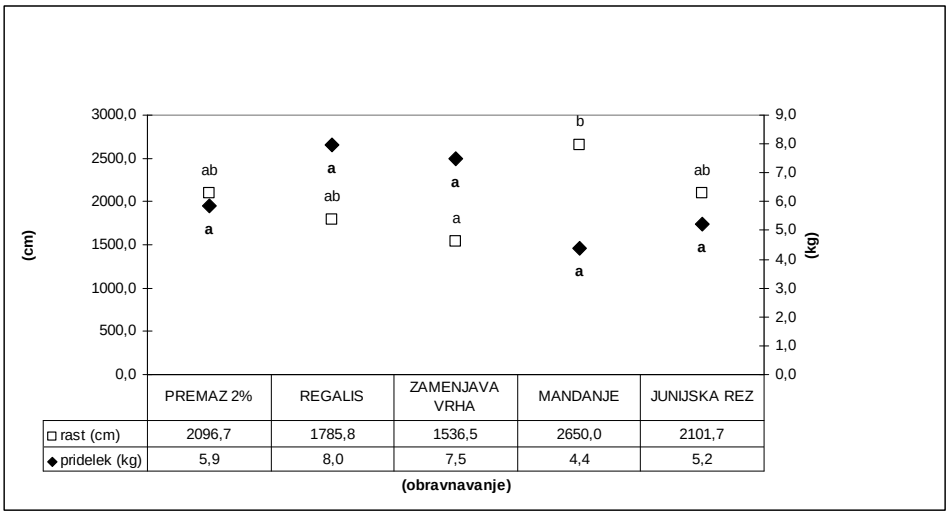
3: Obdelava podatkov: zbrani podatki so bili obdelani in analizirani s pomočjo statističnega programskega paketa Microsoft Excel 2003 ter s pomočjo programa SPSS for Windows 16. Uporabili smo analizo variance (ANOVA). Razlike med obravnavanji smo testirali z Duncanovim testom, kjer smo ugotavljali statistično značilne razlike med obravnavanji v posameznih parametrih ($p < 0,05$). Za izračun indeksa izmenične rodnosti (I) smo uporabili sledečo formulo: $I = 1/n\text{-}1 \times (|a_2\text{-}a_1|/ a_2+a_1) +(|a_3\text{-}a_2|/ a_3+a_2) + (|a_n\text{-}a_{(n-1)}|/ a_n+a_{(n-1)})$.

3 REZULTATI S DISKUSIJO

Preglednica 1: Obseg debla (cm) poskusnih dreves ob postavitvi poskusa (2010), kumulativni prirast obsega 2010-2013 (cm), kumulativni pridelek preračunan v t ha^{-1}, relativno izražen pridelek glede na prvo obravnavanje, indeks izmenične rodnosti (0 -25 nizek, 26 – 50 srednji, 51 – 75 visok in +75 zelo visok indeks izmenične rodnosti), nihanja pridelka v t ha^{-1} in delež pridelka 1. kakovosti plodov za obdobje 2010 – 2013

	obseg 2010	prirast (cm)	Kumulativni (4. letni) pridelek (t ha^{-1})	relativni pridelek	indeks izmenične rodnosti	nihanje (t ha^{-1})	Nihanje deleža 1. kakovosti plodov
PREMAZ	15,6c	5,7a	227	1	29	32 – 75t	77 – 97
REGALIS	12,6ab	4,3a	210	0,93	16	40 – 60t	84 – 96
ZAMENJAVA VRHA	13,5abc	4,6a	194	0,86	31	28 -56t	78 – 97
MANDANJE	14,6bc	5,5a	172	0,76	26	26 – 55t	80 – 99
JUNIJSKA REZ	11,35a	3,6a	150	0,66	35	19 – 48t	90 – 97

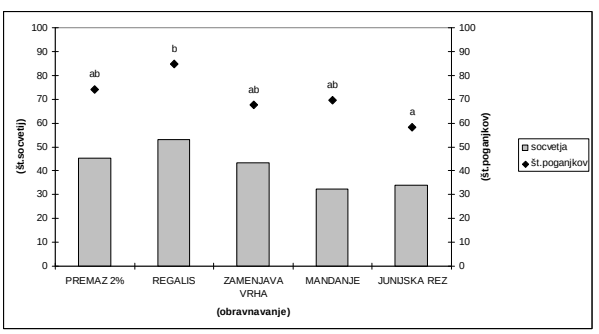
Kljub različnemu začetnemu obsegu dreves v prirastu obsega debla v obdobju 2010 – 2013 let ni bilo statistično značilnih razlik (preglednica 1). Najvišji kumulativni pridelek pri premazovanju vrhov in najnižji pridelek pri junijski rez je bil posledica izbire različno velikih dreves ob postavitvi poskusa (preglednica 1). Na vseh poskusnih drevesih je bila uporabljena enaka tehnologija pridelave jabolk, vendar smo pri tretiranju dreves s pripravkom Regalis dosegli najbolj stabilno rodnost (najnižji indeks izmenične rodnosti) in kakovost plodov.



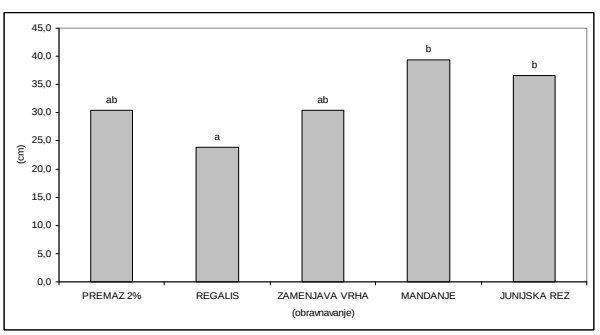
Slika 1: Kumulativni pridelek in kumulativni prirast enoletnih poganjkov v vrhu krošnje za časovno obdobje 2010 – 2013 let

Statistično značilno razliko v rasti vrhov smo dosegli med ukrepom `zamenjava vrha´ in `mandanje´ vrhov (Sl. 1). Z ukrepom zamenjave vrha smo ohranili lastnosti upognjene veje, kot jo opisuje Hann (2007). Dober uspeh umirjene rasti vrhov je bil dosežen tudi z uporabo pripravka Regalis in premazovanje z 2 % raztopino NAA, kar potrjuje trditev Greena (2010), da umirjanje rasti dosežemo s pomočjo regulatorjev rasti.

V kumulativni količini pridelka v vrhu krošnje je bila boljša rodnost vrhov dosežena z uporabo pripravka Regalis.



Slika 2: Povprečno število socvetij in enoletnih poganjkov v vrhu za časovno obdobje 2010 – 2013



Slika 3: Povprečna dolžina enoletnih poganjkov v vrhu za časovno obdobje 2010 – 2013

Skupno število socvetij v vrhu za testno obdobje 2010 – 2013 potrjuje razlike v rodnosti med obravnavanji. Večje število socvetij je bilo na drevesih tretiranih s pripravkom Regalis. Majhno število socvetij pri ukrepu `mandanje´ in `junijska rez´ potrjuje trditev Bangerhta (2000), da z odstranitvijo voditeljice (vrha) v času rasti dreves preselimo nastajanje avksinov v druge dele vrha, kar lahko vodi do zaviranja nastanka cvetnih brstov (Sl. 2). Drevesa so v obdobju 2010 – 2013 tvorila največ enoletnih poganjkov v vrhu pri uporabi pripravka Regalis, pri ukrepu `junijska rez´ pa najmanj. Obratno in posledično temu je bila povprečna rast poganjka v tem obdobju najmanjša pri uporabi pripravka Regalis in največja pri ukrepu `junijska rez´ in `mandanje´ (Sl. 3). Z različnimi metodami umirjanja rasti vrhov smo bili najmanj uspešni pri ukrepu `mandanje´ in `junijska rez´.

4 ZAKLJUČEK

Umirjanje rasti vrhov v času rasti s odstranjevanjem enoletnega prirasta v mesecu maju (`mandanje´) in čiščenje vrhov po zaključku primarne rasti (`junijska rez´), sta najbolj pogosto uporabljeni metodi urejanja vrhov v praksi. V našem poskusu sta se pokazali kot najmanj učinkoviti metodi umirjanja rasti vrhov. Z uporabo pripravka Regalis najbolje umirimo rast vrhov in stimuliramo rodnost v vrhu. Dobri rezultati umirjanja rasti s premazovanjem z 2 % raztopino NAA dokazujejo smiselnost uporabe rastnih regulatorjev za umirjanje rasti vrhov. Z zamenjavo vrha smo uspešno umirili rast in stimulirali rodnost v vrhu za daljše časovno obdobje (4 leta), kar dokazuje, da je počasen in zamuden ukrep v prvem letu na daljše časovno obdobje smiseln in zelo učinkovit. Zamenjava vrha je kot ukrep za umirjanje rasti vrhov primeren v nasadih vključenih ekološki način pridelave, saj uspešno umirimo rast vrhov brez uporabe rastnih regulatorjev.

5 LITERATURA

Baab G. in Lafer G. 2005 Alternanz. V: Kernobst – Harmonisches Wachstum – optimaler Ertrag. aVBUCH im Österriechischen Agrarverlag Druck und Verlags - ges.m.b.H.Nfg. KG: 26 – 27

Bangerth F., Li CJ., Gruber J., 2000 Mutual interaction of auxin and cytoinins in regulating correlative dominance. Plant growth regulation 32,2-3: 205 – 217

Han HH., Coutand C., Cochard H., Trottier C., Lauri PE., 2007. (okt) Effects of shoot bending on lateral fate and changing traits across five apple genotypes. Journal of Experimental botany 58, 13: 3537 – 3547; DOI: 10.1093/jxb/erm200

Medjdoub R., Val J., Blanco A., 2005 Inhibition of vegetative growth in red apple cultivars using prohexadione – cacium. Journal of Horticultural science & biotechnology 80, 2: 263 - 271

Ouellette DR., Young E., 1994 Branch inducment in apple stoolbed shoots summer leaf removal tipping. Hortscience 29,12: 1478 – 1480

Tromp J. 1993. Lateral shoot formation and flower – bud formation in apple in the 1.st year after budding as affected by air-temperature and exposure to red – light. Journal of Hortcultural science 68, 2: 255-260

Tworkoski T., Miller S., 2007. (nov) Endogenous hormone concentrations and bud-break response to exogenous benzyl adenine in shoots of apple trees with two growth habits grown on three rootstocks. Journal of Horticultural science & biotechnology, 82, 6: 960 – 966

Wang SY., Faust M., Line MJ., 1994. (nov) Apical dominance in apple (*malus domestica borkh*) – the possible role of indole – 3 acetic – acid (IAA). Journal of the american society for horticultural science 119, 6: 1215 – 1221

Watanabe M., Suzuki A., Komori S., Bessho H., 2006. (may) Effect of heading-back prunning on shoot growth and IAA and cytokinin concentrations at bud burst of columnar-type apple trees. Journal of the Japanese society for horticultural science75, 3: 224 – 230; DOI: 10.2503/jjshs.75.224

COBISS Code 1.04

DOI: 10.14720/aas.2015.105.1.15
Agrovoc descriptors: juglans regia, walnuts, shell, kernels, nuts, equipment, fruit harvesters, comminution, quality

Agris category code: n20

Preizkusno delovanje stroja za luščenje orehov

Rajko BERNIK[1]

Received August 04, 2014; accepted November 24, 2014.
Delo je prispelo 04. avgusta 2014, sprejeto 24. novembra 2014.

IZVLEČEK

Oreh (*Juglans regia* L.) spada med lupinasto sadje, za katere je značilno, da se slastno jedrce skriva v oleseneli lupini. Na drevesu pa sta jedrce in lupina zavarovana še z zeleno lupino. V Sloveniji, pa tudi drugod se za odstranjevanje lupine v večini še vedno uporablja trda orodja, kot so kamni, kladiva in razni ročni (kuhinjski) drobilniki orehov. Vendar pa v današnjem času, pri intenzivni pridelavi orehov pridejo redko v poštev ali pa sploh ne, predvsem zato, ker današnje gospodarstvo stremi k zniževanju stroškov na vseh področjih gojenja orehov in pridobivanja jedrc. Da bi znižali stroške obdelave orehov in posredno tudi ceno jedrc za potrošnike, se v zadnjem času zelo veliko pojavljajo strojni drobilniki orehov. Raziskava zavzema lastnosti in delovanje stroja za luščenje orehov ter kakovost drobljenja orehov na drobilniku, ki ga je patentiral prof. dr. Rajko Bernik. V raziskavo smo vključili naravno sušene, prisilno sušene in sveže orehe. Naravno in prisilno sušeni orehi so bili razdeljeni v dve podskupini, in sicer na počene ter cele orehe. Pri naravno sušenih celih orehih smo v raziskavo vključili še sortne orehe. V vsaki skupini in podskupini smo označili, analizirali, preučili in ovrednotili približno sto orehov. Namen poskusa je bil ugotoviti, pri kateri vrtilni frekvenci izmetala lahko stroj lupino zdrobi, poči ali delno loči od jedrca, ne da bi ga poškodoval, oziroma pri kateri vrtilni frekvenci izmetala bodo poškodbe jedrca še spremenljive in jedrca primerna za trg, kjer na ceno še največ vpliva videz jedrc.

Ključne besede: oreh, lupina, drobilnik orehov, kakovost jedrc

ABSTRACT

TESTING OF A MACHINE FOR WALNUT CRACKING

Fruit of walnut (*Juglans regia* L.) belongs to stone fruits. This means that the lignified, stony endocarp, a shell, contains delicious kernel. On the tree a nut is surrounded by the green husk.In Slovenia and in the other parts of the world heavy tools (such as stone, hammer and hand-crusher for nuts) are used to remove nutshell. In the intensive walnut production heavy tools are not competitive with today world economy. This is because the priority in walnuts and nuts kernel production is to reduce costs. Walnut shell cracking devices are invented to reduce walnut handling costs and consumer price. Characteristics of this device and walnut kernel quality are presented in this research.

Key words: walnut, shell, walnut cracker, kernel quality

1 UVOD

Pri nas ima pridelava orehov (*Juglans regia* L.) že zelo dolgo tradicijo. Včasih je imela skoraj vsaka hiša svoje drevo. Ljudje so orehe sušili ter iz njih za praznične dni pekli tradicionalno potico, kar je bilo omenjeno že leta 1689 v Valvasorjevi Slavi vojvodine Kranjske (vir). S preteklostjo pa nas povezujejo potomci takratnih orehov ter način ločevanja užitnega dela - jedrca iz olesenele lupine, ki se do današnjih dni ni bistveno spremenilo. Kot včasih, tudi pri nas in drugod za drobljenje orehov v veliki večini še vedno uporabljamo trda orodja kot so kladiva in razni ročni (kuhinjski) drobilniki

[1] prof. dr., Biotehniška fakulteta, Oddelek za agronomijo, Jamnikarjeva 101, SI-1111 Ljubljana; e-mail: rajko.bernik@bf.uni-lj.si

orehov. Vendar pa v današnjem času, pri intenzivni pridelavi orehov, pridejo redko v poštev ali pa sploh ne.

Današnje gospodarstvo stremi h čim cenejši pridelavi in obdelavi pridelkov ter k temu, da jih potrošnikom ponudijo po ceni, ki so jo za pridelke še pripravljeni plačati. Seveda naj bi takšna cena pokrila vse stroške pridelavi ter zagotovila največji možni dobiček pri prodaji orehov. Zaradi znižanja stroškov se v razvitih državah, med katerimi je tudi Slovenija, pojavlja čedalje več orehov, uvoženih iz manj razvitih držav. Čeprav gre tudi tam v večini za ročno drobljenje, se uvoznikom vsekakor obrestuje, predvsem na račun poceni delovne sile.

Da bi znižali stroške obdelave orehov in posredno tudi ceno jedrc za potrošnike, se v zadnjem času zelo veliko pojavljajo strojni drobilniki orehov. Njihova glavna pomanjkljivost so predvsem poškodbe jedrc. Zato je načrtovanje in izdelava novih drobilnikov stalni izziv konstruktorjev, delovanje tovrstnih strojev pa se vsako naslednjo generacijo konstantno izboljšuje.

2 MATERIAL IN METODE DELA

2.1 Tržni standardi za kakovost orehovih jedrc

V prvi kakovostni razred z najvišjo ceno na trgu spadajo nepoškodovane cele polovičke jedrc (Charlot in sod., 1996). Tržni standardi velikosti jedrc, povzeti po ameriški zakonodaji (Cit. po United States Department of Agriculture, 1. september 1968, standard USDA). Za prodajo primerna jedrca se delijo v štiri kakovostne razrede: polovičke, delci in polovičke, delci ter drobni delci. Pri vsaki kategoriji so dovoljena odstopanja. Za kategorijo polovičk (slika 1A) velja, da morajo glede na skupno maso vsebovati minimalno 85 odstotkov celih polovičk. Preostanek skupne mase lahko vsebuje le tri četrtine polovičke jedrca. Skupina polovičk je najvišji (prvi) kakovostni razred. V drugi kakovostni razred sodijo delci in polovičke (slika 1B), ki morajo glede na skupno maso vsebovati vsaj 20 odstotkov celih polovičk, delež preostale količine jedrc pa ne sme pasti čez mrežasto sito s premerom lukenj 9,53 mm^2.

Slika 1: Skupina polovičk (A) in skupina delcev in polovičk (B) (California walnuts…, 2014)

Slika 2: Primer izvedbe mrežastega sita za sortiranje jedrc po velikosti (Alibaba…, 2014)

V skupino delcev jedrc (slika 3C) se razvrščajo vsi deli, ki ostanejo na mrežastem situ z okroglimi odprtinami premera 9,53 mm^2. Drobni delci (četrti kakovostni razred) so vsi tisti, ki padejo čez zgoraj omenjeno sito, a se hkrati ustavijo na manjšem, s premerom lukenj 3,18 mm^2.

Slika 3: Skupina delcev jedrc (C) ter skupina drobnih delcev (D) (California walnuts…, 2014)

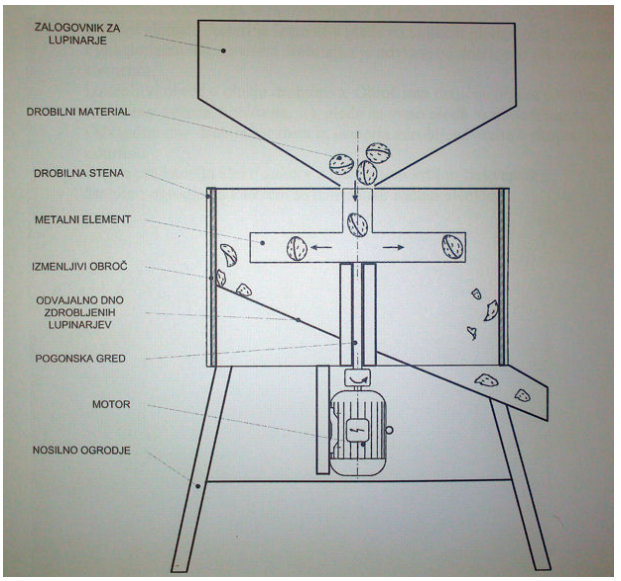
Preglednica 1: Dovoljena odstopanja pri klasifikaciji orehovih jedrc po standardih velikosti (v odstotkih)

Standardi velikosti	Polovice manjše od treh četrtin	Ustavijo na 9,53 mm^2 situ	Padejo skozi 9,53 mm^2 sito	Padejo skozi 6,35 mm^2 sito	Padejo skozi 3,18 mm^2 sito
Polovičke	5%	/	/	1 (od 5 %)	/
Delci in polovičke	/	/	18	3 (od 18 %)	1 (od 3 %)
Delci	/	/	25%	5 (od 25 %)	1 (od 5 %)
Drobni delci	/	10%	/	/	2%

2.2 Rotirajoči centrifugalni drobilnik

Centrifugalni drobilnik celih orehov omogoča drobljenje lupine s pomočjo trka ploda, ki ga povzroči centrifugalna sile, ta pa je rezultat brezstopenjske ali stopenjske nastavljive vrtilne frekvence metalnega elementa in mase ploda ob drobilno steno. Drobilnik omogoča drobljenje velikih količin lupinarjev v kratkem času (Bernik, 2009). Sestavljen je iz elektromotorja, ki preko pogonske gredi omogoča centrifugalno silo, s katero potem oreh trešči ob izmenljivi obroč drobilne stene. Obroč ima različne tipe in oblike površine (orebrena, zobata, koničasta, lesena, kovinska, plastična,…) glede na trdnost luščine in vrsto ploda. Elektromotor je opremljen s frekvenčnim regulatorjem, ki v digitalni obliki prikazuje nastavljivo vrtilno frekvenco metalnega elementa. Frekvenca se spreminja ročno glede na

trdnost lupine (Bernik, 2002). Glavni sestavni deli, poleg zgoraj omenjenih, so še: zalogovnik, v katerega je možno strojno ali ročno dovajanje plodov lupinarjev; zamenljiv metalni element (glede na trdnost lupine in velikost plodov); stabilno ogrodje, na katerem so nameščeni vsi našteti elementi (Bernik, 2009).



Slika 4: Rotirajoči centrifugalni drobilnik orehov (Bernik, 2009)

2.3 Rastlinski material: razvrstitev orehov po skupinah (vrste vzorcev)

Skupaj smo za raziskavo nabrali ter analizirali 694 zrelih plodov oreha. Od tega smo analizirali 594 plodov, pridelanih na sejancih oreha (v nadaljevanju 'sejancev') ter sto plodov, nabranih na drevesih različnih sort (v nadaljevanju 'sortnih orehov'). Vsem orehom, ki še niso padli iz zelene lupine, smo le-to ročno odstranili. Sledila je ročna odbira slabih plodov, ki smo jih zavrgli in jih pustili v nasadu. Nato smo vse izbrane orehe očistili še z navadno vodo.

Slika 5: Sejanci, pripravljeni za čiščenje (Kastelec, 2012)

Prisilno sušeni orehi
Nabrali ter analizirali smo 232 orehov sejancev. Prisilno sušene orehe smo sušili dva tedna na domači sušilnici, pri kateri se orehi posušijo s kroženjem toplega zraka. Zrak kroži s pomočjo vetrnice, ki jo poganja elektromotor. Tako sušeni orehi se posušijo v krajšem času od naravno sušenih. Razdelili smo jih na cele orehe (102 vzorca) in na počene orehe (130 vzorcev).

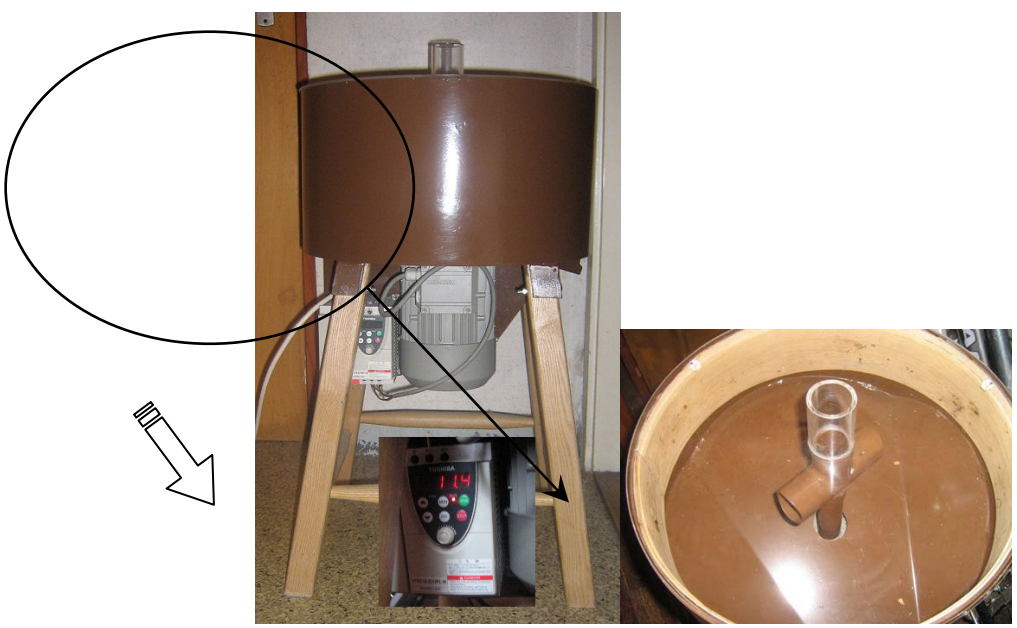
Naravno sušeni orehi
Analizirali smo 238 orehov sejancev ter 100 sortnih orehov. Orehi so se štiri tedne sušili na mreži, dvignjeni od tal za trideset centimetrov. Mreža je bila postavljena na podstrehi v zračnem prostoru brez direktne sončne osvetlitve. Orehe sejance smo ločili na počene (133 vzorcev) ter cele orehe (105 vzorcev).

Počeni orehi
Ovrednotili smo 266 počenih orehov sejancev. Dobili smo jih iz celih orehov, katere smo z navadnim kladivom udarili ravno s takšno močjo, da je trdna lupina počila in da se jedrce ni poškodovalo. Razdelili smo jih na prisilno sušene (130 vzorcev) in na naravno sušene (133 vzorcev).

Sveži orehi
Nabrali ter analizirali smo 124 celih orehov sejancev. Takoj, ko smo orehe nabrali, smo jih ločili od zelene lupine ter očistili. Sledilo je takojšnje merjenje dimenzij, tehtanje ter preizkušanje orehov z drobilnikom.

2.4 Drobljenje orehov

Po končanem sušenju smo vsak oreh označili, mu izmerili širino, višino in debelino ter maso. Izjema so le sveži orehi, ki smo jih merili in preizkušali takoj po čiščenju, da ni prišlo do prevelike izgube vode v jedrcih. Za drobljenje smo uporabili rotirajoči centrifugalni drobilnik orehov. Nekaj neoznačenih orehov smo porabili za to, da smo ročno nastavili primerno začetno vrtilno frekvenco drobilnika. Vse orehe, tako označene kot neoznačene smo posamezno dodajali v metalni element. Zaradi centrifugalne sile in mase oreha je ta priletel ob leseno drobilno steno. Po trku smo analizirali poškodbe trde lupine in jedrca. Naš namen je bil nastaviti tako vrtilno frekvenco, da lupina poči do te mere, da se lahko jedrce izlušči brez raznih pripomočkov oziroma samo z rokami. V primeru, da orehova lupina prvič ni počila, smo isti oreh še enkrat dodali v metalni element, ne da bi spremenili vrtilno frekvenco. Če spet ni prišlo do deformacije lupine, smo frekvenco postopoma povečevali do poškodbe lupine. Po uspelem trku smo vsak počen oreh podrobno preučili.



Slika 6: Drobilnik orehov z regulatorjem vrtilne frekvence (Kastelec, 2012)

Pri počenem orehu smo opisali stanje deformacije lupine in jedrca ter določili odnos med jedrcem in luščino. Na koncu smo tudi ovrednotili skupno kakovost posameznega oreha. Pri tej oceni je bilo odločilno stanje jedrca, upoštevali smo tudi težavnost izluščenja jedrc, vendar ta ni imela bistvene vloge pri ocenjevanju skupne kakovosti. Če jedrca nismo mogli izluščiti z rokami, smo skupno kakovost vzorca opredelili za srednjo oziroma slabo, ne glede na stanje jedrca takoj po testu.

2.4.1 Vrednotenje kakovosti drobljenja (karakteristike drobljenja počenih orehov)

Za lažje razumevanje vrednotenja in kasnejših rezultatov smo opisali nekaj vzorcev počenih orehov z jedrci ter jim dodali slike.

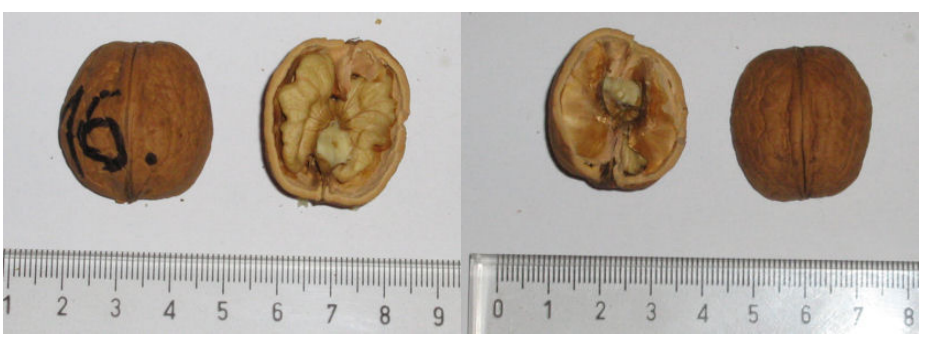
Testni vzorec 1

Lupina sejanca se je razpolovila po širini oziroma po šivu, popolnoma nepoškodovano jedrce pa se lahko izlušči z rokami in spada v najvišji kakovostni razred. Skupna kakovost precej drobnega vzorca je torej odlična.

Slika 7: Prisilno sušen, že počen sejanec (Kastelec, 2012)

Testni vzorec 2

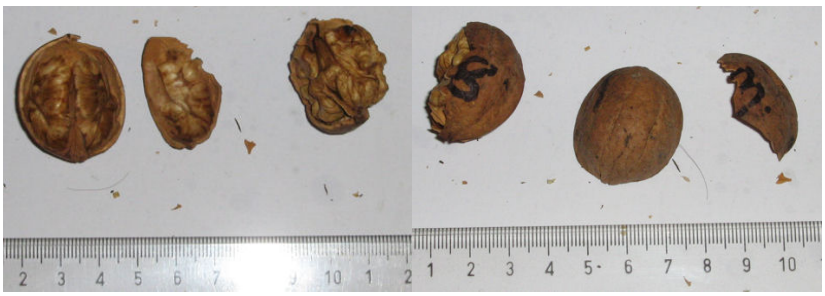
Lupina se je razpolovila po debelini, jedrce pa na dve celi polovički. Pri izluščevanju polovičk smo si morali pomagati z nožem, hkrati pa smo jih tudi malenkost poškodovali. Kljub temu sta polovički ostali cele z nekaj delci. Torej po kakovosti še vedno spadajo v najvišji razred. Vendar je bila zaradi uporabe noža skupna kakovost ocenjena kot srednja.



Slika 8: Prisilno sušen, že počen sejanec – vzorec št. (Kastelec, 2012)

Testni vzorec 3

Od jedrca je odpadlo več kot pol lupine. Jedrce je popolnoma celo in nepoškodovano in se lahko izlušči. Skupna kakovost je odlična.



Slika 9: Prisilno sušen, že počen sejanec (Kastelec, 2013)

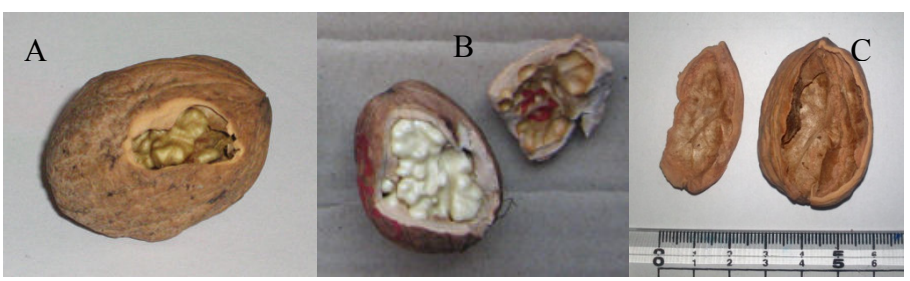
Testna vzorca 4 in 5

Tu bi izpostavili problem z večjimi dimenzijami nekaterih orehov. Da so orehi lahko prešli čez premer odprtine metalnega elementa, smo morali test večkrat ponoviti. Oreh smo zaporedoma spuščali v metalni element, da se je v pravokotnem stičišču le-tega lahko zasukal v pravo smer izmeta in posledično trčil ob steno. Slika prikazuje tudi primer trka, pri katerem približno četrtina lupine oreha odpade.

Slika 10: Naravno sušena, cela sortna oreha – vzorec št. 36 in 40 (Kastelec, 2009)

Ostali vzorci

Predstavili bi še nekaj pogostih primerov drobljenja orehov. Slika A prikazuje oreh, od katerega je po trku odpadla manj kot četrtina lupine, pri sliki C pa je odpadla približno četrtina lupine. Slika B prikazuje nepoškodovano jedrce svežega oreha po trku, od katerega se je odlomila približno polovica lupine.



Slika11: Različni primeri orehov po trku (Kastelec, 2012)

2.4.2 Vrednotenje skupne kakovosti testiranih orehov

Odlična kakovost

Takoj po trku oreha v drobilno steno smo lahko jedrca po videzu umestili v kakovostne razrede. Odlične kakovosti so jedrca najvišjega kakovostnega razreda. Taka jedrca so bila po trku nepoškodovana ali pa deloma oziroma četrtino odlomljena od celega jedrca. Sem smo šteli še dve celi polovički, za izluščitev jedrca pa nismo potrebovali nobenih pripomočkov in nobene sile. V primeru odlične skupne kakovosti do izluščenih jedrc pridemo najhitreje, hkrati pa jih tudi najdražje prodamo.

Srednja kakovost

Sem smo umestili jedrca drugega in tretjega kakovostnega razreda. Polovički jedrc po trku nista bili več celi, ampak sta razpadli na tri ali štiri približno enake dele. V to skupino smo razvrstili tudi vse tiste orehe, pri katerih smo za izluščitev jedrca morali uporabiti pripomoček (nožič), pa čeprav je bilo jedrce po trku nepoškodovano. Za ločitev jedrca od lupine smo uporabili tudi večjo moč. Postopek izluščitve jedrc je zamudnejši, zaradi poškodb pa jih na trg ne moremo ponuditi po najvišji ceni.

Slaba kakovost

Sem smo uvrstili jedrca četrtega (najslabšega) kakovostnega razreda. Ta so se pri preizkusu raztreščila na več drobnih delcev. Če jedrca nismo mogli izluščiti, ne da bi ga zdrobili, smo skupno kakovost prav tako opredelili kot slabo. Za izluščevanje jedrc smo morali uporabiti tudi več moči in pripomočkov hkrati (nožič in šilo, v nekaj primerih pa tudi kladivo) ter se pri tem največ zamudili. Tako zdrobljena jedrca lahko na trg ponudimo le po najnižji ceni. Še najbolje pa je, če jih imamo za lastno uporabo.

3 REZULTATI MERITEV

3.1 Fizikalne karakteristike orehov po skupinah

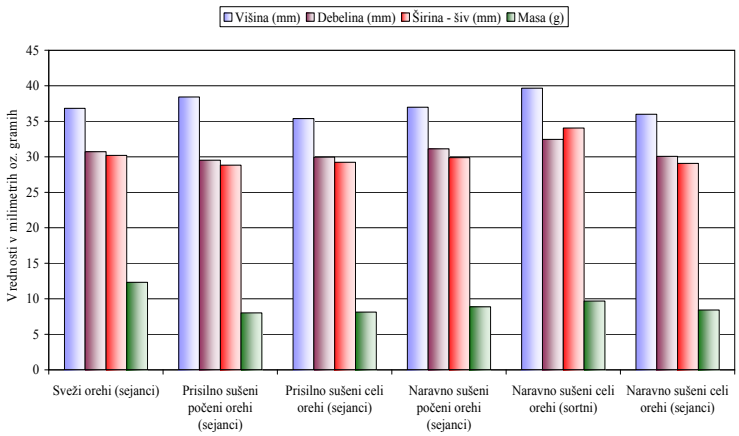
Pri vsakem orehu smo izmerili višino, debelino, širino po šivu ter maso. Dimenzije oreha smo prikazali v milimetrih (mm), maso pa v gramih (g).

Povprečne karakteristike plodov oreha se nanašajo na meritve naslednjih količin vzorcev: 124 svežih sejancev, 130 prisilno sušenih počenih sejancev, 102 prisilno sušena cela sejanca, 133 naravno sušenih počenih sejancev, 105 naravno sušenih celih sejancev ter 100 naravno sušenih sortnih orehov. Skupno smo izmerili 694 orehov.

Izračune srednje vrednosti za posamezno skupino smo predstavili v tabeli ter naredili še grafični prikaz omenjenih karakteristik s stolpnim grafikonom (Slika 12).

Table 2: Povprečna višina, debelina, širina in masa orehov po posameznih skupinah.

Skupine orehov	Višina (mm)	Debelina (mm)	Širina - šiv (mm)	Masa (g)
Sveži orehi (sejanci)	36,8	30,7	30,2	12,3
Prisilno sušeni počeni orehi (sejanci)	38,4	29,5	28,8	8,0
Prisilno sušeni celi orehi (sejanci)	35,4	30,0	29,2	8,1
Naravno sušeni počeni orehi (sejanci)	37,0	31,1	29,9	8,9
Naravno sušeni celi orehi (sortni)	39,7	32,4	34,0	9,7
Naravno sušeni celi orehi (sejanci)	36,0	30,0	29,1	8,4



Slika 12: Srednje vrednosti meritev po posameznih skupinah orehov.

3.2 Rezultati deformacij lupine orehov pri trku

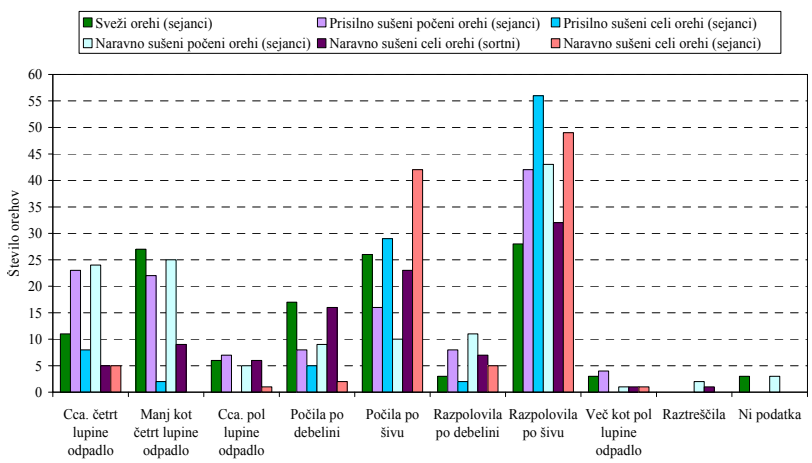
Podatke o deformaciji lupine smo razvrstili v devet glavnih kategorij, deseta pa zavzema orehe prevelikih dimenzij. Posledično pri teh orehih podatka o poškodbi lupine in nadaljnjih rezultatov našega poskusa nismo uspeli pridobiti. Zato smo to kategorijo orehov prevelikih dimenzij označili kar z besedama »Ni podatka«.

Kategorije (»Cca. četrt lupine odpadlo«, »Manj kot četrt lupine odpadlo«, »Cca. pol lupine odpadlo«, »Več kot pol lupine odpadlo«) smo ovrednotili po tem, kolikšen približni delež lupine se je po trku odlomil (odpadel) od jedrca.

Pri nekaterih orehih, ki smo jih s kladivom počili že pred poskusom z drobilnikom, smo deformacijo lupine po trku prav tako ovrednotili za počeno. To smo storili v primerih, ko smo pri trku slišali zvok počene lupine, z nadaljnjim višanjem vrtilne frekvence ali ponovnim poskusom z isto vrt. frekv. pa bi se lupina ali jedrce preveč poškodovala oziroma raztreščila. Lupino smo ovrednotili kot raztreščeno, če delcev po trku nismo uspeli prešteti (zelo drobni delci).

Rezultate po skupinah orehov smo predstavili z grafikonom in tabelo. Rezultati na spodnji sliki prikazujejo količino (število) orehov za posamezno kategorijo deformacij lupine po trku v drobilno steno.

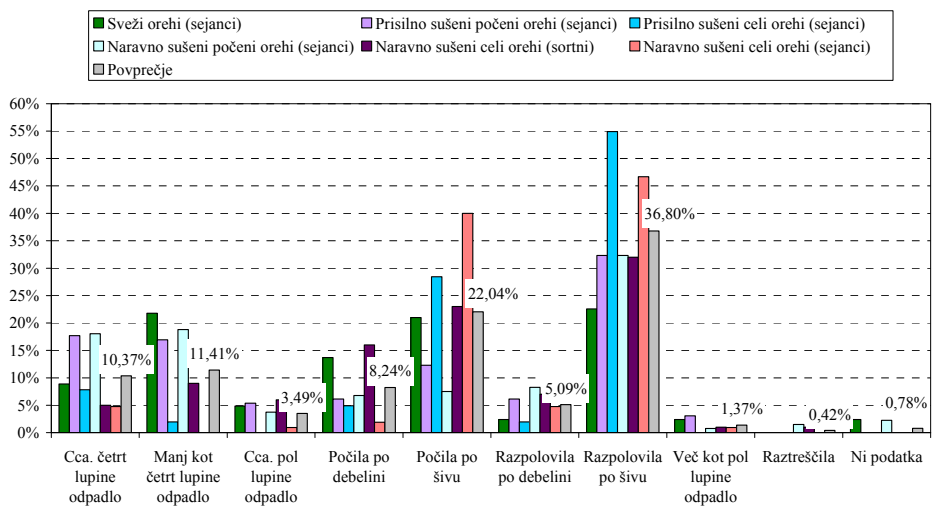
Iz slike 13 je razvidno, da se je pri vseh skupinah orehov lupina največkrat razpolovila po spoju oziroma šivu. Če izvzamemo že pred poskusom počene orehe, je skoraj pri vseh skupinah (razen pri svežih sejancih) pok po šivu druga najpogostejša deformacija lupine.



Slika 13: Različne deformacije orehovih luščin po trku v drobilno steno.

Preglednica 3: Deformacije orehovih lupin po trku v odstotkih (%) za določeno skupino orehov ter srednje vrednosti posameznih deformacij lupine za vse skupine skupaj.

Stanje (deformacija) luščine po trku orehov	**Skupine orehov**						
	Sveži orehi (sejanci)	Prisilno sušeni počeni orehi (sejanci)	Prisilno sušeni celi orehi (sejanci)	Naravno sušeni počeni orehi (sejanci)	Naravno sušeni celi orehi (sortni)	Naravno sušeni celi orehi (sejanci)	*Srednja vrednost (Povprečje)*
Četrt lupine odpadlo	8,87	17,69	7,84	18,05	5	4,76	*10,37*
Manj kot četrt lupine odpadlo	21,77	16,92	1,96	18,8	9	0	*11,41*
Pol lupine odpadlo	4,84	5,39	0	3,76	6	0,95	*3,49*
Počila po debelini	13,71	6,15	4,9	6,77	16	1,91	*8,24*
Počila po šivu	20,97	12,31	28,43	7,52	23	40	*22,04*
Razpolovila po debelini	2,42	6,15	1,96	8,27	7	4,76	*5,09*
Razpolovila po šivu	22,58	32,31	54,9	32,33	32	46,67	*36,8*
Več kot pol lupine odpadlo	2,42	3,08	0	0,75	1	0,95	*1,37*
Raztreščila	0	0	0	1,50	1	0	*0,42*
Ni podatka	2,42	0	0	2,26	0	0	*0,78*



Slika14: Različne deformacije orehovih lupin po trku v odstotkih in srednje vrednosti posameznih poškodb lupine za vse skupine orehov skupaj.

Iz slike 14 je razvidno, da so najpogostejše deformacije lupine pri trku vezane na njeno širino oziroma šiv. V 36,8 % primerov se je lupina razpolovila, v 22,04 % pa počila po šivu. Na drugem mestu so deformacije, pri katerih se odlomi do vključno četrtina lupine oreha. Pri 11,41% orehih se je pri trku odlomil le manjši del lupine, pri 10,37 % pa se je odlomila približno četrtina lupine. Na tretjem mestu po pojavljanju, deformacije lupine potekajo preko debeline oreha. Počenost po debelini se je pojavila v 8,24 odstotkih. Pri 5,09 % primerih pa se je lupina razpolovila po debelini. Najredkeje so se pojavile deformacije lupine, pri kateri se je od jedrca odlomi približno polovica (3,49 %) ali pa več kot polovica (1,37 %). Lupina se je raztreščila v 0,42 % primerih, kar je verjetno posledica prevelike vrtilne frekvence. Podatkov o deformaciji lupine pri šestih orehih (0,78 %) pa zaradi prevelikih dimenzij nismo pridobili.

3.3 Rezultati deformacij jedrc po trku

Poškodbe jedrc so prikazane glede na trk orehov v drobilno steno. Torej brez odnosa med jedrcem in lupino oziroma stopnje izluščitve jedrc. Pri kategoriji raztreščeno jedrce bi poudarili, da so jedrca poleg trka orehov v steno ovrednotena kot raztreščena, če smo jih med samim postopkom izluščitve preveč poškodovali (čeprav so bila jedrca po trku cela).

Stanje jedrca smo razdelili v sedem glavnih kategorij in še v dodatno, pri kateri je bilo šest orehov prevelikih dimenzij (»Ni podatka«), en prisilno sušen počen sejanec pa je bil brez jedrca. Prve štiri kategorije stanja jedrc (»Popolnoma cel«, »Deloma odlomljen«, četrt odlomilo«, »Dve polovici«) so po standardu USDA (1968) najvišje kakovosti. Naslednji dve (»Trije deli«, Štirje deli«) smo umestili v srednjo kakovost (drugi in tretji razred po USDA). V zadnjo kategorijo (»Raztreščil«) smo uvrščali jedrca, pri katerih je bila deformacija takšna, da posameznih delcev ni bilo mogoče natančno prešteti. Taka jedrca so najslabše kakovosti.

Preglednica 4: Deformacije jedrc po trku v odstotkih glede na različne skupine orehov, srednje vrednosti vseh kategorij deformacij jedrc in vsota števila jedrc oziroma orehov za posamezno kategorijo.

Skupine orehov	**Stanje (deformacija) jedrc po trku (%)**							
	Popolnoma cel	Deloma odlomljen	Cca. četrt odlomilo	Dve polovici	Trije deli	Štirje deli	Raztreščil	Ni podatka
Sveži orehi (sejanci)	63,71	12,9	8,87	6,45	2,42	3,23	0	2,42
Prisilno sušeni počeni orehi (sejanci)	62,31	10	3,08	11,54	6,92	3,85	1,54	0,77
Prisilno sušeni celi orehi (sejanci)	26,47	19,61	6,86	12,75	4,9	19,61	9,8	0
Naravno sušeni počeni orehi (sejanci)	36,84	9,77	3,01	18,8	11,28	9,77	8,27	2,26
Naravno sušeni celi orehi (sortni)	52	6	1	17	9	6	9	0
Naravno sušeni celi orehi (sejanci)	53,33	12,38	4,76	10,48	9,52	0,95	8,57	0
Srednje vrednosti	49,11	11,78	4,60	12,84	7,34	7,24	6,2	0,91
Vsota stanja jedrc oz. orehov po trku	*344*	*81*	*32*	*89*	*51*	*49*	*41*	*7*

Največji delež nepoškodovanih jedrc (63,71 %) so po trku imeli sveži orehi, najmanjšega pa prisilno sušeni celi sejanci (26,47 %). Največ nepoškodovanih polovičk so imeli naravno sušeni počeni sejanci (18,8 %), najmanj pa sveži sejanci (6,45 %). Prisilno sušeni celi orehi so imeli največ zdrobljenih četrtin jedrc (19,61 %), najmanj pa naravno sušeni celi sejanci (0,95 %). Delež zdrobljenih tretjin jedrca je bil največji pri naravno sušenih počenih sejancih (11,28 %), za njimi pa so bili naravno sušeni celi sejanci (9,52 %). Največ raztreščenih jedrc smo dobili pri prisilno sušenih celih sejancih (9,8 %), sledijo naravno sušeni celi sortni orehi (9 %). Delež raztreščenih jedrc je bil najmanjši pri prisilno sušenih počenih orehih (1,54 %), pri svežih sejancih pa teh jedrc sploh nismo dobili.

Po trku vseh orehov različnih skupin v drobilno steno smo v povprečju dobili skoraj polovico nepoškodovanih celih jedrc. 546 jedrc in polovičk (78,33 %) smo uvrstili v najvišji kakovostni razred

(kategorije ena, dva, tri in štiri). Sto jedrc (14,58 %) je bilo srednje kakovosti (peta in šesta kategorija stanja jedrc), 41 jedrc (6,2 %) pa se je pri trku raztreščilo ali pa smo jih pri izluščevanju preveč poškodovali (kategorija sedem). Te smo uvrstili v zadnji kakovostni razred. Pri sedmih orehih (0,91 %) podatkov o deformaciji jedrc nismo uspeli pridobiti (osma kategorija deformacij jedrc).

3.4 Rezultati težavnosti izluščitve jedrc

Težavnost izluščitve jedrc po trku orehov v drobilno steno smo razdelili na tri osnovne stopnje (skupine), četrta pa zajema šest orehov prevelikih dimenzij ter en oreh brez jedrca.

Če nam je jedrce od lupine uspelo ločiti brez uporabe pripomočkov in sile, smo ga uvrstili v prvo skupino (»Lahko se izlušči«). Sem smo dodali še orehe, pri katerih se je zaradi sile trka jedrce od lupine ločilo samostojno (brez naše pomoči), oziroma sta se lupina in jedrce raztreščila. Za drugo skupino (»Srednje težko se izlušči«) velja, da smo za izluščevanje jedrc uporabili večjo moč ali pa smo si morali pomagati z nožičem. V tretji skupini (»Težko se izlušči«) pa je bila ločitev jedrca od lupine težavna. Za izluščitev smo morali uporabiti kar precej moči oziroma smo si pomagali z več predmeti hkrati, tudi s kladivom.

Jedrca smo najlažje izluščili pri prisilno sušenih celih sejancih (85,29 %), naravno sušenih celih sejancih (82,86 %) in prisilno sušenih počenih sejancih (82,31 %). Z nožičem smo si največkrat pomagali pri izluščevanju jedrc svežih sejancev (16,13 %), naravno sušenih sortnih orehih (15 %) in naravno sušenih počenih sejancev (14,29 %). Največkrat je bila najbolj težavna izluščitev pri naravno sušenih počenih sejancih (6,76 %), svežih sejancih (6,45 %) in naravno sušenih sortnih orehih (6 %). Pri svežih in naravno sušenih počenih sejancih smo brez težav izluščili najmanjši delež jedrc (75 % oziroma 79 %).

Preglednica 5: Izluščitve jedrc po skupinah orehov v odstotkih ter srednje vrednosti glede na stopnjo izluščitve. V oklepaju je navedeno število analiziranih orehov.

Skupine orehov	**Stopnja izluščitve jedrc**			
	Lahko se izlušči	Srednje težko se izlušči	Težko se izlušči	Brez jedrca oz. Ni podatka
Sveži orehi (sejanci)	75 (93)	16,13 (20)	6,45 (8)	2,42 (3)
Prisilno sušeni počeni orehi (sejanci)	82,31 (107)	12,3 (16)	4,62 (6)	0,77 (1)
Prisilno sušeni celi orehi (sejanci)	85,29 (87)	11,76 (12)	2,95 (3)	0 (0)
Naravno sušeni počeni orehi (sejanci)	76,69 (102)	14,29 (19)	6,76 (9)	2,26 (3)
Naravno sušeni celi orehi (sortni)	79 (79)	15 (15)	6 (6)	0 (0)
Naravno sušeni celi orehi (sejanci)	82,86 (87)	12,38 (13)	4,76 (5)	0 (0)
Srednja vrednost (povprečje)	80,19 (555)	13,64 (95)	5,26 (37)	0,91 (7)

3.5 Skupna kakovost drobljenja orehov

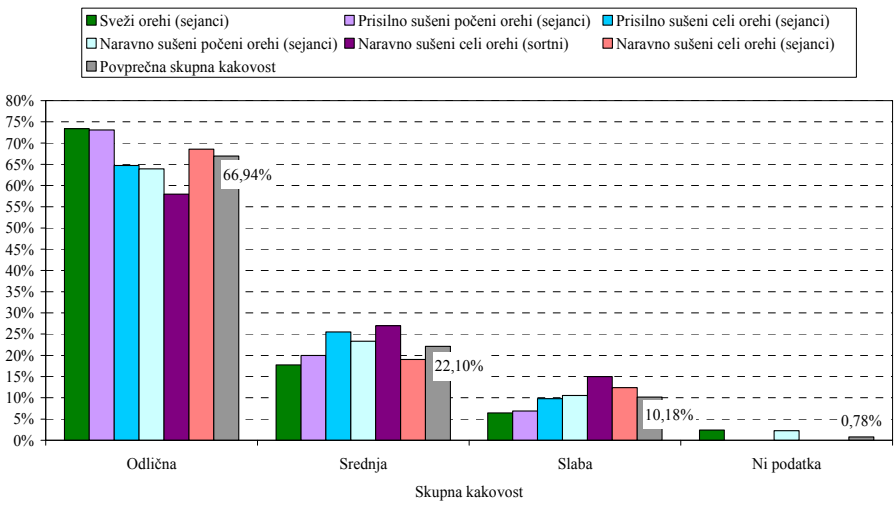
Pri določevanju skupne kakovosti smo upoštevali dva parametra. Večino ocene je prispevala težavnost izluščevanja jedrc po trku orehov. Predvsem zato, ker naj bi z drobilnikom izluščevanje potekalo hitro, brez uporabe pripomočkov ter z minimalno deformacijo jedrca. Za drugi del ocene smo upoštevali deformacijo (stanje) jedrca po trku oziroma po sami izluščitvi, saj smo sami nekajkrat jedrce med postopkom izluščitve poškodovali. Zato je skupna kakovost v primerjavi z deformacijo jedrc po trku orehov malenkost slabša.

Pri svežih in naravno sušenih počenih sejancih trem vzorcem skupne kakovosti nismo uspeli ovrednotiti, zato so na spodnjem grafikonu in v tabeli označeni z »Ni podatka«.

Odlično skupno kakovost smo ovrednotili pri 467 orehih, srednjo pri 152 orehih, s slabo skupno kakovostjo pa smo ocenili 69 orehov. Šest orehov je brez podatka o končni kakovosti. Izmerjeni vzorec je 694 orehov.

Preglednica 6: Skupna kakovost znotraj posameznih kategorij izražena v odstotkih (%) in povprečje vseh skupin orehov glede na kategorijo kakovosti.

Skupine orehov	Skupna kakovost			
	Odlična	Srednja	Slaba	Ni podatka
Sveži orehi (sejanci)	73,39	17,74	6,45	2,42
Prisilno sušeni počeni orehi (sejanci)	73,08	20	6,92	0
Prisilno sušeni celi orehi (sejanci)	64,71	25,49	9,8	0
Naravno sušeni počeni orehi (sejanci)	63,91	23,31	10,53	2,26
Naravno sušeni celi orehi (sortni)	58	27	15	0
Naravno sušeni celi orehi (sejanci)	68,57	19,05	12,38	0
Povprečna skupna kakovost	66,94	22,10	10,18	0,78



Slika 15: Skupna kakovost po skupinah orehov in srednje vrednosti teh po posameznih kategorijah kakovosti v odstotkih vseh izmerjenih 694 orehov.

4 ZAKLJUČKI IN RAZPRAVA

Cilji pri drobljenju orehov s centrifugalnim drobilnikom so minimalne poškodbe jedrc, deformacija lupine pa tako močna, da jedrce lahko izluščimo z rokami oziroma brez dodatnih pripomočkov, kot sta kladivo in nožič. To smo skušali doseči z nastavitvijo prave vrtilne frekvence izmetala za določeno skupino orehov. Za zdrobitev lupine so največjo vrtilno frekvenco (16,9 in 15,5 št. obr./sek.) potrebovali sveži sejanci, ki so bili tudi najtežji (12,3 g) in naravno sušeni sortni orehi s povprečno maso 9,7 g.

Najpogostejša deformacija pri trku orehov je razpolovitev lupine po šivu in velja za vse skupine testiranih orehov. V povprečju se je pojavila v 36, 8 odstotkih vseh orehov.

Najlažje se izluščijo jedrca prisilno sušenih celih sejancev (85,29 %), najtežje pa jedrca naravno sušenih počenih sejancev (6,76 %).

Po klasifikaciji USDA so najvišjo kakovost jedrc po trku (91,93 %) imeli sveži orehi, najslabšo (raztreščena jedrca) pa prisilno sušeni celi sejanci (9,8 %).

Na osnovi podatkov o stopnji (težavnosti) izluščitve in kakovosti jedrc takoj po trku ter po izluščitvi smo določili skupno kakovost. Največji delež orehov odlične kakovosti so imeli sveži (73,39 %) in prisilno sušeni počeni sejanci (73,08 %). Ti so najprimernejši za drobljenje s centrifugalnim drobilnikom. Poleg teh dveh skupin

se za drobljenje priporočajo še naravno sušeni celi sejanci z 68,57 % orehov odlične skupne kakovosti, 19,05 % srednje in 12,38 % slabe končne kakovosti.

S centrifugalnim drobilnikom ni priporočljivo drobiti različnih naravno sušenih celih sortnih orehov, saj so dali najmanjši delež orehov odlične (57 %) skupne kakovosti in največja deleža orehov srednje (27 %) in slabe (15 %) skupne kakovosti.

Iz rezultatov lahko sklepamo, da večja vsebnost vode v svežih jedrcih vpliva na manjše poškodbe le-teh in večjo frekvenco, s katero tak oreh zdrobimo. Nepoškodovani celi orehi za uspešno deformacijo lupine potrebujejo tudi večjo vrtilno frekvenco od počenih.

Rezultati nam pokažejo še, da tudi masa orehov vpliva na vrtilno frekvenco. V povprečju so nepoškodovani (celi) težji orehi za zdrobitev lupine potrebovali večjo vrt. frekv. od lažjih orehov. Izjema so prisilno sušeni celi sejanci, ki so kljub nekoliko manjši povprečni masi (za 0,3 g), za deformacijo lupine potrebovali večjo (za 0,4 št. obr. / s.) povprečno vrtilno frekvenco od naravno sušenih celih sejancev.

Naravno in prisilno sušeni celi sejanci kljub manjši povprečni masi od naravno in prisilno sušenih počenih sejancev, za deformacijo lupine potrebujejo večjo vrtilno frekvenco.

Na podlagi preizkusov drobljenja plodov oreha lahko sklepamo, da so za drobljenje s centrifugalnim drobilnikom najprimernejši sveži in že pred sušenjem z zrakom počeni sejanci. Zato za nadaljnje raziskave drobljenja s centrifugalnim drobilnikom priporočamo, da se pri teh dveh poveča skupna količina vzorca orehov za vsako skupino.

Članek je sestavljen iz raziskav konstruiranja Kmetijskih strojev in naprav na Fakulteti za strojništvo, kjer se je stroj tudi konstruiral in izdelal. Iz omenjenih vsebin in materiala je nastalo tudi diplomsko delo za visokošolski strokovni študij: Analiza delovanja stroja za luščenje plodov oreha (*Juglans regia L.)*, avtor Gašper Kastelec.

5 VIRI

Alibaba. 2014. Round hole perforated metal screen http://www.alibaba.com/product-gs/518174532/Round_Hole_Perforated_Metal_Screen.html (14.1.2014)

Bernik R. 2002. Stroj za drobljenje plodov oreha: patent št. SI 20617. Ljubljana: Urad Republike Slovenije za intelektualno lastnino.

Bernik R., Solar A., Skok D. 2004. Fizikalne lastnosti ploda oreha. Acta agriculturae slovenica, 83-1.

California walnuts. 2014. Shelled walnut sizes http://www.californiawalnuts.eu/314/Walnuts/Product-Types/Shelled-Walnut-Sizes.htm (12.1.2014)

Charlot G., Prunet J.P., Lagrue C., Aleta N. 1996. Noix et cernaux: Qualite et consomaiton. C.T.I.F.L., Paris: 166 s

Ducousso I., Sabatier S., Barthélémy D., Germain E. 1995. Comparaison de quelques caracterristiques morphologiques des pousses annuelles et des branches de la cime de sept varietes de Noyer commun, *Juglans regia* L. (*Junglandaceae*). V: Les coloques, No. 74. Colloque Architecture des arbres fruitieres. Montpellier, France, 23-25 Nov. 1995: 91-108.

Evropski standardi trženja orehov v luščini. Uradni list L 26, 27/01/2001, 24-30 http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32001R0175:SL:HTML (13.1.2014)

FAO. 2003. Inventory of Walnut Research, Germplasm and References. ID: 170653. http://www.fao.org/docrep/007/y5704e/y5704e03.htm (12.1.2014)

Germai E. 1990. Inheritance of late leafing and lateral bud fruitfulness in walnut (*Juglans regia* L.), phenotypic correlations among some traits of the trees. Acta Horticulturae, 284: 125-136.

Košmelj K. 2001. Uporabna statistika. Ljubljana, Biotehniška fakulteta: 249 str.

Ocepek R. 1995. Oreh: pridelovanje in uporaba. Ljubljana, Svet knjige: 3-10: 71-74

Solar A. 2000. Izbor morfometrijskih in polmoloških kazalnikov za žlahtnjenje oreha (*Juglans regia* L.). Doktorska disertacija. Ljubljana, Biotehniška fakulteta, Oddelek za agronomijo: 156 str.

Solar A. 2004. Lupinarji. V: Ambrožič Turk, B. (ur.), Introdukcija in selekcija sadnih rastlin v letu 2003 (Prikazi in informacije, 239). Ljubljana, Kmetijski inštitut Slovenije

Skok D. 2003 Fizikalne lastnosti plodov oreha (*Juglans regia* L.). Diplomsko delo. Ljubljana, Biotehniška fakulteta, Oddelek za agronomijo: 92 str.

USDA. 1968. United States Standards for Grades of Shelled Walnuts (*Juglans regia*). 4-5 http://www.dfaofca.com/Downloadables/Tree%20Nut/walnuts.pdf (12.1. 2014)

Wikipedia. 2013. *Juglans regia*. Original habitat, Introduction around the world. http://en.wikipedia.org/wiki/Juglans_regia (12.1.2014)

Acta agriculturae Slovenica
Volume / Letnik **105** · Number / Številka **1** · **2015**

VSEBINA / CONTENTS

COBISS Code 1.03

DOI: 10.14720/aas.2015.105.1.16
Agrovoc descriptors: biological properties, biological differences, biodiversity, data analysis, data processing, measurement, apples, tomatoes, statistical methods, mathematics

Agris category code: u10

Green mathematics: Benefits of including biological variation in your data analysis

TIJSKENS L.M.M.[1], SCHOUTEN R.E.[1], UNUK T.[2], SIMČIČ M.[2]

Received September 12, 2014; accepted February 27, 2015.
Delo je prispelo 12. septembra 2014, sprejeto 27. februarja 2015.

ABSTRACT

Biological variation is omnipresent in nature. It contains useful information that is neglected by the usually applied statistical procedures. To extract this information special procedures have to be applied. Biological variation is seen in properties (e.g. size, colour, firmness), but the underlying issue is almost always to the variation in development or maturity in a batch of individuals generated by small scale environmental differences.

The principles of assessing biological variation in batches of individuals are explained without putting emphasis on mathematical details. Obtained explained parts increase from about 60 to 80 % for the usual approach to 95 when the biological variation is taken into account. When technical variation or measuring error is small even 99 % can be achieved. The benefit of the presented technology is highlighted based on a number of already published studies covering the colour of apples during growth and storage and the firmness of cut tomatoes during storage.

Key words: biological variation, biological shift factor, mixed effects nonlinear regression, indexed nonlinear regression, colour of apples, firmness of tomatoes

IZVLEČEK

ZELENA MATEMATIKA: KORISTI OD VKLJUČEVANJA BIOLOŠKE SPREMENLJIVOSTI V ANALIZO PODATKOV

Biološka spremenljivost je prisotna povsod v naravi. Vsebuje koristno informacijo, ki je navadno prezrta v navadno uporabljenih statističnih postopkih. Če hočemo izluščiti to informacijo je potrebno uporabiti posebne postopke. Biološko spremenljivost lahko opazujemo v lastnostih kot so velikost, barva, čvrstost itd., kjer je osnova zanjo skoraj vedno razlika v razvoju ali zrelosti vzorca analiziranih primerkov, ki jo povzročajo majhne notranje in okoljske razlike. Osnove ugotavljanja biološke spremenljivosti v naboru analiziranih primerkov so razložene brez poudarjanja matematičnih podrobnostih. Pojasnjen delež spremenljivosti je pri običajenm postopku med 60 in 80 %. Če upoštevamo biološko spremenljivost, se poveča na 95 %. V primeru, da odpravimo še napake meritve, lahko pojasnjen delež spremenljivosti povečamo na 99 %. Takšen način obdelave je dal zelo dobre rezultate v že objavljenih raziskavah razvoja barve jabolk med rastjo in hrambo ter pri meritvah čvrstosti paradižnika med shranjevanjem.

Ključne besede: biološka spremenljivost, biološki šift faktor, mešani učinek nelinearne regresije, indeksirana nelinearna regresija, barva jabolk, čvrstost paradižnika

1 INTRODUCTION

Variation is everywhere, in humans, in animals, in plants, in DNA, in climate, in weather and therefore also in measured experimental data. Nature is very generous in providing variation, but

[1] Horticulture and Product Physiology, Wageningen University, NL; e-mail: Pol.Tijskens@wur.nl

[2] Faculty of Agriculture and Life Science, University of Maribor, SI

[3] Biotechnical Faculty, University Ljubljana, SI

amazingly, in a way nature is also very lazy. It uses the same process mechanisms over and over again but in endless combinations. The presence of all that variation from different sources poses a severe problem on the modelling of processes and on analysing experimental data properly.

In day-to-day horticultural practise, products are sorted and graded to remove the variation in batches. This sorting is very effective, but is only applied to external properties like colour, size and defects. Internal quality attributes like content of sugars, acids, dry matter, Brix, etc., constituting the eating quality, are hardly affected by that kind of grading.

All statistical procedures are built in such a way that the effect of variation is minimal. Mostly this is achieved by making the samples as uniform as possible (sorting) and by using mean values in one way or another.

Variation, however, contains useful information, not only with respect to differences between individuals but also with respect to the real mechanisms in action. Removal of variation, either by sorting or by statistical procedures, also removes the information contained in that variation, and prevents effectively the study and understanding of the dynamics of variation.

What we need is green mathematics, i.e. sustainable mathematics that takes this variation in account, to be used in plant biology and horticulture. But as Kermit the frog of Sesame Street put it: it is not that easy to be green (YouTube). To assess biological variation in experimental data, special lines of thinking have to be used, and data have to be analysed applying special statistical procedures like mixed effects and indexed non-linear regression. In this paper, the reasoning behind the technique (Tijskens et al. 2003, 2005), developed over the last couple of decades (see references) will be highlighted, however, without too much emphasis on equations. The technique will be illustrated based on some examples of skin colour of apples and tomatoes in storage and during growth.

2 VARIATION IN PROPERTIES AND DATA

2.1 Origin of variation

Just like product quality, biological variation is generated exclusively during growth. During the subsequent post-harvest storage, one can only try to minimise the further development of variation and to prevent some of its detrimental effects.

Once formed, plants, trees, organs like leaves and fruit are quite localised. They can't move from one place to another. That means that the small differences (not every day the same but always in the same order / direction) in e.g. micro climate, fertilisation, location in the canopy and soil type etc., accumulate over the entire growth period resulting in a sometimes considerable variation in properties of individuals.

What constitutes an individual depends on the focus of the study or application: fruit, plants, cells, organelles, harvest flights, fruit bins, pallets, containers etc. Scientific studies will (probably) focus on the smaller items (fruit, organs, cells), while commercial application will focus more on the larger items (harvest flights, fruit bins, pallets, containers). The principle however is always the same: determine the biological shift factor.

2.2 How to deal with variation

These sources of variation in growing conditions basically translate in variation in the stage of development of e.g. fruit. In Figure 1, an example is shown for a sigmoidal behaviour, frequently found and applied for colour development. The individual lines represent the individual fruit; the arrows indicate how much a line (individual fruit) has to be shifted in time to fall over the same (central) curve. That shift in time is called the biological shift factor (Tijskens et al. 2005). Irrespective the stage of development (how high or low on the y-axis) that shift factor is the same for each individual fruit: the red arrows are of equal length. The same is true for the green arrows for another individual. In short, the biological shift factor is a property of an individual not to be

attributed to sampling time. The biological shift factors of all the individuals in a batch will show a normal distribution (histogram) invariable in time, independent of the stage of maturity.

So, dealing with variation basically boils down to the estimation of the biological shift of all individuals in a batch. That can only be done, when data are collected repeatedly for the same individual (non-destructive testing). These are so-called longitudinal data: each individual is monitored in time for one or more properties like colour, firmness, size and number of cells in a leaf, size and number of stomata in a leaf, photosynthetic activity in leafs etc.

Applying an appropriate model for the observed behaviour, and using fruit identification, the biological shift factor can be estimated by indexed or mixed effects nonlinear regression for each individual (random effects) along with the other model parameters are estimated in common (fixed effects) for all the individuals (Schouten et al. 2002, 2004, 2009, Hertog et al.2002, 2004, 2007, Tijskens et al. 2003, 2005, 2006, 2008, 2009, 2010, Unuk et al. 2012).

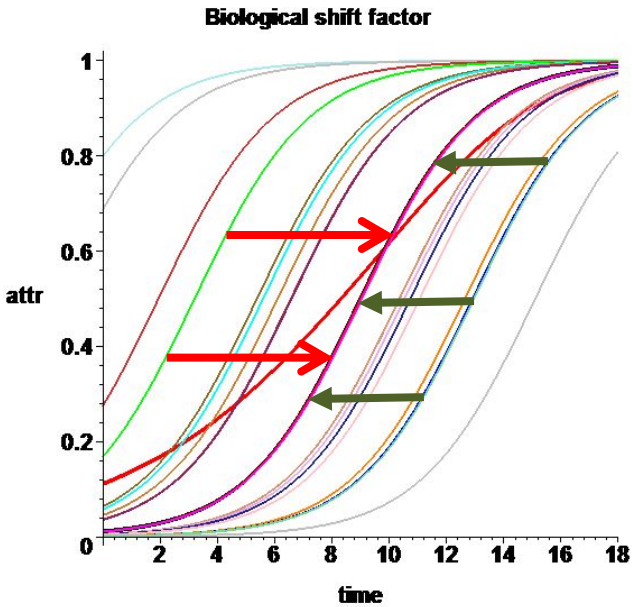


Figure 1: Behaviour of a normalised attribute of several individuals in a batch according a logistic mechanism (e.g. colour). Red and green arrows indicate the shift per individual to fall over the same generic line. The crossing line in the middle is obtained by analysing not the individuals but the mean values per sampling time when the biological variation is not included in the analysis

2.3 Benefits of including biological variation in data analysis

The overall benefit of including biological variation in data analysis is a better understanding of its behaviour, its dynamics and the rules that govern these. The major result of all these dedicated studies, crossing the borders of all disciplines, is that the occurrence, magnitude and behaviour of natural variation are as deterministic as all chemical reactions and reaction mechanisms. Understanding of the mechanisms and dynamics of variation will eventually result in a better prediction of quality and maturity and in ways to deal with variation without or additional to the traditional sorting and grading. Using the statistical procedure of non-linear regression, without explicitly taking care of the variation, explained parts (R^2_{adj}) of 60 to 80 % can be obtained. The unexplained part is a mixture of the technical or measuring error and the biological variation not taken care of. When properly taking care of the variation by including the biological shift factor in the analysis, explained parts can reach as high as 95 to 99%. The unexplained part is now purely the technical or measuring error.

3 EXAMPLES FROM PRACTICE

3.1 Colour in the orchard

Details of this study can be found in Unuk et al. (2012). In 2009, three apple (*Malus domestica* Borkh.) cultivars ('Braeburn', 'Fuji', 'Gala') were grown in an orchard near Maribor (SI). Colour of individual apples was measured with a Minolta colour meter. The apples were selected at three locations in the canopy: shady, partially sunny and sunny. The raw (unprocessed) data for 'Braeburn' are shown in Figure 2. The effect of location (how much sunlight do these apples get) can clearly be observed in the stage of development. Moreover, the difference in and magnitude of the distribution in colour at different moments in time is indicated by the red ellipses.

Applying indexed nonlinear regression one arrives at a generic development curve with numerical information on time axis (biological time). All locations in canopy follow the same curve, with explained parts well above 95% (Figure 3).

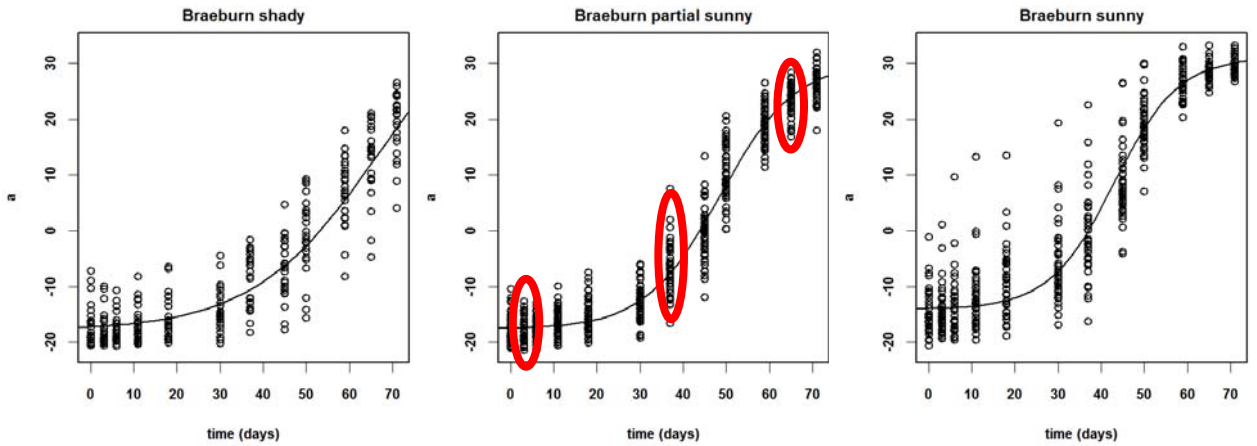


Figure 2: Behaviour of colour a* of 'Braeburn' apples at three locations in the canopy. Clearly the effect of location can be seen in the stage of development (where on the generic sigmoidal curve are the data located): left shady early, partial sunny (middle) and sunny (right) are already more developed.

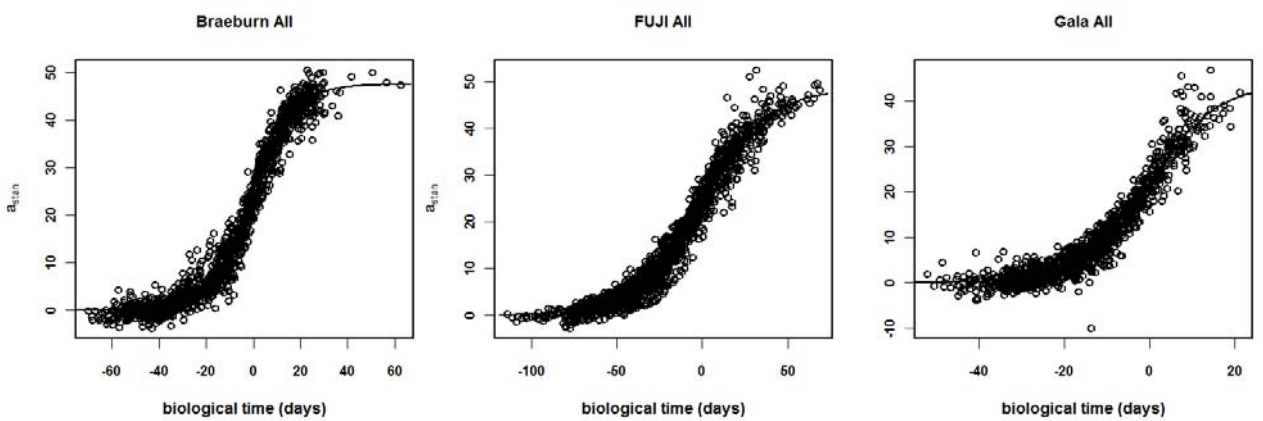


Figure 3: Generic behaviour of colour a* of 'Braeburn', 'Fuji' and 'Gala' apples from the three locations in the canopy versus biological time (calendar time + individual biological shift factor).

3.2 Managing the orchard

Details of this study can be found in Tijskens et al. (2009). 'Golden Delicious' apples were grown near Maribor in 2001 and 2002. Colour of individual apples was measured with a Minolta colour meter. Different levels of crop load and fertilisation were applied. In Figure 4, the raw data are shown along with the estimated behaviour per individual. Again the effect of applied conditions can be seen in the stage of development: The less crop load, the more developed. The effect of fertilisation is less clear (too small number of levels). When analysing the data including biological, all effects of crop load and fertilisation could be attributed to the biological variation. All individuals followed the same generic pattern (Figure 5). Striking is the now very clear difference in behaviour between the two season, both in range of change as in rate of change.

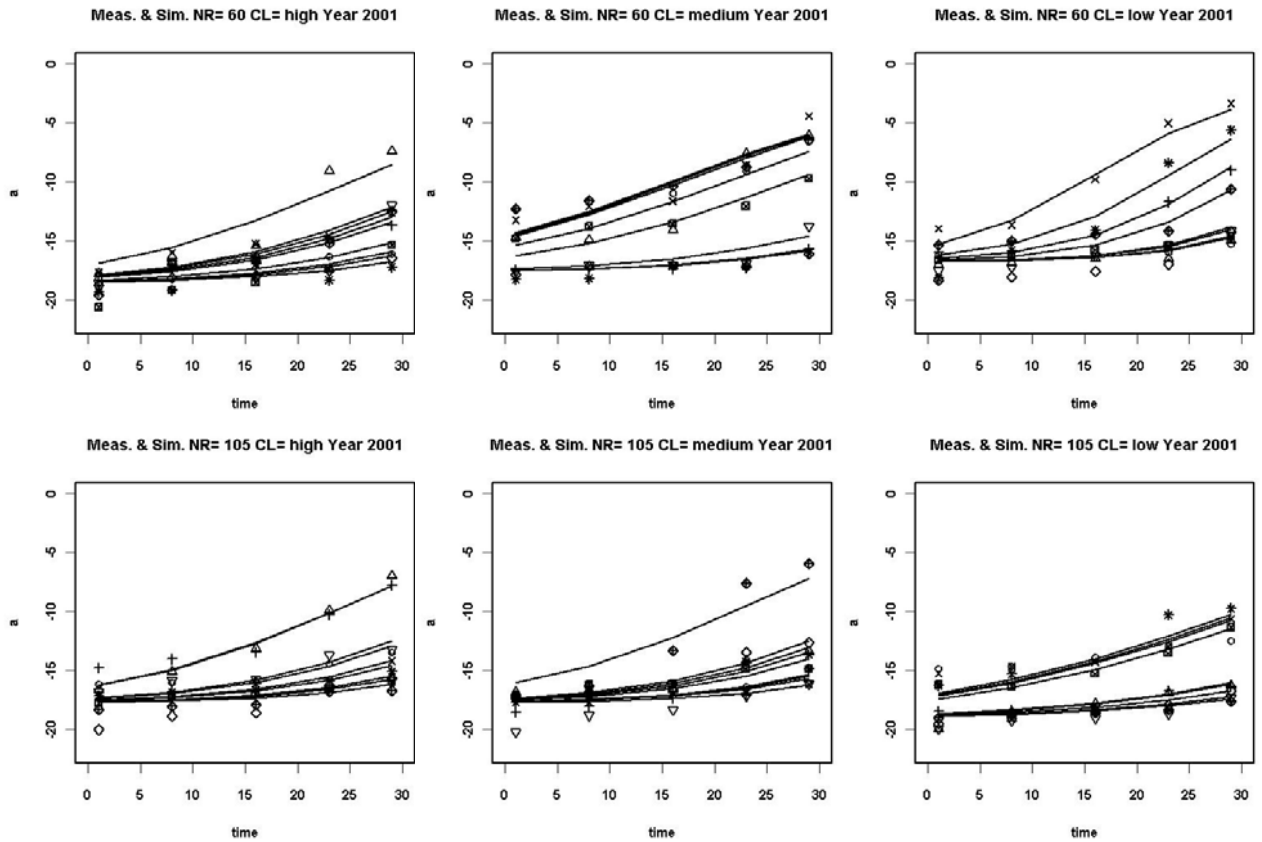


Figure 4: Behaviour of colour a* of individual Golden delicious apples at decreasing levels of crop load (CL: left to right, and increasing levels of fertilisation (NR: top to bottom). Full lines behaviour per individual, estimated using all data combined.

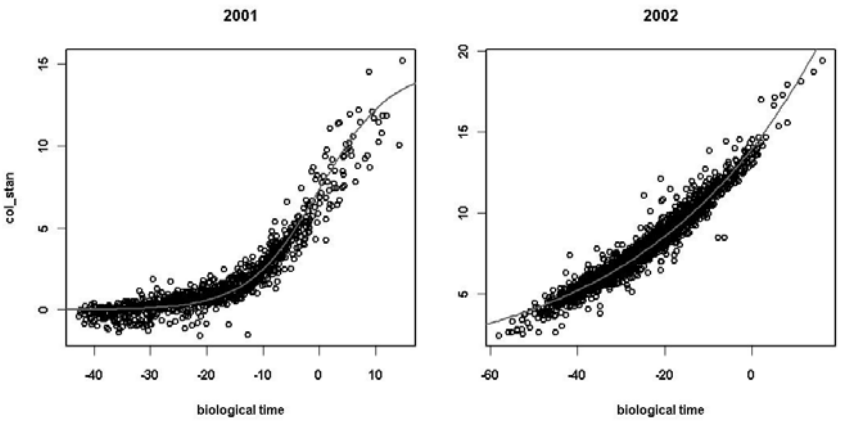


Figure 5: Standardised behaviour of colour a* of 'Golden delicious' apples in two seasons including the effects of crop load and fertilisation. The colour values are 'standardised' (col_stan) to get rid of the different values for the asymptotes at plus and minus infinite time.

3.3 'Granny Smith' in storage

Details of this study can be found in Tijskens et al. (2008). 'Granny Smith' apples were harvested at three orchards in south Slovenia in the season 1997 and stored at three temperatures. The colour of individual fruit was monitored with a Minolta colour meter. In Figure 6, the raw data are shown. The behaviour reflects the lower part of the normally observed sigmoidal behaviour (see e.g., Figure 3). Some clear 'outliers' in rate of change are indicated by the red arrows.

Analysing the data, taking care of the stage of maturity (biological shift factor) and the differences in lower colour values of the individual apples of all three orchards stored at all three temperatures, an explained part of 97 % was obtained. All 'outliers' complied with the model formulation. The generic pattern of development is shown in Figure 7 for the three temperatures separately. A small effect of a chilling injury process can be observed in the rate constants: at 1°C (Figure 7 left) the rate of colour increase is slightly larger than at 10°C (Figure 7 right).

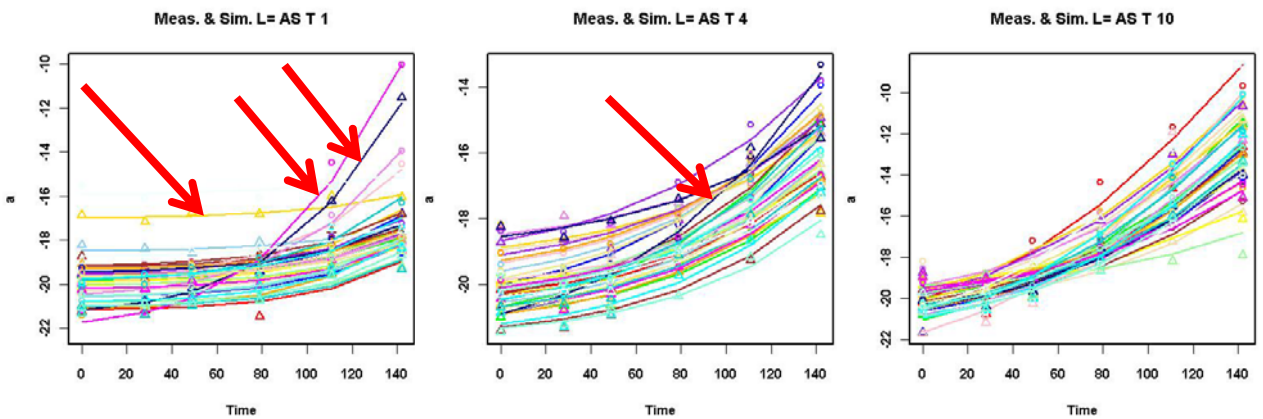


Figure 6: The unprocessed data of apples from the orchard at Arnovo Selo as an example. Individuals that would normally be considered as 'outliers' are indicated by the red arrows.

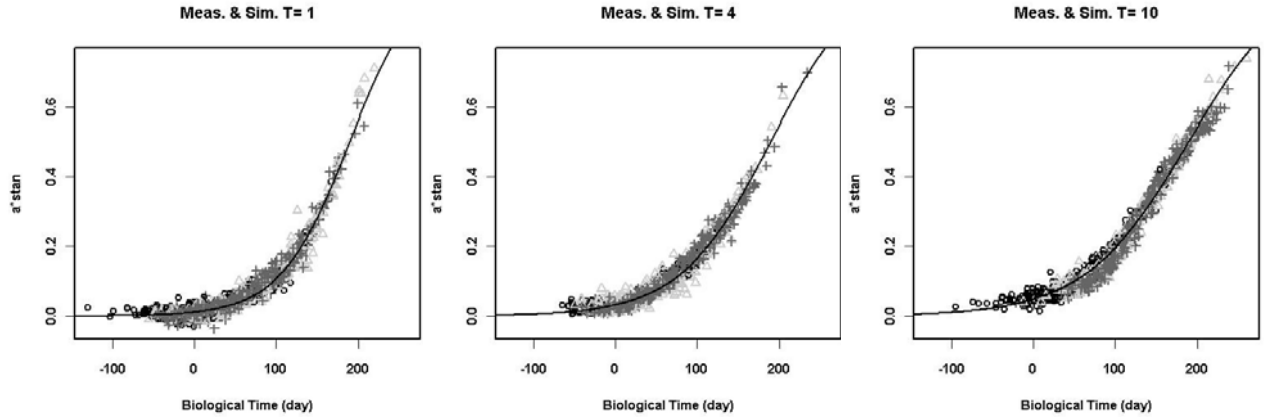


Figure 7: The standardised data of 'Granny Smith' apples all orchards combined for the three temperatures. All 'outliers' comply with the same model (follow the same mechanism), but the value of their biological shift factor is outside the 'normal'.

3.4 Cut Tomatoes

Details of this study can be found in Lana et al. (2005). Tomatoes were harvested at 3 stage of colour development (breaker, pink and red). That actually reflects a grading of the fruit. Tomatoes were sliced and stored at 5 temperatures. Firmness was measured non-destructively by limited compression. The data of all slices were analysed using the same exponential model, and the biological shift factor for the three different stages of maturity was estimated. Clearly, firmness of cut tomatoes decreases according to the same exponential model with the same value of the reaction rate constant, irrespective of the stage of maturity at harvest (Figure 8).

That delivers a direct link between postharvest behaviour and growth of tomatoes.

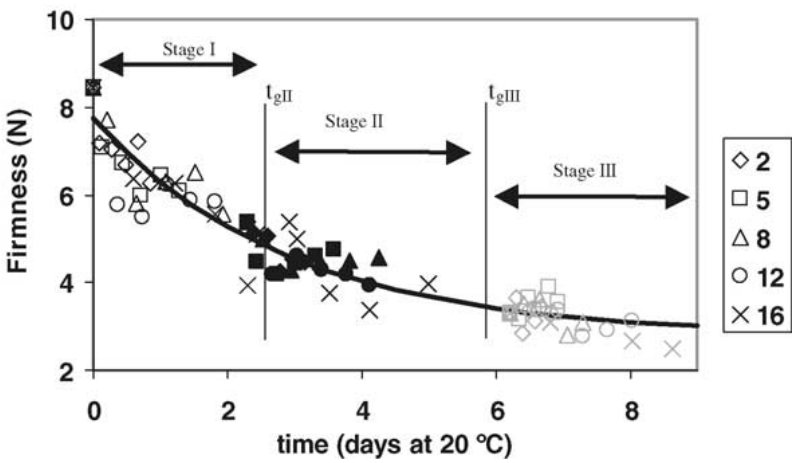


Figure 8: Decrease in firmness of cut tomatoes (outer pericarp) as a function of storage time for the three stages of maturity at harvest. The symbols represent the mean of five replicates at the indicated temperatures. The time on the x-axis has been transformed to a standard temperature of 20 ◦C.

4 CONCLUSION

Biological variation is everywhere. In product properties as well as in experimental data on these properties. Applying mixed effects or indexed nonlinear regression based on fruit identification, the biological shift factor of individuals can be estimated, generating more reliable analyses and a

better understanding of the dynamics and behaviour of that variation.

Biological variation is not random at all, but complies with strict deterministic rules.

The technology is not restricted to fruit in horticulture, but can easily be expanded to cover all kind of areas in agriculture and botany on almost every conceivable property.

5 REFERENCES

Hertog MLATM. 2002. The impact of biological variation on postharvest population dynamics. *Postharvest Biology and Technology* 26:253–63; DOI: 10.1016/S0925-5214(02)00044-3

Hertog MLATM, Lammertyn J, Desmet M, Scheerlinck N, Nicolaï BM. 2004. The impact of biological variation on postharvest behaviour of tomato fruit. Postharvest Biology and Technology 34:271–84; DOI: 10.1016/j.postharvbio.2004.05.014

Hertog, M.L.A.T.M., Lammertyn, J., De Ketelaere, B., Scheerlinck, N., Nicolaï, B.M. 2007. Managing quality variance in the postharvest food chain. *Trends in Food Science and Technology* 18, 320-332; DOI: 10.1016/j.tifs.2007.02.007

Lammertyn J, De Ketelaere B, Marquenie D, Molenberghs G, Nicolaï BM. 2003. Mixed models for multicategorical repeated response: modelling the time effect of physical treatments on strawberry sepal quality. *Postharvest Biology and Technology* 30:195-207; DOI: 10.1016/S0925-5214(03)00100-5

Lana M.M., Tijskens L.M.M., van Kooten O. (2005): Effects of storage temperature and fruit ripening on firmness of fresh cut tomatoes. Postharvest Biology and Technology 35, 87–95; DOI: 10.1016/j.postharvbio.2004.07.001

Schouten RE, Tijskens LMM, van Kooten O. 2002. Predicting keeping quality of batches of cucumber fruits based on a physiological mechanism. *Postharvest Biology and Technology* 26:209–20; DOI: 10.1016/S0925-5214(02)00017-0

Schouten RE, Jongbloed G, Tijskens LMM, van Kooten O. 2004. Batch variability and cultivar keeping quality of cucumber. *Postharvest Biology and Technology* 32:299-310; DOI: 10.1016/j.postharvbio.2003.12.005

Schouten RE, Zhang X, Verschoor JA, Otma EC, Tijskens LMM, van Kooten O. 2009: Development of colour of Broccoli heads as affected by controlled atmosphere storage and temperature. *Postharvest Biology and Technology* 51:27–35; DOI: 10.1016/j.postharvbio.2008.06.005

Tijskens LMM, Wilkinson EC. 1996. Behaviour of biological variability in batches during post-harvest storage. *AAB Modelling Conference: Modelling in Applied Biology - Spatial Aspects*. p. 267-8, 24-27 June 1996, Uxbridge, UK

Tijskens LMM, Konopacki P, Simčič M. 2003. Biological Variance, Burden or Benefit? *Postharvest Biology and Technology* 27:15-25; DOI: 10.1016/S0925-5214(02)00191-6

Tijskens LMM, Heuvelink E, Schouten RE, Lana MM, van Kooten O. 2005. The biological shift factor. Biological age as a tool for modelling in pre- and postharvest horticulture. *Acta Horticulturae* 687:39-46

Tijskens LMM, Herold B, Zude M, Schlüter O, Geyer M. 2006. Biological variation in the apple orchard. In: *Proceedings COST 924 workshop* (ed. J. Ovesná, V. Pouchová, K. Mitrová) *From plant to Human genes*, p27-34, November 2006, Prague, CZ. ISBN 80-86555-99-2.

Tijskens LMM, Konopacki PJ, Schouten RE, Hribar J, Simčič M. 2008. Biological variance in the colour of Granny Smith apples. Modelling the effect of senescence and chilling injury. *Postharvest Biology and Technology* 50:153–63; DOI: 10.1016/j.postharvbio.2008.05.008

Tijskens LMM, Dos-Santos N, Jowkar MM, Obando J, Moreno E, Schouten RE, Monforte AJ, Fernández Trujillo JP. 2009. Postharvest firmness behaviour of near-isogenic lines of melon. *Postharvest Biology and Technology* 51:320-6; DOI: 10.1016/j.postharvbio.2008.06.001

Tijskens L.M.M., Jacob S., Schouten R.E., Fernández-Trujillo J.P., Dos-Santos N., Vangdal E., Pagán E., Pérez Pastor A. 2010. Water loss in horticultural products. Modelling, data analysis and theoretical considerations. Acta Horticulturae 858, 465-471.

Unuk T., Tijskens L.M.M., Germšek B., Zadravec P., Vogrin A., Hribar J., Simčič M., Tojnko S. 2012, Effect of location in the canopy on the colour development of three apple cultivars during growth. *Journal of the Science of Food and Agriculture*. **92**:2450–2458; DOI: 10.1002/jsfa.5651

YouTube Kermit the frog: https://www.youtube.com/watch?v=hpiIWMWWVco&feature=player_detailpage&list=RDhpiIWMWWVco

CONTENT ANALYSIS OF THE PAPERS IN THE ACTA AGRICULTURAE SLOVENICA

VSEBINSKA OBDELAVA PRISPEVKOV V ACTA AGRICULTURAE SLOVENICA let. 105 št. 1

Tomaž BARTOL[a], Karmen STOPAR[b],

SUBJECT INDEX BY AGROVOC DESCRIPTORS
PREDMETNO KAZALO PO DESKRIPTORJIH AGROVOC

a, b: Ph. D., M. Sc.., B. Sc., Jamnikarjeva 101, SI-1000 Ljubljana, P. O. Box 95

VSEBINSKO KAZALO PO SKUPINAH ZNANJA (PREDMETNIH KATEGORIJAH)

NAVODILA AVTORJEM

(letniki z liho številko - rastlinska proizvodnja)

Prispevki

Sprejemamo izvirne znanstvene članke s področja agronomije, hortikulture, rastlinske biotehnologije, raziskave živil rastlinskega izvora, agrarne ekonomike in informatike ter s sorodnih področij - **letniki z liho številko** (npr. 97, 99) - v slovenskem in angleškem jeziku; pregledne znanstvene članke samo po poprejšnjem dogovoru. Objavljamo tudi izbrane razširjene znanstvene prispevke s posvetovanj, vendar morajo taki prispevki zajeti najmanj 30 % dodatnih originalnih vsebin, ki še niso bile objavljene. O tovrstni predhodni objavi mora avtor obvestiti uredniški odbor. Če je prispevek del diplomske naloge, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Navedbe morajo biti v slovenskem in angleškem jeziku.
Prispevke sprejemamo vse leto.
Podrobnejša navodila: http://aas.bf.uni-lj.si/navodila.htm

INSTRUCTIONS FOR AUTHORS

(Odd-numbered volumes - plant production)

Articles

The Journal *accepts original scientific* articles from the fields of agronomy, horticulture, plant biotechnology, plant-related food-and-nutrition research, agricultural economics, information-science, and related research - odd-numbered volumes (for example: 97, 99) - in Slovenian or English language. Review articles are published in advance agreement with the editorial board. Extended versions of selected proceedings-papers can also be considered for acceptance, provided they include at least 30% of new original content, but the editorial board must be notified beforehand. If the article is based on a thesis or dissertation, the thesis-type must be indicated (BSc, MSc, PhD...), along with the role of the candidate and advisor, at the bottom of the first article page.
Manuscripts are accepted throughout the year.
Detailed instructions: http://aas.bf.uni-lj.si/instructions.htm