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Volatile phenolics in Teran PTP red wine

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ABSTRACT

The volatile phenolics, 4-ethylphenol, 4-vinylphenol, 4-ethylguaiacol and 4-vinylguaiacol were quantified in Teran PTP wines that were produced in the Kras winegrowing district. The compounds were determined by using gas chromatography coupled with mass spectrometry after extraction with diethylether. Three years monitoring (2011, 2012, 2013 vintages) showed that all four undesirable compounds were identified in Teran PTP wines, however their content did not influence significantly the sensory characteristics of the wine. The average contents gained over the three-year period (2011-2013; n=82) were 153±193 µg L^{-1} for 4-ethylphenol, 1265±682 µg L^{-1} for 4-vinylphenol, 69±94 µg L^{-1} for 4-ethylguaiacol and 128±106 µg L^{-1} for 4-vinylguaiacol. 7.3 % of samples showed contents of 4-ethylphenol above the odour threshold values. For 4-vinylphenol, 4-ethylguaiacol and 4-vinylguaiacol that percentage was 98.8 %, 25.6 % and 91.5 %, respectively.

Key words: GC/MS, 4-ethylphenol, 4-vinylphenol, 4-ethylguaiacol, 4-vinylguaiacol

IZVLEČEK

HLAPNI FENOLI V RDEČEM VINU TERAN PTP

V vinu Teran PTP, ki ga pridelujejo v vinorodnem okolišu Kras smo merili vsebnost hlapnih fenolov: 4-etilfenola, 4-vinilfenola, 4-etilgvajakola in 4-vinilgvajakola. Spojine smo identificirali in jim merili vsebnost s plinsko kromatografijo sklopljeno z masno spektrometrijo, po ekstrakciji z dietiletrom. Triletno spremljanje (letniki 2011, 2012 in 2013) je pokazalo, da so v pridelanem vinu prisotne vse izpostavljene spojine prisotne v vinih Teran PTP, vendar njihove vsebnosti ne vplivajo na senzorične lastnosti vina. Povprečne vsebnosti, ki smo jih izmerili v vinu v triletnem obdobju so bile 153±193 µg L^{-1} za 4-etilfenol, 1265±682 µg L^{-1} za 4-vinilfenol, 69±94 µg L^{-1} za 4-etilgvajakol in 128±106 µg L^{-1} za 4-vinilgvajakol. 7,3 % vzorcev je imelo vsebnosti 4-etilfenola nad senzoričnim pragom zaznave. Za 4-vinilfenol, 4-etilgvajakol in 4-vinilgvajakol je bil ta delež 98,8 %, 25,6 % in 91,5 %.

Ključne besede: GC/MS, 4-etilfenol, 4-vinilfenol, 4-etilgvajakol, 4-vinilgvajakol

1 INTRODUCTION

One of the most critical problems in the wine production is the appearance of undesirable aromatic compounds which can reduce the wine quality, especially in unpleasant tastes and smells, what can lead to high economic losses. One of these aromatic compounds is the so-called 'Brett' character, which is mainly related to the presence of ethylphenols (4-ethylphenol and 4-ethylguaiacol) and vinylphenols (4-vinylphenol and 4-vinylguaiacol) (Pizarro et al., 2012). Low contents of these compounds contribute positively to the complexity of wine aroma, on the other hand, these same contents, above a certain threshold, can negatively affect the overall aroma of a wine (Silva et al., 2011). The 4-ethylphenol can produce the odour reminiscent of stable, horse sweat, or leather-like (Larcher et al., 2007), while 4-ethylguaiacol reminiscent of toasted bread, smoky, or clove odour (García-Carpintero et al., 2014) 4-vinylphenol, even if its content is under the sensory threshold, can give odours reminiscent of "band-aid" and gouache. However, these

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compounds can be less detrimental if they are present in wine with 4- vinylguaiacol. 4-vinylguaiacol contributes to the spicy note of wine. Various blends of ethylphenols in red wine give the wine unpleasant stable and animal-like odours (Larcher et al., 2007). Odour thresholds published in other papers on this topic are as follows: 440 µg L^{-1} for 4-ethylphenol, 180 µg L^{-1} for 4-vinylphenol, 33 µg L^{-1} for 4-ethylguaiacol and 40 µg L^{-1} for 4-vinylguaiacol (López et al., 2002). Some authors have reported even higher odour thresholds for red wine: 620 µg L^{-1} for 4-ethylphenol and 140 µg L^{-1} for 4-ethylguaiacol (Alañón et al., 2013). For 4-vinylguaiacol, an odour threshold of 10 mg L^{-1} was also reported (García-Carpintero e tal., 2012) and 770 µg L^{-1} (Pour Nikfardjam et al., 2009) was reported for vinylphenols.

Volatile phenolics mainly arise from the metabolism of hydroxycinnamic acids by *Brettanomyces/Dekkera* sp. yeasts, which involves the sequential action of two enzymes. First, a cinnamate decarboxylase cleaves the phenolic acids directly into the corresponding vinylphenol and vinylguaiacol (*p*-coumaric acid is cleaved to 4-vinylphenol and ferulic acid is cleaved to 4-vinylguaiacol). Then, a vinylphenol reductase converts the 4-vinylphenol into 4-ethylphenol and 4-vinylguaiacol into 4-ethylguaiacol (Oelofse et al., 2009; Saez et al, 2011; Silva et al., 2011; Valentão et al., 2007) It is known, that these yeasts can grow during bottle storage over long periods. They can produce 4-ethylphenol and 4-ethylguaiacol contents that exceed critical olfactory thresholds during the first months of storage (Renouf et al., 2007). Careful hygienic precautions and adequately sulphuring the wines and wine containers can prevent the development of these undesirable yeasts (Valentão et al., 2007).

The aim of the present work was to monitor the content of 4 volatile phenols in Teran PTP wine that was produced in the Kras winegrowing district of Slovenia between 2011- 2013. Teran PTP wine is produced exclusively from grapes of the grapevine variety 'Refošk' (*Vitis vinifera* L.) grown on absolute winegrowing sites at characteristic, intensive red colour soil, also known as 'jerina', which gives Teran PTP Recognized Traditional Denomination in EU.

2 MATERIALS AND METHODS

2.1 Samples

Samples of Teran PTP wine were collected from stainless steel tanks or wooden containers from wine producers in the Karst region of Slovenia. During three years of monitoring, 82 wines were sampled from different producers (39 from the 2011 vintage, 22 from the 2012 vintage and 21 from the 2013 vintage). Wines were sampled each year in May, 9 months after alcoholic fermentation and after completion of malolactic fermentation. One bottle of 0.75 L of each wine were sampled directly from the steel tanks or wooden containers. Wines were transported to Agricultural Institute of Ljubljana and kept at 15 °C until analyses. Analyses were performed during one month period after sampling.

2.2 Extraction of volatile phenolics

For extraction of volatile phenolics we used a method proposed by the Central Analytical Facility at Stellenbosch University in South Africa. A 20 ml wine sample was transferred into a glass tube. Then the following was added: 400 µl of internal standard 2,3-dimethyl phenol (99 % purity, dr. Ehrenstorfer) with 5 mg L^{-1} content in a model wine solution (1 g of tartaric acid and 120 ml of absolute ethanol p.a. filled up to 1 L with milliQ and pH adjusted to 3.5 with NaOH). Afterwards 4 ml of diethylether (HPLC purity) was added. Then the tube was closed and sonicated for 30 minutes. The tube was shaken at 5 minutes intervals. After extraction, the organic phase was transferred to a glass vial using a Pasteur pipette. The vial contained sodium sulphate to dry the extract. The organic phase was then transferred to another clean vial and injected into a gas chromatograph coupled with a mass spectrometer (GC/MS).

2.3 Identification and quantification of volatile phenolics

To identify and quantify the volatile phenolics, a method proposed by the Central Analytical Facility at Stellenbosch University was used. The samples were analysed using a gas chromatograph (Agilent

Technologies 7890A, Shanghai, China) equipped with a column DB-FFAP (Agilent Technologies, 60 m, 0.25 mm i.d., 0.5 μm film thickness), with a constant flow of helium at 0.63 ml min^{-1}. The injector was held at 250 °C. The GC oven was programmed as follows: 40 °C for 1 min, from 40 to 150 °C at 20 °C/min, from 150 to 240 °C at 5 °C min^{-1}, held at 240 °C for 8 min. To determine the level of analytes, a mass spectrometer (Agilent Technologies 5975C, upgraded with a triple-axis detector, Palo Alto, CA, USA) was used. The temperature of the ion source was 230 °C, the auxiliary temperature was 250 °C, and the quadrupole temperature was 150 °C. For qualitative determination, retention time and mass spectrum in selective ion monitoring mode (SIM) were used. The ions monitored were m/z 137 and 152 for 4-ethylguaiacol, m/z 107 and 122 for internal standard 2,3-dimethyl phenol, m/z 107 and 122 for 4-ethylphenol, m/z 135 and 150 for 4-vinylguaiacol and m/z 91 and 120 for 4-vinylphenol. Ions 137, 107, 135 and 91 were the target ions used for quantification, whereas other ions were used as qualifier ions. Calibration standards were prepared by extracting model wine solutions with known concentrations of 4-ethylphenol, 4-vinylphenol, 4-ethylguaiacol and 4-vinylguaiacol.

2.4 Validation parameters

The limit of detection and the limit of quantification were estimated from chromatograms of standard solutions with known concentrations of 4-ethylphenol, 4-vinylphenol, 4-ethylguaiacol and 4-vinylguaiacol. The limit of detection (LD) was determined from S/N = 3 and was 1.5 μg L^{-1}. The limit of quantification (LOQ) was determined from S/N = 10 and was 5.0 μg L^{-1}.

Linearity was verified by using extracts of model wine solutions with known concentrations of 4-ethylphenol, 4-vinylphenol, 4-ethylguaiacol and 4-vinylguaiacol (five repetitions for one concentration level, eight concentration levels for the calibration curve). Linearity and range were determined by multiple linear regressions, using the F test. The linear model was fit and remained linear over the range from 5 μg L^{-1} to 2500 μg L^{-1}; R^2 is 0.9977 for 4-ethylphenol, 0.9991 for 4-vinylphenol, 0.9978 for 4-ethylguaiacol and 0.9971 for 4-vinylguaiacol.

Trueness was verified by checking the recoveries. Ten spiked samples were prepared with red wine. The average of recoveries was calculated. The recoveries are presented in Table 1.

Table 1: Recoveries obtained during validation of analytical method for identification and measurement of volatile phenolics content

Preglednica 1: Izkoristki dobljeni med validacijo analizne metode za identifikacijo in merjenje vsebnosti hlapnih fenolov

	Spiking level (mg L^{-1})	Recovery (%)	RSD (%)
4-ethylphenol	0.05	77.6	12.6
4-ethylphenol	2	73.0	3.6
4-vinylphenol	2	103.4	12.3
4-ethylguaiacol	0.05	103.9	17.5
4-ethylguaiacol	2	91.9	3.0
4-vinylguaiacol	0.05	84.6	14.5
4-vinylguaiacol	2	92.2	4.7

2.5 Physico-chemical parameters

The physico-chemical parameters of Teran PTP wine from the 2011, 2012, 2013 vintages were determined by standard EEC (1990) methods (European Union, 1990).

2.6 Statistical analysis

Data were collected and edited using Excel (Microsoft Office Professional Plus 2010) and analysis of variance (one-way ANOVA) was performed on content data for volatile phenolics using Statgraphics® Centurion XVI statistical software package (StatPoint Technologies).

3 RESULTS AND DISCUSSION

The physico-chemical parameters of Teran PTP wine from the 2011, 2012, 2013 vintages are shown in Table 2. Teran PTP is a wine with a moderate alcohol level, elevated acidity and a lower pH (Table 2). The lower pH can be unfavourable for growth of *Brettanomyces/Dekkera* sp. Yeasts (Du Toit et al., 2005). In addition to this, the low alcohol content and low SO_2 content, traditionally used in Teran PTP vinification and aging, can increase the risk of microbiology spoilage (Du Toit et al., 2005). It has been shown by numerous authors that a 0.8 mg L^{-1} molecular SO_2 content is the optimum level to control almost all yeast and bacteria species (Du Toit et al., 2005). However, due to the low free SO_2 contents in Teran PTP wines, the molecular SO_2 content (Table 2) is much lower than proposed for inhibiting any undesired *Brettanomyces/Dekkera* sp. yeasts in the wine.

Table 2: Average standard physico-chemical characteristics with standard deviations of Teran PTP wine for the 2011, 2012 and 2013 vintages
Preglednica 2: Povprečne standardne fizikalno-kemijske značilnosti s standardnimi odkloni vina Teran PTP letnikov 2011, 2012 in 2013

Parameters	2011 vintage (n=39)	2012 vintage (n=22)	2013 vintage (n=21)
alcohol (vol. %)	12.01 ± 0.60	11.95 ± 0.58	12.06 ± 0.46
molecular SO_2 (mg L^{-1})	0.35 ± 0.08	0.41 ± 0.03	0.35 ± 0.12
free SO_2 (mg L^{-1})	13 ± 3	12 ± 1	12 ± 4
total SO_2 (mg L^{-1})	43 ± 6	40 ± 9	35 ± 7
pH	3.37 ± 0.13	3.26 ± 0.12	3.33 ± 0.14
total acidity (g L^{-1} tartaric acid)	7.5 ± 0.7	8.0 ± 0.8	7.5 ± 0.8
volatile acidity (g L^{-1} acetic acid)	0.62 ± 0.17	0.45 ± 0.11	0.73 ± 0.13
reducing sugars (g L^{-1})	2.5 ± 0.7	1.2 ± 0.3	1.3 ± 0.7

The results of the volatile phenolic content and their odour thresholds are presented in Table 3. By comparing results with ANOVA we obtained that there is a statistically significant difference only between contents of 4-ethylguaiacol and 4-ethylphenol and contents of 4-ethylguaiacol and 4-vinylphenol at the 95.0 % confidence level. 7.3 % of samples from the 2011-2013 vintages had content of 4-ethylphenol above odour threshold values described by Alañón et al. (2013) and López et al. (2002). Portion of samples above the odour threshold values were 98.8 %, 25.6 % and 91.5 % for 4-vinylphenol, 4-ethylguaiacol and 4-vinylguaiacol respectively.

Table 3: Contents (µg L^{-1}) of volatile phenolics in Teran PTP wine for the 2011, 2012 and 2013 vintages
Preglednica 3: Vsebnosti (µg L^{-1}) hlapnih fenolov v vinu Teran PTP letnikov 2011, 2012 in 2013

	2011	2012	2013	2011-2013	odour threshold
4-ethylphenol	6 - 465	23 - 593	6 - 953	6 - 953	620 (a)
4-vinylphenol	366 - 3438	423 - 2454	90 - 3376	90 - 3438	180 (b)
4-ethylguaiacol	6 - 441	6 - 479	9 - 250	6 - 479	140 (a)
4-vinylguaiacol	28 - 750	53 - 460	19 - 345	19 - 750	40 (b)

(a) Alañón et al., 2013; (b) López et al., 2002

The average contents found during the 2011-2013 period were 153±193 µg L^{-1} for 4-ethylphenol, 1265±682 µg L^{-1} for 4-vinylphenol, 69±94 µg L^{-1} for 4-ethylguaiacol and 128±106 µg L^{-1} for 4-vinylguaiacol. The average contents of volatile phenolics for each vintage are presented in Figures 1 – 4.

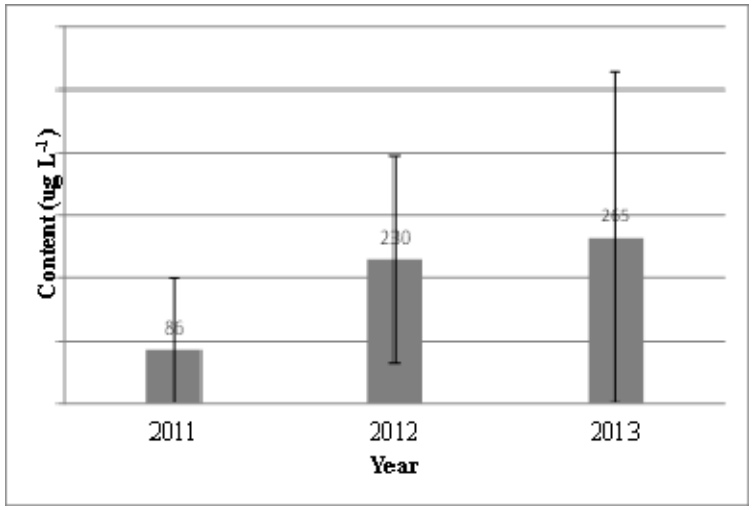

Figure 1: Average content and its standard deviation of 4-ethylphenol in Teran PTP wine from the 2011, 2012 and 2013 vintages
Slika 1: Povprečna vsebnost in njen standardni odklon za 4-etilfenol v vinu Teran PTP letnikov 2011, 2012 in 2013

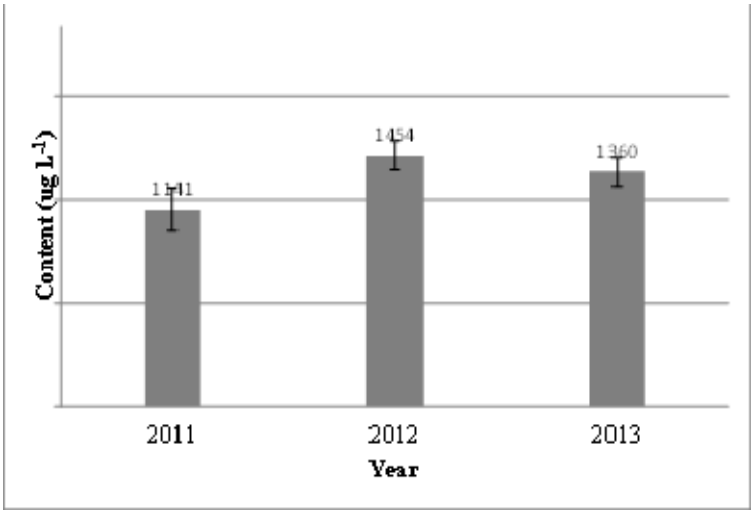

Figure 2: Average content and its standard deviation of 4-vinylphenol in Teran PTP wine from the 2011, 2012 and 2013 vintages
Slika 2: Povprečna vsebnost in njen standardni odklon za 4-vinilfenol v vinu Teran PTP letnikov 2011, 2012 in 2013

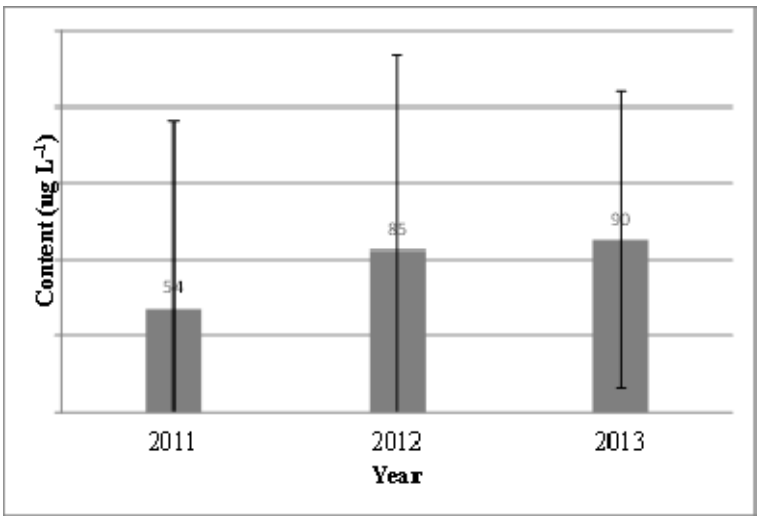

Figure 3: Average content and its standard deviation of 4-ethylguaiacol in Teran PTP wine from the 2011, 2012 and 2013 vintages
Slika 3: Povprečna vsebnost in njen standardni odklon za 4-etilgvajakol v vinu Teran PTP letnikov 2011, 2012 in 2013

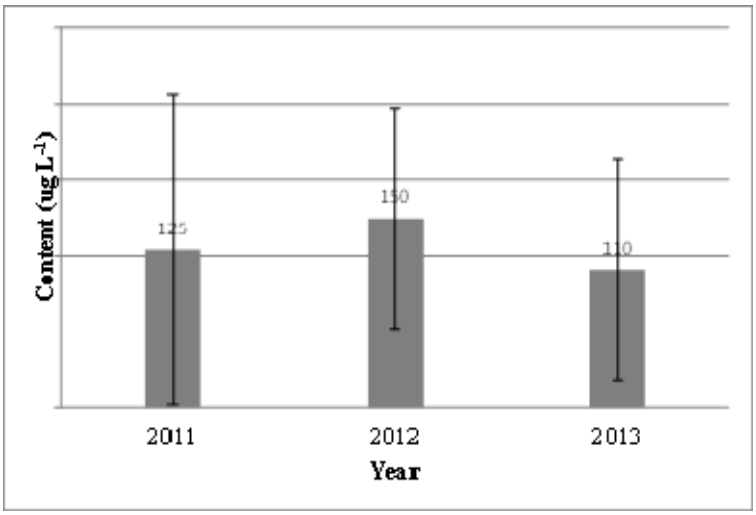

Figure 4: Average content and its standard deviation of 4-vinylguaiacol in Teran PTP wine from the 2011, 2012 and 2013 vintages
Slika 4: Povprečna vsebnost in njen standardni odklon za 4-vinilgvajakol v vinu Teran PTP letnikov 2011, 2012 in 2013

After comparing our results with the data from other published papers on volatile phenolics, we noticed that Spanish oak-aged red wine has similar contents of 4-ethylguaiacol (López et al., 2002). Other wines also have similar contents of 4-ethylphenol, 4-vinylphenol and 4-vinylguaiacol. A comparison of this data is presented in Table 4.

Table 4: Comparison of volatile phenol content (μg L^{-1}) in Teran PTP wine with similar data from other papers
Preglednica 4: Primerjava vsebnosti hlapnih fenolov (μg L^{-1}) v vinu Teran PTP s podobnimi podatki iz drugih člankov

	4-ethylphenol	4-vinylphenol	4-ethylguaiacol	4-vinylguaiacol
Teran PTP red wine	6 - 953	90 - 3438	6 - 479	19 - 750
red wine (a)	101 - 133	n.a.	88 - 105	n.a.
red wine (b)	7 - 84	730 - 4385	42 - 65	49 - 54
red wine (c)	97 - 782	1430 - 2174	72 - 255	282 - 880
Tannat red wine (d)	170 - 1120	n.a.	120	n.a.
Blaufränkisch red wine (e)	149 - 435	n.a.	81 - 152	n.a.
Spanish oak-aged red wine (f)	8.6 - 1500	8.1. - 98	0.53 - 420	5.4 - 236
Moravia Dulce red wine (g)	n.d.	n.a.	n.d.	287 - 473
archive wines (vintage 1909-1981) from Bordeaux region (h)	1040 - 6410	n.a.	122 - 975	n.a.

n.a. - not analysed
n.d. - not detected
(a) Pizarro et al., 2012, (b) Pizarro et al., 2007, (c) Domínguez et al., 2002, (d) Smit et al., 2003, (e) Díez et al., 2004, (f) López et al., 2002, (g) García-Carpintero et al., 2012, (h) Renouf et al., 2007

Due to lower pH, typical for Teran PTP wine, the growth of *Brettanomyces/Dekkera* sp. yeasts can be reduced. This would reduce the possibility of forming volatile phenolics. In our report, more than 90 % of the Teran PTP samples contained 4-ethylphenol below the sensory threshold. However, it should be noted, that the wines were sampled in the spring time, when the temperatures in wine cellars might be still low. Low temperature can suppress the growth of undesirable microorganisms. The content of 4-ethylphenol in bottle-aged Teran PTP wines was reported to be 1016, 678 and 616 μg L^{-1} for the 2007, 2008 and 2009 vintages, respectively (Čuš et al., 2011).

To prevent the growth of *Brettanomyces/Dekkera* sp. yeasts and to prevent the formation of undesirable volatile phenolics it is necessary to either filter the wines during bottling or use SO_2 (Du Toit et al., 2005; Renouf et al. (2007). Renouf et al. (2007) found *Brettanomyces bruxellensis* Kufferath and von Laer to be the most predominant yeast that grows during bottle storage over long periods. This yeast produces 4-ethylphenol and 4-ethylguaiacol contents that exceed critical olfactory thresholds. A 1.0-μm grade filter sheet was sufficient to eliminate all yeasts, and it significantly prevented the increase of volatile phenolics for several years after bottling (Renouf et al., 2007).

4 CONCLUSIONS

Ethylphenols (4-ethylphenol and 4-ethylguaiacol) and vinylphenols (4-vinylphenol and 4-vinylguaiacol), which contribute to undesirable wine taste and smell were identified in Teran PTP wines. During three year monitoring (2011-2013 vintages) we measured contents of 4-ethylphenol and 4-ethylguaiacol in Teran PTP wines mainly below sensory threshold, while contents of 4-vinylphenol and 4-vinylguaiacol were mainly above sensory threshold. Some phenolics were found, especially 4-vinylguaiacol, which can actually have a positive effect on wine aroma, reminiscent of a pepper or clove aroma. However, the volatile phenols that were found in Teran PTP wines were sampled and measured in late spring, when the temperatures in cellars might be still low, which could supress the growth of undesirable yeast. In order to reduce the risk of increased

content of volatile phenolics, Teran PTP producers need to reduce the risk of growing *Brettanomyces/Dekkera* sp. yeasts. This can be accomplished by maintaining hygienic cellar conditions, by filtering the wine with 1.0-µm grade filter sheets, and by proper sulfphiting of wine.

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Agris category code: F03, f06, f40

Changes in seed vigor of safflower (*Carthamus tinctorius* L.) cultivars during maturity in response to water limitation

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ABSTRACT

Seed development and vigor may be influenced by environmental stresses such as water deficit. Thus, this research was carried out to evaluate the effects of different irrigation treatments and harvest times on seed vigor of four spring safflower cultivars. Treatments were irrigations (irrigations after 70, 100, 130 and 160 mm evaporation, respectively) in main plots and safflower cultivars (Faraman, Goldasht, Sina and Soffeh) in sub-plots. Seeds were harvested in 5 days intervals at 7 stages during development and maturity. The highest and the lowest seed and seedling masss at all harvests and irrigation treatments were recorded for Faraman and Sina cultivars, respectively. Seed vigor of safflower cultivars as measured by electrical conductivity of seed leachates and seedling dry mass decreased with decreasing water availability. Maximum seed mass and seedling dry mass and minimum electrical conductivity were obtained at 40-45 days after flowering, depending on irrigation interval and cultivar. Seed moisture content at these stages was 15-20 %, which is suitable for direct and mechanical harvesting and threshing.

Key words: Safflower, seedling and seed mass, seed vigor, water supply

IZVLEČEK

SPREMEMBE V VITALNOSTI SEMEN RAZLIČNIH SORT ŽAFRANIKE (*Carthamus tinctorius* L.) MED DOZOREVANJEM KOT ODZIV NA POMANJKANJE VODE

Razvoj semen in njihova vitalnost sta odvisna od okoljskih stresov kot je tudi pomanjkanje vode. V tej raziskavi so bili ovrednoteni učinki različnih namakanj in časa žetve na vitalnost semen štirih jarih sort žafranike. Obravnavanja so bila namakanje (namakanje po 70, 100, 130 in 160 mm evaporacije) na glavnih ploskvah in štiri sorte žafranike na podploskvah (Faraman, Goldasht, Sina in Soffeh). Semena so bila vzorčena v intervalu petih dni v sedmi stopnji razvojne faze in zrelosti. Največja in najmanjša masa semena in kalic sta bili pri vseh vzorčenjih in časih namakanja zabeleženi pri sortah Faraman in Sina. Vitalnost semen sort žafranike, izmerjena kot električna prevodnost izlužkov vode, v kateri so bila namakana semena in suha masa kalic sta se zmanjševali z upadanjem razpoložljivosti vode. Največja masa semen in suha masa kalic ter najmanša električna prevodnost izlužkov so bile izmerjene 40-45 dni po cvetenju, odvisno od intervala namakanja in sorte. Vsebnost vode v semenih je bila v tem obdobju 15-20 %, kar je ugodno za neposredno mehansko žetev, mlačvo in shranjevanje.

Ključne besede: žafranika, masa semen in kalic, vitalnost semen, oskrba z vodo

1 INTRODUCTION

Environmental factors during seed-filling period and even during flowering stage can widely affect seed yield and quality of oilseed crops (Monotti, 2003). Among all these environmental factors, drought stress is an important limiting factor for plant growth and yield, and may influence the

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quality of safflower. Coincidence of water stress with reproductive stages of different crops reduces duration of flowering and seed filling and consequently lowers the number of seeds per plant, mean seed weight and seed yield per unit area (Ghassemi-Golezani and Mazloomi-Oskooyi, 2008; Ghassemi-Golezani et al., 2010). The deleterious effects of water limitation on field performance of crops may be reduced by cultivation of high vigor seeds (Sun et al., 2007).

High and rapid field emergence are essential to obtain an adequate plant stand in order to gain an advantage of the growing season before the onset of the severe drought stress late in the season (TeKrony and Egli, 1991). Stage of maturity at harvest is one of the most important factors that can influence the vigor of seeds (Demir et al., 2008). Maximum seed vigor on the mother plant may be achieved at the end of seed filling phase (mass maturity) which is also described as physiological maturity (Harington, 1972; Ghassemi Golezani et al., 2011b) or after mass maturity (Ghassemi-Golezani and Mazloomi-Oskooyi, 2008; Ghassemi-Golezani et al., 2011a), depending on species and seed testing methods (Tekrony and Egli, 1997; Ghassemi-Golezani and Hosseinzade-Mahootchi, 2009).

Vieira et al., (1992) reported that drought stress had no significant effect on soybean seed germination and vigor. In contrast, Ghassemi-Golezani et al. (2012b, 2015) showed that water deficit led to significant reductions in seed vigor of soybean and maize. However, the effects of water limitation and harvest time on seed vigor of safflower are poorly understood. Thus, this research was carried out to evaluate changes in seed vigor of safflower cultivars under well and limited irrigation conditions in order to determine the stage at which maximum seed vigor is attained.

2 MATERIALS AND METHODS

The field experiment was conducted at the Research Farm of the University of Tabriz, Iran (latitude 38.05°N, longitude 46.17°E, Altitude 1360 m above sea level) in 2013. The climate was characterized by mean annual precipitation of 245.75 mm, mean annual temperature of 10 ˚C, mean annual maximum temperature of 16.6 ˚C and mean annual minimum temperature of 4.2 ˚C. The experiment was arranged as split plot based on randomized complete block design in three replicates, with irrigation treatments (I_1, I_2, I_3, I_4 for irrigation after 70, 100, 130 and 160 mm evaporation from class A pan, respectively) in main plots and cultivars (C_1; Faraman, C_2; Goldasht; C_3; Sina and C_4; Soffeh) in sub plots. Seeds were harvested in 5 days intervals at 7 stages during their development and maturity. The field plan and experimental arrangement are shown in figure 1.

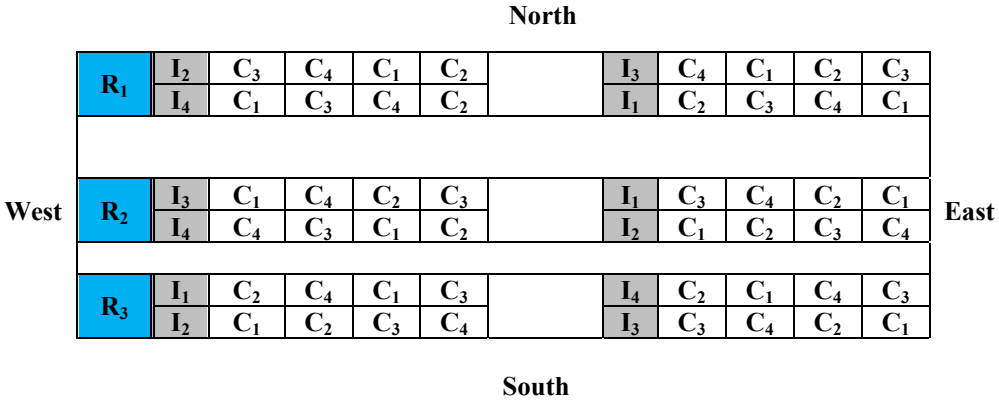


Figure 1: The field plan and experimental arrangement
I_1, I_2, I_3, I_4: irrigation after 70, 100, 130 and 160 mm evaporation, respectively.
C_1, C_2, C_3, C_4: Faraman, Goldasht, Sina and Soffeh cultivars, respectively.

Seeds of Safflower (*Carthamus tinctorius* L.) were sown by hand on 23rd April 2013 in 4 cm depth of a sandy loam soil. Each plot consisted of 10 rows of 3 m length, spaced 25 cm apart. Seeding rate was 100 seeds per m^2. All plots were irrigated immediately after sowing. Subsequent irrigations were carried out on the bases of proposed evaporation from class A pan. Hand weeding of the experimental area was performed as required.

After seed formation, 5 plants from each plot were harvested in 5 days intervals at seven stages. Then seeds were detached from the capitol and seeds were air dried at 18-20 ˚C and mean seed mass of each sample was determined.

Four replicates of 50 seeds from each sample were weighted (SW_1 – SW_4) and then seeds of each replicate immersed in 250 ml deionized water in a container at 20 ˚C for 24 hours. The seed-steep water was then gently decanted and electrical conductivity (EC) of seed leachates was measured, using an EC meter (EC_1 – EC_4). Following equation was applied to calculate the conductivity per gram of seed mass for each sample (Powell et al., 1984).

$$EC\ (\mu s/cm/g) = [(EC_1/SW_1) + \cdots\cdots + (EC_4/SW_4)]/4$$

Seed samples within separate sealed bags were then placed in a refrigerator at 3-5 ˚C. Seed vigor tests were carried out at the seed Technology Laboratory of the University of Tabriz.

Four replicates of 25 seeds from each sample were treated with 2 $g.kg^{-1}$ Benomyl, before testing. Seeds of each replicate were placed between two 30 × 30 cm wetted and rolled filter papers, which were then placed in plastic bags to prevent water loss. These bags were incubated at 20±1 °C and germinated seeds (protrusion of seminal root by 2 mm) were counted every day up to 8 days. Seed germination rate was calculated according to Ellis and Roberts (1980):

$$R = \sum n_i / \sum Dn$$

Where n is number of seeds germinated on each day, D is the number of days from the beginning of the test and R is the mean germination rate. At the end of test, normal seedlings were dried in an oven at 75 ˚C for 48 hours (Perry, 1977) and mean seedling dry mass for each treatment at each replicate was recorded.

Analyses of variance and comparison of means at $P \leq 0.05$ were performed, using SAS 9.1 software. Excel software was used to draw figures.

3 RESULTS

Seed mass and electrical conductivity of seed leachates were significantly affected by Irrigation, cultivar, harvest time and interactions of these factors ($p \leq 0.01$). Seed germination rate significantly influenced by irrigation and harvest time ($p \leq 0.01$) and also by interaction of cultivar × harvest time ($p \leq 0.05$). The effects of cultivar, harvest time and interaction of these factors were also significant for seedling dry mass ($p \leq 0.01$). Thus, regression curves were fitted on mean data of significant interactions in order to show the changes in parameters of seed vigor at different stages of seed development and maturity (Figs. 2-5).

Seed weight of safflower cultivars under different irrigation treatments increased with progressing seed development up to a point where maximum mass was obtained. In most cases, mass maturity (end of seed filling phase) were achieved at about 40 days after flowering and thereafter, no considerable changes were occurred. Seed moisture content at these stages was 15-20 %, depending on water supply. Seed mass at early stages of development under I_3 and I_4 was higher, but at later stages of development was lower than that under well watering (I_1). The highest and the lowest seed mass at all stages of seed development under different irrigation intervals were recorded for Faraman and Sina cultivars, respectively. Changes in seed mass of 'Goldasht' and 'Soffeh' under different treatments were almost similar (Fig. 2).

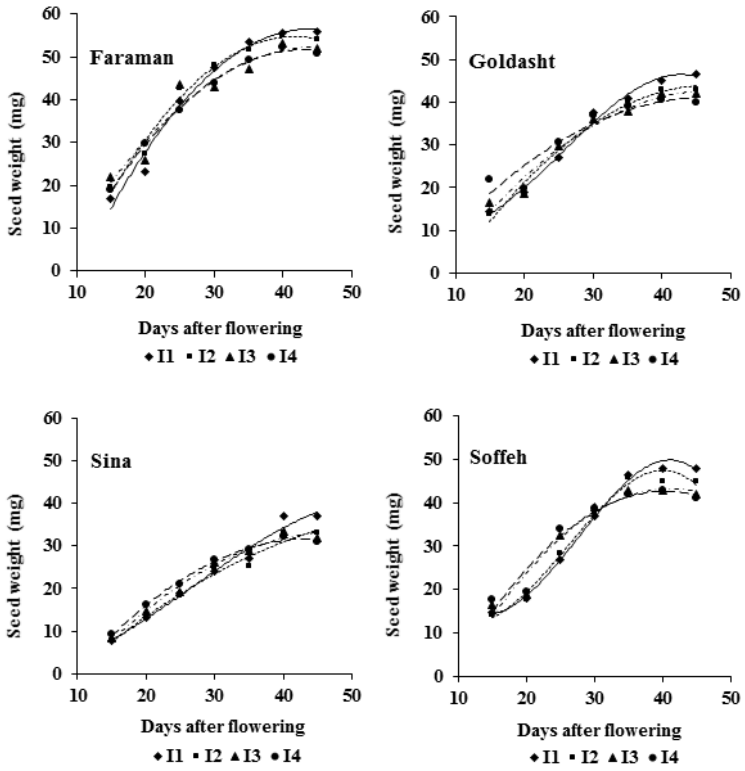
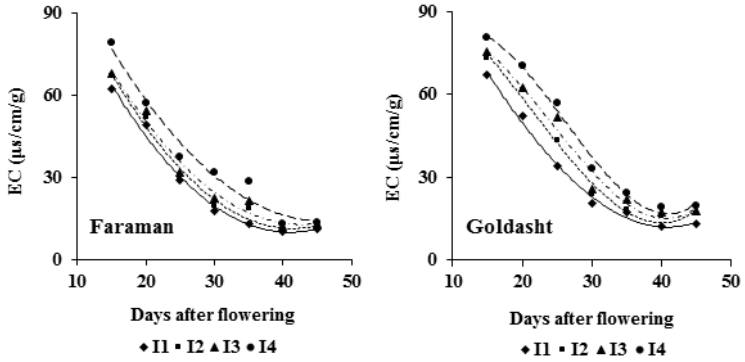


Figure 2: Changes in seed mass of spring safflower cultivars during seed development and maturity under different irrigation intervals
I_1, I_2, I_3, I_4: irrigation after 70, 100, 130 and 160 mm evaporation, respectively.

Electrical conductivity (EC) of seed leachates for all safflower cultivars under different irrigation intervals decreased with improving seed development. In most cases, minimum electrical conductivity of seeds produced for all cultivars under all irrigations were obtained at 40-43 days after flowering, with slight increase thereafter. The highest electrical conductivity of seed leachates was recorded for seeds produced under I_4, which decreased with increasing water supply (Fig. 3).



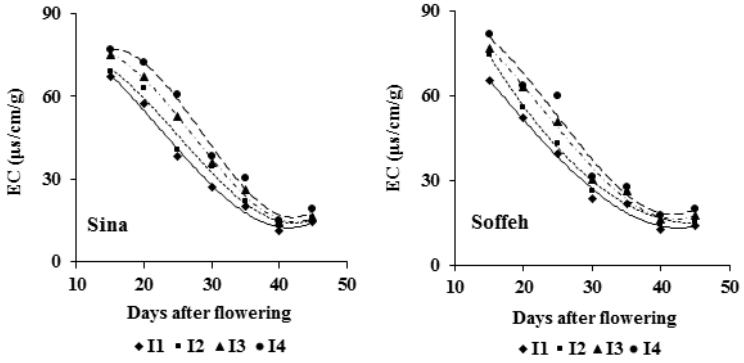


Figure 3: Electrical conductivity of seed leachates for spring safflower cultivars during seed development and maturity under different irrigation intervals
I_1, I_2, I_3, I_4: irrigation after 70, 100, 130 and 160 mm evaporation, respectively

Seed germination rate of all safflower cultivars increased up to final harvest. Therefore, maximum seed germination rate of safflower cultivars was attained at 45 days after flowering. 'Sina' had the highest seed germination rate at two earlier harvest, but the lowest germination rate at three later harvests. Seed germination rate of other three cultivars at later stages of development was almost similar (Fig. 4).

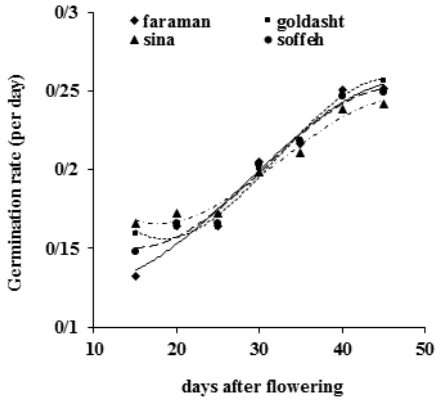


Figure 4: Changes in seed germination rate of safflower cultivars during seed development and maturity

'Faraman' had the highest, but 'Sina' had the lowest seedling dry mass from seeds produced under all irrigation intervals, with no considerable difference between 'Goldasht' and 'Soffeh'. Seedling dry mass of seeds produced under severe water deficit showed an increase for Faraman and a decrease for Sina and Soffeh cultivars (Fig. 5a). Seedling dry mass of safflower cultivars at the early stages of seed development was low, but it was improved progressively with seed development up to 40 days after flowering, and thereafter slightly decreased. Faraman and Sina cultivars had the highest and the lowest seedling dry mass at all stages of seed development, respectively, while seedling dry mass of Goldasht and Soffeh at different harvest times was similar (Fig. 5b).

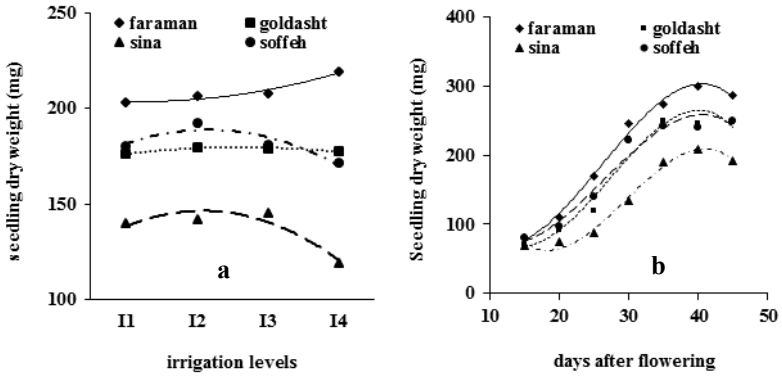


Figure 5: Changes in seedling dry mass for seeds of safflower cultivars produced under different irrigation intervals (a) and harvesting times (b)
I_1, I_2, I_3, I_4: irrigation after 70, 100, 130 and 160 mm evaporation, respectively

Maximum seed mass and minimum electrical conductivity of seed leachates were significantly influenced by irrigation intervals ($p \leq 0.05$) and cultivars ($p \leq 0.01$). Differences in maximum seedling dry mass of safflower cultivars were also significant ($p \leq 0.01$). Maximum seed mass decreased, but minimum electrical conductivity increased with increasing irrigation intervals. The highest seed and seedling dry mass and the lowest electrical conductivity were recorded for seeds of 'Faraman'. In contrast, the smallest seeds and seedlings were observed in Sina cultivar (Table 1).

Table 1: Means of minimum values of electrical conductivity and maximum values of the other seed vigor parameters for different irrigation intervals and safflower cultivars

Treatments	Seed mass (mg)	Electrical conductivity (µs/cm/g)	Germination rate (per day)	Seedling dry mass(mg)
Irrigations				
I_1	47.56 a	11.31 b	0.256 a	262.57 a
I_2	44.96 ab	12.99 ab	0.249 a	266.87 a
I_3	43.03 bc	13.42 a	0.262 a	272.05 a
I_4	41.89 c	14.39 a	0.251 a	259.65 a
Cultivars				
C_1	54.27 a	10.82 b	0.256 a	307.88 a
C_2	44.06 b	14.78 a	0.260 a	266.07 b
C_3	33.79 c	11.70 b	0.248 a	224.78 c
C_4	45.33 b	14.82 a	0.254 a	262.40 b

I_1, I_2, I_3, I_4: irrigation after 70, 100, 130 and 160 mm evaporation, respectively.
C_1, C_2, C_3, C_4: Farman, Goldasht, Sina and Soffeh cultivars, respectively.
Different letters in each column indicate significant difference at $p \leq 0.05$

4 DISSCUSION

Water stress decreases photosynthetic production because of stomata closure and early senescence which ultimately reduces maximum seed mass (Fig. 1, Table 1). Reduction in seed mass due to water limitation is also reported for maize (Ne Smith and Ritchie, 1992), lentil (Erskine and Ashkar, 1993), chickpea (Silim and Saxena, 1993), soybean (Desclaux and Roumet, 1996), wheat (Li et al., 2000) and barley (Samarah, 2005). Variation in seed mass among cultivars under different levels of watering (Fig. 1) is the result of differences in genetic constitution. Different sizes of seeds

having different levels of food storage may be the important factor which influences seed vigor (Perry, 1980).

Maximum seed vigor as measured by electrical conductivity of seed leachate was obtained at about mass maturity or slightly after that stage, depending on cultivar and water supply. Earlier harvests due to immaturity, and later harvests because of aging reduced seed vigor of safflower cultivars under all treatments (Fig. 2). These results were supported by the reports on common bean (Ghassemi-Golezani and Mazloomi-Oskooyi, 2008) and faba bean (Ghassemi-Golezani and Hosseinzadeh-Mahootchy, 2009). Increasing electrical conductivity of seeds with decreasing water availability to the mother plants (Table 1), clearly suggest that the most vigorous seeds of this crop can be produced under well watering. In contrast, Ghassemi-Golezani and Hosseinzadeh-Mahootchy (2009) reported that drought stress had no significant effect on electrical conductivity of faba bean cultivars.

Maximum germination rate of all cultivars was achieved 5 days after mass maturity. Higher germination rate of Sina cultivar at early harvest and the lower germination rate of this cultivar at later harvests may be related with rapid seed development and maturity of that cultivar, compared with other cultivars (Fig. 3). Nevertheless, maximum germination rate was almost similar for all irrigation treatments and cultivars (Table 1).

Water limitation had no significant effect on seed vigor of safflower cultivars, as determined by seedling dry mass (Table 1). The highest and the lowest seedling dry mass of Faraman and Sina under different irrigation treatments (Fig. 4a) directly related with the seed size of these cultivars (Fig. 1). Several reports suggest that large seeds have greater and longer supply of stored reserves to support early seedling growth, leading to the production of larger seedlings (Ghassemi-Golezani, 1992; Ghassemi-Golezani et al., 2012a,b, 2015). Therefore, differences in seedling dry mass among safflower cultivars at different stages of seed development and maturity (Fig. 4b) were associated with variation in seed mass and genetic constitution, which can strongly influence seed and seedling vigor (Perry, 1980). The highest seedling dry weight of all cultivars was recorded at the end of seed filling phase or mass maturity (40 days after flowering) (Fig. 4b). Therefore, maximum seed vigor of safflower cultivars as measured by seedling dry mass was obtained at mass maturity under all irrigation treatments, which can be named physiological maturity as suggested by Harington (1972).

5 CONCLUSION

Seed size of safflower cultivars decreased, but electrical conductivity of seed leachates slightly increased as a result of water deficit. However, maximum germination rate and seedling dry mass were not significantly affected by water limitation. Therefore, high vigor seeds of this crop could be produced under different irrigation intervals, if seeds were harvested at or slightly after the end of seed filling phase (mass maturity) with a moisture content of about 15–20 %. Early and delayed harvests could lead to the production of low vigor seeds. Faraman was a superior cultivar in producing the highest vigor seeds under a wide range of water availability. It seems that safflower cultivars are adapted to semi-arid conditions and water stress has little effect on their seed vigor, provided the seeds are harvested at the right time. Similar research on different crops and environmental conditions may provide more information about producing high vigor seeds.

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Agrovoc descriptors: lamiaceae; essential oil crops; essential oils; chemical composition; chemical compounds; Iran islamic republic

Agris category code: Q04

Chemical constituents of essential oil of *Dracocephalum moldavica* L. and *Dracocephalum kotschyi* Boiss. from Iran

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ABSTRACT

Dracocephalum moldavica L. and *Dracocephalum kotschyi* Boiss. are aromatic plants belonging to Lamiaceae family. The aim of this study was to identify the chemical components of *D. kotschyi* and *D. moldavica* from Iran. The aerial parts of *D. kotschyi* were collected from (Kamu Mountain) Isfahan province and the aerial parts of *D. moldavica* were collected from Sari (Mazandaran province) North of Iran, during 2014. The essential oil was extracted by a Clevenger approach and analyzed using GC/MS. In total, 32 and 24 compounds were identified in the essential oil from the aerial parts *D. kotschyi* and *D. moldavica*, respectively. The results obtained in our study indicated that the major components in the oil *D. kotschyi* were limonene (23.56 %), carvacrol (14.65 %), γ-terpinene (12.99 %), α -pinene (12.62 %), 2-methyl-1-octen-3-yne (9.73 %), camphene (4.66 %), myrcene (3.65 %) and α -terpinene (3.12 %). The major constituents of the oil *D. moldavica* were geranyl acetate (36.62 %), geraniol (24.31 %), neral (16.25 %) and geranial (11.21 %). *D. kotschyi* is one of the important sources of limonene and *D. moldavica* is one of the important sources of geranyl acetate.

Key words: *Dracocephalum moldavica* L., *Dracocephalum kotschyi* Boiss., chemical constitutes of essential oils

IZVLEČEK

KEMIJSKA SESTAVA ETERIČNIH OLJ V DVEH VRSTAH KAČJEGLAVKE (*Dracocephalum moldavica* L., *Dracocephalum kotschyi* Boiss.) IZ IRANA

Vrsti kačjeglavk *Dracocephalum moldavica* L. in *Dracocephalum kotschyi* Boiss.sta aromatični rastlini iz družine ustnatic (Lamiaceae**).** V raziskavi je bila preučevana kemijska sestava obeh vrst iz Irana. Nadzemni deli vrste *D. kotschyi* so bili nabrani na gori Kamu v provinci Isfahan, nadzemni deli vrste *D. moldavica* pa v Sariju, provinci Mazandaran, v severnem Iranu, v sezoni 2014. Eterična olja so bila ekstrahirana po Clevengerjem postopku in analizirana z GC/MS. Celukupno so v nadzemnih delih vrst določili 32, oziroma 24 sestavin eterečnih olj za vrsti *D. kotschyi* in *D. moldavica*. Rezultati te raziskave so pokazali, da so glavne sestavine eteričnih olj pri vrsti *D. kotschyi* limonen (23.56 %), karvakrol (14.65 %), γ-terpinen (12.99 %), α -pinen (12.62 %), 2-metil-1-okten-3-ine (9.73 %), kamfene (4.66 %), mircen (3.65 %) in α -terpinene (3.12 %). Glavne sestavine eteričnega olja vrste *D. moldavica* so bile geranil acetat (36.62 %), geraniol (24.31 %), neral (16.25 %) in geranial (11.21 %). Vrsta *D. kotschyi* je eden izmed pomembnih virov limonene, vrsta *D. moldavica* pa geranil acetata.

Ključne besede: *Dracocephalum moldavica* L., *Dracocephalum kotschyi* Boiss., sestava eteričnih olj

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1 INTRODUCTION

Dracocephalum moldavica L. and *Dracocephalum kotschyi* Boiss. are aromatic plants belonging to Lamiaceae family. *D. moldavica* known as Moldavian balm or Moldavian dragonhead is an annual herb and it is native to central Asia and is naturalized in eastern and central Europe. There is 45 species of herbaceous and shrub's dragonhead in the world and there is 8 species of annual and perennial fragrant herb in Iran, from which three are endemic to Iran (Rechinger, 1986; Mozaffarian, 2008). *D. kotschyi* is an herbaceous plant, endemic in Iran and known as Badrandjboie-Dennaie and Zarrin-Giah. Its oil has been used in folk medicine as an antispasmodic agent. Aerial parts of *D. kotschyi* plants are sources of valuable flavonoids and essential oils (Fattahi et al., 2013). Chromosome number of 2n=2x=10 and 2n=2x=20 are for *D. moldavica* and *D. kotschyi*, respectively (Salehi et al., 2014). *Dracocephalum* is used in folk medicine as painkiller and for treatments of kidney complaints, against toothache and colds as well as antirheumatism, antitumor (Chachoyan and Oganesyan, 1996), antimutagens, antioxidant, antiseptic and stimulant properties (Kakasy et al., 2006; Dastmalchi et al., 2007). Recent findings indicated that some of the medicinal plant characteristics can be affected by genetic and ecological factors, including precipitation, temperature and plant competition. Since essential oils are the product of a predominantly biological process further studies are needed to evaluate if the reported characteristics of each population are maintained at the level of individual plants and along the breeding and selection program when grown under climatic conditions (Ghasemi Pirbalouti and Mohammadi, 2013). Maham et al., (2013) reported the major components of *D. moldavica* essential oil collected from Maragheh of East Azerbaijan province as follows: citral (31.14 %), 3,7- dimethyl -2,6 octadienal (21.43 %), cis-geraniol (17.08 %), neral (9.63 %) and neryl acetate (4.03 %). The major constituents of the essential oil of *D. moldavica* collected from North Iran were limonene (19.8 %), α-pinene (14.4 %), methyl geranate (8.5 %), geranyl acetate (7.9 %), carvacrol (7.8 %) and geranial (5.4 %) (Morteza-Semnani et al., 2007). In studies Saeidnia et al., (2014) reported the folowing main components of the oil of *D. kotschyi* collected from Iran: geranial (37.2 %), limonene-10-al (28.5 %), limonene (20.1 %) and 1,1-dimethoxy decane (14.5 %). Javidnia et al. (2005) reported the main components of the oil of *D. kotschyi* as α-pinene, caryophyllene oxide, terpinen-4-ol and germacrene. Golshani et al., (2004), and also Yaghmai and Tafazzoli (1988) reported citral, myrcene, β-caryophyllene and terpinyl acetate as the main constituents of *D. kotschyi* from northeast mountains. The aim of this study was to identify of the chemical components of *Dracocephalum moldavica* L and *Dracocephalum kotschyi* Boiss from Iran.

2 MATERIALS AND METHODS

2.1 Plant material

The aerial parts of the plant samples of *Dracocephalum kotschyi* Boiss were collected from Kamu Mountain, Isfahan province. Kamu is a city in Qamsar district, Kashan County, Isfahan province, in center Iran (33°, 36′ N and 51°, 14′ E) and the aerial parts of the plant samples of *Dracocephalum moldavica* L. were collected from Sari (Mazandaran province), North of Iran (36°, 39′ N, and 53°, 4′ E) , during 2014. The samples of the plants were identified by regional floras and authors with floristic and taxonomic references, and voucher specimens were deposited at the Herbarium of Agriculture Researches Islamic Azad University, Isfahan (Khorasgan), Iran.

2.2 Essential oil extraction

The fresh aerial part of *D. kotschyi* and *D. moldavica* were dried inside for six days at room temperature (25 ± 5 °C), and ground to fine powder using Moulinex food processor. The essential oil was extracted from 50 g of ground tissue in 1 L of water contained in a 2 L flask and heated by heating jacket at 100 °C for 3 h in a Clevenger–type apparatus, according to producers outlined British Pharmacopoeia. The collected essential oil

was dried over anhydrous sodium sulphate and stored at 4 °C until analyzed.

2.3 GC/MS analysis

Compositions of the essential oils were determined by GC–MS. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. HP-5MS column (30 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas with flow rate of 1.0 mL/min. The oven temperature was kept 20 °C at 50 °C for 4 min and programmed to 280 °C at a rate of 5 °C /min, and kept 20 °C constant at 280 °C for 5 min, at split mode. The injector temperature was at 20 °C at 280 °C. Transfer 20 line temperatures 280 °C. MS were taken at 70 eV. Mass range was from *m/z* 35 to 450. Identification of the essential oil components was accomplished based on comparison of retention times with those of authentic standards and by comparison of their mass spectral fragmentation patterns (Adams 2007).

3 RESULTS AND DISCUSSION

3.1 Compositions of the essential oils

Qualitative and quantitative analysis of the essential oils volatile profile are listed in Table 1. In total, 32 and 24 compounds were identified in the essential oil from the aerial parts *D. kotschyi* and *D. moldavica*, respectively. The results obtained in our study indicated that the major components in the oil *D. kotschyi* were limonene (23.56 %), carvacrol (14.65 %), γ-terpinene (12.99 %), α -pinene (12.62 %), 2-methyl-1-octen-3-yne (9.73 %), camphene (4.66 %), myrcene (3.65 %) and α -terpinene (3.12 %) (Figure 1). The major constituents of the oil *D. moldavica* were geranyl acetate (36.62 %), geraniol (24.31 %), neral (16.25 %) and geranial (11.21 %) (Figure 2).

Structural identification of a number of monoterpene synthases has indicated that they all have similar properties (like molecular mass, a divalent metal ion and neutral pH optimum requirements). Interestingly, a terpene synthase is able to form multiple products (Rajaonarivony et al., 1992; Bohlmann et al., 1998), the pinene synthases (from sage and grand fir) can catalyze the production of both α- and β-pinene (Bohlmann et al., 1997).

The biosynthesis of secondary metabolites, although controlled genetically, is strongly affected by the environmental influences of a particular growing region, and also by the agronomic conditions, harvesting time and the type of processing. In addition, for maximum oil production, long days and high light intensities are required during the maturation period (Thompson, 2003; Golparvar et al., 2015).

For example, Hashemian Ahmadi and Hadipanah (2014) reported that the highest oil content (0.065 %) of *D. moldavica* is obtained at the first sowing date (June 12) and the highest oil content (0.058 %) was obtained at the 30 cm planting density. Davazdahemami (2008) showed that of five major components neral, geraniol, geranial, neryl acetate and geranyl acetate in oil of *D. moldavica* were 92 % and 64 % in spring and summer sowing date and maximum change was seen in geranyl acetate from 35.3 % in spring to 14 % in summer. In studies of Omidbaigi (2010), the highest amount of geranyl acetate (50.10 %), geranial (25.27 %), neral (19.34 %) and geraniol (28.80 %), were obtained from the plants sown on 5th of May, 5th June and 20th of March, respectively. Alaei and Mahna (2013) showed that thirty six and twenty one components were identified from *D. moldavica* in field and greenhouse conditions, respectively. The major constituents of the oil of *D. moldavica* were found as geranyl acetate (46.72 %), geraniol (15.87 %), geranial (8.36 %), neral (5.8 %), cedroxyde (3.39 %), neryl acetate (2.57 %) and hinesol (2.39 %) (totally, 88.49 %) in field condition and geranyl acetate (39 %), geraniol (27.30 %), methyl citronellate (12.92 %) and neral (9.32 %) in greenhouse condition. Aziz et al. (2010) stated that the essential oil of the dragonhead plant grown in newly reclaimed land in Egypt was generally characterized by a high percentage of oxygenated compounds and the major constituents under all agricultural sulfur and ammonium sulfate treatments were geraniol (29.11–42.56 %), geranial (14.08–30.94 %), geranyl acetate (15.08–23.51 %) and neral (10.96–15.35 %).

In the study of Abd-El-Baky and El-Baroty (2007) on the dragonhead they found that 44 combination of essential oils was obtained which consist 97.18 % of essential oil and 90 % of them was combined with oxygenated monoterpenes and consisted less than one percent of the weight of the plant which include compounds such as: gerannyl acetate, neryl acetate, geranial, geraniol, neral, nerol, linalool.

Table 1: Chemical compositions of essential oils of *Dracocephalum moldavica* L. and *Dracocephalum kotschyi* Boiss

NO	Compound	RI	*D. kotschyi* %	*D. moldavica* %
1	α-thujene	923	1.71	-
2	α - pinene	928	12.62	-
3	Camphene	944	4.66	-
4	Sabinene	974	1.83	0.42
5	β-pinene	978	-	0.86
6	Myrcene	992	3.65	0.04
7	α -phellandrene	1008	0.31	-
8	α -Terpinene	1016	3.12	-
9	*p*-Cymene	1021	-	0.92
10	limonene	1035	23.56	1.35
11	Cis-β-ocimene	1041	1.03	-
12	γ-Terpinene	1056	12.99	0.17
13	Trans-sabinene hydrate	1066	0.81	-
14	Linalool oxide	1076	-	0.64
15	α -Terpinolene	1091	0.26	-
16	Linalool	1101	-	0.81
17	Cis-sabinene hydrate	1104	0.21	-
18	E,Z-alloocimene	1137	0.57	-
19	Camphor	1149	0.22	-
20	Cis chrysanthenol	1162	-	0.48
21	Borneol	1170	2.22	-
22	R-terpinen-4-ol	1180	0.44	-
23	α -Terpineol	1193	0.13	0.36
24	3-Methylene-1,5,5-trimethylcyclohexene	1198	0.21	-
25	2-Methyl-1-octen-3-yne	1206	9.73	-
26	Nerol	1221	-	0.35
27	Neral	1236	0.83	16.25
28	Carvone	1241	-	1.14
29	Geraniol	1257	0.81	24.31
30	Geranial	1270	-	11.21
31	Bornyl acetate	1287	0.41	-
32	Thymol	1300	0.23	1.41
33	Carvacrol	1311	14.65	-
34	Neryl acetate	1360	-	0.91
35	Geranyl acetate	1379	-	36.62
36	α -copaene	1378	0.54	0.12
37	β-bourbonene	1387	0.61	-
38	Beta- elemene	1392	-	0.15
39	2-Cyclohexen-1-ol, 2-methyl-5-(1-m ethylethenyl)	1415	0.14	-
40	β-caryophyllene	1422	0.95	0.51
41	γ -muurolene	1479	0.11	-
42	Germacrene-D	1482	-	0.47
43	γ -cadinene	1517	0.21	-
44	delta-cadinene	1526	0.17	-
45	Caryophyllene oxide	1580	-	0.17
46	Viridiflorol	1590	-	0.02
	Total		99.9	99.6

RI: Retention indices determined on HP-5MS capillary column.

Terpene synthases have been cloned from different species and also the phylogenetic distances among them have been well documented. *D. moldavica* contains 0.06–0.92 % essential oil, with the maximal level during flowering. Its lemon-like scented essential oil consists mainly of oxygenated acyclic monoterpenes, geraniol, geranyl acetate, geranial, neral and nerylacetate (Kakasy et al., 2006).

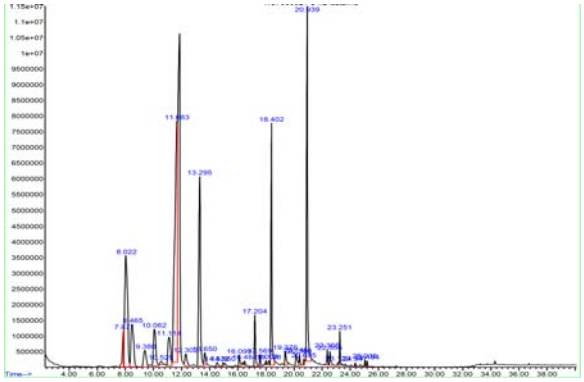

Figure 1: The chromatograms found in essential oils of *D. kotschyi.*

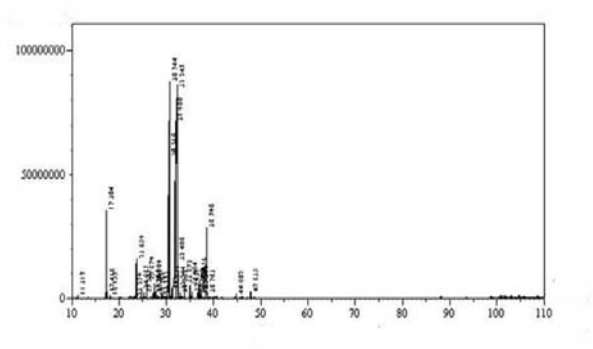

Figure 2: The chromatograms found in essential oils of *D. moldavica*.

Chu et al., (2011) reported that the major constituents of the flowering aerial parts essential oil of *D. moldavica* from Beijing, China were 1,8-cineol (31.25 %) and 4-terpineol (22.82 %), cumin alcohol (4.29 %), α-terpineol (4.21 %) and sabinene (3.62 %). Also in Egypt, Aziz and El-Sherbeny (2003) observed that the essential oil of *D. moldavica* was characterized by a high percentage of oxygenated monoterpenes (81.84 %–96.05 %), with the major compounds being geranial (22.82 %–55.83 %), geranyl acetate (9.75 %–31.48 %), neral (16.08 %–22.02 %) and geraniol (0.42 %–16.59 %). In China, *D. moldavica* essential oil from Xinjiang autonomous region contained citral (31.43 %), n-hexadecanoic acid (16.48 %), and geraniol ester (9.02 %) (Tian et al., 2009). The major constituents of the oil of *D. moldavica* extracted by hydro distillation were found to be geranyl acetate, geranial, neryl acetate, geraniol, neral and nerol (Li, 2001). Hawthorne et al., (1993) identified geranyl acetate (65.8 %), carvacrol (14.9 %) and thymol (7 %) as the major components of the oil of *D. moldavica*. But Shatar and Altantseg (2000) introduced linalool (67 %) and carvone (5.9 %) as the main components of the oil of *D. moldavica*.

4 CONCLUSION

In conclusion, the results obtained in our study indicated that the major components of oil of *D. kotschyi* were limonene, carvacrol, γ-terpinene and α –pinene. The major components of oil of *D. moldavica* were geranyl acetate, geraniol, neral and geranial. A comparison of our results with different reports ibndicates that differences in the volatile oil composition of the plants could be attributed to genetic (genus, species, and ecotype), chemotype, distinct environmental and climatic conditions, seasonal sampling periods, geographic origins, plant populations, vegetative plant phases, and extraction and quantification methods.

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Agris category code: F40, f60, f62

Effect of foliar-applied silicon on photochemistry, antioxidant capacity and growth in maize plants subjected to chilling stress

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ABSTRACT

Low temperature is one of the major adverse climatic factors that suppress plant growth and sustainable agricultural development. In these climate conditions, silicon (Si) can mitigate various abiotic stresses including low temperature. In this study, the roles of foliar-applied silicon (10 mM potassium metasilicate) in enhancing tolerance to chilling stress were investigated in maize (*Zea mays* 'Fajr') plants. The low temperature stress caused significant reduction of plant growth and relative water content; however, Si ameliorated these effects. Si supply in maize exhibited a significantly positive effect on accumulation of free amino acids, and reduced the necrotic leaf area. The decrease in maximum quantum yield of PSII (F_v/F_m) was reversible during recovery, but not in the non-Si-treated leaves. This can be explained by enhancement of protective pigments; carotenoid and anthocyanin leading to the protection of PSII from damage. Additionally, analysis of OJIP transients revealed that Si reduced cold damaging effect on performance index (PI_{abs}) and F_v/F_m through improvement of excitation energy trapping (TR_0/CS) and electron transport (ET_0/CS) per excited cross-section of leaf. The malondialdehyde (MDA) concentration, which was significantly increased under chilling stress, was decreased by Si. The reduced glutathione and ascorbate concentrations were higher in Si-treated plants as compared to those without application of Si under chilling stress. These results indicated that Si could enhance the chilling stress tolerance of maize plants through improving the biomass accumulation, maintaining a high level of glutathione, ascorbic acid, protein, protective pigments, and enhancing the photochemical reactions. This study also suggests that the foliar-applied Si increases recovery ability from chilling injury.

Key words: chilling stress, lipid peroxidation, non-photochemical quenching, silicon, *Zea mays*

IZVLEČEK

UČINEK FOLIARNEGA DODAJANJA SILICIJA NA FOTOKEMIČNO IN ANTIOKSIDACIJSKO UČINKOVITOST TER RAST KORUZE V RAZMERAH HLADNEGA STRESA

Nizka temperatura je eden izmed glavnih neugodnih klimatskih dejavnikov, ki zavira rast rastlin in trajnostni razvoj kmetijstva.V takšnih klimatskih razmerah lahko silicij oblaži abiotski stress vključno z učinki nizke temperature. V tej raziskavi je bila preučevana vloga foliarnega dodajanja Si (10 mM kalijevega metasilikata) pri povečevanju odpornosti koruze (*Zea mays* 'Fajr') na hladni stres. Stres zaradi nizkih temperature je značilno zmanjšal rast in vsebnost vode v rastlinah, kar je dodajanje Si oblažilo. Dodatek silicija je sprožil v koruzi značilne pozitivne učinke v kopičenju prostih amino kislin in v zmanjšanju nekrotičnosti listov. Zmanjšanje v fotokemični učinkovitosti PS II (F_v/F_m) je bilo povratno med okrevanjem, vendar ne pri rastlinah, ki niso bile tretirane s silicijem. To bi lahko razložili s povečanjem vsebnosti zaščitnih pigmentov karotenoidov in antocianinov, kar vodi v zaščito PSII pred poškodbami. Dodatno so analize prehodne fluorescence klorofila a (OJIP) odkrile, da je dodatek Si zmanjšal učinek poškodb zaradi hlada na fotosintetski elektronski transport (PI_{abs} in F_v/F_m) preko boljšega prestrezanja ekscitacijske energije (TR_0/CS) in boljšega elektronskega transporta (ET_0/CS) na presek ekscitiranega lista. Hladni stres je povzročil poškodbe membran, kar se je odrazilo v povečani koncentraciji malondialdehida. V rastlinah tretiranih s Si koncentracija malondialdehide ni dosegla ravni rastlin, izpostavljenih hladnem stresu. Koncentracije reduciranega glutationa in askorbata so bile večje v rastlinah, tretiranih s Si v razmerah hladnega stresa v primerjavi s tistimi, ki s silicijem niso bile tretirane. Ti rezultati nakazujejo, da bi lahko silicij povečal odpornost na hladni stres pri koruzi z izboljšanjem prirastka biomase, vzdrževanjem visoke ravni glutationa, askorbinske kisline, beljakovin, zaščitnih pigmentov in v povečevanju fotokemičnih reakcij. Raziskava nakazuje, da foliarno dodajanje silicija povečuje sposobnost okrevanja iz od hlada nastalih poškodb.

Ključne besede: hladni stres, peroksidacija lipidov, nefotokemična pretvorba svetlobe, silicij, *Zea mays*

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1 INTRODUCTION

Because of global climate change, we can expect increased damage to plants, such as increased spring frost. Cold stress includes chilling (< 20 °C) and/or freezing (<0 °C) temperatures, and negatively affects the growth and development of plants (Waśkiewicz *et al*., 2014). Exposure to cold increases the production of reactive oxygen species (ROS) (Zhang *et al*., 2011), resulting in cytotoxic conditions that affects plant metabolism by stimulating oxidative damage to lipid, proteins, and nucleic acid (Suzuki *et al*., 2012).

To alleviate the effect of cold stress, plants adapt various approaches for their survival. Proper plant nutrition is one of the strategies in mitigating the stress induced damage in plants (Habibi, 2014a). Si is the second most common element in the lithosphere after oxygen and has been proved to be beneficial for the healthy growth and development of many plant species; particularly Gramineae plants (Broadley *et al*., 2011). Si application to crops has been reported to enhance their tolerance of multiple stresses (Guntzer, 2011), including pests and pathogens (Dallagnol *et al*., 2012), salt and water stress (Liu *et al*., 2014). However, the effects of Si on plant resistance to cold stress and the underlying mechanisms have not been well identified (Liang *et al*., 2008). It has been reported that the protective role of Si in plants exposed to cold-stress conditions in most cases has been attributed to increase water use efficiency and antioxidant activity in winter wheat (Liang *et al*., 2008) and cucumber leaves (Liu *et al*., 2009). In previous work, we concluded that supplementation of water-deficient pistachio (Habibi and Hajiboland, 2013) and canola (Habibi, 2014b) plants with Si alleviates the adverse effects of drought due to its enhancement of photochemical efficiency and photosynthetic gas exchange, as well as an activation of the antioxidant defense capacity in these plants.

The maize (*Zea mays*) is one of the most important crops, and adaptation of this plant to early annual planting dates requires improvement of chilling tolerance (Battal et al. 2008). One of the major problems arising in some maize cultivation areas includes different levels of injuries caused by lower temperatures in early spring. Because of the fact that the yield of maize was reduced due to chilling damage, the understanding of the physiological and biochemical mechanisms improving chilling tolerance of this species is very significant. Alleviating this growth suppression requires a further improvement of the maize chilling stress tolerance. There is no information about the physiological responses of the maize to Si under chilling stress, which may increase cold tolerance. In this work, we investigated photosynthetic and chlorophyll fluorescence parameters in chilling shocked maize plants, in order to determine the mechanisms of chilling tolerance and survival ability after chilling exposure.

2 MATERIALS AND METHODS

2.1 Plant growth and treatments

Seeds of maize (*Zea mays*'Fajr') were sown in top of the cylindrical plastic pots; four seeds were planted in each pot. 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 every 5 days to maintain at 90 % field capacity (FC). 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 25-28 ºC /17-19 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. Four weeks after sowing, half of the plants were sprayed with 10 mM Si (as potassium metasilicate, pH adjusted to 5.8). The volume of the spray was 400 ml per pot. A drop of Tween 20 (0.05 %, v/v) as surfactant was added to 500 ml of the spray solutions. Control plants were sprayed with Tween 20 and equimolar concentrations of KCl for balancing K amounts. Ten 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 (3±1°C) light (at 300 µmol m^{-2} s^{-1} photosynthetic photon flux)/12 h (2±1°C) dark cycle at 65 % relative humidity for 2 days. After the chilling treatment, all plants were returned to normal conditions as described above, to allow leaves to recover from 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 harvested 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 Habibi and Hajiboland (2012). The percentage of necrotic area was determined by measuring separately green and necrotic leaf area according to Irigoyen *et al.* (1996).

2.3 Measurements of chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters were recorded using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK) for both dark adapted and light adapted leaves. Leaves were acclimated to dark for 30 min using leaf clips before measurements were taken. Initial (F_0), maximum (F_m), variable ($F_v = F_m – F_0$) fluorescence as well as maximum quantum yield of PSII (F_v/F_m) were recorded. Light adapted leaves were used for measurement of steady-state (F_s) and maximum (F'_m) fluorescence. Calculations were made for F'_0 ($F'_0 = F_0/[(F_v/F_m) + (F_0/F'_m)]$), photochemical quenching, q_P $[(F'_m – F_s)/(F'_m – F'_0)]$ and non-photochemical quenching, NPQ $(F_m – F'_m)/F'_m$ (Krall and Edwards, 1992).

2.4 Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence transients (OJIP transients) were measured with a Plant Efficiency Analyser (PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 32 1JL, England) in dark-adapted (for at least 20 min) leaves. The OJIP transients were induced by a red light (peak at 627 nm) of 3500 µmol m^{-2} s^{-1} (sufficient excitation intensity to ensure closure of all PSII reaction centers to obtain a true fluorescence intensity of Fm) provided by the PEA through an array of six light-emitting diodes.

2.5 The JIP-test (The analysis of the fluorescence rise O-J-I-P)

The JIP-test (Strasser and Strasser, 1995; Strasser *et al.*, 2004) was used to analyse each OJIP transient. The following data from the original fluorescence measurements were used: maximal fluorescence intensity (F_m); fluorescence intensity at 50 µs (considered as F_0); the specific energy fluxes (per reaction center) for absorption (ABS/RC), trapping (TR_0/RC), dissipation at the level of the antenna chlorophylls (DI_0/RC) and electron transport (ET_0/RC); the flux ratios or yields, i.e. the maximum quantum yield of primary photochemistry ($\varphi_{Po}=TR_0/ABS=F_v/F_m$), the efficiency ($\psi_0=ET0/TR_0$) with which a trapped exciton can move an electron into the electron transport chain further than *Q*A, the quantum yield of electron transport ($\varphi_{Eo}=ET_0/ABS$); the phenomenological energy fluxes (per excited cross-section of leaf, CS) for absorption (ABS/CS), trapping (TR_0/CS), dissipation (DI_0/CS) and electron transport (ET_0/CS). The fraction of active PSII reaction centeres per excited cross-section (RC/CS) is also determined. In addition to above parameters, a multi-parametric expression, the performance index (PI_{abs}), is also calculated (Strasser et al., 2000). The PI_{abs} regards the three main steps that regulate photosynthetic activity by a PSII reaction centre (RC) complex, namely absorption of light energy (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport (ET).

2.6 Determination of total chlorophyll, anthocyanin, Si content and total free amino acids

Leaf concentration of total chlorophyll and carotenoid was determined after extraction of pigments in the cold acetone and allowing the samples to stand for 24 h in the dark at 4 ºC (Lichtenthaler and Wellburn, 1985). Determination of anthocyanin contents was carried out using the method of Wagner (1979). To calculate the amount of anthocyanins, the extinction coefficient 33,000 mol^{-1} cm^{-1} was used and anthocyanin content were expressed as µmol g^{-1} FM. Leaves were prepared for determination of Si (Jaiswal, 2004) using Inductively-Coupled Plasma-Atomic Emission

Spectrometry (ICP-AES, INTEGRA XL2, GBC Australia). Content of total free α-amino acids was assayed using ninhydrin colorimetric method. Glycine was used for production of standard curve (Hwang and Ederer, 1975). Soluble protein was estimated spectrophotometrically by the Bradford method (1976).

2.7 Determination of antioxidants and malondialdehyde

Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid according to methods described elsewhere (Habibi and Hajiboland, 2012). The level of glutathione (GSH) was determined according to Singh *et al*. (2006) with few modifications. Samples of 0.5 g were homogenized in 6 % *m*-phosphoric acid (pH 2.8) containing 1 mM EDTA. Two solutions were then prepared. Solution A consisted of 110 mM $Na_2PO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 15 mM EDTA, 0.3 mM 5, 5'–dithiobis (2-nitrobenzoic acid) and 0.4 ml l^{-1} BSA (final pH 7). Solution B consisted of 1 mM EDTA, 50 mM imidazole, 0.2 ml l^{-1} BSA and an equivalent of 1.5 units GR activity (Sigma). The absorbance at 412 nm was read after 2 min. The GSH concentration was determined from a standard curve by preparing solutions of 0.5–16 mM GSH. Levels of AsA followed the procedure described by Singh *et al*. (2006) with few modifications. Briefly, fresh leaf sample of a known weight (1 g) was extracted with 10 ml of 5 % (v/v) *m*-phosphoric acid and centrifuged at 12,000 × g for 15 min. AsA was determined in a reaction mixture consisting of 0.2 ml of supernatant, 0.5 ml of 150 mM phosphate buffer (pH 7.4, containing 5 mM EDTA) and 0.2 ml of deionized water. Colour was developed in reaction mixtures with the addition of 0.4 ml of 10 % (w/v) TCA, 0.4 ml of 44 % (v/v) phosphoric acid, 0.4 ml of α, α-dipyridyl in 70 % (v/v) ethanol and 0.2 ml of 3 % (w/v) $FeCl_3$. The reaction mixtures were incubated at 40 °C for 40 min and quantified spectrophotometrically at 525 nm. Ascorbate standards were between 1 and 50 mmol ascorbate in 5 % (v/v) *m*-phosphoric acid.

2.8 Statistical analysis

Experiment was performed according to a factorial design on the basis of Completely Randomize Design (CRD) with 10 pots as 10 independent replications. Statistical analyses were carried out using Sigma Stat (3.5) with Fisher LSD test ($P < 0.05$).

3 RESULTS

In the absence of chilling stress, Si had no effect on the growth of maize seedlings (Table 1). A significant loss of FM was observed in maize plants under cold stress, i.e., in 96 h after recovery. However, the decrease extent in the Si treatment was less than that in the non-Si treatment. Chilling stress caused significant reduction of RWC, although Si application ameliorated this effect and decreased significantly damaging effects of cold on RWC. Reduction of RWC under chilling stress was alleviated by Si application, accompanied by an increase in FM (Table 1). Chilling stress dramatically increased the necrotic leaf area, while Si supplementation significantly decreased it. As shown in Table 1, cold alone increased necrotic leaf area by 8.6 % after treatment for 96 h recovery, but the increase was only 2.2 % when Si was applied. Concentration of Chl*a* and *b* were significantly decreased when the plants were exposed to cold shock in comparison with the non-stressed plants. Si-supplied plants showed the higher anthocyanin and carotenoid concentration as compared with those without application of Si under chilling-stress conditions. The concentration of soluble sugars in the leaves was increased by chilling stress (Table 1). The concentration of starch decreased significantly after 96 h of chilling stress. No significant increase of starch was found by Si application under normal temperature. Under chilling-stress conditions, plants showed an increase in amino acid concentration in the leaves when treated with Si while this change for protein content was negligible. Si supplementation dramatically increased the leaf Si concentration, but the Si concentration was not affected by chilling stress during all treatment periods.

Table 1: Effect of Si supplementation on the shoot fresh mass (SFM), shoot dry mass (SDM), relative water content (RWC), necrotic leaf area, and the concentration of chlorophyll *a* and *b*, carotenoid, anthocyanin, soluble sugars, starch, total free α-amino acids, protein and Si in maize plants grown with or without Si under chilling-stress conditions

	Non-stressed		Chilling-stressed	
	−Si	+Si	−Si	+Si
SFM (g plant^{-1})	13.9±1.22 ^{a}	14.1±1.36 ^{a}	10.6±0.84 ^{c}	12.1±1.12 ^{b}
SDM (g plant^{-1})	2.87±0.36 ^{a}	2.94±0.44 ^{a}	2.43±0.47 ^{a}	2.66±0.83 ^{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}	8.60±1.30 ^{a}	2.20±0.82 ^{b}
Chl *a* (mg g^{-1} FM)	4.10±0.72 ^{a}	3.90±0.47 ^{a}	3.04±0.26 ^{b}	2.97±0.32 ^{b}
Chl *b* (mg g^{-1} FM)	1.78±0.32 ^{ab}	2.46±0.57 ^{a}	1.06±0.22 ^{b}	1.18±0.39 ^{b}
Carotenoid (mg g^{-1} FM)	0.98±0.33 ^{b}	1.16±0.87 ^{b}	1.21±0.41 ^{b}	2.35±0.72 ^{a}
Anthocyanin (μmol g^{-1} FM)	4.78±0.78 ^{b}	5.46±0.57 ^{b}	6.06±1.00 ^{b}	7.98±1.07 ^{a}
Soluble sugars (mg g^{-1} FM)	12.3±2.6 ^{c}	14.2±3.35 ^{bc}	18.0±2.65 ^{ab}	20.7±3.12 ^{a}
Starch (mg g^{-1} FM)	164±17.6 ^{a}	172±19.6 ^{a}	121±15.6 ^{b}	118±21.6 ^{b}
Amino acids (μmol g^{-1} FM)	3.45±0.65 ^{bc}	3.02±1.04 ^{c}	5.00±0.89 ^{b}	6.80±1.67 ^{a}
Protein (mg g^{-1} FM)	10.7±2.11 ^{a}	11.4±2.01 ^{a}	7.22±1.27 ^{b}	8.98±1.99 ^{ab}
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}

Samples were taken 96 h after recovery after cold treatment. Data of each row indicated by the same letters are not significantly different ($P < 0.05$).
Data are the mean ± SD (n = 10).

Si supplementation had no effect on leaf F_v/F_m ratio under the normal condition, but it was decreased by cold treatment after 2 h recovery in the chilling-stressed leaves (Fig. 1*a*). However, Si quickly and significantly increased the F_v/F_m ratio after 2, 46 and 96 h recovery after cold treatment. The photochemical quenching (q_P) of non-Si-supplemented plants showed little change in response to chilling treatment, and Si-supplemented plants showed a marked increase after 2 h recovery. In contrast, the non-photochemical quenching (NPQ) of the maize plants increased significantly after chilling, and the most marked increase in NPQ was occurred for Si-supplemented leaves during 2 h after chilling stress. During recovery, NPQ gradually reduced in the Si-supplemented leaves, but not in the non-Si-treated leaves.

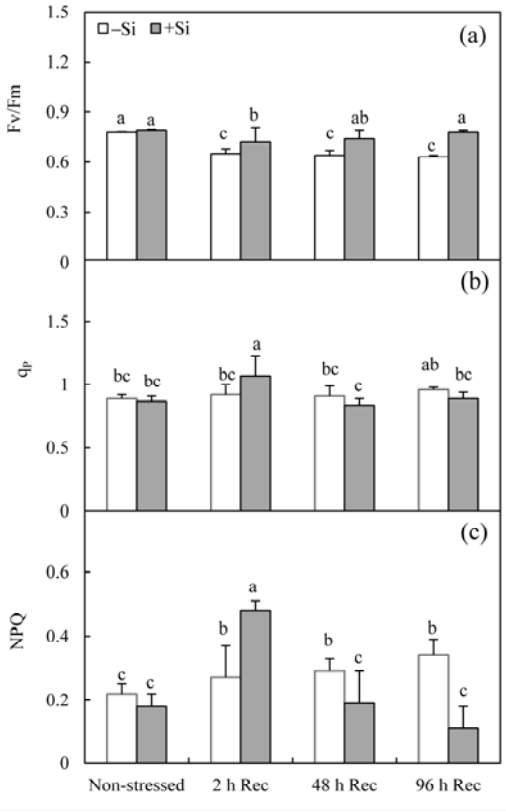

Figure 1: Changes in the maximum quantum yield of PSII (F_v/F_m) (a), photochemical quenching (q_P) (b) and non-photochemical quenching (NPQ) (c) in maize plants grown with or without Si under chilling-stress conditions. Samples were taken 2, 46 and 96 h after recovery (Rec) after cold treatment. Symbols with error bars are the mean ± SD (n = 10), and significant differences ($P < 0.05$) are indicated by different letters.

To understand the precise effects of Si on the kinetics of recorded OJIP transients, data collected at water stress periods were analysed in Fig. 2. The effects of drought stress on the maximum quantum yield of primary photochemistry (F_v/F_m) and the specific and phenomenological energy fluxes for light absorption, excitation energy trapping and electron transport are also showed in the form of a radar plot (Fig. 2). Chilling stress resulted in the deactivation of reaction centers (RC/CS) and decreased excitation energy trapping (TR_0/CS) and electron transport (ET_0/CS). Non-Si plants had performance indexes (PI_{abs}) of 2-3, however, in Si-supplemented leaves, the PI_{abs} values were respectively 200 % higher than those recorded in non-Si-supplemented leaves (Fig. 2). Si-supplemented plants showed higher PI_{abs} with compared to those without application of Si under cold conditions.

Chilling-stressed plants displayed an increase in lipid peroxidation (Fig. 2) determined by the accumulation of MDA. However, the MDA level was decreased by foliar application of Si under chilling-stress conditions. Under chilling-stress conditions, plants were able to maintain higher GSH and AsA levels, as compared with non-stressed plants, but consistent with the decrease in lipid peroxidation seen in Si-supplied plants during stress, the levels of GSH showed an increment with a concomitant increase in AsA content (Fig. 2).

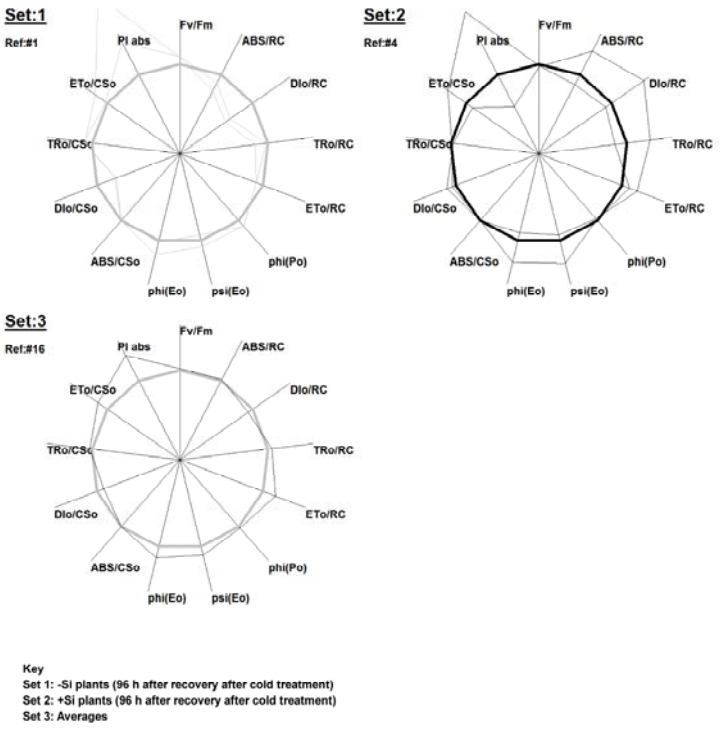

Figure 2: Radar plots depicting changes in the phenomenological (per CS) and specific (per RC) energy fluxes of absorption (ABS) excitation energy trapping (TR) and electron transport (ET). The changes in quantum efficiency (F_v/F_m) and the performance indexes (PI_{abs}) are also shown.

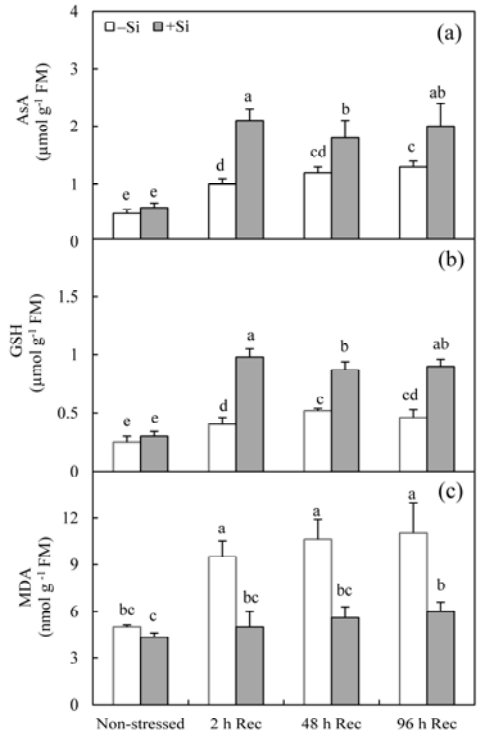

Figure 3: Changes in the concentrations of reduced ascorbate (AsA) (a) and glutathione (GSH) (b), and malondialdehyde (MDA) (c) in maize plants grown with or without Si under chilling-stress conditions. Samples were taken 2, 46 and 96 h after recovery (Rec) after cold treatment. Symbols with error bars are the mean ± SD (n = 10), and significant differences ($P < 0.05$) are indicated by different letters.

4 DISCUSSION

Our results showed that chilling-induced growth inhibition in maize seedlings was partly reversed by Si supplementation (Table 1). These results are in agreement with previous reports regarding the beneficial effects of Si on the growth and yield of *Sorghum bicolor* (L.) Moench under salt stress (Yin *et al*., 2013) and wheat cultivars under cold-stress (Liang *et al*., 2008) conditions. Chilling-sensitive plants exposed to low temperature often exhibit 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). We have already shown that the RWC in the non-Si-treated plants was significantly reduced under chilling stress. In this study, addition of Si helped the plants to maintain a high RWC, and dry matter production. 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 (Cooke and Leishman, 2011; Sonobe *et al*., 2011). Leaf necrosis is a typical external sign of chilling injury in chilling-sensitive plants. In this study, pre-Si treatment reduced the leaf necrosis under chilling stress conditions.

In the present study, though an expected enhancement in free amino acid level under chilling stress, Si application caused a significant stimulation in free amino acid level. Accumulation of these metabolites can function as osmolytes to preserve cell turgor and have the ability to protect membranes from stress damage (Krasensky and Jonak, 2012).

F_v/F_m is an indication of overall photosynthetic capacity (Balouchi, 2010). Significant reduction of F_v/F_m in chilling-stressed maize indicated that a proportion of PSII reaction centers is damaged or inactivated following photoinhibition, commonly observed in plants under stress (Baker and Rosenqvist, 2004). In the present study, there was a remarkable decrease in F_v/F_m ratio of Si-treated leaves after chilling treatment and rapid increase during recovery, indicating that the capacity of electron transport was inhibited by chilling stress, and the damage in chlorophyll reaction center was reversible, but not in the non-Si-treated leaves. Both Si and non-Si treatments showed a significant increase in NPQ just after cold shock, but then declined in Si-treated leaves during recovery, indicating that NPQ capacity of photosynthetic apparatus is changeable over different environments. It may be also suggested that the ability to cope with excess energy and photoinhibition was much improved in Si-treated plants. Based on the current results, it can be concluded that Si application not directly enhance chilling tolerance of maize plants, but it increased recovery ability from chilling injury. Plants have evolved a variety of protective mechanisms against the stress induced damage to cellular components, such as the dissipation of excess excitation energy and the synthesis of protective pigments, such as carotenoids and anthocyanins (Marczak *et al*., 2008; Huang *et al*., 2010). In this study, Si application caused a significant stimulation in these pigments under chilling stress. This finding is consistent with other published reports suggesting that the accumulation of carotenoids and anthocyanins is generally well correlated with chilling tolerance (Marczak *et al*., 2008).

The results presented that the decrease in the PI_{abs} and down-regulation of photochemical activity during chilling stress conditions may be interpreted as evidence for PSII RC deactivation (Ivanov *et al*., 2006), and the PI_{abs} was much more sensitive than the F_v/F_m ratio. These results indicated that PSII RC's are functionally altered by Si application through increase in density of active reaction centers, RC/CS.

The magnitude of oxidative damage is usually measured by MDA (an end product of membrane lipid peroxidation), as a marker for the ROS-mediated cell membrane damage (Liu *et al*., 2009). In the present work, chilling stress caused membrane damage, as assessed by lipid peroxidation. However, Si could enhance antioxidant defense activity in maize plants under chilling stress, resulting in decreased membrane oxidative damage, and improved stability of cell membranes and enhanced stress tolerance. Fu *et al*. (2014) reported a consistent increase in reduced glutathione and ascorbate in *Elymus nutans* Griseb.in response to an increase in the intensity and duration of chilling stress. In agreement with

the above, in our study, the contents of total GSH and AsA increased under chilling stress. GSH and AsA concentrations were higher in Si-treated plants under chilling stress compared with –Si ones. Our results suggest that improvement of maize tolerance to chilling stress by Si supplementation may be achieved by maintaining a relative high content of GSH and AsA as well as activation of antioxidant defense capacity in cold-stressed plants.

In conclusion, results from this study showed that the foliar application of Si alleviated effects of chilling on plant growth. In the present study, there was a remarkable decrease in F_v/F_m ratio of Si-treated leaves after cold treatment and rapid increase during recovery, indicating that the damage in chlorophyll reaction center was reversible, but not in the non-Si-treated leaves. This can be explained by enhancement of efficiency for dissipation of excess photon energy in the PSII antenna, determined as non-photochemical quenching as well as accumulation of protective pigments, such as carotenoid and anthocyanin leading to the protection of PSII from photo-damage. Our results suggest that improvement of maize tolerance to chilling stress by Si supplementation is achieved by maintaining a relative high content of antioxidants and photochemical reactions.

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Influences of various factors on hairy root induction in *Agastache foeniculum* (Pursh) Kuntze

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ABSTRACT

Agrobacterium rhizogenes is known as a natural tool of genetic engineering in many plant species. For the first time, hairy root induction in *Agastache foeniculum* using *A. rhizogenes*, rosmarinic acid content and the effect of different culture media and inoculation methods on hairy root growth rate were investigated. Hairy root culture of *A. foeniculum* was established by inoculation of the 1-month-old leaf explant with A4 strain of *A. rhizogenes* and the effectiveness of light – dark conditions and two inoculation methods (immersion and injection) were tested. Furthermore, in immersion method, the effects of inoculation time (3, 5 and 7 min) on root induction were investigated. In the second part of the study, the hairy root culture of *A. foeniculum* was studied using different basal culture media (MS, 1/2 MS and B5). Rosmarinic acid content in hairy roots and non- transformed roots was analyzed using high-performance liquid chromatography (HPLC). There was no significant difference between various inoculation methods in the ability of hairy roots induction. Observations showed that percentage of hairy root induction was higher when the explants were immersed for 5 min in bacterial suspension. Light conditions displayed the highest hairy root induction rates compared with dark condition. Various culture media are different in terms of types and amounts of nutrients and have influence on growth rate. The maximum growth rate (1.61 g fr wt/50 ml) of hairy roots were obtained in 1/2 MS medium. Rosmarinic acid content in transformed roots (213.42 µg/g dry wt) was significantly higher than non-transformed roots (52.28 µg/ g dry wt).

Key words: *Agastache foeniculum*, *Agrobacterium rhizogenes*, culture medium, hairy roots, immersion, injection, rosmarinic acid

IZVLEČEK

VPLIV RAZLIČNIH DEJAVNIKOV NA INDUKCIJO LASASTIH KORENIN PRI JANEŽNEM OŽEPU (*Agastache foeniculum* (Pursh) Kuntze)

Bakterija *Agrobacterium rhizogenes* je znana kot naravno orodje genskega inženiringa pri mnogih rastlinskih vrstah. V tej raziskavi so prvič preučevali indukcijo lasastih korenin pri janežnem ožepu (*Agastache foeniculum* (Pursh.) Kuntze**)** z uporabo bakterije *A. rhizogenes*, vsebnost rožmarinske kisline in vpliv različnih gojišč ter inokulacijskih metod na rast lasastih korenin. Kulturo lasastih korenin janežnega ožepa so vzgojili iz izsečkov enomesečnih listov inokuliranih z bakterijo *A. rhizogenes* sev A4 z uporabo dveh inokulacijskih metod – potapljanje in vbrizgavanje, v razmerah na svetlem in v temi. Pri metodi potapljanja izsečkov v bakterijsko suspenzijo so preučevali tudi vpliv časa (3, 5 in 7 min) na indukcijo korenin. V drugem delu raziskave so preučevali vpliv različnih osnovnih gojišč (MS, 1/2 MS in B5) na kulturo lasastih korenin. Vsebnost rožmarinske kisline v transformiranih lasastih koreninah in netransformiranih koreninah so analizirali s HPLC metodo. Značilna razlika v indukciji lasastih korenin v odvisnosti od metod inokulacije ni bila ugotovljena. Opazovanja so pokazala, da je bil odstotek indukcije lasastih korenin večji, če so izsečke listov potopili v bakterijsko suspenzijo za pet minut. Indukcija lasastih korenin je bila največja v razmerah gojitve na svetlobi v primerjavi z gojenjem v temi. Različna gojišča so se razlikovala v vrsti in količini hranil in so vplivala na rast korenin. Največja rast lasastih korenin (1.61 g sveže mase/50 ml) je bila dobljena pri polovični koncentraciji osnovnega gojišča (1/2 MS). Vsebnost rožmarinske kisline je bila v transformiranih koreninah značilno večja (213.42 µg/g suhe mase) kot v netransformiranih (52.28 µg/ g suhe mase).

Ključne besede: *Agastache foeniculum*, *Agrobacterium rhizogenes*, gojišče, lasaste korenine, potapljanje, vbrizgavanje, rožmarinska kislina

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1 INTRODUCTION

Anise hyssop (*Agastache foeniculum* (Pursh) Kuntze) is a perennial herbaceous plant belonging to Labiatae family (Omidbaigi and Mahmoodi, 2010). This plant is native to the United States and Canada (Mallavarapu et al., 2004) and is grown in all regions of the Mediterranean, in Northen and Central Europe (Omidbaigi et al., 2008). Clinical trials have shown that anise hyssop extract is effective in treatment of heart, lung, cough diseases and induction sweating to reduce fevers (Mallavarapu et al., 2004). Essential oils of this plant have anti-bacterial and anti- fungal properties. Anise hyssop has many useful constituents, including monoterpenes and phenyl propanoids. Among these constituents, rosmarinic acid, chlorogenic acid, rutin, apigenin and galangin are important. The antioxidant activity of *A. foeniculum* (98.6 mg.g dry plant) depends on the total content of polyphenols (Matei, 2012).

Many valuable medicinal compounds are found in plant roots; root systems play an important role in determining the temporal and spatial patterns of the activity and synthesis of macromolecules: it has been revealed that in many species of Solanaceae alkaloids are synthesized in plant roots and are transported through vascular tissues to shoots where they accumulate or are converted to other compounds (Hector et al., 1999).

In vitro conditions provide the environmental control and the possibility for addition of precursors needed to increase yields and production of specific secondary metabolites (Hu and Du, 2006). However, low yields and reproducibility of the cultures are the limiting factor in this context so these limitations have led to the development of new techniques in tissue culture, one of them is hairy root culture (Hu and Du, 2006). Rapid growth, low doubling time, easy maintenance and the ability to synthesize a range of chemical compounds in hairy roots are including the advantages that have made them a permanent source of valuable secondary metabolites production (Hasanloo et al., 2008). *Agrobacterium rhizogenes* (Riker et al., 1930) Conn. 1942 is a gram – negative soil bacterium, which is known as a natural tool of genetic engineering in many plant species, particularly in dicotyledon plants. *A. rhizogenes* is the causative agent for hairy root induction in wound site and for some biochemical changes in plant metabolism (Gandi and Giri, 2012).

Gene transfer is a powerful tool for increasing productivity and production of secondary metabolites that are produced in low amounts in normal plants (Sharafi et al., 2013). T-DNA transfer from *A. rhizogenes* Ri plasmid to plant cells and its integration into the host genome leads to the induction of hairy roots (Srivastava and Srivastava, 2007). Hairy roots have rapid growth, high branching and plagiotropic growth in hormone-free medium and can increase the production of secondary metabolites compared to intact plants (Srivastava and Srivastava, 2007).

Contact between the bacteria and plant cells could be increased by direct injection of bacteria suspension into explants or with immersion plant tissue in bacteria suspension culture (Tomilov et al., 2007). In *Arachis hypogaea* L. hairy roots induction was obtained by injection method (Geng et al., 2012). The effects of two different inoculation methods (co- cultivation and injection) on induction of hairy roots were evaluated in *Trigonella foenum*-graecum L.; the results showed that transformation performance was 26 % in injection method and 6 % in co- cultivation method (Akbarian et al., 2011). *Amaranthus spinosus* L. explants were inoculated using both injection and immersion in bacterial suspensions and highest transformation (98.57 %) was achieved using the immersion method (Ajantaa et al., 2012). Light plays a key role in the growth and production of secondary metabolites and hairy roots formation is strongly dependent on the light sensitivity of plants and light conditions applied (Wu, 2007).

For the use of *A. rhizogenes* to transfer genes into plants, various factors such as culture conditions and inoculation time must be optimized (Kabirnetaj et al., 2012). Optimization of nutrient compounds of hairy roots culture medium is necessary to enhance the growth and production of secondary metabolites (Sharafi et al., 2013). The responses to various culture media have been reported in many studies, In *Eucommia ulmoides* Oliver effect of different culture media (B5, WPM and MS) on the hairy roots growth of were studied

and the results showed that the maximum amount of hairy roots dry weight (0.46 g) was found in roots grown on MS media as compared to roots grown on WPM media (Wu, 2007). The effect of four culture media (1/4 SH, 1/2 SH, SH, 2 SH) on the growth rate of *Angelica gigas* Nakai hairy roots was studied and 1/2 SH was the best medium for the growth of hairy roots (Xu et al., 2009).

Technology of rosmarinic acid (RA) production with cell culture and hairy root culture, have been reported in several plant species (Petersen and Simmonds, 2003). Production of RA in *Agastache rugosa* (Fisch. & C.A.Mey.) Kuntze hairy roots increased 14 days after culturing (Lee et al., 2008). Increased production of RA in hairy roots of *Plectranthus barbatus* Andrews (*Coleus forskohlii*) and *Salvia miltiorrhiza* Bunge has been reported (Li et al., 2005; Yan et al., 2006).

In the present study for the first time, hairy roots were successfully induced from leaves of *A. foeniculum* by *A. rhizogenes* A4 and synthesis of RA in transformed and non-transformed roots was investigated. In addition, the effects of culture media and inoculation methods were also evaluated for the growth enhancement of hairy roots.

2 MATERIALS AND METHODS

2.1 Seed culture and preparation of explant

Agastache foeniculum (Pursh) Kuntze seeds were obtained from the botanical garden of the Urmia University - Iran and were surface sterilized using 70 % ethanol for 1 min and 2.5 % (v/v) sodium hypochlorite (NaClO) for 10 min, rinsed three times with distilled sterile water. The seeds were cultured on MS (Murashige and Skoog, 1962) medium containing 7 g/l agar. Cultures were maintained at temperature of 25 ± 2 ^{oC} under a 16-h light/8-h dark photoperiod for seed germination and explants preparation.

2.2 Establishment of hairy roots and culture conditions

Agrobacterium rhizogenes (Riker et al., 1930) Conn 1942 agropine A4 strain (bank of microbes at the National Institute of Genetic Engineering and Biotechnology, Tehran-Iran) was used in transformation experiments. Bacteria were grown for 48 h (OD_{600} = 0.5– 0.6) in liquid LB (Luria-Bertani) medium at 28 ^{o}C on a rotary shaker at 180 rpm. Leaf segments from 1–month- old seedling of *A. foeniculum* were used as explants for co-cultivation and hairy root induction. The explants were immersed in a suspension of *A. rhizogenes* strain A4. All explants were then placed in MS hormone free media fortified with 3 % sucrose for co-cultivation. After 48 hours co-cultivation in the dark at 28 $^{\circ}$C, the explants were transferred to the fresh MS medium containing 200 mg/l cefotaxime. The emerging hairy roots were excised and transferred to 50 ml of 1/2 MS (half – strength MS) liquid medium supplemented with 3 % sucrose, in 250 ml conical flasks and grown at 25 ± 2 ^{o}C on a rotary shaker (Hei-dolph, Germany) in darkness for 2 months.

2.3 Effect of inoculation method on hairy root induction

The effects of two inoculation methods (injection and immersion) on hairy root induction by *A. rhizogenes* were evaluated. In the injection method, suspensions of A4 strain was injected in different parts of 1–month- old leaf explants by 0.5 ml insulin syringes (Exel – America). In the immersion method, explants after wounding with scalpel were immersed into a beaker containing the same strain of bacterial suspension. Furthermore, in the immersion method, the effect of inoculation time (3, 5 and 7 min) on hairy root induction was examined.

2.4 Effect of light-dark on hairy root induction

To determine the effects of light and dark conditions on hairy root induction, the explants were immersed in a suspension of *A. rhizogenes* A4 strain and then incubated separately under dark or light at 28 $^{\circ}$C. After 48 hours incubation, the explants were rinsed with sterile water and placed in Petri dishes containing 25 ml of solidified MS medium containing 200 mg/l cefotaxime.

2.5 Influence of various culture media on hairy roots growth

To optimize suitable culture medium for growth of hairy roots, they were cultured in full strength MS, 1/2 MS and B5 (Gamborg 5) basal media containing vitamins. Thereafter, 1 g of fresh roots were excised and transferred to 50 ml of the corresponding fresh liquid media in 250 ml conical flasks and grown at 25 ± 2 ^{o}C on a rotary shaker (100 rpm) in darkness. After 21 days of cultivation, the roots were washed with sterile distilled water and dried with sterile filter paper, then recorded their fresh mass.

2.6 HPLC analysis of hairy roots

Transformed and untransformed roots were oven dried (45°C) for one day. Then, approximately 0.5 g of dry tissue was used to determine RA content. 5 ml of extraction solution (2.5 ml methanol and 2.5 ml water) was added to each sample and RA was quantified by an Agilent / 1100 series HPLC system with DAD, model G1315B (USA) and a C18 reverse phase column (4.6 × 250 mm) at room temperature. The mobile phase (solution B) was acetonitrile / 5 % acetic acid (7: 3 v/v) with a linear gradient of 50 % solution B to 30 % solutions. RA was detected at 254 nm. Retention time of rosmarinic acid was 5.34 min.

2.7 Statistical Analysis

The experiment was carried out in randomized completely design (RCD) with three replicates. The data collected were subjected to analysis of variance test. The means were compared using Duncan's multiple range tests. All statistical analyses were performed using SAS 9.1 software.

3 RESULTS AND DISCUSSION

3.1 Establishment of hairy roots

In the present study, hairy roots were successfully induced (51.1 %) by infection of 1–month- old leaf explants of *A. foeniculum* with *A. rhizogenes* strain A4. Hairy roots emerged from wounded sites 10 days after infection. Hairy root induction using 1–month- old leaves as explants can be related to the high sensitivity of these explants to bacteria that depends on the physiological status of explants tissue (Pawar and Mathesh wari, 2003). Selection of an appropriate strain of bacteria is very important to produce hairy roots (Lee et al., 2010; Avansyans, 2009). The selected transformed roots showed rapid growth rate and tendency for profuse branching and active elongation on MS medium.

3.2 Effect of inoculation method on hairy root induction

ANOVA results indicated no statistically significant differences between the two inoculated methods in hairy root induction (Figure 1, 2). Effect of inoculation time (in immersion method) was investigated and it was found that immersion of explants for 3 min in the bacterial suspension was not sufficient to induce hairy roots. Long - term exposure to the bacterial suspension for 7 min lead to necrosis of explants, so inoculation of explants for 5 minutes was detected as the best time in the immersion method (Figure 3). Contact between bacteria and plant cells can be increased by direct injection of bacterial suspensions into the explants or by immersion (Tomilov et al., 2007). In *Arachis hypogaea* L., embryonic axes along with cotyledons were injected with a suspension culture of. *A. rhizogenes* using microliter syringes (Geng et al., 2012). In *Leucaena leucocephala* (Lam.) de Wit hairy roots were induced by injection of *A. rhizogenes* into the hypocotyls explants (Saifuddin et al., 2013). Various methods of inoculation have huge impact on the rate of entry of foreign genes and their integration into the host genome (Akbarian et al., 2011). Our results are in agreement with other studies, in *Arachis hypogaea* L. the highest rate of hairy root induction was achieved by immersion for 5 min and rooting percentage decreased with increasing time of inoculation time (Karthikeyan et al., 2007).

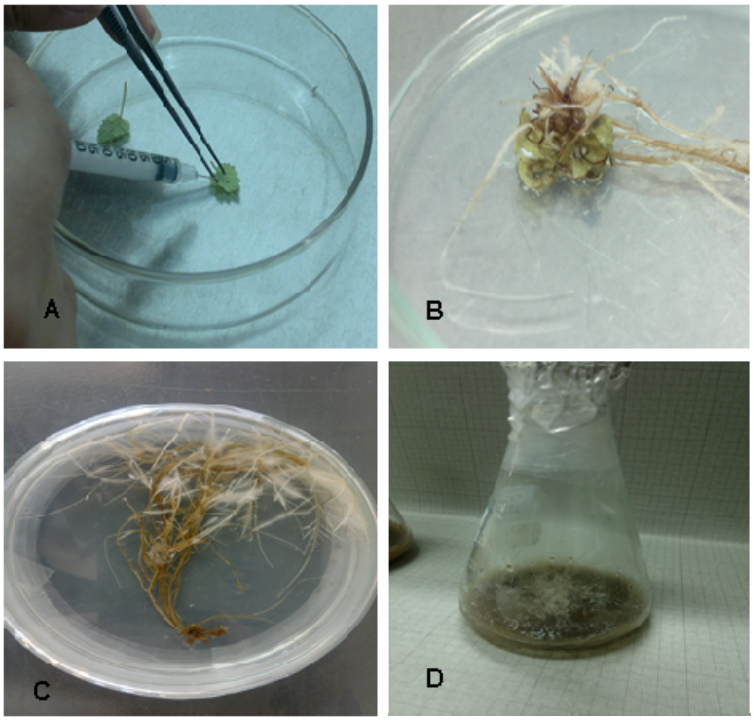

Figure 1: Transformed hairy roots of *A. foeniculum* by *Agrobacterium rhizogenes*. (A) Injection of bacteria suspension in the explant (B) Production of transformed roots on 1 – month – old leaf explant (C) Extension of transformed roots in MS solid medium including cefatoxim (D) fast growth of transformed roots in 1/2 MS liquid medium.

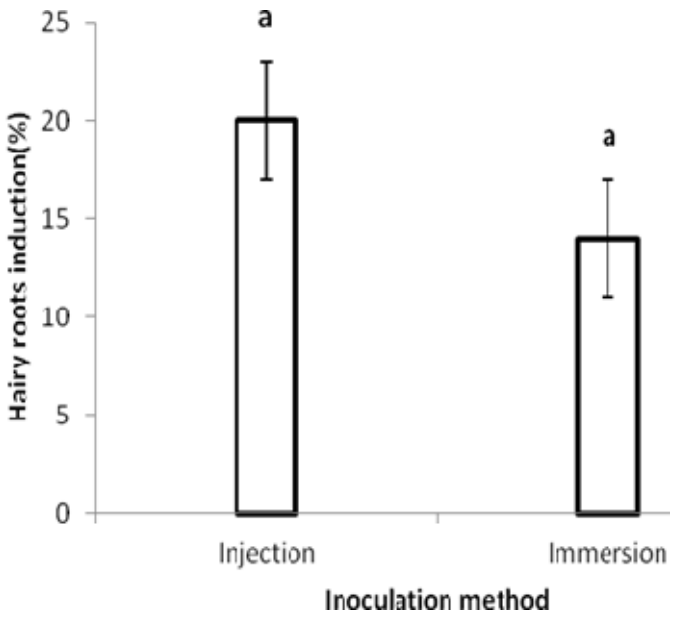

Figure 2: Comparison of immersion and injection methods to induce hairy roots on 1 – month – old leaf explants in *A. foeniculum*. The data were obtained as mean of three replications. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test.

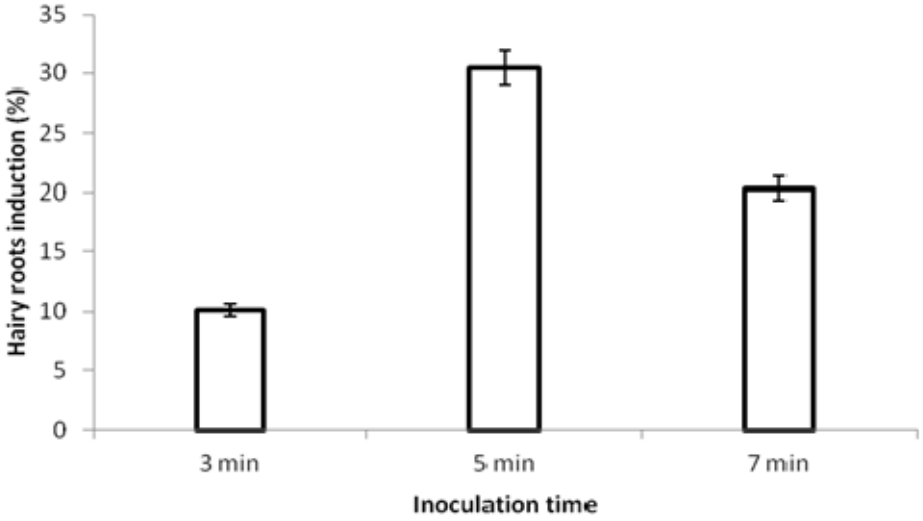

Figure 3: Effect of different co-cultivation times on the hairy root induction in *A. foeniculum*. The experiment was performed in triplicate and each experiment contained 8 explants.

3.3 Effect of light- dark condition on hairy root induction

In the present study, the induction of hairy roots in dark and light incubation was measured (Figure 4). Exposure of transformed explants in light condition improved the hairy root induction and finds it more favorable to hairy root formation than in dark conditions. The highest (39.81 %) and the lowest (23 %) hairy root induction frequency produced in light and dark conditions, respectively. ATP is required for hairy root elongation through cell division. Therefore, optimal photosynthetic capacity of explants under light environment is primary causes for high transformation (Taiz and Zeiger, 2006).

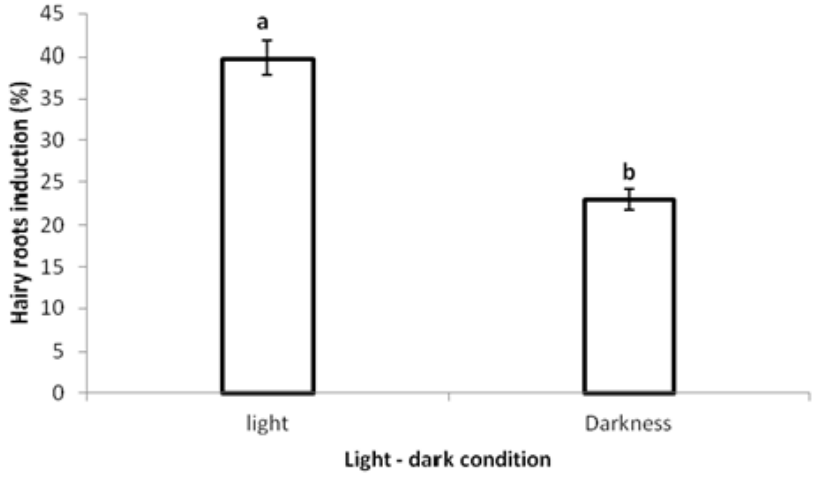

Figure 4: Effect of different light – dark condition on the hairy roots induction in *A. foeniculum*. The experiment was performed in triplicate and each experiment contained 8 explants. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test.

3.4 Effect of basal medium on hairy root growth

Media composition could have a significant impact on hairy root induction and growth in culture systems (Sivakumar et al., 2005). ANOVA results after 21 days showed significant difference between treatments. It was found that 1/2 MS medium produced more biomass (1.61 g fr wt/50 ml) compared to MS and B5 culture media and there was no statistically significant difference between MS and B5 media (Figure 5). The medium type has an important influence on the induction and growth rate of hairy roots. Culture media with high salt such as, MS and LS (Linsmaier and Skoog, 1965) are suitable for the formation of hairy roots in many plants (Giri and Narasu, 2000). Optimization of the hairy root induction and stabilization of the hairy root cultures is important to increase biosynthetic capacity of hairy root cultures (Sharafi et al., 2013). Nutritional factors may influence the number and length of hairy roots (Hilton et al., 1990). It is inferred that the difference in ions of the medium is the primary factor that affect the growth of hairy roots (Wu, 2007). In all three media (MS, 1/2 MS and B5) there is the same amount of calcium and phosphorus, while the nitrogen content was different. MS and B5 medium have the highest amount of nitrogen (approximately 5.22 and 8.26 mM) but the amount of nitrogen in the 1/2 MS medium is less. The growth of hairy roots was limited by high concentration of nitrogen (Wu, 2007). Thus, the nitrogen may be a factor that affects the growth of hairy roots. The effect of different culture media (MS, 1/2 MS, B5, 1/2 B5, WP, 1/2 WP) on the hairy roots of *Pueraria candollei* var. *mirifica* Airy Shaw et Suvat was studied and the results showed that, 1/2 MS medium produced the highest dry weight of hairy roots (0.13 ± 0.03 g) and the lowest dry mass was produced in 1/2 B5 medium (Udomsuk et al., 2009). SH and MS culture media were suitable for the growth of *Angelica gigas* Nakai hairy roots compared with B5 medium (Xu et al., 2009). *Glycyrrhiza glabra* L. hairy roots showed the best growth (fresh mass) in NB medium compared with MS, B5 and WP media (Mehrotra et al., 2008).

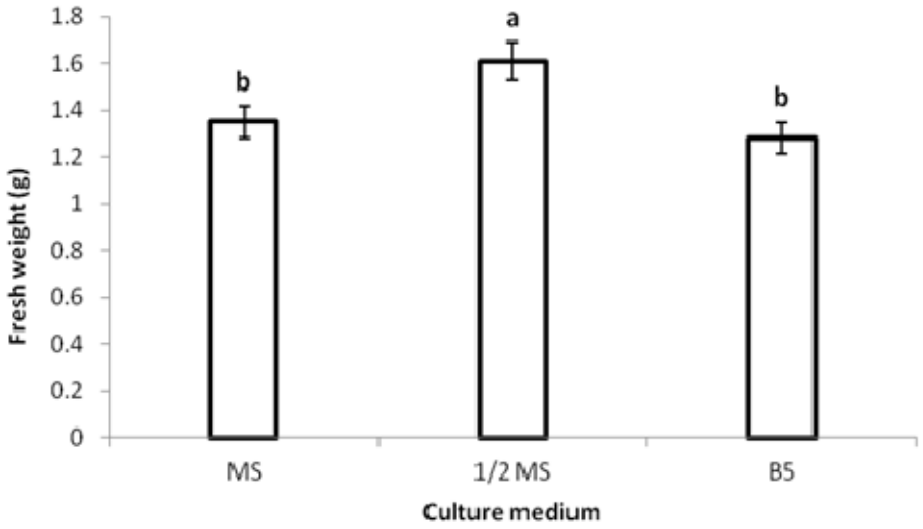

Figure 5: Growth of transformed roots of *A. foeniculum* in different liquid media (MS, ½ MS and B5) after 21 days. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test. Vertical lines represent SE.

Hairy roots were cultured in 1/2 MS liquid medium for 2 months and RA content was investigated by harvesting 3 flasks at intervals. RA Content in transformed roots (213.42 µg/g dry wt) was significantly higher than in non-transformed roots (52.28 µg/ g dry wt) (Figure 6, 7).

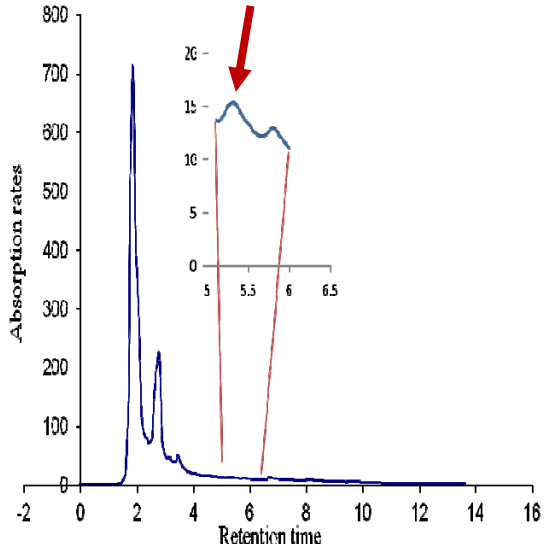

Figure 6: The rosmarinic acid content in hairy roots of *A. foeniculum* retention at 5/34 Min.

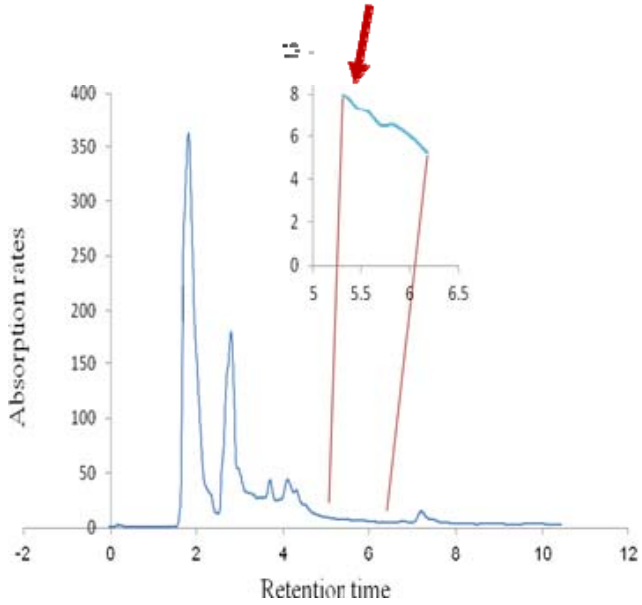

Figure 7: The rosmarinic acid content in non-transformed roots of *A. foeniculum* retention at 5/34 Min.

RA production by hairy root cultures have been reported in many plant species, such as: *Coleus forskohlii* (Li et al., 2005), *Salvia officinalis* L. (Grzegorczyk et al., 2006), *Agastache rugosa* (Fisch. & C.A.Mey.) Kuntze (Lee at al., 2008), *Nepeta cataria* L. (Lee at al., 2010). In present study it has been shown that hairy roots can be a valuable source for the production of RA, which is in agreement with the results of other researches. The increase of the amount of RA in hairy roots is probably due to some biochemical changes in plant metabolism by *A. rhizogenes* (Gandi and Giri, 2012). It has been determined that the *rol* genes are active participants in the production of secondary metabolites, it is known that the *rol* genes act via transcriptional activation of defense genes (Bulgakov, 2008). Therefore optimization of culture media and other influencing factors on hairy root induction and formation can be used as an efficient method for continuous and high secondary metabolites production in important medicinal plant under *in vitro* conditions (Sharafi et al., 2013).

4 CONCLUSION

The main outcome of the present study is the development of a reliable and well defined protocol for hairy root induction and increased growth rate in hairy root cultures in *A. foeniculum*. In the present study, transgenic hairy root cultures of *A. foeniculum* using *A. rhizogenes* A4 were established. Suitable explants for hairy root induction proved to be 1 – month – old leaf explants. The effects of injection and immersion methods on the hairy root induction were investigated. Hairy roots were induced with both methods. Hairy root induction was increased in light comparable to dark condition. ½ MS medium was the most appropriate medium for culture of hairy roots. Finally, hairy root techniques may be considered as a useful system for large-scale production of valuable secondary metabolites in cultures of *A. foeniculum*.

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DOI: 10.14720/aas.2016.107.1.06
Agrovoc descriptors: fragaria ananassa; protected cultivation; irrigation systems; trickle irrigation; cost benefit analysis; emitters (irrigation)

Agris category code: F06, f08

Technical and economical evaluation of tape drip and drip line irrigation systems in a strawberry greenhouse

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ABSTRACT

This study was done in a strawberry greenhouse to examine the technical and the economical evaluation of two drip irrigation systems including the tape and the drip line in the northern part of Iran. The result showed that all of the technical indices with tape were higher than drip line, and due to statistical analysis reveal a significant difference ($P < 0.05$). Yield and water productivity (WP) with tape were higher than drip line ($P < 0.05$). Benefit per drop (BPD) and net benefit per drop (NBPD) with tape were higher than drip line. Net present value, internal rate of capital return and benefit to cost ratio in drip line were higher than tape. In general, regarding technical evaluation tape was better than drip line, besides according to the economical evaluation the drip lines were better than tape.

Key words: drip irrigation, emitter efficiency, benefit to cost ratio, strawberry (*Fragaria x ananassa* Duchesne)

IZVLEČEK

OVREDNOTENJE TEHNIČNE USTREZNOSTI IN EKONOMIČNOSTI DVEH SISTEMOV KAPLJIČNEGA NAMAKANJA (z namakalnim trakom in z linijo s kapljači) JAGODNJAKA (*Fragaria x ananassa* Duchesne) V RASTLINJAKU

Raziskava je bila opravljena z namenom ovrednotenja tehnične ustreznosti in ekonomičnosti dveh sistemov kapljičnega namakanja: z namakalnim trakom in z linijo s kapljači pri gojenju jagodnjaka v rastlinjaku, v severnem delu Irana. Rezultati so pokazali, da so bili pri sistemu namakanja z namakalnim trakom vsi tehnični kazalci boljši kot v sistemu z linijo s kapljači in statistične analize kažejo značilno razliko ($P < 0.05$). Pridelek in produktivnost vode (WP) sta bila večja pri sistemu z namakalnim trakom kot z linijo s kapljači ($P < 0.05$). Prihodek na m^3 vode (BDP) in dobiček na m^3 vode (NBDP) sta bila večja v sistemu z namakalnim trakom kot v sistemu linija s kapljači. Neto sedanja vrednost, interna stopnja donosnosti kapitala in indeks donostnosti so bili večji v sistemu linije s kapljači kot v sistemu z namakalnim trakom.V splošnem je bil tehnološko sistem z namakalnim trakom boljši kot linija s kapljači, a pri ovrednotenju ekonomičnosti je bilo ravno obtratno.

Ključne besede: kapljično namakanje, učinkovistost kapljača, indeks donosnosti, jagodnjak (*Fragaria x ananassa* Duchesne)

1 INTRODUCTION

With water resources deficiency, optimum use of water in the agricultural sections is very important. In countries that face water deficiency, producing crops with minimum water consumption is very important. Iran is located in an arid and semiarid zone and water is a limiting factor in crop

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production. In recent years due to water resources deficiency, greenhouse cultivation of products on one hand and applying drip irrigation on the other hand have received a lot of attention. Currently the best irrigation method for greenhouse productions is drip irrigation which along with fertigation increases water productivity and fertilizers efficiency and improves yield quantity and quality. Due to the expansion of drip irrigation methods in greenhouses being familiar with the designing principles, performance and proper management of irrigation systems in greenhouses seems very indispensable. Beside expansion of drip irrigation systems in greenhouses, system quality should be considered. By technical evaluation of drip irrigation systems, distribution uniformity indices, emitter efficiency, discharge variations and variation coefficients of emitters should be measured. Then system performance can be determined and by proposing relevant solutions system defects can be eliminated which results in improving and maximizing system performance. The high investment of installation and implementation of drip irrigation systems and focusing on their profitability justify the importance of economical evaluation of irrigation systems. Therefore, revenues, costs and net income of drip irrigation in greenhouse would be calculated and then the most cost effective irrigation system in greenhouse can be determined. In recent years the use of tape drip irrigation and drip lines in greenhouse has become common. Both systems have advantages and disadvantages and have caused doubt in experts and farmers on selecting the desired system.

According to Picha (1999) tapes of drip irrigation for strawberry production is optimum. Ortega et al (2002) evaluated 100 drip irrigation systems and reported that the amount of distribution uniformity in systems was good and estimated the average of emitter uniformity (EU) as 84.3 %. Noshadi and Ghaemi (2012) evaluated drip irrigation systems in 124 orchards in Fars province, Iran and calculated the amounts of application efficiency of low quarter (AELQ) and potential efficiency of low quarter (PELQ) being of 72 and 65 %, respectively. Cetin et al (2004) evaluated drip irrigation in olive gardens of Turkey and used net present value index and reported that drip irrigation is profitable and have the economic justification. Azizi (2007) by examining three parameters including: net present value, internal rate of capital return and benefit to cost ratio, reported that banana cultivation and mix cultivation of banana and asparagus have not got any economic justification in Kishestan Greenhouse Complex, Guilan province, Iran, whereas, the production of some other plants such as cut flowers and vegetables have economic justification. Rhea et al (2001) obtained net profit for tomato and tobacco as 10372.52 and 12121.22 US \$ ha^{-1}, respectively.

As far as in recent studies have been dedicated to the technical and the economical evaluations of irrigation systems in the fields and gardens separately according to the product itself, therefore, the technical and the economical evaluations of the aforementioned irrigation systems have been the center of attention. The results of this study would lead to a better status along with developing the drip line system, improving water consumption to an acceptable level, and finally reducing the production costs in the greenhouses. Moreover, it would be applied by farmers, the experts on irrigation system designing as well as those who are interested in establishing a greenhouse.

2 MATERIALS AND METHODS

This study was done in a strawberry greenhouse in Kishestan Greenhouse Complex located in Some'sara, Guilan province in the northern part of Iran (Figure 1) in 2014-2015. The approximate area of greenhouse was 500 m^2 (the width of greenhouse was 9 m and the length of that was 55 m). This study was done in a completely randomized design. The physical and chemical characteristics of the soil are presented in Tables 1 and 2.

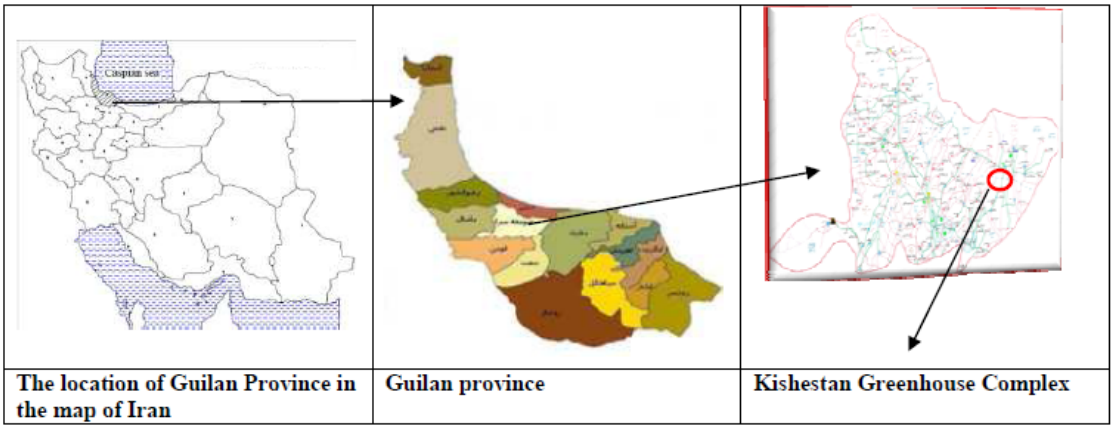


Figure 1: The location of Kishestan Greenhouse Complex in the Guilan province and in Iran

Table 1: Physical characteristics of the soil in Kishestan Greenhouse Complex (Iran)

Sand (%)	Silt (%)	Clay (%)	Soil texture	θs* (%)	Field capacity (%)	Wilting point (%)
66	20	14	Sandy Loam	51.51	20.44	11.44

*volumetric saturated water content

Table 2: Chemical characteristics of the soil in Kishestan Greenhouse Complex (Iran)

Cations sum	Cations (meq l^{-1})				Anions sum	Anions (meq l^{-1})				pH	EC (mmhos cm^{-1})
	Mg^{2+}	Ca^{2+}	Na^{+}	K^{+}		CO_3^{2}	HCO_3^{-}	Cl^{-}	SO_4^{-2}		
44.1	14.1	15.3	14.7	-	44.4	-	11.3	17.8	15.3	7.9	403

Irrigation water was classified according to the Wilcox classification in C2S1 class (C2 i.e. EC: 250-750 mmhos cm^{-1} and S1 i.e. SAR: 0-10). Chemical analysis of irrigation water showed the deposition risk of calcium carbonate and calcium sulfate in irrigation systems is absent. In general, irrigation water quality parameters were in an acceptable range. Soil preparation has been done using a plough and then a disc. Six plots have been prepared and in each one 8 furrows have been prepared to transplant strawberry seedlings. Irrigation water for the greenhouse has been provided by a 2000-liter-tank from Pasikhan River and pumped to the irrigation system. To supply necessary pressure in the irrigation system a CAM-100 Pentax pump has been applied with a discharge of 10-50 l min^{-1}. In order to prevent tapes and drip lines clogging a disc filter (130 micron) was installed. Polyethylene pipes with 32 mm of diameter for the main pipe, 25 mm as the manifold and 16 mm as tapes and drip lines were used. Water was transferred from the pump to the main pipe having a length of 5 m. Six manifolds were branched from the main pipe. In the beginning of each manifold a valve was installed to adjust the amount of inflow to the system, and then the required pressure in the system was controlled by placing a manometer and a pressure regulator after the electro-pump. Drip lines and tapes were branched from the manifolds. In the beginning of every drip lines and tapes, a valve was installed in order to stop the water from being wasted. Inflow water volume was measured by a flow-meter. The soil surface was covered with black polyethylene mulch to keep away weed and to preserve soil moisture. The strawberry seedlings ('Selva' variety, a common variety in the region) were transplanted to the greenhouse in the beginning of December and have been planted in two-row lines. Required fertilizers and pesticides were applied in both of the treatments as recommended by the expert and used by the farmers in the region.

Greenhouse environmental factors such as temperature and heat were controlled by thermometer, heater and thermostat. The treatments were two drip irrigation systems including tape and drip line with three replications. Tapes with a diameter of 16.1 mm, a discharge of 8 l $h^{-1}m^{-1}$, an emitter intervals of 20 cm and drip lines with a diameter of 16 mm, a discharge of 2.2 l h^{-1}, an emitter intervals of 40 cm both of them under 100 kPa of pressure have been applied which were manufactured by AZUD company and have been provided for this study.

For the technical evaluation, coefficient of variation (CV) for emitter manufacturing, coefficient of uniformity (CU_1) for water distribution at the soil surface, coefficient of uniformity in soil (CU_2) for water distribution in the soil, distribution uniformity (DU) for water distribution at the soil surface, statistical coefficient of uniformity (UC) for water distribution at the soil surface which has a statistical meaning and depends on the standard error and the average of dripper discharge in a drip irrigation system, emission uniformity (EU), a measure of the uniformity emissions from all the drippers within a drip irrigation system, and efficiency of application (EA) for irrigation water in a drip irrigation system have been measured. DU is related to CU1 and DU=100-1.59(100-CU1) whereas EU is the field test emission uniformity (%) and is defined as the ratio of the average rate of discharge of the lowest one-fourth of the field data emitter discharge reading to the average rate of all the emitters checked in the field (Merriam and Keller, 1978).

In order to determine CV in drip line, emitter discharge of 50 emitters at a pressure of 50, 80, 100 and 160 kPa were measured and used to calculate the mean discharge i.e. emitter discharge was calculated as the ratio of measured water volume in the elapsed time, with embedded graduated cylinders below emitters the volume of water was measured within a known time. For tape, emitter discharge of each meter of tape at a pressure of 50, 80, 100 and 120 kPa were measured, a pressure higher than 120 kPa would cause ruptures in the tape so the maximum pressure was 120 kPa. Measurements were carried out in the first and last one meter of the tape as well as in one meter of the one third and two thirds of tape length (Merriam and Keller, 1978; Shojaeian and Ghaemi, 2010).

To determine other technical indices, 2-3 meters from the beginning of the pipe was waived due to discharge changes. Four emitters at first, a third, two-thirds and at the end of the drip line length were selected [Juana et al, 2007]. In the tapes, four 1-meter sections were selected. An hour after the start of the system and reaching the hydraulic equilibrium in the system, sampling containers were placed at designated locations and the amount of irrigation water was measured in a known time. To evaluate the uniformity of water distribution in the soil, 24 hours after irrigation soil samples were taken with an auger in sampling locations near the emitter and in the root zone (0-15 cm) and weighted average of soil moisture content was determined (Juana et al, 2007). All measurements were replicated three times.

For the economical evaluation yield, water productivity indices (WP, BPD and NBPD), net present value (NPV), internal rate of capital return (IRR) and benefit to cost ratio (B/C) were determined.

Strawberry harvest was done from mid-March to mid-June for a period of four months and the average yield of the iterations (three replications in each treatment) and total harvested yield during this period was considered as the strawberry yield in the greenhouse.

The statistical analysis was done using SPSS software package version 18, and two sample t-test has been done. The data has been evaluated by the Kolmogorov–Smirnov test. The variance has been examined by Leven test.

3 RESULTS AND DISCUSSION

CV for drip lines under pressures of 50, 80, 100 and 160 kPa in average was calculated as 0.08 and for tape under pressures of 50, 80, 100 and 120 kPa in average was calculated as 0.07 (tables 3 and 4).

The CV in tape was less than drip lines. According to American Society of Agricultural Engineering (ASAE) classification (Alizadeh, 2011), drip lines were in the medium class (0.07 < CV < 0.11) and tapes were in the excellent class (CV < 0.1). The study of Shojaeian and Ghaemi (2010) showed that the coefficients of variation in tape was less than drip line; therefore, this system improved DU at the soil surface as well as in the soil profile.

CV in drip lines under 100 kPa of pressure was 0.07 and classified in the medium class. Sadrghaen et al (2012) and Umara et al (2011) reported that CV in drip lines according to ASAE classification was in the medium class which is in agreement with the results of the present study. The coefficient of variation in tape under 100 kPa of pressure was calculated as 0.03 that was classified in the excellent class. Duta (2008), Hasanzadeh-Arnayi and Fathi (2012) and Karimi et al (2004) determined the coefficient of variation in tape and classified it in the excellent class according to ASAE classification being in agreement with our results. Coefficient of variation under 100 kPa of pressure in tape was less than drip lines. Therefore, it can be concluded that the coefficient of variation under 100 kPa of pressure in tape had a higher uniformity than drip lines. Coefficient of variation in drip lines increased by increasing the pressure from 100 to 160 kPa and by reducing the pressure from 100 to 80 kPa. Coefficient of variation did not change by reducing the pressure from 100 to 50 kPa. As a result, it can be concluded that drip lines under 100 kPa of pressure had higher distribution uniformity than 80 and 160 kPa. The coefficient of variation in tape increased by increasing the pressure from 100 to 120 kPa. Besides, the coefficient of variation increased by decreasing the pressure from 100 to 80 and 50 kPa that causes non uniformity in less and higher pressures than the nominal pressure (100 kPa). Generally, the distribution uniformity in both systems under 100 kPa of pressure was more than the other pressures. This finding is similar to the results of Shojaeyan and Ghaemi (2010).

Table 3: Coefficient of variation for emitter manufacturing in drip lines in Kishestan Greenhouse Complex (Iran) experiment (N = 3)

Pressure (kPa)	50	80	100	160
CV	0.07	0.11	0.07	0.08
Classification	average	bad	average	average

Table 4: Coefficient of variation for emitter manufacturing in tape in Kishestan Greenhouse Complex (Iran) experiment (N = 3)

Pressure (kPa)	50	80	100	120
CV	0.13	0.06	0.03	0.05
Classification	good	great	great	great

Calculated uniformity coefficients of the two irrigation systems are presented in table 5. Coefficient of uniformity (CU1) in drip lines and tape were calculated as 91.22 and 97.38 %, respectively that according to Bralts's method of classification (Bralts, 1986), both irrigation systems are in the excellent class. Statistical analysis showed that there is a significant difference between those two irrigation systems ($P < 0.01$). Safi et al (2007), Tagar et al (2010), Duta (2008), Ghaemi et al (2008) and Karimi et al (2004) estimated the amount of uniformity coefficients (CU_1) from 95 up to 98 % in tape that is in agreement with the results of the current study. Distribution uniformity in soil (CU_2) in drip lines and tape were calculated as 81.40 and 92.45 % respectively, showing that the two irrigation systems have a significant difference

($P < 0.01$). Distribution uniformity in soil on tape was more than drip lines which is similar to the results of the coefficient uniformity in these two systems. Shojaeyan and Ghaemi (2010) reported a better water distribution in tape than drip line at soil surface and in the soil profile. DU was calculated 86.04 in drip lines and 95.76 % in tape. According to Noshadi and Ghaemi (2012) in valuable plants with shallow root system such as strawberry, the most economical system should have a high uniformity and in other words more than 80 %, and according to Ghassemzadeh-Mojaveri (1990) a DU of 50 % is acceptable. Consequently, both irrigation systems in terms of distribution uniformity are appropriate, however, statistical analysis showed a significant difference in distribution uniformity between the two systems ($P < 0.01$). The results of the present study are in the range of distribution uniformity reported by Umara et al (2011), Hanson and Bendixen (2004) and Ashiri et al (2013) which varies between 81 to 96 %. Statistical coefficient (UC) in both drip lines and tape systems calculated as 93.24 and 96.72 %, respectively. According to Bralts's classification and ASAE classification both systems are in the excellent class but statistical analysis showed a significant difference between these two systems ($P < 0.05$).

EU was estimated in drip lines and tape systems as 84.11 and 95.84 %, respectively and statistical analysis showed that there is a significant difference ($P < 0.05$) through which according to Merriam and Keller (1978) and Bralts (1986), drip lines systems were in the good class and tape irrigation system was in the excellent class. According to Erida classification (Bamdad-Machiani et al., 2014) drip lines was in the acceptable and tape irrigation system was in the excellent class and according to Capara and Scicolone (1998) classification both of the systems have a high EU. So totally according to Merriam and Keller (1978), Bralts (1986) and Erida (Bamdad-Machiani et al., 2014) tape was in the excellent class and drip line according to Merriam and Keller (1978), Erida (Bamdad-Machiani et al., 2014) and Capara and Scicolone (1998) was respectively in good, acceptable and high classes where these differences were due to different units of categorizing in the three above classification methods.

Table 5: Uniformity indices in both drip lines and tape irrigation systems in Kishestan Greenhouse Complex (Iran) experiment (N = 3)

Treatments	CU_1	CU_2	DU	UC	EU
Drip lines	91.22	81.4	86.04	93.24	84.11
Tape	97.38	92.45	95.76	96.72	95.84
Significant level according to t-test	**	**	**	*	*

*Significant at the level of five percents, **Significant at the level of one percent

EA was calculated by two methods (Sahafamin and Farshi, 1999) and (Anonymous, 2004) in both irrigation systems (table 6). The amount of EA in drip lines and tape drip irrigation calculated with the formula provided by Sahafamin and Farshi (1999) was slightly higher than those of calculated with Anonymous (2004) method being 0.104 and 0.102 % higher, so calculated EA amounts in these two method were not much different. In general, EA in tape was more than drip lines, and there is a significant difference between the two systems in both calculating methods at the level of five percent, which is due to more uniformity in tape drippers than drip lines drippers.

Table 6: Application efficiency (EA) in drip lines and tape in Kishestan Greenhouse Complex (Iran) experiment (N = 3)

Treatments methods	Drip lines	Tape	t-test
Sahaf-Amin and Farshi (1999)	76.54	87.61	*
Anonymous (2004)	76.46	87.52	*

*Significant at the level of five percents

The technical evaluation in two irrigation systems showed that tape was better than drip lines in the greenhouse. As far as farmers and capital owners prefer more net profit, and also a plan should be justified in terms of its economic achievements, therefore, the economical evaluation was done. After estimating the costs, the net profit, the water productivity indices, the net present value, the internal rate of capital return and the benefit to cost ratio, appropriate system in greenhouse in terms of the technical and the economic aspects can be recommended.

The amounts of applied water, yield and WP for two systems are presented in table 7. Strawberry yields with drip lines and tape drip irrigation were measured as 3327 and 4242 kg ha^{-1}, respectively. Strawberry yield with the tape drip irrigation was higher than the drip lines, but statistical analysis showed that there is not any statistical difference among both of the treatments. Applied water with the drip lines and the tape drip irrigation were 1873 and 2109 m^3 ha^{-1}, respectively. Amount of applied water in both treatments was not statistically different.

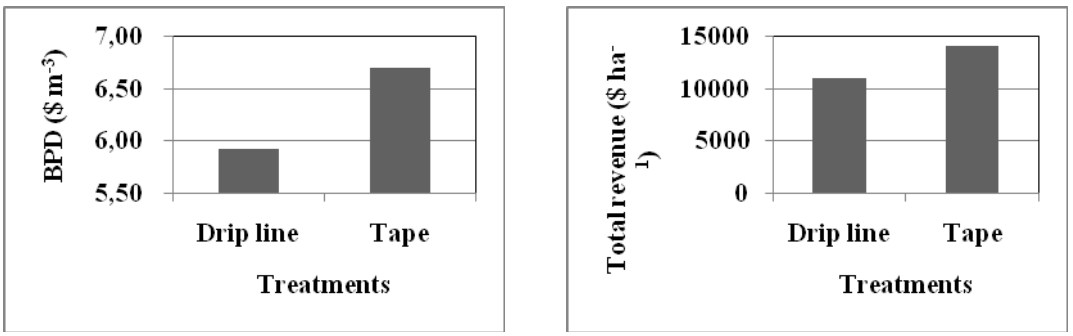
However, by increasing the applied water in the tape as compared with the drip lines yield was increased. It can be seen that the effect of both irrigation systems on strawberry yield was not significant. The effect of both yield and applied water caused the increase of the WP with tape as compared with drip lines.

Table 7: Applied water, yield and water productivity (WP) in two irrigation systems in Kishestan Greenhouse Complex (Iran) experiment (N = 3)

Treatments	Yield (kg/ha)	Applied water (m^3/ha)	WP (kg/m^3)
Drip lines	3327	1873	1.78
Tape drip irrigation	4242	2109	2.01
t-test	ns	ns	ns

ns: not significant at the five percent level

Total revenue and BPD index are presented in figure 2 (a and b). Sales price of strawberry was considered as 3 US $ kg^{-1}. The total revenues in the drip lines and the tape drip irrigation were calculated as 10082 and 12855 US $ ha^{-1}, respectively. The yield was higher with tape as compared with drip lines. As a result, the total revenue with tape was higher than drip lines. BPD index with drip lines calculated as 5.45 and with tape drip irrigation as 6.06 US $ m^{-3}, being 11 % higher with tape. The concurrent effects of both applied water and total revenue increased the BPD index with the tape as compared with the drip lines.



a b

Figure 2: a) BPD index in drip lines and tape drip irrigation in Kishestan Greenhouse Complex (Iran) experiment (N = 3) and b) Total revenue (US $ ha^{-1})

As in BPD index gross profit is considered, therefore, NBPD index in which the net profit is included, is more important. The cost of central control station, installation, irrigation system operation, pipe (main and manifold), fittings and valves are the same in both irrigation systems, whereas, the costs of irrigation water and lateral (the drip lines and the tape) are different in two treatments. The cost of applied water for each cube meter was considered as 0.002 US $ (reported by Regional Water Company). 162 m of tape and drip lines was used in the greenhouse. The prices of tape and drip lines were 0.55 and 2.27 US $ m^{-1}, respectively.

Calculated NBPD index in both systems are presented in table 8. It can be seen that NBPD with tape and drip lines were calculated as 5.15 and 3.94 US $ m^{-3}, respectively. The NBPD with tape was higher than the drip lines because of higher total revenue with tape as well as a lower cost of irrigation water as compared with the drip lines. As a result, net profit was higher with tape than with that of the drip lines.

Table 8: Net benefit per drop NBPD index in studied two irrigation systems in Kishestan Greenhouse Complex (Iran) experiment (N = 3)

Treatments	Costs (US $ ha^{-1})	Net profit (US $ ha^{-1})	NBPD (US $ m^{-3})
Drip lines	87.18	246	0.13
Tape drip irrigation	59.33	365	0.17

Calculated net present value (NPV), IRR and B/C ratio indices are presented in table 9 for both irrigation systems. The NPV in the drip lines and tape was calculated as 33.48 and 11.17, respectively, being higher than zero in both irrigation systems, therefore, both of the systems are justified according to the economic aspects. The IRR in the drip lines and the tape was calculated as 50.61 and 47.27 which are both more than the interest rate of 22 % (official rate in Iran in 2014) and showed economical justification of irrigation systems in the greenhouse. The B/C ratio with the drip lines and tape was calculated as 1.46 and 1.21, respectively where in both of the irrigation systems is more than one, in which shows an economic justification of both of the irrigation systems. In the benefit to cost ratio method for comparison between treatments, the present value of benefits to the present value of investment ratio was calculated being 2.2. As this ratio is higher than one, so the drip line was chosen as an economical method which is in agreement with Oskounejad (2009) results.

Table 9: Net present value (NPV), internal rate of capital return (IRR) and benefit to cost ratio (B/C) in both irrigation systems in Kishestan Greenhouse Complex (Iran) experiment (N = 3)

Indices	Drip lines	Tape drip irrigation
NPV	33.48	11.17
IRR (%)	50.61	47.27
B/C	1.46	1.21

4 CONCLUSION

In this study the technical and the economical evaluations of the two drip irrigation systems i.e. the tape and the drip lines were done in a strawberry greenhouse in Kishestan Greenhouse Complex located in Some'sara, Guilan province, in the northern part of Iran. The result showed that the tape irrigation system was better in terms of the technical evaluation, whereas, drip lines were better according to economical indices. It should be said that both systems have acceptable standard level of economical and technical evaluations. Consequently, if the farmers are those who pay for the irrigation system, as they have not enough capital resource, so it is better to use tape because of lower investment costs, but if the government pays subsidy, it is better to use drip lines because of its being more beneficial. Generally, the government pays 85 % of the costs to implement a pressurized irrigation system to address water resources issues in the country.

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Agris category code: F01, F04, f08, q04

Effects of nitrogen treatment and intra-row spacing on the morphological and physiological characteristics in pumpkin (*Cucurbita pepo* L.)

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ABSTRACT

Pumpkin (*Cucurbita pepo* L.) is a medicinal plant recently under common cultivation in Iran. The seeds and its products are used in the treatment of some diseases. Due to the lack of information about the intra-row spacing and nutritional demands of the plant a factorial experiment with Randomized Complete Block Design with three replications was planned in Azarbaijan Shahid Madani University Research Field in 2013. The treatments were 5 levels of Urea fertilizer (0, 50, 100, 150, 200 kg/ha nitrogen), and intra-row spacing in 3 levels (30, 60 and 90 cm). The result showed that interaction effects of intra-row spacing and nitrogen treatment had significant effect on some morphological traits such as plant height, number of auxiliary branches and leaf number per plant. Application of 200 kg/ha nitrogen increased the plant height, the number for auxiliary branches and leaves as well as the leaves dry mass, mainly due to the prolonged vegetative growth period and delayed flowering and fruiting. 150 kg/ha nitrogen combined with 60 cm intra-row spacing hold the highest number of female flowers, fruit number and intact fruit number and fruit fresh weight, and the highest dry weight of the intact seeds. The results for some physiological traits showed that interaction effects of nitrogen and intra-row spacing had significant effects on chlorophyll b at the late stages of flowering period and the contents for chlorophyll a and carotenoids were significant at the end of growing season. The highest oil content was recorded in 150 kg/ha nitrogen and 60 cm intra-row spacing. Based on results obtained, 150 kg/ha nitrogen and 60 cm intra-row spacing were the best regime for the production of this plant.

Key words: *Cucurbita pepo* L., nitrogen, intra-row spacing, yield, oil content

IZVLEČEK

UČINKI OBRAVNAVANJA Z DUŠIKOM IN MEDVRSTNIH RAZDALJ NA MORFOLOŠKE IN FIZIOLOŠKE LASTNOSTI BUČ (*Cucurbita pepo* L.)

Gojenje buč (*Cucurbita pepo* L.) kot zdravilnih rastlin je v zadnjem času splošno razširjeno v Iranu. Semena in pripravki iz njih se uporabljajo za zdravljenje različnih bolezni. Zaradi pomankanja informacij o vplivu medvrstnih razdalj setve in potrebah po hranilih na pridelek je bil izveden naključni faktorski poskus s tremi ponovitvami na Azarbaijan Shahid Madani University Research Field v letu 2013. Obravnavanja so obsegala 5 ravni gnojenja z ureo (0, 50, 100, 150, 200 kg N/ha), in 3 medvrstne razdalje setve (30, 60 in 90 cm). Rezultati kažejo, da so imela ta obravnavanja značilen učinek na nekatere morfološke znake kot so dolžina rastlin, število stranskih poganjkov in število listov na rastlino. Uporaba 200 kg N/ha je povečala višino rastlin, število stranskih poganjkov in število listov kot tudi suho maso listov predvsem zaradi podaljšane vegetativne faze rasti in zakasnjenja cvetenja in tvorbe plodov. 150 kg N/ha v kombinaciji s 60 cm medvrstno razdaljo setve je dalo največ ženskih cvetov, največ plodov, največjo maso svežih plodov in največjo maso suhih semen. Rezultati meritev nekaterih fizioloških parametrov so pokazali značilne učinke inteakcije obravnavanja z dušikom in medvrstne razdalje na vsebnost klorofila b v zadnji fazi cvetenja in na vsebnost klorofila a in karotenoidov na koncu rastne sezone. Največja vsebnost olja je bila zabeležena pri150 kg N/ha in 60 cm medvrstni razdalji setve. Na osnovi dobljenih rezultatov lahko zaključimo, da je za največji pridelek buč najprimernejše gnojenje s 150 kg N/ha in 60 cm medvrstna razdalja setve.

Ključne besede: *Cucurbita pepo* L., gnojenje z dušikom, medvrstna razdalja setve, pridelek, vsebnost olja

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1 INTRODUCTION

Nowadays, the importance of medicinal plants is thoroughly known to everyone. Millions of people worldwide are active in planting, harvesting and processing of medicinal plants (Stepleton et al., 2000). Medicinal pumpkin (*Cucurbita pepo* L.) is an important annual plant that belongs to the Cucurbitaceae family (Ebadi et al., 2008). The seeds of pumpkin contain medicinal raw materials that are used for producing some pharmaceutical products such as people to overcome prostatic hypertrophy and urinary tract irritation (Horvath and Bedo, 1998; Younis and Al-Shihry, 2000). Studies on plant density have shown that this agronomic criterion can influence plant development, growth and the marketable yield (Khasmakhi-Sabet et al., 2009). Plant density is one of the main factors determining seed yield in pumpkin (Ebadi et al., 2008). Abdi et al. (2012) tried to study the sowing date and plant density in *Cucurbita pepo* and showed that the highest grain yield belonged to the density of 12500 plant/ha. The other agricultural practices are the fertilizer application, especially nitrogen in terms of its type and rate. It had been observed that nitrogen fertilizer is an essential component of any system in which the aim is to maintain good yield (Law and Egharevba, 2009). The productivity of pumpkin is highly responsive to N fertilizer (Moradi et al., 2014). Sara et al. (2002) reported that nitrogen fertilizer increased fruit mass, yield and fruit number of *Cucurbita maxima* Duchesne. Therefore, the aims of this experiment were to find the best intra-row spacing and nitrogen level for *Cucurbita pepo* plants cultivated under field condition in Northwest Iran.

2 MATERIALS AND METHODS

This study was carried out at the Research Farm of Azarbaijan Shahid Madani University, Iran, in the growing season of 2013. A factorial experiment with Randomized Complete Block Design with three replications was planned to study the effects of nitrogen fertilizer and intra-row spacing in pumpkin. The treatments were 5 levels of nitrogen fertilizer (0, 50, 100, 150 and 200 kg/ha), and intra-row spacing in 3 levels (30, 60 and 90 cm). Each plot had three rows and spacing was 3 m between rows. Each row had 8 plants. There was considered a one row between treatments as a border. After the preparation of the field in early June, according to the planting pattern, seeds (*Cucurbita pepo* L. convar. *pepo v*ar. *styriaca*) were planted in rows. Three seeds were located in every hole. After germination, the strongest plant was kept. The first irrigation was done after planting and the subsequent irrigations were performed based on the climatic conditions. Weeds control was done manually during the growth period. Nitrogen fertilizer (urea) was divided into three equal parts and used at three different phases (planting time, flowering time and fruit set) before irrigation on narrow and at the uniform (10 cm) depth. To study the effect of different levels of nitrogen fertilizer and planting density, after discarding margins, three random samples were selected from each experimental unit and were analyzed for the following characteristics: plant height, number of auxiliary branches, leaves, the number of male and female flowers, intact fruits of each plant, fruits mass, fruit yield, mass of the intact dry seeds, chlorophyll a, chlorophyll b and carotenoids contents.

2.1 Plastid pigments measurements

Chlorophyll (Chl) and carotenoids were extracted from 0.5 g of fresh leaves by grounding in 0.5 mL of acetone (80 % V/V). The absorption was recorded at 645 nm (Chl *α*), 663 nm (Chl *b*) and 470 nm (carotenoids) in a spectrophotometer (PG Instrument LTD $T80^{+}$ UV/VIS. Measurements were carried on the fully expended leaves. Photosynthetic pigment contents were calculated from the following equations as described by Lichtenthaler and Wellburn (1983).

Chl *α* ($mg.g^{-1}$ fr. Wt.) = $11.75 \times A_{663} - 2.35 \times A_{645}$
Chl *b* ($mg.g^{-1}$ fr. Wt.) = $18.61 \times A_{645} - 3.96 \times A_{663}$
Carotenoids ($mg.g^{-1}$ fr. Wt.) = $4.69 \times A_{470} - 0.268 \times (20.2 \times A_{645} + 8.02 \times A_{663})$

2.2 Seed oil extraction and quantification

Oil was extracted from the crushed seed powder (20 g) of the plant by petroleum ether (300 ml) as solvent at 60 °C using soxhlet method according to American Oil Chemists' Society (1983). The obtained extracts were filtered through the Whatman No. 1 filter paper under vacuum. Thereafter, solution was collected and concentrated with a rotary evaporator at 45 °C to reach the pure oil, and then weighted precisely and the percentage of seed oil was determined accordingly.

2.3 Statistical analysis

The data were analyzed using the SAS statistical software. A factorial experiment based on randomized complete block design was carried out with three replicates (n=3). Duncan's Multiple Range Test ($P < 0.01$) was used to compare the means.

3 RESULTS AND DISCUSSION

3.1 Plant height

The interaction effects of nitrogen fertilizer and intra-row spacing on the height of the pumpkin was significant at 1 % level of probability (Table 1). The maximum height of plants was obtained at 150 & 200 kg/ha of nitrogen and 30 or 90 cm intra-row spaces, respectively (Table 2). The result of this experiment was in agreement with the studies of Moazzen et al. (2006). Plant height depends on plant vigor, plant growth habits, and soil fertility. It seems that the plant height was increased when fertilizer rates increased.

3.2 Number of auxiliary branches

The number of auxiliary branches in pumpkin was affected by the interaction of experimental factors (Table 1). The maximum number of auxiliary branches was observed in intra-row spacing of 90 cm and 200 kg/ha nitrogen fertilizer (Table 2). Moazzen et al. (2006) showed that there was not a significant effect between planting density and auxiliary branches of pumpkin at the early flowering stage.

3.3 Number of leaves and leaf dry mass

There was a significant interaction effect between nitrogen and intra-row spacing on the number of pumpkin leaves (Table 1). The maximum number of leaves was related to 90 cm planting distance and when N fertilizer rates applied was 200 kg/ha (Table 2). The additive effect of nitrogen levels on the pumpkin leaves was associated to the plant metabolism that resulted in increasing of the photosynthetic products, and consequently, increased biomass (Omidbeigi, 2000). Moazzen et al. (2006) demonstrated that with reducing planting density, due to increasing the spacing for each plant, the availability of nutrients and other growth factors increased which in turn increased the plant height, number of nodes, auxiliary branches and the leaves compared with high planting densities.

3.4 The number of male and female flowers

Different levels of nitrogen and intra-row spacing had significant effect on the number of male and female flowers (Table 1). The highest number of male and female flowers was observed at 150 kg/ha nitrogen fertilizer and within row-spacing of 60 and 90 cm (Table 2). Appearance of male and female flowers in pumpkins is controlled by endogenous hormones of plant which is severely affected by the environmental conditions (Stepleton et al., 2000). Studies by Hafideh (2002) on *Cucurbita pepo* showed that by reducing the distance within a row from 30 to 20 and from 20 to 10 cm in two consecutive years, the number of flowers and yield of plants were increased. In the Cucurbitaceae family, high density of plant with narrow distances may increase male flowers. This is possible by reducing the light incidence to the plant due to the high density (Lower, 1983).

3.5 The total number of fruits per plant

The total number of fruits per plant was significantly affected by intra-row spacing and nitrogen fertilizer (Table 1). The highest number of fruits per plant was related to 50 and 150 kg/ha nitrogen fertilizer within row-spacing of 60 and 90 cm (Table 2). According to Edelstein et al. (1989) there is negative relationship between the high density and the number of fruits per plant in

pumpkin. Result of this experiment is in agreement with the finding of Edelstein et al. (1989).

3.6 Fresh mass of fruit

Nitrogen treatments have significant effects on the mass of fresh pumpkin fruit. The maximum fresh mass of fruit was observed in 150 kg/ha N fertilizer (Table 2). The results of this experiment corresponded with the findings of the experiment of Gholipoori et al. (2007) on the term of increasing fruit mass by nitrogen. Any increase in nitrogen applications are associated with increase in leaf area and mass, chlorophyll content, increase in light capture, photosynthetic activities of leaf and ultimately leads to the increase in the number and size of fruit cells (Marcelis, 1992). Yadeghari and Barzegar (2010) stated that there was a negative correlation between the mass of the fruit and number of fruits. In other words, increasing plant density, led to competition between the plants. Fruit mass was reduced and the yield was correspondingly affected.

3.7 Number of intact fruits per plant

Number of intact fruits per plant was significantly affected by nitrogen fertilizer and intra-row spacing (Table 1). The maximum number of safe fruits was obtained in 50 kg N/ha of applied fertilizer within row-spacing of 90 cm (Table 2). Effect of nitrogen fertilizer on plants was related to its role in plant metabolism and photosynthetic products (Omidbeigi, 2006). Chlorophyll is required to absorb light and to precede photosynthesis, and N fertilizer had a positive role in this area. An adequate availability would cause disruptions in the vital metabolism of plants (Salisbury and Ross, 1991).

3.8 Fruit yield

Fruit yield was significantly affected by the intra-row spacing, nitrogen, and the interaction of these two treatments (Table 1). Highest fruit yield was obtained when fertilizer and intra-row spacing were 150 kg N/ha and 60 cm, respectively (Table 2). The application of fertilizers amount above 150 kg N/ha increased plants height, auxiliary branches and leaf number. Reducing the plant density went to a reduction in plant competition and enhanced the yield of fruits. More possibly reducing the fruit yields in 200 kg N/ha nitrogen application has been the result of excessive vegetative growth due to high level of applied fertilizer. Fertilizer application has to be restorable interns of production cost (Khajehpoor, 2010). Since higher levels of nitrogen in addition to the increase in production costs, yield and fruit quality decreases.

3.9 Seed dry weight

Seed dry weight was significantly affected by nitrogen fertilizer and intra-row spacing (Table 1). The highest fruit yield was obtained when fertilizer and intra-row spacing were 150 kg N/ha and 60 cm, respectively (Table 2). The application of 150 kg N/ha increased number seed per plant (data not shown) and also seed dry weight. It had been observed that the productivity of pumpkin is highly responsive to N fertilizer (Moradi et al., 2014).

Table 1: Analysis of variance for some traits investigated in pumpkin in response to nitrogen fertilizer rates & intra-row spacing

Source		Means of Square										
	d.f.	**Plant height**	Number of auxiliary branches per plant	Number of leaves	Leaf dry mass	Number of female flowers	number of male flowers	Fresh mass of fruit	Fruit yield	Total number of fruits per plant	Number of intact fruits per plant	mass of the intact dry seeds
block	2	0.18^{ns}	16.02^{ns}	2.86^{ns}	0.07^{ns}	11.26^{ns}	30.86^{ns}	105325.32^{ns}	2450.94^{ns}	4.20^{ns}	0.35^{ns}	308.74^{*}
Factor a (nitrogen fertilizer levels)	4	2.91^{**}	941.41^{**}	3626.13^{**}	12.20^{**}	178.97^{**}	4835.63^{**}	1414167.41^{**}	5680.21^{**}	58.66^{**}	6.07^{**}	5436.52^{**}
Factor b (intra-row spacing)	2	0.23^{*}	3202.48^{**}	850.86^{**}	0.19^{*}	44.60^{**}	962.60^{**}	173056.42^{ns}	52948.5^{*}	23.26^{*}	0.95^{ns}	161.56^{*}
Interaction a*b	8	0.48^{**}	183.87^{**}	55.78^{**}	0.03^{ns}	2.29^{ns}	32.93^{ns}	63952.34^{ns}	571.72^{*}	5.26^{*}	0.84^{*}	77.43^{ns}
Error	28	0.07	7.92	10.24	0.40	3.52	17.79	8751.19	189.57	2	0.35	48.66

**and * significant at 0.01 and 0.05 probability levels, respectively; ns, non significant

Table 2: Means values for some traits investigated in pumpkin in response to nitrogen fertilizer rates & intra-row spacing

Experimental Factors	Plant height (m)	Number of auxiliary branches per plant	Number of leaves	Leaf dry mass (g)	number of female flowers	Number of male flowers	Fresh mass of fruit (g/plant)	Fruit yield (tha^{-1})	Total number of fruits per plant	Number of intact fruits per plant	mass of the intact dry seeds (gr per fruit)
nitrogen treatment(kg N/ha)											
0	3.07 c	24.88 d	8.88 e	2.59 e	11.77 d	24 e	1582.2 a	36.73 c	5.55 c	2.33 b	9.21 d
50	3.47 b	30.55 c	30.55 d	2.94 d	14.44 c	36.44 d	1901 b	76.76 ab	11 a	4 a	30.27 c
100	3.67 b	34.11 b	43.33 c	3.72 c	16.66 b	49.66 c	2026.3 b	70.58 b	10.55 a	3.44 a	37.09 b
150	4.37 a	48.33 a	54.66 b	4.48 b	22.22 a	84.77 a	2445.6 a	86.77 a	11.66 a	3.55 a	75.43 a
200	4.35 a	46.66 a	58.22 a	5.46 a	11.22 d	59.11 b	c1434.9	31c	7.88 b	2.11 b	25.46 c
Intra- row spacing treatment (cm)											
30	3.74 ab	24.64 c	31 c	3.71 b	13.40 b	43.66 c	1759.1 a	51.32 b	8 b	2.8 b	32.67 b
60	3.69 b	33.26 b	40.53 b	3.87 a	15.60 a	49.26 b	1968 a	66.60 a	9.53 a	3.2 ab	39.10 a
90	3.93 a	53 a	45.86 a	3.93 a	16.80 a	59.46 a	1907 a	62.83 a	10.46 a	3.26 a	34.71 ab

Within columns, mean values followed by the same letter are not significantly different at the 0.05 level, according to Duncan's multiple range test.

3.10 Leaf chlorophyll and carotenoid content during the pumpkin seed development (early flowering and late flowering)

The studied treatments factors effects and their interactions on chlorophyll a, chlorophyll b and carotenoid in the early flowering stage were not significant (Table 3). The content of chlorophyll b in late flowering stage was influenced by the interaction of experimental treatments (Table 3). There was a significant difference between the control and 150 kg N/ha nitrogen on chlorophyll b in late flowering stage (Table 4). The highest content for chlorophyll b was obtained in intra-row spacing of 30 cm (Table 4). Different rates of nitrogen fertilizer and intra-row spacing didn't have significant effect on the amount of chlorophyll b in the last stage of growth in pumpkin. Content of chlorophyll a and carotenoid were affected by the interaction of studied treatments (Table 3). Chlorophyll b is highly unstable under high temperature conditions, its activity is strongly reduced and also heat stress denaturates 33 kDa proteins responsible for the stability of Mn^{2+} in the reaction center of photosystem II (oxygen-evolving complex in photosystem II). As a result, Mn^{2+} atoms are released from the reaction center and this causes the instability of chlorophyll b (Tiaz and Zeiger, 2010). Nitrogen is one of the essential structural elements of chlorophyll, therefore the increase in the rate of N in growth environment results in increasing the levels of chlorophylls (Gross, 1991).

Table 3: Means values for some traits investigated in pumpkin in response to nitrogen fertilizer rates & intra-row spacing

Source	Mean of Square									
	d.f.	Chlorophyll a in early of flowering	Chlorophyll b in early of flowering	Carotenoid in early of flowering	Chlorophyll a in late of flowering	Chlorophyll b In late of flowering	Carotenoid in late of flowering	Chlorophyll a In last stage of growth	Chlorophyll b In last stage of growth	Carotenoid in last stage of growth
Block	2	0.006^{ns}	0.979^{ns}	0.067^{ns}	0.058^{ns}	0.061^{ns}	0.200^{ns}	0.002^{ns}	0.016^{ns}	0.008^{ns}
Factor a (nitrogen fertilizer levels)	4	0.335^{ns}	0.530^{ns}	0.260^{ns}	0.184^{ns}	0.360^{ns}	0.088^{ns}	0.077^{ns}	0.558^{ns}	0.351^{**}
Factor b (Intra-row spacing)	2	0.078^{ns}	0.076^{ns}	0.063^{ns}	0.061^{ns}	0.993^{ns}	0.556^{ns}	0.405^{ns}	0.109^{ns}	0.467^{**}
Interaction a*b	8	0.088^{ns}	0.499^{ns}	0.057^{ns}	0.217^{ns}	0.167^{**}	0.343^{ns}	0.308^{**}	0.492^{ns}	0.393^{**}
Error	28	0.150	0.390	0.108	0.144	0.165	0.117	0.073	0.138	0.027

**and * significant at 0.01 and 0.05 probability levels, respectively; ns, non significant. Units of pigments is mg.g-1 fr. Wt..

Table 4: Means values for some traits investigated in pumpkin in response to nitrogen fertilizer rates & intra-row spacing

	Chlorophyll a in early of flowering	Chlorophyll b in early of flowering	Carotenoid in early of flowering	Chlorophyll a in late of flowering	Chlorophyll b in late of flowering	Carotenoid in late of flowering	Chlorophyll a in last stage of growth	Chlorophyll b in last stage of growth	Carotenoid in last stage of growth
Nitrogen treatment (kg N/ha)									
0	0.82ab	1.46ab	0.83 a	0.88ab	0.85 a	0.38 a	0.36 a	0.37 bc	0.20 d
50	0.83ab	1.34ab	0.78ab	0.02 a	0.87ab	0.57 a	0.39 a	0.79 a	0.53 b
100	0.96 a	1.63 a	0.92 a	0.91ab	0.62ab	0.57 a	0.49 a	0.50 abc	0.37 c
150	0.46 a	1.02 b	0.48 b	0.65 b	0.93 a	0.60 a	0.60 a	0.16 c	0.73 a
200	0.65ab	1.58ab	0.65ab	0.97ab	1.77 b	0.63 a	0.47 a	0.67 ab	0.51 b
Intra- row spacing treatment (cm)									
30	0.69 a	1.37 a	0.67 a	0.94 a	1.03 a	0.39 b	0.63 a	0.59 a	0.67 a
60	0.72 a	1.39 a	0.73 a	0.90 a	0.60 b	0.77 a	0.30 b	0.49 a	0.33 b
90	0.83 a	1.49 a	0.80 a	0.82 a	0.57 b	0.49 b	0.46ab	0.42 a	0.41 b

Within columns, mean values followed by the same letter are not significantly different at the 0.05 level, according to Duncan's multiple range test. Units of pigments is $mg.g^{-1}$ fr. Wt.

3.11 Seed oil percentage

Effect of nitrogen fertilizer treatments on seed oil content was significant (Table 5). The highest seed oil content was related to 150 kg N/ha and 60 cm intra-row spacing (Table 6). Nitrogen is considered as effective element contributing to the increase in leaf area and photosynthesis rate. Therefore, the appropriate amount of nitrogen can increase fatty acids & plant oil content and yield. Moradi *et al*. (2014) have studied the effects of different levels of nitrogen and planting distance on oil content of pumpkin. The results showed that the highest percentage of pumpkin oil was related to 250 kg/ha of nitrogen fertilizer and density of 1.25 plant/m^2.

Table 5: Analysis of variance for the seed oil percent in pumpkin in response to nitrogen fertilizer rates

Source	d.f.	Means of Square
		Seed oil percent
block	2	192.1
Factor a (nitrogen level)	4	292.46*
Error	2	0.381

**and * significant at 0.01 and 0.05 probability levels, respectively; ns, non significant

Table 6: Means values for the seed oil percent in pumpkin in response to nitrogen fertilizer rates

nitrogen treatment (kg N/ha)	Seed oil percent
0	40 c
150	84.47 a
200	31.44 b

Within columns, mean values followed by the same letter are not significantly different at the 0.05 level, according to Duncan's multiple range test.

4 CONCLUSIONS

The main goal of planting pumpkin was the use of its seeds. According to the results of the experiment, in the tested ecological region at 150 kg/ha of nitrogen fertilizer & intra-row spacing of 60 cm, maximum number of female flowers, fruit number, intact fruit number, fruit diameter, fruit mass, fruit yield and seed dry weight was observed. The highest content of chlorophyll b and a, as well as carotenoid in the late growing pumpkin were related to 150 kg N/ha fertilizer treatments and intra-row spacing of 30 cm. The highest percent of seed oil was obtained from 150 kg/ha fertilizer treatments and intra-row spacing of 60 cm. Based on the results observed, it would be the best to apply nitrogen fertilizer at the rate of 150 kg N/ha & intra-row spacing of 60 cm for pumpkin production.

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Agrovoc descriptors: microwave radiation; seed treatment; disease control; *aspergillus niger;* seed vigour; seed viability ; seed characteristics; germinability; oil crops; rapeseed; soybeans; safflower

Agris category code: F03, h01, q04

Effect of microwave radiation on seed viability, survival of *Aspergillus niger* van Tieghem and oil quality of oilseeds crops canola, soybean and safflower

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ABSTRACT

The effect of microwave's radiation on seed viability of three different oilseed crops, spores of *Aspergillus niger* and quality of extracted oil from treated seeds over various exposure times was evaluated. The seeds were exposed to 2450 MHz. at five different power levels of 0, 100, 200, 400, 600 and 800 W for two exposure times of three and five minutes. At a given time, a direct negative relationship between seed viability and microwave's radiation power level was detected. Substantial variation in the lethality of tested seeds to microwave's power levels was apparent in the fiducial limits of the estimated LD_{50} values in probit analysis approach. A similar trend of *A. niger* spores' susceptibility to microwave radiation was detected. The microwaves' radiation and exposure time did not impact one another and a significant interaction was not detected. Short term fungal infection did not cause substantial quantitative and qualitative damage to the oilseeds. The oil quality was generally unaffected by microwave radiation and fungal infestation for tested oilseeds. Moreover, microwave radiation decreased seed germination percentage and vigor index. The microwave radiation could provide an effective and friendly environmental treatment technique for improving the dietary consumption of the oil in any seed disinfestation program.

Key words: disinfestation, microwave, *Aspergillus niger*, vigor index, oil composition, canola, soybean, safflower

IZVLEČEK

VPLIV MIKROVALOVNEGA SEVANJA NA VIABILNOST SEMEN, PREŽIVETJE GLIVE *Aspergillus niger* van Tieghem IN KVALITETO OLJA OLJNE OGRŠČICE, SOJE IN ŽAFRANIKE

V raziskavi je bil preučevan učinek mikrovalovnega sevanja na viabilnost semen treh oljaric, preživetje spor glive *Aspergillus niger* in kakovost olja, pridobljenega iz tretiranih semen v odvisnosti od časa izpostavitve. Semena so bila izpostavljena mikrovalovnemu sevanju 2450 MHz s petimi stopnjami moči (0, 100, 200, 400, 600 in 800 W) za čas treh in pet minut. Po določenem času tretmaja je bila ugotovljena neposredna negativna povezava med viabilnostjo semen in močjo mikrovalovnega sevanja. Očitna je bila znatna variabilnost v smrtnosti testiranih semen glede na moč mikrovalovnega sevanja, statistično ovrednotena kot standardne LD_{50} vrednosti. Podoben trend občutljivosti na mikrovalovno sevanje so pokazale tudi spore glive *A. niger*. Mikrovalovno sevanjem in čas izpostavitve semen nista vplivala drug na drugega, značilna interakcija ni bila ugotovljena. Kratkotrajna okužba semen z glivami ni povzročila večjih kakovostnih in količinskih izgub. Kvaliteta olja vseh tretiranih semen oljaric je bila v glavnem nespremenjena po mikrovalovnem obsevanju in okužbo z glivo. Mikrovalovno obsevanje je zmanšalo odstotek kalitve in vitalnostni indeks vseh tretiranih semen. Mikrovalovno obsevanje lahko nudi učinkovit in okolju prijazen način izboljšanja ohranjanja olj v prehrani v kateremkoli dezinfekcijskem programu semen.

Ključne besede: dezinfekcija, mikrovalovi, *Aspergillus niger*, vitalnostni indeks, sestava olj, oljna ogrščica, soja, žafranika

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1 INTRODUCTION

Microwave's energy is not persistent in the environment and does not have hazardous impacts or damage to a foodstuff (Vadivambal et al., 2007). Radiation has been recognized as a cheap, safe and effective technique for food preservation among all existing techniques (Bangash et al., 2007). In this line, Bangash et al. (2007) as well as Kumar and Viswanathan (2013) stated that the stability of many edible oils such as canola has been improved by irradiation treatment. Temperature is one of the principal factors delimitating survival and reproduction of fungal spores. Lethal temperatures are those above or below the suboptimum which will eventually kill the organism. Decontamination through temperature manipulation is receiving renewed interest as a non-chemical method with lack of a residue problem (Hallman and Denlinger, 1999).

Fungi can be controlled by manipulating the physical environment or by applying physical treatments to the infested oilseed. Fungal spores are abundant in the atmosphere and settle on to surfaces, can grow and form colonies depending on the physicochemical environmental conditions (Christofi et al., 2008; Ehrensing, 2008). Many surfaces, including oilseeds become infested; therefore, it is a requirement for effective disinfestations (Christofi et al., 2008). In this line, Afolabi et al., (2015) stated that microwave radiation could detoxify seed oil and improve its dietary and industrial use. Organisms under microwave's irradiation are prone to some types of stress such as controlled atmosphere and cold ambient (Wang and Tang, 2001). The warehouse environment is usually one that is enclosed, allowing for the manipulation of temperature. Thus, the use of temperature to restrict fungal growth is an excellent tool for the stored-product industry. Exposure to temperatures only 5 °C above the optimum are capable of slowing down or stopping fungal activity and development and depending on the species, are capable of causing death. The review of the literature revealed the scarcity of information concern over optimal power levels of microwave's radiation combined with the exposure period in oilseeds disinfestations and fungal control programs. To clarify the interaction issue, the present investigation was undertaken. The purpose of this study is specifically to evaluate the effect of microwave radiation on the viability *of A. niger* spores, the quality of oil, oilseed germination, and seedling vigor index.

2 MATERIALS AND METHODS

The study was conducted at the Urmia University, Urmia (37.34 °N 44.58 °E and altitude 1365 m) a town in Iran in 2015. All the experiments were conducted using a kitchen type, 2450 MHz microwaves oven (Butane, BC 320 W, Butane Co., Tehran, Iran) with capability of producing 100 through 1000 W microwave power. The experimental units and bioassay procedures were identical in all trials. In each trial, the control petri plates were treated identically except that no microwave radiation treatment was employed.

2.1 Fungal isolate

An isolate of *Aspergillus niger* van Tieghem, Ani109, was provided by mycology laboratory of Urmia university which was isolated from canola (*Brassica napus* 'Okapi') seeds. The fungus was grown on potato dextrose agar (PDA, Merck) medium (9 cm diameter Petri plates) and incubated at 25 °C for 7 days in the dark for full sporulation. Spores were scrapped with a sterilized scalpel from the medium and suspended in distilled sterile water containing 0.1 % Trition X100. Suspension was passed through double layer sterile cheese cloth to remove mycelia and medium debris and the concentration of the suspension was adjusted to 1×10^6 spore/ml with a neubauer haemocytometer.

2.2 Bioassays

Seeds of three oilseed crops viz. canola (*Brassica napus* 'Okapi'), soybean (*Glycine max* 'Williams') and safflower (*Carthamus tinctorius* 'Goldasht') were treated with a suspension of 1×10^6 spore/ml for 15 minutes and then allowed to dry. The amount of the spore suspension was adjusted enough to cover all the seeds and seeds were

agitated three times with a sterile glass rod in order to complete contact with fungal spores. In controls, seeds were treated with distilled water containing 0.1 % Trition X100. To commence oilseed microwave's irradiation, each petri plate (15 cm diameter) containing 50 treated seeds was placed in the microwave's oven with capability of producing 100 through 1000 W microwave's power. For microwave's radiation, five power outputs of the generator were set at 100, 200, 400, 600 and 800 W. The exposure times were 3 and 5 minutes. After treatments, seed were allowed to germinate under their usual growth conditions for 7 days and the mean shoot length of fifty seedlings was determined at 8 days. Vigor index was calculated using the formula: Vigor index = Germination seed percentage × seminal root length.

In the case of the effect of irradiation on fungal spore viability, all the treated seeds were transferred into 15 cm sterilized screw cap test tubes containing 5-10 ml distilled water and 0.1 % Trition X100. Test tubes were agitated vigorously for 5 minutes. One ml of the suspension was taken with a sterile Pasteur pipette and was sprayed over the PDA medium in 9 cm Petri plates. Petri plates were incubated at 25 °C in the dark for 48 h and were seen under the 40X magnification. Where the length of the germ tube was more than spore diameter, the spores were counted as germinated, otherwise they were considered dead. Two hundred spores were examined each time. Each test was replicated four times. Mortality data from the replicates were pooled, and mortality response was determined. In order to evaluate the combination's effect of the microwave's power and exposure duration, a factorial experiment with 6 power levels and 2 exposure duration was performed.

2.3 Oil Extraction and Composition

For each oil seed crop, a sample of 20 g of clean seeds was isolated to measure the oil concentrations. Soxhlet extraction technique was employed to determine the total oil concentration of the canola seed and the oil concentration was expressed as mg/g (Movahhedy-Dehnavy et al., 2009). The oil concentrations were reported as percent of the seed weight standardized to 9% moisture. Lipid was extracted from 20 g of ground seed three times at room temperature by homogenization with hexane/isopropanol (3:2 v/v) (St. John and Bell, 1989). The formation of FAME was carried out according to the procedure described by Desvilettes et al (1994). The sample was saponified with methanolic sodium hydroxide and the fatty acids were esterified with methanolic sulfuric acid. FAME were analyzed with a 6890 NGC-FID (Agilent Technologies, Wilmington, DE, USA) fitted with a J & WDB-Wax capillary column (30 m, 0.25 mm i.d., 0.25 mm film thickness), a split-split less inject or with Agilent tape red liner (4 mm i.d.) and flame ionization detector. The initial column temperature was maintained at 100 ºC for 1min and then raised at 25 ºC/min to 190 ºC and held for 10 min. Nitrogen was used as carrier and make up gas, at flow rates of 1.0 and 45 mL/min, respectively. The injector and detector temperature were held at 250 ºC and 260 ºC, respectively. Chem Stations Software was used for online data collection and processing. Individual FAME was identified by comparison with known standards (Sigma, Chemical Co. St. Louis).

2.4 Data analysis

Mortality data from all bioassays were analyzed with SPSS Software (SPSS Inc., 1993). Probit analysis was used to estimate LD_{50} and LD_{95} values (watt) and the slopes of the regression lines. The values and significance of χ^2 and the 95 % CL were determined according to Robertson et al., (2007). Parallel regression lines were also compared using overlapping confidence limits ($p \leq 0.05$) of relative potencies as the criterion to detect significant differences in mortality. In Factorial experiments and germination tests, the data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test to determine statistical differences between means at $\alpha = 0.05$.

3 RESULTS

3.1 Susceptibility of canola, soybean and safflower to microwave radiation

The present study revealed that there is a reduction in germination rate of oilseeds after exposure to microwave power (Table 1). Based on overlapping fiducially limits, three oilseeds tested showed similar susceptibility to microwave radiation; however, slope of the probit-log dose line reveals that safflower has more natural mortality rate compared to the other oilseeds.

Table 1: Lethality of microwave radiations at 5-minute exposure duration to oilseeds

Oilseed	LD_{50} (watt)	95 % FL	Slope ± SEM	Chi-Square	df	Sig.
G. max	516	403-729	1.077±0.183	0.618	3	0.892
C. tinctorius	535	405-811	0.957±0.179	0.997	3	0.801
B. napus	622	483-913	1.129±0.12	0.185	3	0.681

3.2 Susceptibility of *Aspergillus niger* spores to microwave radiation

This study proved that *A. niger* is susceptible to microwave radiation (Table 2). Based on the fiducially limit of LD_{50} values due to overlapping these limits, the three oilseeds' *A. niger* showed similar susceptibility to microwave stimuli. The slopes of regression lines provide sufficient evidence that the regression lines are parallel with different intercepts.

Table 2: Susceptibility of *Aspergillus niger* to microwave radiation at 5-minute exposure duration

Oilseed	LD_{50} (watt)	95 % FL	Slope ± SEM	Chi-Square	df	Sig.
G. max	1063	816-88901	2.651±1.312	1.687	2	0.43
C. tinctorius	716	189-1179	1.315±0.413	2.220	2	0.329
B. napus	1175	862-79021	1.969±0.925	0.343	2	0.842

The unit 'watt' is a power rating in its own

3.3 Interaction between power level and exposure duration

The result reveals that a statistically significant interaction does not exist between microwave power level and exposure duration in the current study. Apparently, power levels have been differently hazardous to seed germination; and are more important to germination compared to exposure duration (Table 3). The lack of interaction of two factors implies that the power level with exposure period impact the *A. niger* independently.

Table 3: Lethality of various microwave power levels and exposure duration on oilseeds germination

Oilseed	Source	df	Mean Square	F	Sig.
G. max	Corrected Model	11	937.111	113.58	0.000
	Intercept	1	21121.778	2560.215	0.000
	A	5	2046.378	248.046	0.000
	B	1	9.000	1.091	0.307
	A×B	5	13.467	1.632	0.190
	Error	24	8.250		
	Corrected Total	35			
	R Squared = 0.981				
C. tinctorius	Corrected Model	11	1088.657	72.443	0.000
	Intercept	1	20832	1386.240	0.000
	A	5	2381.044	158.433	0.000
	B	1	25.000	1.664	0.209
	A×B	5	9.00	0.599	0.701
	Error	24	15.028		
	Corrected Total	35			
	R Squared = 0.957				
B. napus	Corrected Model	11	961.947	91.132	0.000
	Intercept	1	19460.250	1843.603	0.000
	A	5	2104.783	199.401	0.000
	B	1	23.361	2.213	0.150
	A×B	5	6.828	0.647	0.667
	Error	24	10.556		
	Corrected Total	35			
	R Squared = 0.977				

A: radiation power levels; B: radiation times

3.4 Effect of microwave radiation on germination percentage and vigor index

The germination percentage and vigor index of oilseeds were reduced as compared to healthy seeds of all the three types of tested seeds (Table 4). The reduction in germination percentage was observed after 7th day of both infested and healthy seeds. The reduction of seed germination compared to control group was 13 %, 14 % and 15 % for soybean, canola, and safflower, respectively. Concerning vigor index of infested seeds, the index was 487, 136, and 106, respectively. While for control seeds, vigor index for soybean, canola, and safflower was 910, 694 and 203. As it is obvious there has been drastic reduction in seedling vigor indexes of infested and healthy seeds.

Overall, this study proved the ability of the microwaves period to kill the fungal colonies and do not allow for the growth of fungal spores, meaning that the rate of growth of fungal colonies is positively proportional to the microwaves power level.

Table 4: Effect of microwave radiation on germination percentage and vigor index of three oilseed crops

Oil-seed		*G. max*	*C. tinctorius*	*B. napus*
Germination percentage after 7th day	Control	99	94	86
	Infested	88	80	44
Vigor index after 7th day	Control	910	203	694
	Infested	487	106	136

3.5 Microwave radiation and oil composition

In 35 out of 36 cases, the effects of microwave radiation on oil composition were similar to those of control treatment (Table 5). Moreover, results display that for soybean and safflower; mean oil content is different from control cohorts, while in the case of canola no significant difference was detected between infested and healthy seeds (Table 6).

Table 5: Mean comparisons of oil compositions of *G. max*, *C. tinctorius,* and *B. napus* infested with *A. niger* and control group

Traits	Fatty acid profile (%)											
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3n	C20:0	C20:1	C22:0	C24:0	C24:1
G. max	0.079	11.17	0.099	3.747	17.44	55.46	9.913	0.322	0.232	0.336	0.064	0.163
Control	0.135	11.09	0.144	3.741	18.33	54.84	9.642	0.381	0.279	0.344	0.236	0.160
t	-0.99	0.28	-0.68	0.10	-1.16	3.92	3.01	-0.76	-0.83	-0.31	-1.79	0.027
p	0.42	0.80	0.36	0.92	0.36	0.059	0.09	0.52	0.49	0.78	0.215	0.98
C. tinctorius	0.14	6.47	0.069	2.351	13.79	73.99	0.368	0.215	0.43	0.69	0.27	0.364
Control	0.15	6.63	0.095	2.36	14.05	73.65	0.401	0.281	0.26	0.69	0.23	0.254
t	1.33	1.30	0.79	0.64	0.38	-0.45	0.58	0.78	-7.25	0.04	-0.25	-0.86
p	0.31	0.32	0.51	0.58	0.74	0.69	0.61	0.51	0.01	0.97	0.82	0.48
B. napus	0.060	4.51	0.22	2.13	62.31	17.98	9.05	0.66	1.39	0.32	0.17	0.208
Control	0.063	4.44	0.22	2.10	62.64	17.64	9.12	0.67	1.39	0.33	0.23	0.166
t	-1.05	1.20	-0.07	1.95	-0.88	0.99	-1.48	-0.14	0.07	-0.64	-1.51	1.26
p	0.40	0.35	0.94	0.19	0.46	0.42	0.27	0.90	0.94	0.58	0.27	0.33

C14:0 myristic acid; C16:0 palmitic acid; C16:1 palmitoleic acid; C18:0 stearic acid; C18:1 oleic acid; C18:2 linoleic acid; C18:3n linolenic acid; C20:0 arachidic acid; C20:1 gadoleic acid; C 22:0 behenic acid; C24:0 lignoceric acid; C24:1 nevonic acid.

t= t-test, *p*= probability

Table 6: Effect of *Aspergillus niger* on oil content of three oilseed crops

Oilseed	% Oil content in oilseeds	
	Control (Mean ±S.E.)	Infested (Mean±S.E.)
G. max	19.64 ± 0.41	14.34±0.53*
C. tinctorius	32.27±0.43	29.27±0.39*
B. napus	38.77±1.16	$37.32±1.03^{ns}$

*$p \leq 0.05$; ns=not significant

4 DISCUSSION

Distinct characteristics such as faster heating rate and greater penetration in food stuff depth have made microwaves a unique tool for many industrial sanitation applications such as sterilization and disinfestations (Knutson et al., 1987). The mechanism of devastation of pathogens through microwaves is controversial. However, there is an anonymous agreement about the

destructive effect of microwaves. In this line, Datta and Davidson (2000) reported that no pathogen had been reported to be microwave resistant. Nevertheless, similar to any other process, microwave applications have their own challenges. For instance, uneven temperature distribution is one of the problems with microwave applications under field conditions.

The test oilseeds are the main important source of vegetable oil in the world. For instance, soybeans and canola oils are first and second important edible oil resources worldwide. These oils are considered healthy for human nutrition due to their lowest content of saturated fatty acids among vegetable oils and moderate content of poly-unsaturated fatty acids. In the present study, the oil quality of oilseeds was generally unaffected by microwave radiation. Although fatty acid profiles vary somewhat from sample to sample, they are widely used to characterize vegetable oils from particular species or varieties of plants (Ehernsing, 2008).

There is a drastic reduction in seedling vigor indexes of infested and healthy seeds. The results of current study confirmed the statement of Nargund et al. (2003) who argued that *A. niger* reduces germination percentage and vigor index in all genotype of *Arachis hypogea.* In order to analyze the influence of the factors (exposure time and power level) on the destruction rate for oilseed and spores, the empirical data were statistically analyzed. The probit analysis revealed that microwave radiation had a deleterious impact on oilseed viability (Table 3). The lack of statistically significant interaction most likely is due to short term exposure time. The reasoning behind is the lack of significant difference between two levels of the exposure period. Therefore, the extension of the exposure period is imperative and even inevitable action and must be done. The distinction between live seeds and germinated seeds is important since microwave radiation may cause injury by retarding germination as well as destruction of germinative capacity. Therefore, decreases in germination rate or seminal roots length after radiation was adequate to prove a deleterious effect of microwave radiation on oilseeds seed viability. A new approach in fungal control research could be the use of less hazardous substances or control methods, which are more compatible with environment. Method for the control strategies that are environmentally sustainable and avoid the use of conventional is of paramount important. Disinfestations fungal infestation stored-products with physical control methods such as using microwaves energy treatment can be an alternative measure to fungicide in controlling fungi.

Microwave power, as a powerful decontamination tool showed acceptable biological effectiveness against *A. niger*. With retrospect, due to sorption characteristics and deleterious effects on oilseed viability, microwave radiation may have only limited use in oilseed decontamination. Nevertheless, since microwave is highly toxic to *A. niger,* and because it is compatible with the environment, it could be considered as a potential compound for oilseed disinfestations. It is well established that a good control agent must kill the target organism with an acceptable level of the agent in a short period of time. Since, microwave's power is lethal to the *A. niger*, it could be considered as a potential tool which helps to reduce *A. niger's* development in any disinfestations programs.

5 CONCULSIONS

From the present study it was evident that *Aspergillus niger* was sensitive to microwave radiation. Its spores were killed. Oil quality was generally unaffected by microwave radiation, but microwave radiation had a deleterious impact on oilseeds seed viability. Also it can be concluded that more detailed studies are required to explore a safer and economic technique to evaluate seed borne fungi infestation potentials, to that exposed seeds' oil might be safe guarded.

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Influence of exogenous polyamines on antioxidant defence and essential oil production in valerian (*Valeriana officinalis* L.) plants under drought stress

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ABSTRACT

The objective of this study was to determine the effects of foliar application of polyamines (PAs) on antioxidant defence and essential oil production of valerian (*Valeriana officinalis* L.) grown under different drought stress treatments (100, 70, 50 and 30% available water content). This study was carried out using pots in greenhouse condition. Drought–stressed valerian seedlings were sprayed with 1 mM concentration of each putrescine (Put), spermidine (Spd) and spermine (Spm). The results showed that drought stress significantly affected most biochemical characteristics of valerian plants. Characteristics including leaf relative water content, chlorophyll a and b contents were decreased, while carotenoids and electrolyte leakage were increased with the increase of water stress. In this research, defensive characteristics comprising proline content, soluble sugars, catalase, and ascorbate peroxidase were increased followed by drought stress to ameliorate the adverse effect of it. Results revealed that foliar application of Spd and Spm provoked the antioxidant enzymes activity as well as proline accumulation in valerian which alleviate the membrane damages. Consequently, Spd and Spm increased photosynthetic pigments which act as energy supply for plant growth and production. Here, putrescine had detrimental effects on CAT activity and Chl a content. Albeit, PAs presented remarkable effects under moderate drought stress condition but it showed reverse trends in severe drought stress condition. In terms of quantity and quality yield, drought stress reduced root growth but increased the concentration of essential oils. PAs are able to alleviate water deficit-induced diminish root growth. These results suggest that in moderate drought stress, growers can use PAs to increase productivity valerian.

Key words: biochemical characteristics, drought stress, essential oils, polyamines, *Valeriana officinalis*

IZVLEČEK

VPLIV DODAJANJA POLIAMINOV NA ANTIOKSIDATIVNO OBRAMBO IN PRODUKCIJO ETERIČNIH OLJ PRI ZDRAVILNI ŠPAJKI (*Valeriana officinalis* L.) V RAZMERAH SUŠNEGA STRESA

Namen raziskave je bil določiti učinke foliarnega dodajanja poliaminov (PAs) na antioksidacijsko obrambo in tvorbo eteričnih olj pri zdravilni špajki (*Valeriana officinalis* L.) gojeni pri različni oskrbi z vodo (100, 70, 50 in 30 % vsebnosti razpoložljive vode). Raziskava je bile izvedena kot lončni poskus v rastlinjaku. Sadike špajke v sušnem stresu so bile škropljene z 1 mM raztopino putrescina (Put), spermidina (Spd) in spermina (Spm). Rezultati so pokazali, da je sušni stres značilno vplival na večino analiziranih biokemičnih parametrov v zdravilni špajki. Parametri kot so relativna vsebnost vode, vsebnost klorofila a in b so se zmanjšali, medtem ko sta se vsebnost karotenoidov in iztok elektrolitov povečala z večanjem sušnega stresa. Obrambni mehanizmi rastline, ki obsegajo vsebnost prolina, topne sladkorje, aktivnost katalaze in askorbat peroksidaze so se z večanjem sušnega stresa povečali, da bi omilili škodljive učinke stresa. Rezultati so pokazali, da je foliarno dodajanje Spd in Spm sprožilo antioksidacijsko aktivnost encimov in akumulacijo prolina v zdravilni špajki, kar je zmanjšalo poškodbe membrane. Posledično sta Spd in Spm povečala vsebnost fotosinteznih barvil, ki rastlini omogočajo oskrbo z energijo za rast in produkcijo. Dodajanje putrescina je imelo negativen učinek na aktivnost katalaze (CAT) in vsebnost klorofila a. Pozitivni učinki dodajanja poliaminov so bili opazni le v razmerah zmernega sušnega stresa in so dobili nasproten trend ob njegovi zaostritvi. Z vidika kakovosti in količine pridelka je sušni stres zmanjšal rast korenin, a povečal vsebnost eteričnih olj. Poliamini lahko oblažijo zmanjšano rast korenin, ki jo povzroča pomanjkanje vode. Ti rezultati nakazujejo, da lahko pridelovalci v razmerah zmernega sušnega stresa z dodatkom poliaminov povečajo pridelek zdravilne špajke.

Ključne besede: biokemični parametri, eterična olja, poliamini, *Valeriana officinalis*, sušni stress

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1 INTRODUCTION

Valerian (*Valeriana officinalis* L.) which belongs to the family Valerianaceae is an important medicinal plant that is, due to its mild sedative and sleep aid, widely used in traditional medicine (Bos *et al*., 1996). The subterranean organs of valerian contain several compounds including the essential oils and their sesquiterpenoid derivatives (valerenic acids), epoxy iridoid esters (valepotriates) and their decomposition products such as baldrinal and homobaldrinal, and alkaloids (Isetts, 2010). Valerian is the 8th top-selling herbal supplement in North America (Blumenthal, 2001), making it very attractive to improve agronomical practices and in general its production increases. Therefore, eliminating the unfavourable circumstance conditions for valerian is one of the primary and most important strategies in it`s production. The genus *Valeriana* contains about 200 species, which are mostly found in the cold and temperate regions and majority of species possessed high water requirement (Bernatb, 1999). It is reported that the lower water saturation levels had an adverse effect on valerian production (Berbec, 1965). Considering literature, plant growth and functionality suppression under drought stress condition result from morphological, physiological and molecular changes. In limiting water states, plants tend to close their stomata, resulting in reduction of CO_2 flow into the leaves for fixation. As result, far less amounts of $NADPH^+H^+$ and ATP are consumed within the Calvin cycle. Consequently, the concentration of $NADP^+$, and thus that of potential electron acceptors for electron transport chain decreases. Thereafter, electron flow on free oxygen and finally oxygen radicals are induced. The ROS are highly reactive and can seriously damage plants by protein degradation, DNA fragmentation, lipid peroxidation, membrane destruction, and ultimately cause cell death (Beligni and Lamattina 1999).

In plants, several defence systems contribute with together to alleviate effects of any stress factor. One of the important defensive systems is the involvement of phytohormons (Farooq *et al*., 2009). Literatures have emerged that abiotic stress modulated the accumulation of polyamines in many plant species (Alcázar *et al*., 2011; Shi and Chan, 2014), and these observations revealed the possible implication of polyamines to increase plant stress tolerance. Polyamines (PAs) including putrescine (Put), spermidine (Spd), and spermine (Spm), are mainly accumulated in plants exposed to environmental stresses, and play a key role in stress tolerance which depends on species and stress intensity and duration (Tavladoraki *et al*., 2012). Limited water resources beside progressive demand for food production encouraged researchers to improve water use in agriculture. Recently, researches pursue to apply polyamines exogenously to ameliorate the adverse effect of environmental stresses such as drought stress (Mustafavi *et al*., 2015). For instance, Nayyar *et al*., (2005) found that exogenous application of Put and Spd substantially improved the drought tolerance in soybean. It was also shown that plants mainly change range of essential oils production as defense system when exposed to stress conditions (Ramakrishna and Ravishankar, 2011). Saeidnejad *et al*., (2013) reported that drought stress increased essential oil content of *Bunium persicum* (Boiss.) B. Fedtsch. plants. Recently researches have deliberately applied drought stress during the cultivation of medicinal plants in order to stimulate the accumulation of pharmaceutical products such as essential oils. In this regard there is a fundamental question which exogenously applied substance can trigger in plant the accumulation of secondary metabolites with no impact on medical use. Therefore, in this study we followed the hypothesis that by providing polyamines exogenously, the synthesis of pharmaceutical metabolites would be increased.

This study was aimed to determine the effects of several drought stress states accompanied with exogenous application of PAs on biochemical characteristics and essential oil production of valerian plants.

2 MATERIALS AND METHODS

A pot experiment was conducted in greenhouse condition at the University of Maragheh, Maragheh, Iran. Pots with 8 L content were filled with 12 kg of soil (clay: clean sand; 2/1 v/v). The soil had been passed through a mesh number 10. Then, 20 days-old valerian seedlings were obtained from medicinal plants institute, Tehran, Iran. Uniform seedlings were collected and then transplanted into each pot. The pots were placed under a rain-shelter based on completely randomized design. During the next 30 days after transplantation, plants were irrigated with tap water, and then divided into four lots subjected to different water levels. Drought stress levels were measured using control plants with 100 % water availability (L1), 70 % (L2), 50 % (L3) and 30 % (L4) of available water. The pots were weighed daily and water was added to maintain soil moisture content. In this project, polyamines including putrescine, spermidine and spermine, (Sigma Chemical Co.) were sprayed 2 times in 1 mM concentration. In order to eliminate the effect of water from test results, untreated plants were sprayed with distilled water. The first spray was made 35 days after transplanting and the second repeated after 15-days. The plants were sprayed with a manual pressure pump at an average of 10 ml. All of the observations, except essential oil content, were taken three days after later foliar application of PAs.

Water status of leaves was determined by measuring relative water content (RWC). The leaves were subsequently rehydrated in distilled water for 4 h to obtain the turgid mass (TM), and dried for 48 h at 72 ◦C to obtain the dry mass (DM). RWC was calculated by the ratio: RWC = [(FM − DM)/(TM − DM)] × 100. For measurement of electrolyte leakage, 20 leaf discs from the young fully expanded leaves were placed in 50 mL glass vials, rinsed with distilled water to remove electrolytes released during leaf disc excision. Vials were then filled with 30 mL of distilled water and allowed to stand in the dark for 24 h at room temperature. Electrical conductivity (EC_1) of the bathing solution was determined at the end of incubation period. Vials were heated in a temperature-controlled water bath at 95°C for 20 min, and then cooled to room temperature and the electrical conductivity (EC_2) was measured. Electrolyte leakage was calculated as percentage of EC_1/EC_2.

Chlorophyll-a, -b, and carotenoid were determined according to the method of Arnon (1949). Fresh leaves were taken from the plants and triturated in 80 % acetone. The absorbance of the extracts was measured at 663, 642 and 472 nm using a spectrophotometer (BioTek, PowerWave, USA). Proline content of leaf tissues was estimated spectrophotometrically following the ninhydrin method described by Bates *et al*., (1973). Total soluble sugars were extracted and determined by the anthrone method of Riazi *et al*., (1985).

The shoot tissues (0.5 g fresh mass) were homogenized in 2 mL of 100 mM potassium phosphate buffer, pH 7 containing 1 mM of EDTA and 1 % (w/v) polyvinylpyrrolidone (PVP). The extract was then centrifuged at 4°C for 15 min at 12.000 ×g in a cooled centrifuge. This supernatant was used to measure the activities of guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and catalase (CAT). Activity of GPX was determined at 25°C with guaiacol (Bergmeyer, 1974). Activity of APX was measured by following the rate of hydrogen peroxide-dependent oxidation of ascorbic acid (Nakano and Asada 1981). Finally, the activity of CAT was assayed following H_2O_2 decomposition.

Finally, 50 g of dry root was taken for determination of essential oil percentage using Clevenger instrument (European Pharmacopoeia, 2005). Essential oil yield was determined using the following formula: Essential oil yield = Essential oil percentage × Root yield. Analysis of variance appropriate to the experimental design was conducted using SPSS software (version 16). Means of each trait were compared according to Duncan multiple range test at $P<0.05$ by means of MSTATC software.

3 RESULTS AND DISCUSSION

3.1 Relative water content (RWC) and electrolyte leakage

Regarding Table 1, relative water content (RWC) was significantly decreased with decreasing the irrigation from 100 % to 30 % AWC. Levels of 70 %, 50 % and 30 % of the available water content (AWC) lead to 14 %, 34 % and 35 % of RWC to respectively (Table 1). Leaf RWC reflected the metabolic activity in tissues and it significantly declined due to water stress (Table 1). It seems that decrease in leaf RWC could have been due to unavailability of water in the root zones, which is not able to compensate for water, lost by transpiration. In this project, based on RWC measurements, foliar application of polyamines especially putrescine had not any ameliorate effects against drought stress. Paralleled with our findings, Kubis *et al*. (2014) reported that application of spermidine did not improved water status of water-stressed cucumber plants. Similar results were reported by Bolat *et al.*, (2014) who found that leaf RWC decreased with intensifying stress.

Cell membrane stability is considered to be one of the best physiological indicators of drought stress tolerance. Electrolyte leakage (El) level increased from 20 to 42 %, when AWC dropped from 100 to 30 %. Except for Put, plants treated with PAs showed a significant reduction of stress-induced electrolyte leakage depending on its type and drought stress level (Table 1). Under moderate drought stress (70 % AWC), application of Spd and/or Spm vigorously decreased El but with increasing the water shortage their positive effect was markedly reduced (Figure 1a). Similar results were also reported by Shaddad *et al*., (2011) who reported that at very severe drought stress condition, application of polyamines did not alleviate the adverse effect of drought stress. Results reported in the present paper showed that exogenous Spd and Spm could effectively alleviate membrane damage induced by water shortage up to 50 % AWC (Figure 1). The observation is in harmony with the results of Chattopadhayay *et al*., (2002) on rice and Li *et al.*, (2014) on white clover plants. They proposed that exogenous spermidine and spermine effectively protected cells against damage by inhibition of protease and RNAse activities, which probably helped in maintaining membrane integrity. In addition, polyamines may mediate a decrease in ion fluxes across the vacuolar membrane by blocking fast-activating vacuolar channels under salt stress, as suggested by Pottosin *et al.* (2014). Besford *et al*., (1993) reported that dicotyledons mainly accumulate Spd and Spm with a decline in Put. Therefore, according to their findings it seems that Put had detrimental effect on dicotyledons plant such as valerian.

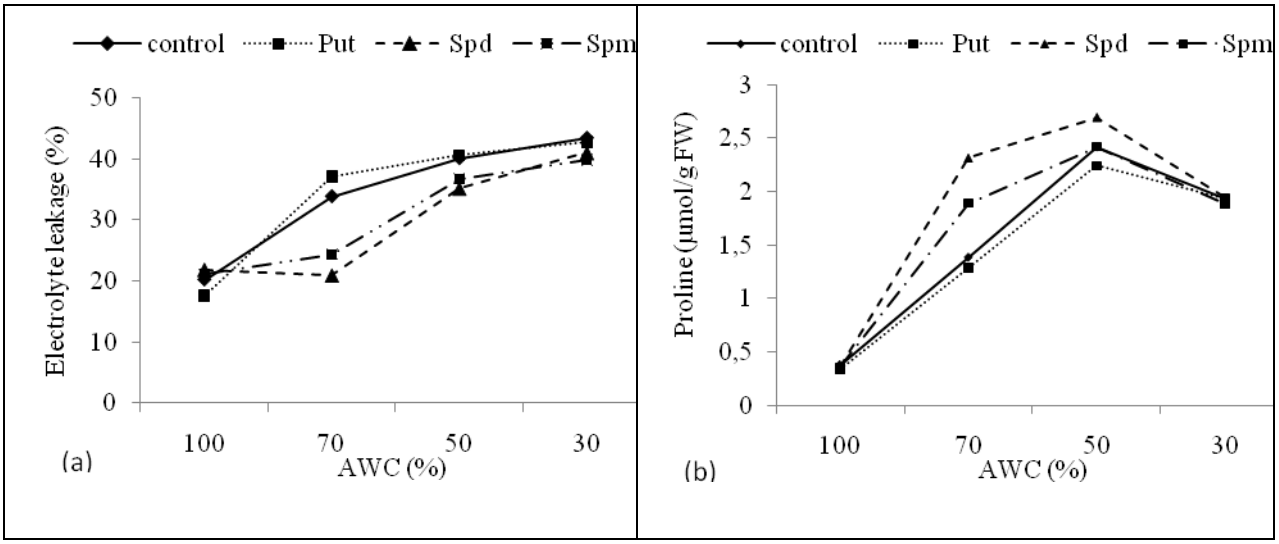

Figure1: Effect of foliar PAs treatments on valerian leaves (a) electrolyte leakage and (b) proline concentration under different available water content levels (AWC).

3.2 Osmotic adjustment

Proline is considered to act as a compatible osmoticum and is perhaps involved in drought tolerance (Bandurska, 2000). In the present study, in response to drought stress, proline concentration was gradually increased reaching a peak value at 50 % AWC and then commenced to reduce (Table 1). The lowest proline content was obtained with normal irrigation (control), while the highest was obtained at irrigation with 50 % AWC. Proline accumulation is in accordance with the fact that many higher plants accumulate free proline in response to drought stress (Farooq *et al.*, 2009). Several possible roles have been attributed to accumulation of proline: osmoregulation, detoxification of free radicals, conservation of nitrogen and energy for the post-stress period and regulating the stress protective proteins (Khedr *et al.*, 2003). Although stressed plants sprayed with Spd and Spm exhibited 19.7 and 7.2 % higher accumulation of proline compared to PA-untreated shoots, their effects mainly depend on soil AWC (Table 1). In this respect, Spd and/or Spm treatments stimulated proline accumulation whenever soil AWC lowered and reached to 50 % (Figure 1b). Our results are in line with the results of Kubis *et al.*, (2014). They concluded that cucumber seedlings treated with PAs exhibited a definitely higher stress-evoked proline accumulation. Shi and Chan (2014) reported that polyamine and proline biosynthesis pathways might share some common substrates, and exogenous application of polyamines might result in more substrates for proline biosynthesis, especially under stress conditions.

Total soluble sugars concentration increased with increasing the drought stress levels up to 50 % AWC, however, differences were not significant, and then they reduced markedly. Foliar application of PAs failed to revert changes in carbohydrates concentration caused by the water shortage treatments (Table 1). In general, the inability of PAs in reducing water loss (Table 1) suggests that osmotic adjustment in valerian leaves cannot be regulated by PAs under drought conditions.

3.3 Antioxidant enzymes activity

Results indicated that drought stress had a significant effect ($P < 0.01$) on antioxidant enzymes activity in leaves of valerian plants. The activities of catalase (CAT) and ascorbate peroxidase (APX) were increased with the increase of drought stress from the control (100 % AWC) to 50 % AWC. However, further increase in water shortage reduced CAT activity, but maximum value of APX activity was achieved at 30 % AWC. In contrast to CAT and APX, the activity of guaiacol peroxidase (GPX) tended to decrease by increased water deficit. Maximum GPX (10.7 units/min·mg.pro) contents were noted from well-watered valerian plants, which were decreased significantly upon exposure to drought stress (Table 1). Liu *et al.*, (2011) reported that catalase activity was higher under mild drought stress than under severe drought stress and well-watered treatments. The increase of CAT and APX activity in plants under drought stress was also reported in other studies (Anjum *et al.*, 2012; Huseynova, 2012).

In current study, except for Put, exogenous PAs significantly promoted activities of CAT relative to untreated plants. High and low CAT activities were recorded from 1 mM Spd and Put, respectively (Table 1). Efficacy of PAs application on APX and GPX activities depends on water stress levels. According to Figure 2a, under moderate drought stress, only Put induced marked changes in APX activity, but by further increase in drought stress levels, treatments had not statistically significant differences with control, and adverse effects of drought stress eclipsed PAs effects. In the case of GPX activity, at all drought stress levels, exogenously applied Put and/or Spd had no significant effect on GPX activity, while Spm had dual effects, under moderate and severe drought stress, positive and negative, respectively (Figure 2b). The same result was obtained in study of Li *et al.*, (2014) who showed that exogenous Spd significantly promoted activities of antioxidant enzymes under drought stress. These findings revealed that enzymatic antioxidant activities of valerian leaves were substantially induced by Spd and Spm application (Table 1). Enhanced antioxidant defense system in PAs treated plants resulted in improving cell membrane stability, as demonstrated by lower electrolyte leakage (Table 1). Pottosin and Shabala (2014) reported that polyamines may play a dual role in the ROS scavenging process. First, PA may play a critical

role in drought stress signalling to confer adaptive responses (Toumi *et al*., 2010). On the other hand, PAs are known to significantly enhance activity of both enzymatic (Radhakrishnan and Lee, 2013) and non-enzymatic (Asthir *et al*., 2012) antioxidants. Therefore, the PA control over the balance between ROS production and scavenging may "shape" H_2O_2 signal, conferring differential stress responses between species and genotypes.

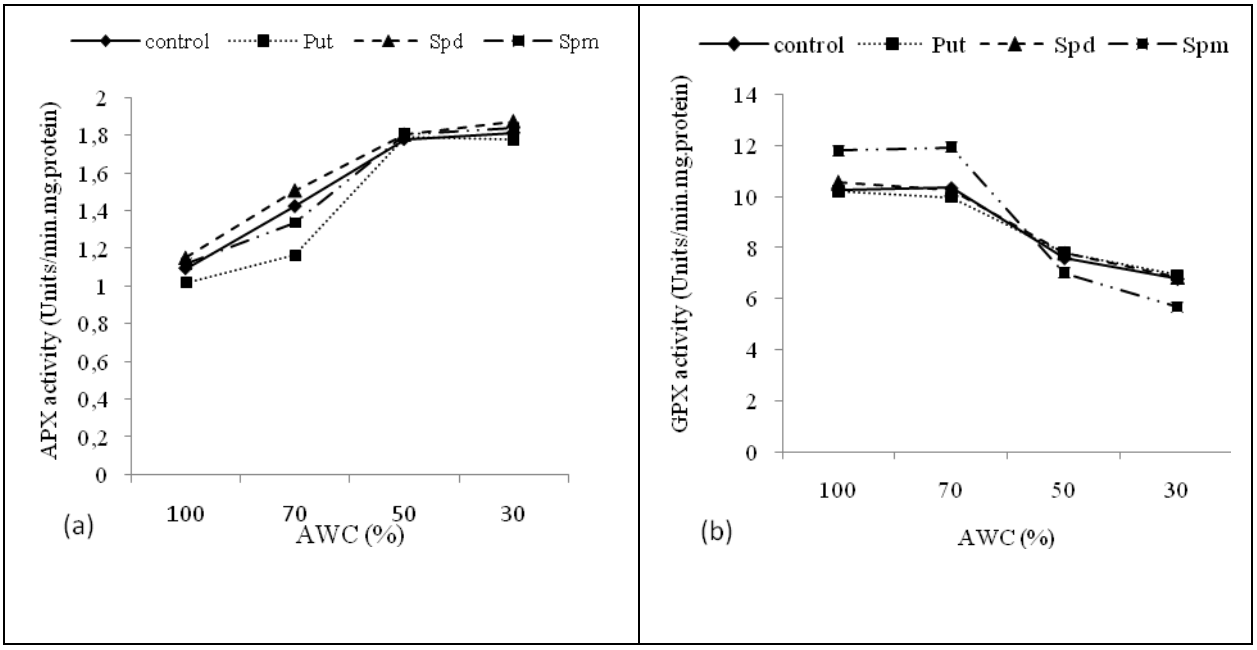

Figure 2: Effect of foliar PAs treatments on Ascorbate peroxidase (APX) activity (a) and Guaiacol peroxidase (GPX) activity (b) in leaves of valerian under different available water content levels (AWC)

3.4 Photosynthetic pigments

Drought stress significantly affected chlorophyll a, b, and carotenoids contents (Table 1). The results revealed that Chl. "a, b" contents decreased by decreasing the soil moisture content. Drought stress up to 70 % AWC induced a slight effect on the chl-a, chl-b of valerian leaf, then it reduced them markedly by the further increase in the level of drought stress. Contrary to these results, plants carotenoid values increased by drought stress increasing up to 50 % and then commenced to reduce (Table 1). Our results about chl-a and chl-b changes in response to drought stress are in agreement with reports of Liu *et al*., (2011). Reduction in chlorophyll pigments concentration can be as a drought response mechanism in order to minimize the light absorption by chloroplasts (Pastenes *et al*., 2005). Since carotenoid plays an important role in photo-protection (Munne-Bosch and Penuelas, 2003), the increased carotenoid content under drought conditions, indicate a higher need of photo-protection by carotenoid (Elsheery and Cao, 2008).

PAs foliar application had significant effect on chlorophyll a and b contents depending on available water content levels. According to Figure 3a, with increasing water shortage up to 50 % AWC, exogenously applied PAs had significant effects on chl.a content, but under severe conditions, differences among the treatments were not statistically significant. Under moderate drought stress, Spd and Spm application increased chl.a content, while Put had detrimental effect. In the case of chl.b content, application of Spd had positive effect when sprayed on stressed-plants. At all water shortage levels, Spm foliar application slightly increased chl. b content (Figure 3b). In normal plants, moderate drought stress lead to increasing in chla/b ratio while this ratio tends to decrease followed by severity of drought stress (Figure 3c). Results (Figure 3c) depicted that foliar application of PAs did not make any remarkable changes on chla/b ratio. Our findings manifested that Spd could positively influenced total chlorophyll (a + b) content comparing to other PAs

in all drought stress states (Figure 3d). All PAs treatments had not significant effect on carotenoid content (Table 1). In connection with these results, Chattopadhayay *et al*., (2002) found that the exogenous application of polyamines enhanced the total chlorophyll level of salt-stressed rice plants. This enhancement effect of Spd and Spm may be attributed to increased stability of thylakoid membranes (Chattopadhayay *et al*., 2002). Thus polyamines could bind to the negatively charged phospholipid head groups on membranes, thereby influencing stability and permeability characteristics of these membranes.

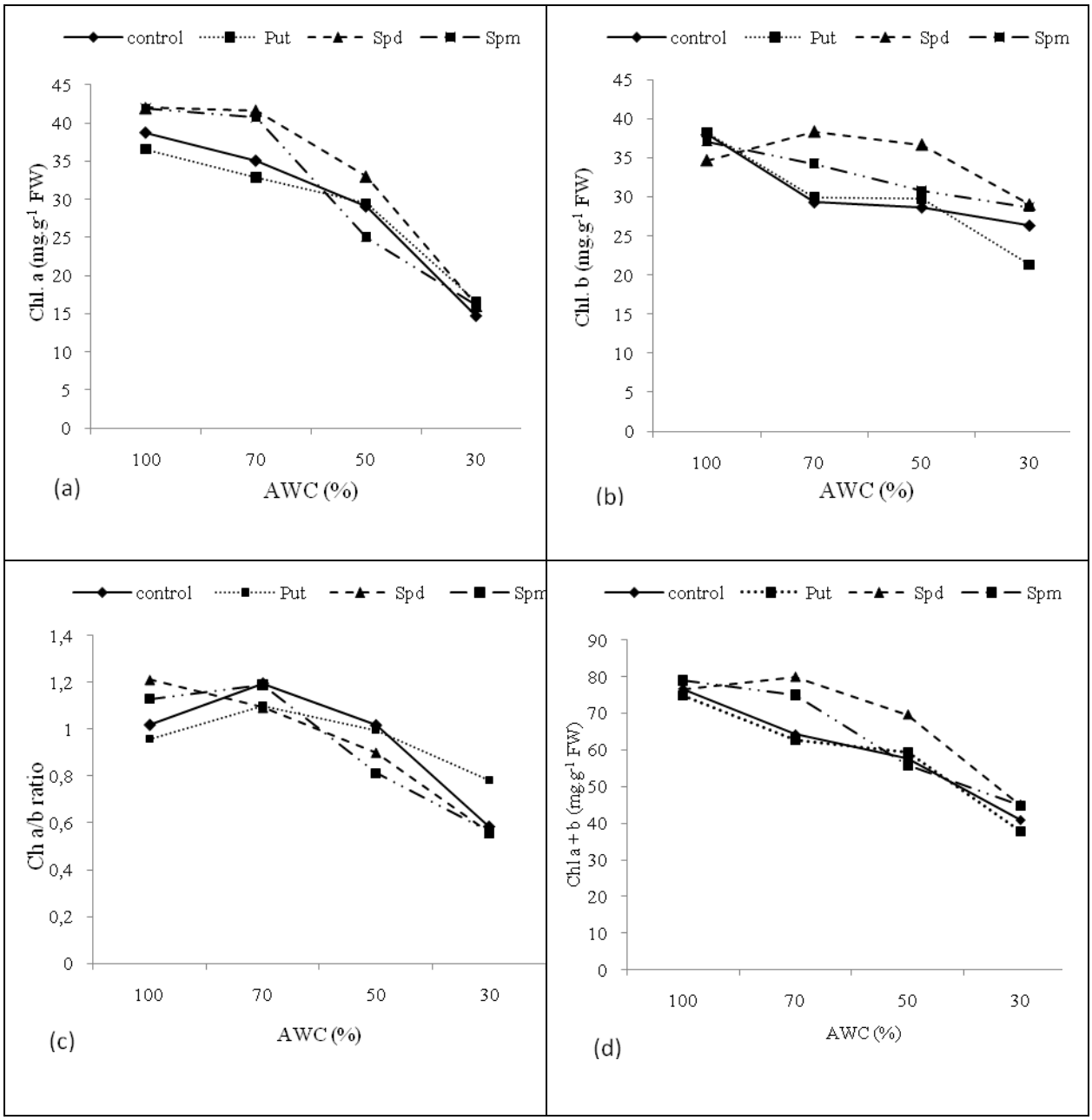

Figure 3: Effect of foliar PAs treatments on chlorophyll a content (a), chlorophyll b content (b), chlorophyll a/b ratio (c) and total chlorophyll (a + b) content in leaves of valerian under different available water content levels (AWC).

3.5 Growth and Essential oil production

Plant aerial biomass as well as root biomass was severely inhibited under drought stress, and both of them decreased with increasing the water shortage (Table 2). Aerial parts growth decreased by 28-50 % while subterranean organs growth decreased by 21-35 %. Therefore, roots were less affected by drought stress than aerial parts (Table 2). Root is the first organ to come into contact with water stress in the rhizosphere, thus supplying assimilate for this organ may be a best strategy for their resistance. Application of PAs significantly improved root and above ground organs growth under drought stress (Table 2). Maximum root biomass (economical part) was recorded from Spm foliar application, which was at par with Spd treatment (Table 2). PAs exogenously applied to plants grown under drought stress mitigated the adverse effect of stress on plant growth which may be attributed to stimulatory effects of PAs on defence systems and chlorophyll protection as as our data indicated (Table 1). Our results are in line with previous studies (Shaddad *et al.*, 2011; Amooaghaie, 2011) where exogenous PAs alleviate stress-induced growth inhibition possibly due to protection of membranes and minimization of oxidative damage.

Essential oil percentage increased when soil AWC dropped to 50 %, and then it declined with drought stress intensifying (Table 1). The highest essential oil content was achieved at 50 % AWC, which accumulated by 63 % when compared with control. As shown in Table 1, increasing of drought stress level from 50 % to 30 % had an adverse effect on essential oil accumulation in valerian. Bernatb (1999) reported that sufficient and continuous water supply did not only enhance essential oil content of valerian plant but also it could decrease essential oil content. Our results are in agreement with those previously documented by Rebey et al., (2012) on *Cuminum cyminum* L. and Hassan and Ali (2014) on *Coriandrum sativum* L. who revealed that the essential oil content was positively affected by drought stress treatments. Exogenously applied PAs remained generally ineffective on essential oil accumulation (Table 2). It seems that the products of polyamine catabolism, more involved in the synthesis of proline and did not contribute in terpen biosynthesis. Essential oil yield is a dependent variable determined by root yield and essential oil percentage. The highest essential oil yield (301 mg) was achieved in plants exposed to 50 % AWC. The lowest value for essential oil yield was achieved when plants exposed to very severe drought stress (30 % AWC). In PA-treated valerian plants, the essential oil yield increased by 18 %, 20 % and 19 % with Put, Spd and Spm, respectively. Essential oil improvement by PAs mainly attributed to positive effects of these growth regulators to root growth.

Table 1: Effect of foliar PAs treatments on some physiological and biochemical traits of valerian under drought stress

	RWC (%)	El (%)	Chl.a	Chl.b	Car	CAT	APX	GPX	Proline (µmol. g^{-1} FW)	Total soluble sugars (mmol.g^{-1} DW)
			(mg.g^{-1} FW)			Units/min·mg.protein				
AWC (%)										
100	78.17a	20.15d	39.77a	37.0a	1.27c	12.08d	1.09c	10.7a	0.35d	99.8a
70	67.44b	29.02c	37.55b	32.9b	1.32b	18.56b	1.35b	10.6a	1.71c	108.2a
50	51.59c	38.12b	29.12c	31.4b	1.39a	21.80a	1.79a	7.55b	2.44a	110.0a
30	51.05c	41.74a	15.81d	26.3c	1.24c	14.30c	1.82a	6.55c	1.92b	80.58b
A	**	**	**	**	**	**	**	**	**	**
Control	63.05ab	34.36a	29.33bc	30.5bc	1.30a	14.96c	1.52b	8.74a	1.52c	93.58a
Putrescine	58.35b	34.49a	28.83c	29.8c	1.31	14.63c	1.43c	8.72a	1.45c	100.6a
Spermidine	65.61a	29.69b	33.16a	34.6a	1.32a	20.03a	1.58a	8.86a	1.82a	100.2a
Spermine	61.24ab	30.49b	30.93b	32.7ab	1.29a	17.13b	1.52b	9.10a	1.63ab	104.1a
B	*	**	**	**	ns	**	**	ns	**	ns
A*B	ns	**	**	**	ns	ns	**	**	**	ns

Different letters in each column indicating significant difference at $p \leq 0.05$.
ns: non-significant Significant at: *P>0.05, **P>0.01, # P>0.1

Table 2: Effect of foliar PAs treatments on quantitative and qualitative yield of valerian under drought stress

	Aerial biomass (g/plant)	Root biomass (g/plant)	Essential oil content (%)	Essential oil yield (mg/pod)
AWC (%)				
100	116.9a	64.40a	0.44c	283a
70	83.66b	50.30b	0.54b	274a
50	64.16c	45.46c	0.72a	301a
30	58.25c	41.74c	0.42c	193b
A	**	**	**	**
Control	76.5b	44.38c	0.53a	230b
Putrescine	81.5ab	49.34b	0.56a	271a
Spermidine	80.1ab	53.45ab	0.52a	276a
Spermine	84.8a	54.74a	0.51a	273a
B	#	**	ns	#
A*B	ns	ns	ns	ns

Different letters in each column indicating significant difference at $p \leq 0.05$.
ns: non-significant Significant at: *P>0.05, **P>0.01, # P>0.1

4 CONCLUSION

To sum up, present study suggest that foliar application of Spd and Spm could ameliorate the deleterious effects of drought stress by stimulating the antioxidant enzymes activity and increasing the accumulation of proline, which protect cell membrane integrity and decrease chlorophyll loses. Albeit, PAs possessed less tangible effects on valerian plants in severe drought stress state but, it is more effective in moderate drought stress state through induction of biochemical changes. It is inferred that PAs which is economical and environment friendly alternative can be implicated to improve productivity of valerian in current scenario of drought and climate change.

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Agrovoc descriptors: biological control; biological control agents; zea mays; maize; plant pests; ostrinia nubilalis; ichneumonidae; insect pests; parasitoids

Agris category code: H10, h01

Parasitoid inventarisation of European corn borer (*Ostrinia nubilalis* Hübner, 1796) and options for its biological control in Slovenia

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ABSTRACT

European corn borer (*Ostrinia nubilalis*) (ECB) is an important maize pest in central and northern Europe. Presently it is controlled by insecticides or biological agents such as *Trichogramma brassicae* in several European countries, excluding Slovenia, where the pest's pressure is highly variable and no appropriate mechanization is available. Lessening the dependence on chemical pesticides is an integral part of the European Union's agenda for agriculture. Mass release of *Trichogramma* spp. could be seen as a promising alternative for ECB control in countries with a highly fluctuating ECB pressure and no mechanization for insecticide applications. However, no records of naturally occurring hymenopteran parasitoids of ECB exist in Slovenia. To address this important under-researched topic and provide the expert basis for potential introduction of ECB egg parasitoids in Slovene maize production, a systematic inventarisation programme of ECB parasitoids was launched in 2010. Additionally, ECB flight was monitored in 2011 and 2012 at two locations in Slovenia: Jablje and Rakičan. In both locations two ECB generations were observed. ECB was fist observed at the end of May in Rakičan. During the five years of the systematic survey we discovered two ECB parasitoid species. ECB egg masses were parasitized by *Trichogramma brassicae*, whereas ECB pupae were parasitized by *Tycherus nigridens*, with 6 or 7 % parasitation rate, respectively. *T. nigridens* represents a new taxon report for Slovenia. We conclude that there is a strong need for undertaking systematic surveys of natural enemies of agricultural pests.

Key words: biological control; corn; Ichneumonidae; insect pests; maize; new records; parasitoids; *Tycherus nigridens*; Trichogrammatidae; *Trichogramma brassicae*; *Zea mays*

IZVLEČEK

INVENTARIZACIJA PARAZITOIDOV KORUZNE VEŠČE (*Ostrinia nubilalis* Hübner, 1796) IN MOŽNOSTI NJENEGA BIOTIČNEGA ZATIRANJA V SLOVENIJI

Koruzna vešča (*Ostrinia nubilalis*) (ECB) je pomemben škodljivec koruze v srednji in severni Evropi. V številnih evropskih državah jo obvladujejo z insekticidi, kar pa ne velja za Slovenijo, saj poleg tega, da je populacijski pritisk tega škodljivca zelo spremenljiv oziroma nepredvidljiv, tudi ni na voljo ustrezne škropilne tehnike. Glede na zastavljene cilje EU, ki so usmerjeni v zmanjševanje tveganja zaradi rabe fitofarmacevtskih sredstev, je uporaba biotičnih agensov, kot je na primer množični izpust parazitoidov iz rodu *Trichogramma* proti ECB dobrodošla in obetavna alternativa kemičnim sredstvom. Znanje o zastopanosti in razširjenosti parazitoidov ECB iz reda kožekrilcev je v Sloveniji še vedno pomanjkljivo. Za preučitev možnosti obvladovanja ECB z biotičnimi agensi smo leta 2010 začeli sistematično spremljati navzočnost in razširjenost parazitoidov ECB na koruznih poljih. Dodatno smo v letih 2011 in 2012 spremljali nalet ECB v Jabljah in Rakičanu. Na obeh lokacijah smo odkrili dva rodova ECB. Škodljivec se je navadno pojavil najprej v Rakičanu konec maja. V petih letih raziskave smo v Sloveniji naleteli na dve vrsti parazitoidov ECB, in sicer na vrsto *Trichogramma brassicae*, ki smo jo izolirali iz parazitiranih jajčec ECB in na vrsto *Tycherus nigridens*, ki smo jo izolirali iz parazitiranih bub ECB. Stopnja parazitizma je bila 6 % za *T. brassicae* in 7 % za *T. nigridens*. Vrsta *T. nigridens* predstavlja novo taksonomsko najdbo za Slovenijo. Zaključujemo, da je izvajanje sistematičnega iskanja in inventarizacije naravnih sovražnikov kmetijskih škodljivcev nujnega pomena za Slovenijo.

Ključne besede: biotično varstvo; Ichneumonidae; koruza; nove najdbe; parazitoidi; *Tycherus nigridens*; škodljivci koruze; Trichogrammatidae; *Trichogramma brassicae*; *Zea mays*

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1 INTRODUCTION

Corn borers represent an important biotic stressor for maize (*Zea mays* L.) crops in Europe (Meissle *et al*., 2010). European corn borer (*Ostrinia nubilalis* (Hübner 1796)) (ECB) is the most important maize pest in central and northern Europe, while pink stem borer (*Sesamia nonagrioides* (Lef., 1827) is predominant in warmer areas of southern Europe (Velasco *et al*., 2002; Malvar *et al*., 2004; Meissle *et al*., 2010). ECB mostly causes damage by larvae that enter into the maize stalk after hatching and feed on the stalk pith. Yield is affected by ECB tunnelling which interferes with vascular system and increases the risk of stalk lodging and breakage (Gomboc *et al*., 1999). In addition, corn borer damage can affect plant health by vectoring *Fusarium moniliforme* J. Sheld. and facilitating fungal infections (Sobek and Munkvold, 1999). The yield loss produced by corn borer attack of the ear is sometimes less important than yield reduction associated to stalk tunnelling, as this kind of damage has been described as an important factor for favouring high levels of fumonisins in maize kernels (Sobek and Munkvold, 1999; Butrón *et al*., 2009).

Foliar insecticide applications in maize production are used in most European regions (i.e. Spain, Hungary, Poland, Germany, Italy, France and Denmark). The most commonly used active ingredients are pyrethroids and organophosphates (Meissle *et al*., 2010). However, Pons and Albajes's (2002) results illustrate how broad spectrum insecticides can have undesirable effects regarding ECB control: although treating maize seeds with imidacloprid reduced the incidence of cutworms (*Agrotis segetum* Denis & Schiffermüller, 1775), wireworms (*Agriotes lineatus* Linnaeus, 1767), pink stem borer and leafhoppers (*Zyginidia scutellaris* Herrich-Schäffer, 1838), treated plots were attacked significantly more by ECB. In Slovenia however, chemical measures in corn crops are not undertaken against ECB, partly due to highly fluctuating ECB pressure, partly due to non-existent spraying mechanization. It is important to mention that ECB in Slovenia occurs also in vegetables like tomato, peppers and others, where the pest can be controlled with insecticides (Carlevaris *et al.,* 2003).

Lessening the dependence on chemical pesticides is an integral part of the European Union's (EU) agenda for agriculture (European Parliament, 2009). The objective of this Directive is to reduce dependence on, as well as the risks and adverse impacts of, pesticide use on human health and the environment, and a key element to reach this goal is to promote the implementation of Integrated Pest Management (IPM), which has become compulsory in the EU in 2014. In sync with this directive and the previously mentioned reasons mass release of *Trichogramma* spp. is a promising alternative for ECB control in countries with a highly fluctuating ECB pressure and no insecticide applications. In Europe, the parasitoid wasps are already released mainly against ECB on about 150 000 ha per year with the largest area in France (Meissle *et al*., 2010). Encouraging results of biological control of ECB also exist from Asia (Zhang *et al*., 2010) and North America (Hoffmann *et al*., 2002; Hoffmann *et al*., 2006). An important ECB parasitoid, *Trichogramma brassicae* Bezdenko, 1968, was only recently discovered in Slovenia by Bohinc *et al.,* (2015).

Despite encouraging results of biological control of ECB, records of naturally occurring hymenopteran parasitoids of ECB in Slovenia are limited. To address this important under-researched topic and provide the expert basis for potential introduction of ECB egg parasitoids in Slovene maize production, a systematic inventarisation programme of ECB parasitoids was launched in 2010. In this paper we report the first findings.

2 MATERIALS AND METHODS

2.1 Locations

From 2010 to 2014 a systematic search for natural enemies of ECB was carried out at different locations in central Slovenia (Jablje near Ljubljana) and the north-eastern part of Slovenia (Rakičan, Prekmurje) with predominant maize

production. Several fields within Agricultural Institute's Jablje experimental station were monitored (46°08'17.1"N, 14°34'15.2"E; 46°08'25.3"N, 14°34'39.8"E; 46°09'05.8"N, 14°34'52.5"E and 46°08'20.6"N, 14°34'15.0"E) and two locations near the Biotechnical School Rakičan (46°38'53.2"N, 16°13'21.3"E and 46°39'17.1"N, 16°11'33.1"E). The exact parasitoid finding dates are listed in Table 1.

Table 1: Dates and locations of parasitoid discoveries in the years 2010-2014

	Jablje			Rakičan		
Year	*T. brassicae*	*T. nigridens*	Maize plants surveyed	*T. brassicae*	*T. nigridens*	Maize plants surveyed
2010	/	/	200	/	/	/
2011	Jul. 8	Aug. 3	770	Jul. 7 and Aug. 1	/	760
2012	Aug. 7. and Aug. 21	/	520	/	Avg. 21	320
2013	/	/	400	/	/	/
2014	/	/	400	/	/	/

2.2 ECB flight monitoring

In the sampled areas (Jablje, Rakičan) ECB flight was monitored with a fluorescent light trap (Grote Lichtval zonder lamp, Entomologie-speciaalzaak, Vermandel, Hulst, Nederlands) from May to September in the years 2011-2012. One trap per location was used.

2.3 Scouting for ECB egg masses and pupae in the field

Our search for natural enemies of ECB was focused on egg and pupal parasitoids. Search for ECB egg parasitoids was performed by scouting for ECB egg masses on the abaxial side of maize leaves. Scoutings were carried out during the oviposition period of the second ECB flight. All potentially parasitized ECB egg masses were collected and transferred to the entomological laboratory of Agricultural Institute of Slovenia for observation of parasitoid emergence. Pupal parasitoids were searched for in plants with evident ECB damage (stalk tunnelling and breakage, frass on leaves, holes in leaves). ECB damaged plants were dissected to collect ECB pupae, which were also transferred to the entomological laboratory of Agricultural Institute of Slovenia for observation of pupal parasitoid emergence.

2.4 Observation of ECB egg masses and pupae for parasitoid emergence

ECB egg masses on 10 cm^2 pieces of maize leaves and ECB pupae were put into individual 50 mL centrifuge tubes. The tubes were kept at room temperature for observation until ECB larva/imago or parasitoid emerged.

2.5 Parasitoid classification

The morphological classification of the ECB egg mass parasitoids was based on Cônsoli *et al*. (2010), Ferriere and Kerrich (1958), Goulet and Huber (1993) to family and genus level. For species identification, Pintureau (2011) was used. Identification of *Trichogramma* species relies very heavily on examination of the male genitalia and to a lesser extent the male antennae. For this reason a slide-mounting technique modified from Platner *et al*. (1999) was necessary to allow the examination of male genitalia. Therefore, freshly emerged individuals of the egg parasitoid were killed and incubated in 90 % lactic acid for three days in order to allow their clearing. Then specimens were transferred, dorsum-up, to a small drop of Hoyer's medium on a slide and carefully covered with a small round coverslip. During this process the male genital capsule was gently pressed out of the gaster. After drying, the slides were examined at 100 x and 400 x magnification under the light microscope and morphological characters were determined for species identification according to Pintureau (2011). The morphological classification of pupal parasitoids was based on Goulet and Huber (1993) and a detailed description by Smith (1932).

3 RESULTS

3.1 ECB flight and parasitation levels

The ECB pressure varied between northeast Slovenia (Rakičan) and central Slovenia (Jablje). Higher catches were recorded in Rakičan. ECB first occurred in Rakičan at the end of May, early June. In Jablje, ECB was first observed in mid-June. In both localities two ECB flights were observed, although the two-flight trend was more evident in Rakičan. The first ECB flight peaked from 17-20 June, and the second from 15-20 July. In 2012 in Jablje the first ECB flight was not very distinct. ECB flight waned in August (Figure 1).

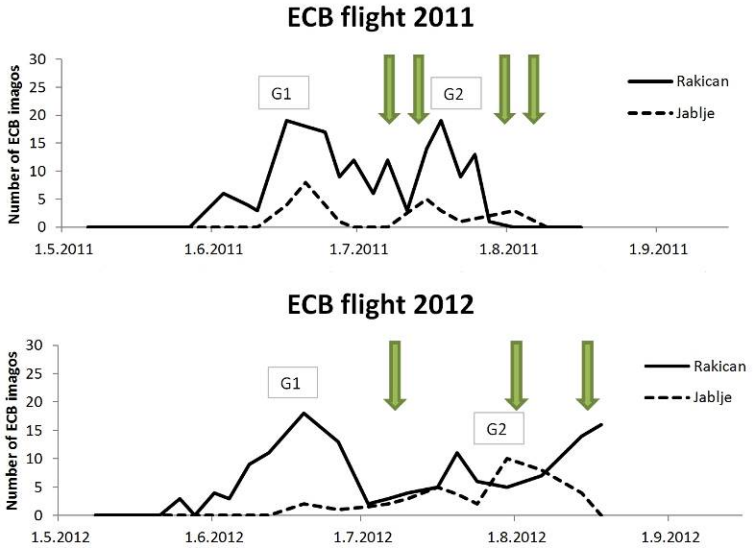

Figure 1: ECB flight in Jablje and Rakičan in 2011 2012. Green arrows show approximate times of ECB egg mass and pupae sampling. *G1* or *G2 – first or second ECB flight.*

During the five years of the survey over 3000 maize plants were inspected for the presence of second generation ECB egg masses. Altogether 84 ECB egg masses were discovered. Five of these 84 egg masses were parasitized, resulting in a percentage parasitism of nearly 6 %. Approximately 150 ECB-damaged plants were dissected to obtain potentially parasitized second generation ECB pupae. Approximately 30 pupae were transferred to the laboratory. Two of them were harbouring parasitoids (percentage parasitism: nearly 7 %). The egg masses were parasitized by *Trichogramma brassicae* Bezdenko, 1968, whereas the two parasitized pupae were parasitized by *Tycherus nigridens* Wesmael, 1845. Taxon *T. nigridens* had not been reported previously in Slovenia according to our knowledge.

3.2 *Trichogramma brassicae* Bezdenko, 1968

The parasitized ECB egg masses were typically discovered on the abaxial side of 3rd or 4th maize leaf from stem base up. ECB eggs usually formed aggregates of 20-35 eggs and were located as a single cluster near the central leaf vein. Some parasitized egg masses were completely parasitized, whereas some only partially. After transfer to the lab, minute wasps emerged in 3-5 days from the uniformly black ECB egg masses (Figures 1 and 2). The wasps were minute, ca. 0.9 mm long, yellow or yellow and black / brown with bright red eyes, short antennae, compact bodies and severely reduced wing venation. Based on morphological characteristics described in Cônsoli *et al*. (2010), Ferriere and Kerrich (1958), Goulet and Huber (1993), they were classified to the genus *Trichogramma*. The genus *Trichogramma* contains more than 200 described species (Pinto, 2006). Mostly they are generalists parasitizing lepidopteran, dipteran, neuropteran and coleopteran egg masses (Querino *et al*., 2010).

They are commonly used as an alternative measure to control major agronomical pests via inundative releases (Kölliker-Ott *et al*., 2004). For more detailed classification to the species level, Pintureau (2011) was used. Based on morphological characteristics of male genitalia the emerged *Trichogramma* wasps were classified as *Trichogramma brassicae* (Figure 3). The hymenopteran parasitoid *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) was introduced into Western Europe to control ECB more than 40 years ago (Babendreier, 2003b). The introduced strain originates from Moldavia. Initially it was described under the name *T. maidis* Pintureau and Voegelé (Pintureau and Voegelé, 1980) and later assigned to be the neotype of *T. brassicae* Bezdenko by Pintureau (1987). Its present day areal extends throughout European mainland, including, but not only, Austria, Belgium, Bulgaria, Germany, France, Italy, Moldova, Romania, Spain, Switzerland, The Netherlands and Ukraine (Fauna Europea, http://www.faunaeur.org/, accessed on June 4th, 2015). The species develops idiobiontically inside the egg of the host (Boivin, 2010), where it also overwinters (Stengel *et al*., 1977).

Figure 1: *Ostrinia nubilalis* (ECB) non-parasitized hatching egg mass (left). Parasitized ECB egg mass (right). Scale bar on the right picture – 1.0 mm. Photos: Š. Modic.

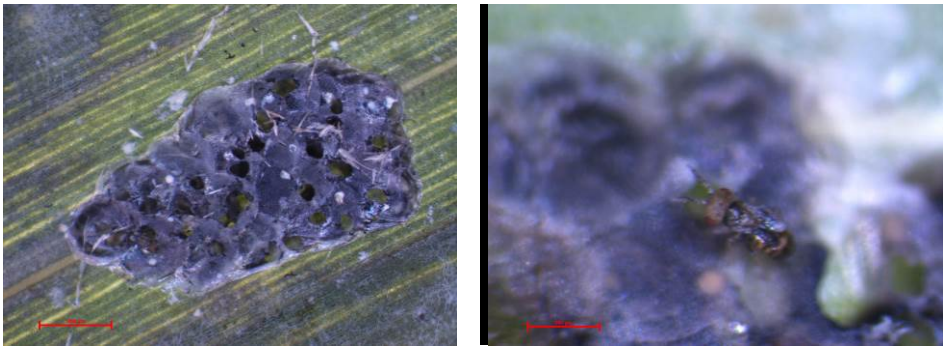

Figure 2: *Ostrinia nubilalis* parasitized egg mass with *Trichogramma brassicae* emergence holes (left). Freshly emerged *Trichogramma brassicae* female (right). Scale bar represents 1.0 mm on the left and 0.5 mm on the right picture. Photos: J. Razinger.

Figure 3: Left picture: *Trichogramma brassicae* female (left) and male (right). Right picture: microscopic preparation of a *Trichogramma brassicae* male. Scale bar represents 0.1 mm on the left and 0.2 mm on the right picture. Photos: J. Razinger.

3.3 *Tycherus nigridens (Wesmael, 1845)*

The pupae sampled for pupal parasitoid inventarisation were collected from maize plants evidently infested by ECB, exhibiting breakage, holes and / or frass. Most often, the pupae were discovered near the stem base. They were carefully removed from the plants using a Swiss army knife and tweezers. Upon transfer to the lab, the pupae were observed on a daily basis for ECB or parasitoid emergence. When an ECB emerged it was discarded. The emerged parasitoids were classified according to Goulet and Huber (1993) and Smith (1932) to *Tycherus nigridens*. *T. nigridens* (Wesmael, 1845) (Hymenoptera: Ichneumonidae; synonym: *Tycherus planifrons* Wesmael, 1845, *Phaeogenes nigridens* Wesmael) is an internal solitary parasite which attacks the pupal stage of ECB (Smith, 1932). It prefers 1- and 2-day old pupae, which it perforates with its ovipositor and lays the eggs free in the body cavity of the host (Baker *et al*., 1949). The earliest definite reference to this species in literature appeared in 1844, when Wesmael first described it as *Phaeogenes nigridens*. In addition to the corn borer the only other host from which *Tycherus nigridens* has been reared is *Tortrix pronuboena* Hübner (Smith, 1932). The adult is distinguished from the other parasites of ECB by its robust appearance and its rusty red-brown abdomen with black terminal portion. The larva has at least four, maybe five, distinct instars. The adults have long life spans; some females were kept alive at low temperature for 10 months (Smith, 1932). It overwinters as an adult female (Baker *et al*., 1949). Originally described from Belgium, this species was initially reported from Sweden, Germany, Spain and France (Smith, 1932). Its present day areal includes most of the EU countries, with the exception of the following countries for which no data exists: Albania, Belarus, Bosnia and Herzegovina, Bulgaria, Croatia, Greece, Iceland, Ireland, Kosovo, Latvia, Lithuania, Macedonia, Moldova, Montenegro, Portugal, Serbia, Slovakia, Ukraine and Vojvodina (Fauna Europea, http://www.faunaeur.org/, accessed on June 4th, 2015).

Figure 4: *Tycherus nigridens* (left) and a detail of forewing venation (right). Scale bar represents 1.0 mm on both pictures. Photos: J. Razinger.

4 DISCUSSION

During the five years of the systematic survey we discovered two ECB parasitoid species. ECB egg masses were parasitized by *Trichogramma brassicae*, already reported in Slovenia by Bohinc *et al*. (2015), whereas ECB pupae were parasitized by *Tycherus nigridens* (new finding for Slovenia), with 6 or 7 % parasitation rate, respectively. More effort was invested in searching for egg mass parasitoids (> 3000 plants examined). Thus, to be sure of the exactness of *T. nigridens's* observed natural parasitation rate of 7 %, we would need to sample a much greater number of pupae.

Trichogramma occurs in all vegetated terrestrial habitats that have been sampled in all six biogeographic regions: Palearctic, Oriental, Nearctic, Neotropical, Afrotropical and Australasian (Querino *et al*., 2010). *T. brassicae* was isolated from several host species (Pintureau, 2011). In Slovenia it was first discovered on August 18, 2014, on *Mamestra brassicae* (Linnaeus, 1758) eggs (Bohinc *et al*., 2015). However, due to their small size, species of *Trichogramma* can potentially be transported by wind and intentionally or otherwise, by man from one country to another in a short period of time. Because of this it is often difficult to determine individual species' natural range of distribution (Querino *et al*., 2010).

Attempts at classical biological control of ECB employing *Tycherus nigridens* release were performed already in 1924 in the USA when a single colony was released in Massachusetts. Attempts intensified by releasing more numerous parasitoids imported from Europe and Japan, totalling more than 50.000 *T. nigridens* between years 1926-1933 (Baker *et al*., 1949). In their report Baker *et al*. (1949) conclude that the biocontrol program was successful as entomologists were able to recover *T. nigridens* in localities where it was not released in the previous three years, but that more knowledge on the biology of the parasitoid would be needed in order to improve the storage conditions of the adults enabling better synchronization of the releases so the parasitoid would establish broader. Smith *et al*. (1932) reported that *T. nigridens* was the most effective ECB parasite in Europe with the maximum parasitism rate of 17.5 % recorded in 1930 in the fields around Padova in northern Italy. Factors that may limit its effectiveness as an effective biocontrol agent are the small number of eggs produced by each female; the mortality of some ovarian eggs, especially among hibernating females; the rather long oogenetic period; and the generally unsuitable oviposition conditions in the spring (Smith *et al*., 1932). Two related *Tycherus* species are reported in literature as pupal parasitoids of agricultural or forestry pests: cherry bark tortrix, *Enarmonia formosana* (Scopoli, 1763), an invasive orchard pest in northwest US, is parasitized by *Tycherus vagus* (Berthoumieu, 1899) (Jenner *et al*., 2004), whereas *Cydia strobilella* (Linnaeus, 1758), a Holarctic pest of spruce cones, is parasitized by *Tycherus fuscibucca* (Berthoumieu, 1901) (Brockerhoff and Kenis, 1996).

T. nigridens represents a new taxon report for Slovenia. However, this taxon has been previously reported from many EU countries, including some of Slovenia's neighbouring countries like Austria, Italy and Hungary, but not Croatia. This fact reveals the problem of reliability of such taxon distribution information, as the real, biological distribution is probably underestimated due to insufficient funding allocated to natural enemy inventarisation studies, especially in countries with a lower gross domestic product per capita. In line with the present Slovenian legislature (Official Gazette of the RS, 83/12, 36/10 40/14, 62/07 and 45/06), potential introduction of commercially reared natural enemies and their release can only be done after obtaining concrete evidence on the autochthonous presence of these species as the pests' natural enemies. Thus our study clearly illustrates the need for undertaking systematic surveys of natural enemies of agricultural pests, so that concrete evidence for their autochthonous presence can be confirmed and their introduction possible.

Specific risks exist when releasing large number of Lepidopteran egg parasitoids. Laboratory experiments demonstrated that *T. brassicae* can attack many non-target butterflies including endangered species (Babendreier *et al*., 2003a). However, non-target butterflies were very seldom attacked under natural conditions in Switzerland (Babendreier *et al*., 2003b). Besides having

adverse effects on non-target organisms, inundatively released non-indigenous *Trichogramma* may compete with locally occurring *Trichogramma*. Therefore preference should always be given to indigenous strains or species, when developing a new IPM program based on *Trichogramma* (Herz *et al*., 2007).

Despite the mentioned risks, one should consider the benefits of parasitoid-based biological control strategies. The released parasitoids do not harm autochthonous population of generalist predators and other beneficial arthropods (Chapman *et al*., 2009; Zhang *et al*., 2010); the application has no negative effect on farmers' health; the environment, especially the ground- and surface water is not burdened by pesticides and their breakdown or transformation products (Zhang *et al*., 2010), and the food or feed is free from insecticide residues.

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Calcium application mitigates salt stress in Date Palm (*Phoenix dactylifera* L.) offshoots cultivars of Berhi and Sayer

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ABSTRACT

The effectiveness of exogenous application of calcium in ameliorating the adverse effects of salt stress (15.9 dS m^{-1}) on date palm offshoots (*Phoenix dactylifera* L. cultivars of Berhi and Sayer) was investigated. Ca-fertilisers Polixal and Rexene were applied either as soil amendments or foliar spray. The results showed that Polixal at 30 ml $offshoot^{-1}$ significantly increased plant height, leaf area, total chlorophyll content, RWC, proline concentration, peroxidase activity, IAA content, K^+ and K^+/Na^+ ratio in leaves of Berhi cultivar, whereas catalase activity, ABA and Cl^- content were decreased. Also Berhi cultivar responded to soil amendments more than to foliar spray. However, Ca-fertilisers mitigated salt stress in the two cultivars and Berhi cultivar was more salt stress tolerant than Sayer cultivar by maintaining the high ratio of K^+/Na^+ and regulating levels of IAA to ABA, in silty clay loam soil. These results suggest that calcium application can improve the defense system under salt stress conditions.

Key words: antioxidant enzymes, Date Palm, salt stress, IAA, ABA, calcium application, proline, RWC

IZVLEČEK

DODAJANJE KALCIJA ZMANJŠUJE SLANOSTNI STRES PRI KOKOSOVI PALMI (*Phoenix dactylifera* 'Berhi' IN 'Sayer')

Pri dveh sortah kokosove palme (*Phoenix dactylifera* 'Berhi' in 'Sayer') je bil preučevan blažilni učinek dodajanja kalcija na negativne učinke slanostnega stresa (15.9 dS m^{-1}). Ca-gnojili Polixal in Rexene sta bili dodajani ali kot talni dodatek ali kot listno pršilo. Rezultati so pokazali, da je dodatek Polixal-a v količini 30 ml na rastlino značilno povečal višino rastlin, listno površino, vsebnost celokupnega klorofila, relativno vsebnost vode (RWC), vsebnost prolina, aktivnost peroksidaze, vsebnost IAA in K^+ ter razmerje K^+/Na^+ v listih sorte Berhi, aktivnost katalaze, vsebnost ABA in Cl^- so se zmanjšali. Sorta Berhi se je bolje odzvala na dodatek gnojil v tla kot na foliarna gnojila. Calcijeva gnojila so pri obeh sortah zmanjšala slanostni stres na bogatih peščeno-ilovnatih tleh, vendar se je sorta Berhi izkazala nanj odpornejša kot sorta Sayer saj je ohranjala večje K^+/Na^+ razmerje, kar je izboljšalo tudi razmerje med IAA in ABA. Rezultati te raziskave nakazujejo, da gnojenje s Ca izboljša obrambni sistem rastlin v razmerah slanostnega stresa.

Ključne besede: antioksidacijski encimi, dateljeva palma, slanostni stres, IAA, ABA, gnojenje s Ca, prolin, RWC

1 INTRODUCTION

Inhibition of plant growth by high amounts of Na^+ and Cl^- is one of the main deleterious effects of salt stress. When present in excess amount, Na^+ and Cl^- ions enter into plant cells and can exert toxic effects on cell membranes and metabolic activities in the cytosolic part of the cell (Hasegawa *et al*. 2000; Zhu, 2001; Türkan and Demiral, 2009). The resultant effect of osmotic

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stress and ionic toxicity may lead to secondary effects in plants such as decreased cell expansion, production of assimilate and membrane functions, decreased cytosolic metabolism with raised production of ROS, including singlet oxygen ($^1O_2$), superoxide (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) (Lindberg *et al.,* 2012). Calcium plays a fundamental role in plant growth and development. Many extracellular signals and environmental cues including light, abiotic and biotic stress factors, elicit change in the cellular calcium levels, termed as calcium signatures (Wu *et al*., 2013). Calcium ions ameliorate the effect of salt stress by competing with sodium ions for membrane-binding sites. Salt stress reduces N, P, K, and Ca content in tissues; however, the addition of Ca restored the levels of these nutrients. In general, as external Ca^{2+} concentrations increase, Na^+ uptake and concentrations decrease while Ca^{2+} uptake and concentrations increase because Ca^{2+} interferes with non-selective cation channels and restricts Na^+ uptake. In addition, as the salt concentration in the root zone increases, the requirement for Ca^{2+} increases. However, the uptake of Ca^{2+} from the soil may be reduced as a result of ion interactions, precipitation, and increased ionic strength. These factors reduce the activity of Ca^{2+} in solution, which reduces the availability of Ca^{2+} (Grattan and Grieve 1999, Reddy, 2001 and Louchli and Grattan, 2007). Also, Na/Ca interactions can affect growth, photosynthesis, plant nutrition, water and ion transport in plants. The nature of the response will vary depending on the plant genotype (Cramer, 2002).

Zekri and Parsons (1990) found that the addition of 1, 5, or 7.5, but not 13.5, mM $CaSO_4$ to the saline solution significantly decreased the adverse effect of NaCl on shoot growth of sour orange seedlings. In salt stressed wheat (*Triticum aestivum*'Samma') Ca^{2+} improved plant height and proline content compared with treatment of 90 mM of NaCl alone (Al-Whaibi *et al.* 2011). El-Khawaga, (2013) found that the anti-salinity agents as calcium alleviated the adverse effects of salt stress on the growth of Sewy, Zaghloul and Hayany date palm cultivars. Keeping in view all these aspects, a study was planned to test the effectiveness of Ca-fertilisers in alleviating the undesirable effects of salt stress by evaluating morphological, physiological and biochemical attributes change in date palm offshoots cultivars of Berhi and Sayer.

2 MATERIALS AND METHODS

2.1 Field experiment

The experiment was carried out at the General Authority of Palm station, in Hartha region – Basrah, Iraq (30°36.54'N& 30° 38.60'N to 47°44.42'E to 47° 45.18'E), 24 km from center of Basrah, in 2014. 30 uniform, girth ± 10 cm vigorous 4-5 years-old 'Berhi' and 'Sayer' date palm offshoots were used in the experiment. The selected offshoots were planted at 5x5 m by 15 offshoot for each cultivar. Drip irrigation system was installed. Soil samples were taken from untreated offshoots; also samples of water were taken weekly. Each treatment was replicated three times, with one offshoot for each replicate. The selected offshoots were subjected to spraying foliar and addition to soil treatments to both cultivars at the first week of March as the following:

2.2 Treatments

C: Foliar spray of water as control

P1: Soil addition of POLIXAL 20-8* (15 ml offshoot^{-1})

P2: Soil addition of POLIXAL 20-8 (30 ml offshoot^{-1})

R1: Foliar spray of Rexene ca 10∗ (1000 ppm offshoot^{-1})

R2: Foliar spray of Rexene ca 10 (2000 ppm offshoot^{-1})

2.3 Ca-fertilisers compounds

***POLIXAL 20-8**: (liquid) 8 % calcium oxide polyhydrocarboxyl (organic acids) 20 % organic material 20 % total nitrogen 4.70 % for alleviation soil salinity and calcium deficiency from Company of ABONOSUALENCIA .co., Spain

∗ **Rexene ca 10**: (Solid) Chelated Calcium EDTA 9.7 % Functional Chemicals B.V. AKzoNobel, Mexio.

2.4 Average of some Environment factors at field

Average of electrical conductivity (EC) for soil in study was 15.9 dS m^{-1}, pH was 8.10. Also, average of water EC was 4.55 dS m^{-1} and pH 7.91, average of field temperature was 41.6°C.

2.5 Parameters of study

Were taken on October 15; all the physiological measurements were performed as following:

2. 5.1 Parameters of vegetative growth

2. 5. 1.1 Increase in offshoot height (cm)
Measured by a measuring tape to third fully expanded leaf.
The increase in plant height = plant height when sampling - plant height before treatment

2. 5.1. 2 Leaf area (m^2)
Leaf area (m^2) was determined according to Ahmed and Morsy, (1999) in four pinnae taken from the middle parts of each leaf, following the equation:
Leaf area (m^2) = (0.37 (length × width) + 10.29 × No. of pinnae) / 1000

2.5.2 Biochemical constituents

2.5.2.1 Total chlorophyll content
The extraction of total chlorophyll was carried out according to Lichtenthaler and Wellburn, (1983). The fresh tissue of leaves was collected and froze then; the leaves (0.25 g) were homogenized with 80 % acetone. The optical density (O.D.) of the extracted chlorophyll was measured at 645 and 663 nm by using spectrophotometer PD-303. Total chlorophyll content was calculated by the following formulae:

Total chlorophyll (mg/g) = 20.2 (OD 645) + 8.02 (OD 663) x (Vol. / Wt.)
Vol = the final volume (ml)
Wt. = sample weight (g)

2.5.2.2 Relative water content (RWC)
Leaf samples were weighed (fresh biomass) immediately after harvesting, soaked in distilled water at 25°C for 24 hr to determine the turgid mass then ,the samples were dried in an oven at 80°C for 48 hr and their dry biomass was determined. RWC was calculated by the following equation:

RWC = (fresh mass- dry mass) / (turgid mass- dry mass) x 100.

2.5.2.3 Determination of proline concentration according to Irigoyen *et al.*, (1992).

2.5.3 Antioxidant enzyme activity assays

2.5.3.1 Enzyme extraction after Luhova *et al.* (2003)

2.5.3.2 Enzyme activity was determined spectrophotometricaly.

2.5.3.3 Peroxidase activity was measured by using a guaiacol assay Angelini *et al.* (1990).

2.5.3.4 Catalase activity was measured by hydrogen peroxide assay based on formation of its stable complex with ammonium molibdate (Goth, 1991). 0.2 ml of plant extract was incubated in 1 ml reaction mixture containing 65 mM hydrogen peroxide in 60 mM potassium phosphate buffer, pH 7.4 at 25°C for 4 min. The enzymatic reaction was stopped with 1 ml of 32.4 mM ammonium molibdate and the concentration of the yellow complex of molibdate and hydrogen peroxide was measured at 405 nm. Activity was expressed on a fresh mass basis (Units mg $protein^{-1}$FW).

2.5.4 Extraction and purification of IAA and ABA
Extraction, purification and quantitative determination of free and bound IAA and ABA were done, with minor modifications, according to the methods of Rastegar *et al.* (2011). Spectrophotometric techniques were used to determine the amounts of IAA and ABA. One gram fresh weight of each sample was taken and extracted with 60 ml of methanol: chloroform: 2N ammonium hydroxide mixture (12:5:3 v/v/v). Each extract (60 ml) was kept in a bottle in deep freeze for further analysis. Extract was then treated with 25 ml of distilled water and the chloroform phase was discarded. The water-methanol phase was evaporated. The water phase was adjusted to the extract pH value of 2.5 or 7 or 11 with 1N HCl or 1N NaOH respectively and 15 ml ethyl acetate was added at each of three steps. This procedure provided the isolation of free-form IAA and ABA

from the extraction solvent. After an incubation period of 1 hour at 70 °C, the same procedure was used for the isolation of bound-form of IAA and ABA from the extraction solvent. Evaporation of ethyl acetate was performed at 45 °C using a rote-evaporator system (B.chi Instruments). Thin-layer chromatography (TLC) was done using silica gel GF254 (Merck Chemicals, Germany) according to the method of Rastegar *et al.,* (2011). TLC separated IAA and ABA were isolated from the glass plaques according to the standard synthetic IAA and ABA Rf values. IAA and ABA were dissolved in 2 ml of methanol for filtration and separation from silica using cotton-glass filled transferring pipettes. Spectrophotometeric assay was done at 280 nm for IAA and 263 nm for ABA and for all standard synthetic IAA and ABA and isolation samples.

2.5.5 Determination of potassium and sodium concentration was according to Creser and Parsons, (1979). This solution became transparent and used for determinations of K and Na concentrations by emission flame photometer (model 129, Shanghai Lingguang int. trade co., ltd.)

2.5.6 Determination of chloride concentration Chloride (Cl^-) in plant tissue extracts was determined by potentiometric titration. With use 0.2 g of dried ground leaf tissue and addition of 50 ml 2 % acetic acid with shacking through 30 min and filtered by Whatman No.1, tritrated against 0.01 N silver nitrate using potassium chromate as an indicator to a bricked end point (Kalra, 1998).

2.6 Statistical analysis

Randomized completely block design of two date palm cultivars and five treatments of calcium replicated three times were used to conduct the experiment. Experimental data on all variables were subjected to analysis of variance (ANOVA) procedures using a statistical package, SPSS version 16.0 (SPSS, Chicago, IL). Revised Least Significant Differences (R.L.S.D.) among treatments was considered at the $P \leq 0.05$ levels.

3 RESULTS AND DISCUSSION

3.1 Effect of calcium on plant height, leaf area, total chlorophyll and RWC under salt stress

Results presented in Table 1 revealed that calcium treatments significantly ($P \leq 0.05$) increased the height and leaf area of offshoots compared with control. Using polixal at 30 ml offshoot^{-1} to Berhi cultivar gave the highest values of height and leaf area (34.3 cm, 1.20 m^2), respectively, whereas control with Sayer cultivar recorded the lowest value in this respect (11.3 cm, 0.7 m^2), respectively. However, the increase of growth may be attributed to the expansion of cells and activation of photosynthesis by increased total chlorophyll and RWC, proline concentration and peroxidase activity. Protective role common to concentration, of Ca^{+2} might be attributed to its role in the maintenance of the structural integrity of the plasma membrane and thus controlling the uptake of Na^+ and Cl. Larkindale and Knight, (2002) suggested that calcium role is in protecting against oxidative damage by the protection of calcium channel blockers and calmodulin inhibitors under heat stress. During our experiments field temperature was up to 40 °C. The resultant transient Ca^{2+} increase caused potential stress signal transduction and led to salt adaptation (Gul and Ajmal, 2006). Effect of calcium on height of the plant is in agreement with that obtained by Al-Whaibi *et al.,* (2011) on wheat plant and effect of calcium on leaf area is in concordance with findings of El-Khawaga, (2013) on date palm. The increased offshoot height of Berhi cultivar may be attributed to its ability to restrict Cl^- movement into the shoot more effectively than the Sayer cultivar. Thus, the concentrations of potentially harmful Cl^- ions would be lower in the photosynthetically active tissues, or different in the genotype of vigorous the growth rate in term of Berhi cultivar, and the largest in Sayer cultivar. Table 1 reveals that the total chlorophyll content and RWC of leaves was increased by polixal at 30 ml offshoot^{-1}. Analysis of Berhi cultivar gave the highest values of total chlorophyll and RWC (1.8 mg.g^{-1}, 6.5 g, 83.7 %), respectively, whereas control with Sayer cultivar gave the lowest value in this respect (0.8 mg.g^{-1},

65.3 %). Ca^{2+} retarded the loss of chlorophyll, protein and intercellular space, suggesting that the ion plays a regulatory role in maintaining and controlling membrane structure and function (Hepler, 2005). From those studies and our results suggesting that the Ca^{2+}plays a regulatory role in maintaining of chlorophyll, it acts as an antioxidant system regulator by increase proline in chloroplasts for scavenging of ROS. Effect of Ca^{2+} is in agreement with that obtained by Jafari *et al.,* (2009) on sorghum plant by calcium. The role of Ca^{2+} in increased relative water content might be attributed to its ability to regulate the compatible solutes and osmotic adjustment, subsequently increasing turgor. Jafari *et al*. (2009) suggested that the protective effect of Ca^{2+} in salinized plants is probably due to its role in maintaining membrane integrity, because one of the primary effects of salt stress is a disruption of membrane integrity caused by displacement of Ca^{2+} from the cell surface by Na^{+}.

Table 1: Averages of plant height (cm), leaf area **(**m^2**)**, total chlorophyll (mg g^{-1}) and RWC (%) of Berhi and Sayer

Cultivars	Treatments	Plant height (cm)	Leaf area **(**m^2**)**	Total chlorophyll (mg g^{-1})	RWC (%)
Berhi	C	15.0 ± 5.0^{d}	0.8 ± 0.02^{ef}	0.9 ± 0.02^{fg}	67.3 ± 1.2^{d}
	P1	26.6 ± 1.5^{b}	0.9 ± 0.06^{cde}	1.4 ± 0.05^{b}	74.3 ± 0.7^{c}
	P2	34.3 ± 2.0^{a}	1.2 ± 0.16^{a}	1.8 ± 0.03^{a}	83.7 ± 0.8^{a}
	R1	25.0 ± 2.0^{bc}	0.9 ± 0.04^{cde}	1.0 ± 0.05^{de}	78.9 ± 0.5^{b}
	R2	31.6 ± 2.5^{ab}	1.1 ± 0.23^{ab}	1.1 ± 0.0^{c}	83.3 ± 0.5^{a}
Sayer	C	11.3 ± 1.5^{d}	0.7 ± 0.0^{f}	0.8 ± 0.07^{g}	65.3 ± 0.9^{e}
	P1	21.6 ± 2.5^{c}	0.9 ± 0.01^{cde}	1.0 ± 0.00^{d}	75.0 ± 1.2^{c}
	P2	30.3 ± 0.57^{ab}	1.0 ± 0.02^{bc}	1.1 ± 0.02^{c}	79.5 ± 0.6^{b}
	R1	25.0 ± 2.0^{bc}	0.8 ± 0.01^{def}	0.9 ± 0.02^{ef}	74.6 ± 0.9^{c}
	R2	28.6 ± 2.0^{b}	0.9 ± 0.02^{bcd}	0.9 ± 0.02^{f}	78.1 ± 0.6^{b}
R.L.S.D. ($P \leq 0.05$)		4.1	0.16	0.06	1.4

cultivars response to Ca-fertilisers under salt stress

Value represents mean ± standard error of three replicates.

C: Foliar spray of Water as control, P1: Soil addition of POLIXAL 20-8* (15ml offshoot^{-1}), P2: Soil addition of POLIXAL 20-8 (30 ml offshoot^{-1}), R1: Foliar spray of Rexene ca 10∗ (1000 ppm offshoot^{-1}), R2: Foliar spray of Rexene ca 10 (2000 ppm offshoot^{-1})

3.2 Effect of calcium on proline concentration, POD and CAT activities, IAA and ABA under salt stress

Table 2 reveals that calcium treatments resulted in significantly ($P \leq 0.05$) higher proline concentration and peroxidase activity of treated leaves compared to control. The application of polixal 30 ml offshoot^{-1} to Berhi cultivar led to increase proline concentration and peroxidase activity (15.4 mg g^{-1}, 7.2 unit mg^{-1} FW), respectively, compared with control to Sayer cultivar which had the lowest values in this respect (9.1 mg g^{-1} ,4.2 unit mg^{-1} FW), when using polixal at 30 ml offshoot^{-1}, Experiment with Berhi cultivar gave the lowest value of catalase activity (0.7 units mg protein^{-1} FW) compared to control to Sayer cultivar, which gave the highest value of catalase activity (2.3 units mg protein^{-1} FW). Effect of calcium in the alleviation of salt stress and the increase of proline reflects the ability of salt-tolerant offshoot to prevent damage of ROS by maintaining better enzymatic (POD and CAT) and non-enzymatic (proline) defense systems. Proline plays a major role in osmoadaptation through an increase in osmotic stress that shifts the dominant osmolyte from glutamate to proline (Tripathi *et al*., 1998). From the results in this work, it seems that proline might confer salt stress tolerance to

offshoots by increasing the antioxidant system and osmotic adjustment mediator. Thus proline facilitates water uptake. Effect of calcium is in agreement with that obtained by Al-Whaibi *et al.*, (2011) on wheat plant by used calcium. Also, it has been commonly reported that salt stress is one of the major causes of oxidative damage to plant tissues. Though, plants can reduce the damaging effects of reactive oxygen species by developing a physiologically powerful defense system together with antioxidant enzymes like POD and CAT (Rao *et al.* 2013). Salinity intensity leads to reduce water accessibility and/or absorption and therefore lowered leaf turgor and, at last, leads to stomata closure (Azizpour *et al.* 2010). CO_2 attainment influenced by stomata closure is a basis for fluctuations and imbalances in ongoing light reactions and CO_2 fixation. The final result of these nonstandard conditions would be reduced $NADP^+$/NADPH, H^+ ratio and increased ROS production (Esfandiari *et al.* 2007). Our results showed that peroxidase to activity increased more in salt-stressed plants supplied with calcium than salt stress alone, whereas catalase activity decreased in calcium treatments. The mechanism of CAT and POD activities regulated by external calcium is still vague. Whereas peroxidase has ability to stimulate growth and improve tolerance to salt stress, its primary function is to oxidize molecules at the expense of hydrogen peroxide. They play a major role in four physiological processes; auxin metabolism, lignin fortification, defense mechanisms against pathogens and some respiratory processes (Baaziz, 1989), it is also a part of antioxidant defense systems which work in concert to control cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS. Also, data in Table 2 indicate that two date palm cultivars had significant differences in IAA content. The maximum value was presented in using polixal at 30 ml offshoot^{-1} to Sayer cultivar (84.6 μg g^{-1}), whereas control of Berhi cultivar recorded the minimum value in this respect (43.3 μg g^{-1}). While the results showed a reverse effect on the content of ABA that the treatment of polixal at 30 ml offshoot^{-1}to Berhi cultivar gave the lowest values of ABA content (54.2 μg g^{-1}), and control of Sayer cultivar recorded the highest value in this respect (85.8 μg g^{-1}). The positive action of calcium compounds on increased endogenous IAA and decreased endogenous ABA content might be attributed to mitigated the adverse effects of salt stress on offshoots by osmoregulation which is possibly mediated by increased production of carbohydrates (data not shown) as well as increased proline concentration by regulating the membrane stability, photosynthetic pigments and modify the balance between these hormones and metabolites. Further, protection under salt stress was achieved through enhanced activities of antioxidant enzymes, to POD and CAT. Thus enhanced recover and stimulate growth. These phytohormones can positively or adversely affect preceding plant growth, while interacting with each other (Fahad *et al.* 2014). One of the fast and sensitive auxin-induced reactions is an increase of Ca^{2+} cytosolic concentration, which is suggested to be dependent on the activation of Ca^{2+} influx through the plasma membrane and auxin increases plasma membrane permeability to Ca^{2+} (Kirpichnikova *et al.* 2014). The role of calcium in increase IAA and decrease ABA content might be to IAA acts as a signal for increases plasma membrane permeability to Ca^{2+}, thus maintains and modifies the balance between these hormones for the purpose of reducing the damage by salinity and stimulate growth by alleviation of ABA content.

Table 2: Averages of proline (mg g^{-1}), POD (unit mg^{-1} FW), CAT (unit mg $protein^{-1}$ FW), IAA (μg g^{-1}) and ABA (μg g^{-1}) of Berhi and Sayer cultivars response to Ca-fertilisers under salt stress

Cultivars	Treatments	Proline (mg g^{-1})	POD (Unit mg^{-1}FW)	CAT (Unit mg $protein^{-1}$FW)	IAA (μg g^{-1})	ABA (μg g^{-1})
Berhi	C	12.4±0.04 ^{d}	5.1±0.11 ^{c}	1.3± 0.10^{c}	43.3±3.9^{e}	80.6±5.8 ^{ab}
	P1	13.3±0.13^{c}	6.6±0.21^{b}	0.9±0.02 ^{ab}	70.7±4.5^{d}	67.9±3.0 ^{cd}
	P2	15.4±0.35 ^{a}	7.2±0.23 ^{a}	0.7±0.01 ^{a}	81.0±2.0 ^{ab}	54.2±4.3^{e}
	R1	13.4±0.25 ^{c}	6.5±0.08^{b}	1.3±0.05 ^{c}	73.8±2.8 ^{cd}	77.1±4.5 ^{b}
	R2	14.9±0.34 ^{b}	7.1±0.06 ^{a}	1.2±0.10 ^{bc}	78.3±1.9 ^{bc}	63.2±3.3 ^{d}
Sayer	C	9.1 ±0.07 ^{h}	4.2±0.18 ^{e}	2.3±0.09 ^{e}	46.1±1.6 ^{e}	85.8±3.4 ^{a}
	P1	11.1±0.01 ^{g}	4.6±0.03 ^{d}	1.6±0.07 ^{d}	73.6± 2.0^{d}	71.5±4.1 ^{bc}
	P2	11.2 ±0.06^{fg}	5.0±0.07 ^{c}	1.3±0.11 ^{c}	84.6±2.0 ^{a}	66.9±2.9 ^{cd}
	R1	11.5±0.12 f	4.6 ±0.02^{d}	1.8±0.57 ^{de}	69.6±2.9 ^{d}	69.9±2.8^{c}
	R2	11.9±0.12 ^{e}	5.0 ±0.11^{c}	2.1± 0.26^{e}	81.8±1.5^{ab}	68.4±1.8 ^{cd}
R.L.S.D. ($P \leq 0.05$)		0.3	0.2	0.3	4.6	6.4

Value represents mean ± standard error of three replicates.
C: Foliar spray of Water as control, P1: Soil addition of POLIXAL 20-8* (15 ml $offshoot^{-1}$), P2: Soil addition of POLIXAL 20-8 (30 ml $offshoot^{-1}$), R1: Foliar spray of Rexene ca 10∗ (1000 ppm $offshoot^{-1}$), R2: Foliar spray of Rexene ca 10 (2000 ppm $offshoot^{-1}$)

3.3 Effect of calcium on Na^+, K^+, Cl^- and Na/K ratio under salt stress

Results presented in Table 3 revealed that calcium treatments significantly ($P \leq 0.05$) decreased Na^+ and Cl^- content of leaves compared with control. The using of polixal at 30 ml $offshoot^{-1}$to Berhi cultivar gave the lowest values of Na^+ and Cl^- content (4.0 mg g^{-1}, 4.0 mg g^{-1}), respectively, whereas, control treatment of Sayer cultivar recorded the highest value in this respect (11.6 mg g^{-1}, 15.1 mg g^{-1}), respectively. Also, data in Table 3 showed that the using polixal at 30 ml $offshoot^{-1}$ to Sayer cultivar gave the highest values of K^+ content (18.0 mg g^{-1}) compared with control treatment to Berhi cultivar (10.6 mg g^{-1}). Calcium treatments significantly increased K^+/Na^+ ratio of leaves compared with control. Using polixal at 30 ml $offshoot^{-1}$ to Berhi cultivar gave the highest values of K^+/Na^+ ratio (4.0) compared with control treatment of Sayer cultivar (1.2). Increasing K^+ concentrations under saline conditions may help to decrease sodium uptake required for maintaining the osmotic balance (Tuteja and Mahajan, 2007). Grattan and Grieve, (1999) reported that addition of Ca restored the levels of N, P, K in tissues. Our results cleared that increasing K^+ related with increase Na^+ in both cultivars and the cultivar is more salt tolerant when accumulates less Na^+, Cl^- and K^+ ions. The role of calcium in decreased Na^+ content and promoted uptake of potassium, thus increased K^+/Na^+ ratio might be attributed to that Ca^{2+} promoted the uptake of K^+ in the presence of sodium. Thus, Ca^{2+} by some mechanisms imparts selectivity to the ion transport process (Hepler, 2005). Ca^{2+} decreased roots Na^+ accumulation, increased shoots K^+ accumulation, and enhanced the selective absorption and transport capacity for K^+ over Na^+ in thr plant (Wu and Wang, 2012). The obtained results go in line with the findings of Zekri and Parsons, (1990) on sour orange seedlings. K^+/Na^+ and Ca/Na ratios are useful indicators of the degree of plant resistance to salinity that a greater degree of salinity tolerance in plants are associated with a more efficient system for selective uptake of K^+ and/or Ca^{2+} over Na^+ (Wu and Wang, 2012; Wu *et al.* 2013). It is suggested that K^+ and Ca^{2+} play key roles in several physiological processes, such as stabilization of membranes and control of enzyme

activity, Na^+ does not function as a macro-nutrient, and thus the substitution of K^+ by Na^+ and the decrease in Ca^{2+} concentration may cause ion imbalances (Tuna *et al.* 2007). Therefore, control of Na^+ accumulation, and high K^+/Na^+ and Ca^{2+}/Na^+ ratios may enhance salinity tolerance in plants (Wu *et al.* 2013). However, Berhi cultivar had higher K^+/Na^+ ratio than Sayer cultivar. This was due to the result of both a higher K^+ and a lower Na^+ concentration in Berhi cultivar, indicating that Berhi cultivar has a better capacity to maintain intracellular K^+ and Na^+ homeostasis, and thus is subjected to less damage under salt stress. The relatively stronger tolerance of this cultivar to salinity may be related to the ability of plants to accumulate high levels of proline, RWC and maintain the high ratio of K^+/Na^+ and regulate levels of IAA to ABA.

Table 3: Averages of Na^+, K^+, Cl^- (mg g^{-1}) and K^+/Na^+ ratio of Berhi and Sayer cultivars response to Ca-fertilisers under salt stress

Cultivars	Treatments	Na (mg g^{-1})	K (mg g^{-1})	Cl (mg g^{-1})	K/Na Ratio
Berhi	C	8.3±1.7 ^{b}	10.6±2.2 ^{b}	9.6±0.5 ^{de}	1.3±0.4 ^{c}
	P1	5.5±0.7 ^{a}	11.0±0.6 ^{d}	6.1±0.7 ^{b}	2.0± 0.1^{c}
	P2	4.0±1.1 ^{a}	15.6±0.5 ^{bc}	4.0±0.5 ^{a}	4.0±0.9 a
	R1	4.9±1.0 ^{a}	12.3±1.5 ^{d}	8.0±0.5^{c}	2.5±0.8 ^{bc}
	R2	4.8±1.0 ^{a}	14.8±0.8 ^{bc}	6.0±0.5^{b}	3.1±0.4 ^{ab}
Sayer	C	11.6±2.0 ^{c}	14.7±0.6 ^{c}	15.1±1.0 ^{g}	1.2±0.2 ^{c}
	P1	6.8±0.5 ^{b}	15.0±2.0 ^{bc}	10.0±0.5^{e}	2.2±0.4 ^{bc}
	P2	6.0±1.0 ^{ab}	18.0±1.0 ^{a}	8.9±0.6 ^{cd}	3.0± 0.4^{b}
	R1	8.0±1.0 ^{b}	15.5±1.7 ^{bc}	11.5±0.5 ^{f}	1.9±0.4 ^{c}
	R2	6.6±0.5 ^{b}	17.1±1.0 ^{ab}	10.5± 0.5^{ef}	2.6±0.4 ^{bc}
R.L.S.D. ($P \leq 0.05$)		2.0	2.3	1.0	0.9

Value represents mean ± standard error of three replicates.
C: Foliar spray of Water as control, P1: Soil addition of POLIXAL 20-8* (15ml $offshoot^{-1}$), P2: Soil addition of POLIXAL 20-8 (30 ml $offshoot^{-1}$), R1: Foliar spray of Rexene ca 10* (1000 ppm $offshoot^{-1}$), R2: Foliar spray of Rexene ca 10 (2000 ppm $offshoot^{-1}$)

4 CONCLUSIONS

The study involving calcium with different concentrations and type of fertilisers indicated that the addition of POLIXAL 20-8 (30 ml $offshoot^{-1}$) to the soil is more effective than foliar spray of Rexene ca 10 (2000 ppm $offshoot^{-1}$) in all the parameters of study. We can deduce from the results that calcium effect was due to the protection against oxidative damage by protecting calcium channel blockers and calmodulin inhibitors under salt and heat stress. Also, maybe Berhi cultivar is more salt stress tolerant than Sayer cultivar by maintaining a high ratio of K^+/Na^+ and regulating levels of IAA to ABA. We suggest also that there is a common link between the peroxidase enzyme and calcium ion in stimulating growth and improving salt stress tolerance.

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Germination and seedling characteristics of drought-stressed corn seed as influenced by seed priming with potassium nano-chelate and sulfate fertilizers

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ABSTRACT

Effect of seed-priming with potassium (K) sources (K-nano-chelate, KNC, and sulfate (0, 2 and 4 %)) under drought stress (DS) conditions (0, -0.3, -0.6, -0.9, -1.2 and -1.5 MPa water potential) on the corn seedling traits was studied. Drought stress decreased the germination indices and seedling vigor. The highest germination, seminal root fresh and dry mass (RFM and RDM) was obtained in KNC primed seeds at -0.3 MPa DS. Mean germination time increased under DS conditions mainly in non-primed seeds. Increasing DS to -1.2 MPa led to decrease in RFM and RDM. Influence of DS on the fresh mass of shoots was more severe than on seminal roots. The highest shoots and seminal roots length was observed in 4 % KNC without any DS. Proper priming can be suggested to increase the plant tolerance under DS.

Key words: germination percentage, vigor index, seed priming, corn, shoots and seminal roots fresh and dry mass

IZVLEČEK

VPLIV PREDTRETIRANJA SEMEN KORUZE K KALIJEVIM NANO HELATOM IN SULFATOM NA KALITEV IN LASTNOSTI KALIC V RAZMERAH SUŠNEGA STRESA

V raziskavi so bili preučevani učinki predtretiranja semen koruze s kalijevim nano helatom (KNC) in K-sulfatom(K) (0, 2 in 4 %) na kalitev in lastnosti kalic v razmerah sušnega stresa (SS) (0, -0.3, -0.6, -0.9, -1.2 in -1.5 MPa vodnega potenciala). Sušni stres je zmanjšal indekse kalitve in vitalnost kalic. Največja kalitev, največja sveža in suha masa semenskih korenin (RFM in RDM) so bile dosežene pri predtretiranju semen s KNC pri -0.3 MPa SS. Povprečni čas kalitve se je v razmerah sušnega stresa povečal v glavnem le pri ne predtretiranih semenih. Povečanje sušnega stresa na -1.2 MPa je vodilo k zmanjšanju sveže mase semenskih korenin in poganjkov, vendar je bilo zmanjšanje pri poganjkih večje. Največji dolžini semenskih korenin in poganjkov sta bli doseženi pri predtretiranju s 4 % KNC in brez sušnega stresa. Na osnovi te raziskave lahko zaključimo, da primerno predtretiranje semen lahko poveča njihovo toleranco na sušni stress.

Ključne besede: odstotek kalitve, vitalnostni indeks, predtretiranje semen, koruza, sveža in suha masa semenskih korenin in poganjkov

1 INTRODUCTION

In arid and semiarid regions, drought, as an abiotic stress, is one of the major factors limiting plant growth at various stages of their life. The plant growth and development is restricted under water stress conditions so that under prolonged drought stress conditions, many plants will dehydrate and die. Water stress in plants reduces the water potential and turgor and increases concentration of solutes in cytosol. Consequently, cell enlargement, gas exchange, transpiration, plant nutrients uptake

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and transport are decreased (Lisar et al. 2012). Hegarty (1978) demonstrated that if water potential of the growth medium is reduced, due to low water availability or high soil salt concentration, germination may be delayed or prevented depending upon the extent of reduction in water potential. Osmotic solutions of poly ethylene glycol (PEG) are often used to control water potential in seed germination studies (Young et al., 1983). Mexal et al. (1975) demonstrated that PEG amendment reduces oxygen solubility and diffusivity, which have been shown to decrease as a function of PEG solution concentration. A PEG-induced reduction in oxygen availability, however, does not appear to be the limiting factor to germination response. The critical aspect of oxygen availability may simply be the distance between the seeds and the air/water interface over which oxygen would have to diffuse (Hardegree and Emmerich, 1994).

Seed germination is critical stage of plant growth which is affected by water stress. Nowadays, different methods are used to improve plant growth especially in adverse environmental conditions. One of these is seed priming which is aimed to improve seed performance under stress conditions such as salinity and drought stress (Sedghi et al., 2010). This method is defined as the uptake of water to initiate the early events of germination but not sufficient to permit seminal root protrusion, followed by drying. Application of priming increases germination rate and percentage of germination under different environmental conditions and improves seedling vigor and growth (McDonald, 2000). Bradford (1990) believed that seed priming sometimes decreases the basic water potential towards more negative values, increasing the ability of the seed to germinate under lower water availability. Activation of cell respiration and cycling, repair of macromolecules, assimilated materials translocation and weakening of seed coat structure for root emergence are reasons to increase mass of primed seeds (Bewley and Black, 1994; Osborn, 1993; Gallardo et al., 2001; Vasquez-Ramos and Sanchez, 2004 and Cantliffe et al., 1984). Four techniques are currently used to perform seed priming. These include hydropriming (soaking seeds in water and redrying them before they complete germination), osmopriming (soaking seeds in aerated osmotica of low water potential to control the amount of water absorb), matripriming (use of solid carriers with low matric potentials) and pregermination (seed hydration to the point of seminal root protrusion) (McDonald, 2000)].

Ahmed et al. (2014) indicated that with increasing drought stress, sunflower traits such as germination percentage, shoot length, germination rate and seedling fresh biomass decreased significantly; they reported the adverse effects of drought stress at -0.12 MPa were more drastic than -0.06 MPa. Patane et al. (2009) illustrated that at 10 and 15 °C, germination percentage of sweet sorghum declined with decreasing water potential in comparison to control (no water stressed plants). Their results showed that in primed seeds, germination percentage were significantly higher than unprimed seeds. Furthermore, mean germination time at 15 and 35 °C increased significantly by reduced water potential. The increase of germination time was higher in unprimed seeds than in primed seeds. Jisha et al. (2013) believed that seed priming protects plants against different abiotic stresses. Finding appropriate priming agents that might be used to increase the tolerance of plants under adverse field conditions is very considerable concept for seed industry (Job et al. 2000). Adverse environmental stress such as drought, salinity and high temperature are interrelated and their detrimental effects on plants are similar. Moreover, they stimulate cell signaling pathways and cellular responses. Whereas, seed priming can put these ways in the early growth stages and result in faster plant defense responses. Potassium is one of the major plant essential nutrient elements that play key roles in the water and energy relationships of plants and can mitigate the adverse effect of drought stress on plants. The positive effect of two sources of potassium, K (nano-chelate and sulfate) on mitigating the adverse effect of salinity stress on germination and seedling characteristics of corn seeds have been shown in our previous study (Zahedifar, 2013). But from the best of our knowledge there was no published research about the effect of aforementioned K sources on reducing the harmful impacts of drought stress on seed germination and seedling characteristics. Therefore, the main objective of this research was to evaluate the effect of seed priming with two nano-chelate and sulfate sources of potassium (K) on germination and seedling characteristics of corn seeds under drought stress conditions.

2 MATERIALS AND METHODS

A factorial laboratory experiment of completely randomized design with three replicates was carried out to study the effect of seed priming with potassium nano-chelate and sulfate on germination and emergence traits of corn under drought stress conditions. Treatments consisted of drought stress by using poly ethylene glycol (PEG, mol wt 6000) solution, prepared as described by Michel and Kaufmann, 1973), at six osmotic potential levels of 0, -0.3, -0.6, -0.9, -1.2 and -1.5 MPa, two sources of potassium (K-nano-chelate including 27 % K and K-sulfate) and three levels of potassium (0, 2 and 4 %) for seed priming.

Seeds of corn (*Zea mays* 'SC 704')) were sterilized with 5 % sodium hypochlorite for three minutes then washed with distilled water. Seeds were placed in pots saturated with solutions of aforementioned K-nano-chelate and K-sulfate for six hours. Ten seeds were placed on filter paper in glass petri dish with 9 cm diameter soaked with 26 ml solution (about of 0.4 mm solution in each petri dish) of desired treatment. Seed germination was recorded daily up to nine day, after the beginning of experiment (a seed was considered as germinated when its seminal root emerged by about 2 mm in length). Finally, seminal root and shoot lengths and their fresh mass were measured. Then seedlings' seminal roots and shoots were dried in oven for 48 hours at 72 °C and their dry masses were measured. Germination percentage (GP), germination rate (GR), mean germination time (MGT), coefficient velocity of germination (CVG) and vigor index (VI) were calculated using Eqs. 1 to 5 (Table 1).

Table 1: Some equations for measuring the characteristics of seedlings

No.	Equation†	References
[1]	$\text{Germination Percentage (GP)} = (\frac{\text{number of normally germinated seeds}}{\text{total number of seeds}}) \times 100$	(ISTA, 1996)
[2]	$\text{Germination Rate (GR)} = \sum_{\text{i}-1}^{\text{j}} \frac{\text{n}_i}{\text{D}_i}$	(ISTA, 1996)
[3]	$\text{Mean Germination Time (MGT)} = \frac{\sum \text{n.d}}{\sum \text{n}}$	(Ellis and Roberts, 1981)
[4]	$\text{Coefficent Velocity of Germination (CVG)} = \frac{G_1 + G_2 + G_3 + \cdots + G_n}{(1 \times G_1) + (2 \times G_2) + \cdots + (n \times G_n)}$	(Scott, et al.1984)
[5]	$\text{Vigor Index (VI)} = (\text{Final germination percentage} \times \text{seedling weight})$	

†Where n_i is the number of seeds emerged on i^{th} day and D_i is the number of days counted from the beginning of the experiment. J is set to 9 days in this experiment, n is the number of seeds germinated on day and d is the number of days from the beginning of experiment, G_1-G_n is the number of germinated seeds from the first to the last day.

Data were analyzed statistically by application of ANOVA using MSTATC (Michigan State University, East Lansing, MI, USA) software packages and the mean values of seed traits were compared statistically using Tukey-Kramer's Multiple Range Test at the probability level of 0.05. The Tables and Figures were prepared using Excel (Microsoft, Redmond, WA, USA) software packages.

3 RESULTS AND DISCUSSION

Results of variance analysis (ANOVA) for all of studied germination parameters have been summarized in Table 2. Findings indicated that effect of applied water potentials, seed priming as

well as their interaction effects are statistically significant at the probability level of 0.01 ($P<0.01$). The results of mean comparison for studied germination attributes using Tukey-Kramer's Multiple Range Test at the probability level of 0.05 are described in detail as the following subsections.

3.1 Germination percentage (GP) and germination rate (GR)

Effect of drought stress and seed priming with potassium nano-chelate and sulfate on germination percentage of corn has been summarized in Table 3. Percentage of germinated seeds was dependent on water availability. Comparison of means showed that GP was reduced by 24 and 55 % as compared to that of control at -1.2 and -1.5 MPa water potential (Ψ), respectively. The maximum GP (78 %) was observed at water potential of -0.3 MPa. In other words, seed germination in PEG solution was not influenced by decreasing water potential less than -0.9 MPa (GP at this Ψ was even higher than that of control). But at -0.6 and -0.9 MPa water potential, GP was lower than that of 0.3 MPa. The GP in no primed seeds decreased significantly as the water potential decreases toward -1.5 MPa. However, the maximum GP in no primed seeds was observed when the water potential was -0.3 MPa. Patane et al. (2012) showed that seed germination of sweet sorghum was reduced by water stress at Ψ < -0.6 MPa. Furthermore, they revealed that the lowering of Ψ to -0.8 and -1.0 MPa negatively affected final germination percentage. Keshavarz et al. (2013) showed that GP of *Brassica rapa* L. decreased significantly with decreasing water potential (0 to -12 bar). Maraghni et al. (2010) demonstrated that seed germination of *Ziziphus lotus* (L.) Lam. was inhibited with increasing water stress and the highest GP were obtained under control conditions without PEG. De and Kar (1994) concluded that water absorption by seeds of mung bean is impaired, and this may result in reduced seed germination in these circumstances. It seems that followed by drought stress and reduced seed water uptake, seed physiological process decline. As a result plant nutrient availability is affected. It has been reported that drought stress reduces growth, delays maturity and reduces biomass and grain yield of corn (Cakir, 2004). Results showed that at the lowest water potential (Ψ = -0.3 MPa), GP was not only decreased but also increased by about 76 % as compared to that of control. From the results, it can be concluded that the seeds of studied corn variety is not very susceptible to drought stress at the early growth stages. Chapman et al. (1997) also reported that the corn crop is particularly sensitive to drought stress several weeks before and after flowering. It should be noted that the results of our study cannot be fully discussed with respect to those obtained at mature plant and field conditions which were reported by aforementioned researchers.

Our results were also in close agreement with the findings of Saeidi et al. (2007) who reported that wheat germination percentage until water potential of -0.8 MPa was not changed, significantly. After that GP decreased toward -1.2 MPa. They believed that wheat is relatively resistance to drought stress at germination stage. Based on these results it may be concluded that the studied corn variety was relatively sensitive to drought stress at germination stage. Jajarmi (2012) showed that GP of wheat seeds decreased with increasing drought stress and the minimum GP was observed at -1.2 Mpa water potential. Karavani et al. (2013) showed that GP of *Tanacetum polycephalum* (L.) Schultz-Bip seeds decreased significantly with increase in severity of drought stress. They believed that moisture deficit conditions can affect enzymatic activity and consequently GP decreases under more negative osmotic potential.

Results showed that the highest mean value of GP was obtained in seeds soaked in 2 and 4 % potassium nano-chelate (Table 3). Seed priming with 2 or 4 % of K-nano-chelate increased the GP as compared to that of no primed seeds when seeds were not under drought stress conditions, whereas seed priming with K-sulfate decreased this trait under the aforementioned conditions, significantly. Furthermore, the GP of seeds at almost all of the applied water potential treatments was the highest when seeds were primed with 2 or 4 % K-nano chelate. Positive effect of seed priming on GP was only observed with potassium nano-chelate. In the other word potassium sulfate did not promote germination (increase GP). Application of 2 and 4 % potassium nano-chelate increased GP by 61 % as compared to that of control. Zahedifar (2013) showed that the mean value of GP in corn seeds subjected to priming with K-nano chelate was

significantly more than that of potassium sulfate under salinity stress conditions. The reduction of GP as influenced by potassium sulfate application would be probably due to the presence of anion and cation amounts more than usual that having toxic effects on growth and decrease the water potential. As a results plant cannot absorb water. The highest GP (about 78 %) was observed in seed primed with 4 % K-nano chelate at -0.3 MPa water potential. At lower water potentials (-1.2 and -1.5 MPa) seeds primed with potassium sulfate, failed to germinate.

Table 2: ANOVA for the effect of water stress (WS), priming (P), and their interaction effects (WS x P) on the germination parameters of corn seed

		Mean square				
Source of variation	Degree of freedom	Germination Rate	Germination Percentage	Mean Germination Time	Vigor Index	Coefficient Velocity of Germination
WS	5	13.57**	2648**	13.26**	5263**	8183**
P	4	2.61**	2389**	2.58**	1744**	1031**
WS x P	20	1.02**	468**	3.73**	493**	768**
Error	60	0.024	7.99	0.006	0.141	0.493

* and ** means statistically significant at probability levels of 0.05 and 0.01, respectively and ns means not significant at probability level of 0.05.

		Mean square					
Source of variation	Degree of freedom	Seminal Root Length (mm)	Shoot Length (mm)	Seminal Root Fresh Mass (mg)	Shoot Fresh Mass (mg)	Seminal Root Dry Mass (mg)	Shoot Dry Mass (mg)
WS	5	25508^{**}	5843^{**}	1010434^{**}	541120^{**}	5042^{**}	2850^{**}
P	4	3268^{**}	928^{**}	194527^{**}	103097^{**}	1269^{**}	1424^{**}
WS x P	20	1600^{**}	197^{**}	69169^{**}	31875^{**}	492^{**}	259^{**}
Error	60	0.933	0.933	0.933	0.933	0.933	0.933

* and ** means statistically significant at probability levels of 0.05 and 0.01, respectively and ns means not significant at probability level of 0.05.

Table 3: Effect of K priming treatments and drought stress (water potential) on germination rate and percentage of corn seeds

Priming treatment† (%)	Water potential (MPa)						Mean
	0	-0.3	-0.6	-0.9	-1.2	-1.5	
	Germination percentage (GP)						
0	33 e††	44 d	23 f	22 f	22 f	22 f	28 B
NK2	34 e	67 b	56 c	33 e	56 c	11 g	43 A
NK4	33 e	78 a	56 c	56 c	11 g	33 e	45 A
SK2	22 f	22 f	43d	22 f	11 g	0	20 D
SK4	22 f	44 d	22 f	33 e	11 g	0	22 C
Mean	29 D	51 A	40 B	33 C	22 E	13 F	
	Germination rate (GR)						
0	3.00 b	2.62 c	0.83 ghi	0.41 klm	1.00 gh	0.52 jkl	1.39 B
NK2	2.51 c	3.94 a	1.46 ef	0.91 ghi	1.37 f	0.20 mn	1.73 A
NK4	2.99 b	2.94 b	1.66 e	1.39 ef	0.20 mn	0.78 hij	1.66 A
SK2	0.66 ijk	1.50 ef	2.12 d	0.25 lmn	0.21 mn	0	0.79 D
SK4	2.00 d	2.58 c	1.01 gh	1.08 g	0.50 kl	0	1.19 C
Mean	2.23 B	2.72 A	1.42 C	0.81 D	0.65 E	0.30 F	

† Treatments consisted of 0: control, NK2: 2 % potassium-nano-chelate, NK4: 4 % potassium-nano-chelate, SK2: 2 % potassium sulfate and SK4: 4 % potassium sulfate.

†† Means in each row or column followed by the same lowercase or capital letters are not significantly different ($p < 0.05$) by Tukey-Kramer's Multiple Range Test.

Effect of drought stress and seed priming with potassium nano-chelate and sulfate on germination rate (GR) of corn has been summarized in Table 3. Results showed that the maximum mean value of GR over the studied priming treatments was obtained at -0.3 MPa water potential. However, the maximum GR value of unprimed seeds was obtained at normal conditions (without any water stress, Ψ = 0 MPa). In no primed seeds, the GR values decreased significantly with increasing the degree of applied water stress conditions (from 0 to -1.5 MPa). In general, imposition of drought stress except -0.3 MPa resulted in a significant decreases in the mean value of seed germination rate (GR) over all of applied priming treatments, also the highest decline about 86 % as compared to that of control in GR occurred at the highest drought stress (water potential of -1.5 MPa). Gill et al. (2003) demonstrated that application of strong water or salt stresses in sorghum increased suger levels of embryos. This helps in osmoregulation under stress conditions. Our results are in close agreement with the findings of Jajarmi (2012) who reported that GR of wheat declined significantly with the decrease in water potential (Ψ< -0.3 MPa). According to the finding of Keshavrz Afshar et al. (2012), the minimum GR was obtained at -1.2 MPa water potential. Saedi et al. (2007) showed that GR of different wheat genotypes against the applied osmotic potential, were not similar. They reported at first level of applied osmotic stress (-0.4 MPa), germination rate of the most studied genotypes was induced and after that declined in more negative potential.

Findings indicated that when seeds are in normal conditions (without drought stress) application of all priming treatments, K-nano-chelate or sulfate, decreased GR value significantly as compared to that of control (except for 4 %K-nano chelate that had no significant effect). Our results also showed that the highest mean value of GR was obtained in seeds soaked in 2 and 4 % potassium nano-chelate. Whereas, seed priming with potassium sulfate decreased the mean value of GR by about of 24 and 19 % as compared to that of control, significantly probably due to the toxic effect of cation and anion. Patane et al. (2009) showed that seed priming of sorghum with PEG increased germination and shortened the delay in germination time due to the increase in saline stress. They believed that in primed seeds, water absorption were faster than unprimed, irrespective of salt concentration of the solution. According to

McDonald (2000), primed seeds obtain the potential to rapidly absorb and revive the seed metabolism thus improving the germination rate. Bradford (1990) demonstrated that seed priming increased seed germination under low water availability due to avoid more negative water potential. Our results revealed that the maximum GR was observed at -0.3 MPa water potential with application of 2 % potassium nano-chelate. Our findings revealed that the reduction in GR because of drought stress, decreased as seed primed with 2 and 4 % k-nano-chelate, except at -1.5 MPa.

3.2 Mean germination time (MGT) and coefficient velocity of germination (CVG)

The effects of corn seed priming with K-nano chelate and sulfate under drought stress on MGT have been shown in Table 4. The MGT values of non-primed seeds increased significantly from 1 to 4.67 as the severity of drought stress conditions increased (the applied water potential decreased from 0 to -1.5 MPa). Results showed that the mean value of MGT at -1.5 MPa water potential was 4.67 fold higher than control. In fact, drought stress increased the mean value of MGT and the highest MGT was obtained when water potential decreased to -1.5 MPa. Findings indicated that when seeds were under normal condition (without waters tress condition), application of K-nano-chelate for priming decreased the MGT values, significantly as compared to that of control (non-primed seeds), but seed priming with K-sulfate increased it, significantly. In addition, MGT of primed and non-primed seeds increased under drought stress but the effect of drought stress on MGT of primed seeds was smaller than that of no-primed seeds. Indeed, the mean value of MGT in seeds subjected to priming with 2 % K-nano chelate was the lowest in comparison to that of other levels of potassium nano chelate or sulfate. The same results were also reported by Zahedifar (2013) for corn seeds but under salinity stress conditions. Patane et al. (2009) showed that germination time of sorghum increased by increasing salinity stress, but this germination time in PEG primed seeds was lower than that of unprimed seeds at 10 and 15° C. Moradi et al. (2012) showed that hydro and osmo priming of tall wheat grass decreased MGT. In this case, priming with urea and distilled water has more reducing effect on MGT as compared to that of PEG. Elouaer and Hannachi (2012) reported that MGT of safflower seeds increased by salinity stress for both of primed and non-primed seeds.

Table 4 highlights the effect of drought stress and potassium priming as nano-chelate and sulfate on CVG of corn. The CVG values of non-primed seeds decreased significantly from 45 to 20 as the severity of drought stress conditions increased (the applied water potential decreased from 0 to -1.5 MPa). Results also showed that with increasing drought stress the mean CVG values decreased significantly, so that the maximum and minimum CVG values (54.5 and 21 germinated seeds day^{-1}, respectively) of corn seeds were obtained at control and -1.2 MPa water potential, respectively. Our results were in close agreement with the findings of Masarat et al. (2014) who reported that the highest CVG value of corn seeds was obtained for control and increasing osmotic potential from Ψ of 0 to -1.2 MPa, declined CVG significantly. Jajarmi (2012) showed that CVG of wheat decreased significantly when drought stress increased from 0 to -1.2 MPa (19.39 and 11.90 germination seeds per day respectively); although it was not significant difference between control and -0.3 MPa drought level.

Findings indicated that when seeds were under normal condition (without waters tress condition), application of K-nano-chelate for priming increased the MGT values, significantly as compared to that of control (non-primed seeds), but seed priming with K-sulfate decreased it (for 2 % K-sulfate) or did not change it (for 4 % K-sulfate), significantly. Findings also revealed that the mean CVG values of primed seeds were higher than unprimed seeds. Furthermore, this positive effect was sharper at low applied level of potassium nano-chelate i. e. 2 %. It was 39.5 % more than control. The highest CVG (about 75) was observed in seeds primed with 2 % K-nano chelate without any drought stress. Elouaer and Hannachi (2012) illustrated that CVG of safflower in NaCl seed priming was higher than control seeds.

Table 4: Effect of K priming treatments and drought stress (water potential) on mean germination time (day) and coefficient velocity germination of corn seeds

Priming treatment † (%)	Water potential (MPa)						Mean
	0	-0.3	-0.6	-0.9	-1.2	-1.5	
	Mean Germination Time (MGT)						
0	1.00 n††	2.16 j	3.01 g	3.32 f	4.01 d	4.67 a	3.03 A
NK2	0.83 o	1.78 kl	2.50 h	2.16 i	3.50 e	3.33 f	2.35 C
NK4	0.67 p	2.05 ij	3.58 e	4.16 c	3.67 e	3.33 f	2.91 B
SK2	2.00 j	1.33 m	3.50 e	4.33 b	3.67 e	0	2.96 B
SK4	1.85 k	1.33 m	3.45 ef	3.50 e	4.00 d	0	2.82 B
Mean	1.27 F	1.73 E	3.21 C	3.49 B	3.77 A	3.77 A	
	Coefficient velocity of germination (CVG)						
0	45.0 d	33.3 f	27.8 g	20.0 h	20.0 h	20.0 h	27.7 C
NK2	75.0 a	42.9 de	38.8 e	30.0 fg	25.0 gh	25.0 gh	38.6 A
NK4	67.0 b	28.0 g	31.3 fg	22.7 h	20.0 h	25.0 gh	32.3 B
SK2	33.3 f	55.7 c	30.8 fg	25.5 gh	20.0 h	0	33.1 B
SK4	52.0 cd	44.4 d	35.0 ef	33.3 f	20.0 h	0	34.7 B
Mean	54.46 A	40.86 B	32.75 C	24.10 D	21.00 E	23.33 E	

† Treatments consisted of 0: control, NK2: 2 % potassium-nano-chelate, NK4: 4 % potassium-nano-chelate, SK2: 2 % potassium sulfate and SK4: 4 % potassium sulfate.

†† Means in each row or column followed by the same lowercase or capital letters are not significantly different ($p < 0.05$) by Tukey-Kramer's Multiple Range Test.

3.3 Seminal root fresh mass (RFM) and seminal root dry mass (RDM)

Table 5 shows the effect of drought stress and seed priming with K treatments on RFM of corn. Results showed that increasing drought stress from Ψ of -0.3 to -1.2 MPa led to significant decrease in RFM of non-primed seeds. Also the same trend was obtained for the mean value of RFM over all of primed and non-primed seeds. The maximum and minimum mean value of PFM were obtained at Ψ of -0.3 and -1.5 MPa. Our findings showed that the mean value of RFM decreased significantly by about of 49, 58, 77 and 93 % compared to that of control at drought levels of -0.6, -0.9, -1.2 and -1.5 MPa. Whereas, at -0.3 MPa drought level, RFM increased about 93 % compared to that of control. Khakshoor et al. (2011) reported that drought stress decreased RFM of *Anethum graveolens* L. significantly (about 65.1 %). The reduction in fresh plant mass might be associated with declined cell growth and enlargement due to the low turgor pressure under drought stress (Rane et al., 2001). Primed seed showed better performance than non-primed seeds (Table 5).

As results indicated the RFM values of seeds at normal conditions (without water stress) subjected to priming with the high level (4 %) of K- nano-chelate or sulfate were significantly more those of control or those of seeds primed with low level (2 %) of K- nano-chelate or sulfate. Furthermore, the mean value of RFM in seeds subjected to priming with 4 % K-nano chelate was significantly more than that of seed priming with potassium sulfate. Furthermore, the highest levels of applied K were more effective than the lower levels. The highest RFM (about 1080 mg) was observed in seeds primed with 4 % K-nano chelate at -0.3 MPa water potential, whereas, the lowest RFM (about 10 mg) was observed with application of -1.2 and -1.5 MPa water potential without priming. Results showed that the reduction of RFM in seeds primed with K-nano chelate and potassium sulfate at the high level of applied drought stress (-1.2 MPa) was lower than that of seeds without any priming treatment.

Table 5: Effect of K priming treatments and drought stress (water potential) on seminal root fresh and dry mass (mg) of corn seed

Priming treatment† (%)	Water potential (MPa) 0	-0.3	-0.6	-0.9	-1.2	-1.5	Mean
			Seminal root fresh mass (RFM)				
0	350 g††	745 d	235 j	140 q	10 x	10 x	248 D
NK2	305 h	927 b	140 q	156 o	175 n	40 w	290 C
NK4	620 e	1080 a	217 l	220 k	60 v	90 t	381 A
SK2	210 m	647 e	150 p	110 r	70 u	0	237 D
SK4	505 f	820 c	280 i	210 m	150 p	0	327 B
Mean	398 B	734 A	204 C	167 D	93 E	28 F	
			Seminal root dry mass (RDM)				
0	19 kl	53 d	18 lm	17 m	5 rs	4 s	19 C
NK2	37 f	64 c	44 e	29 g	21 j	5 rs	33 A
NK4	64 c	78 a	12 o	24 i	6 qr	9 p	32 A
SK2	23 i	19 kl	15 n	15 n	11 o	0	14 C
SK4	27 g	48 e	26 h	20 jk	12 o	0	22 B
Mean	30 B	56 A	23 C	21 C	11 D	4 E	

† Treatments consisted of 0: control, NK2: 2 % potassium-nano-chelate, NK4: 4 % potassium-nano-chelate, SK2: 2 % potassium sulfate and SK4: 4 % potassium sulfate.

†† Means in each row or column followed by the same lowercase or capital letters are not significantly different ($p < 0.05$) by Tukey-Kramer's Multiple Range Test.

Findings revealed that increasing drought stress from Ψ of -0.3 to -1.2 MPa led to significant decrease in RDM of non-primed seeds. Also the same trend was obtained for the mean value of RDM over all of primed and non-primed seeds. Application of drought stress at -0.6, -0.9, -1.2 and -1.5 MPa water potential, decreased the mean RDM values by about 23, 30, 63 and 87 % compared to that of control. Khakshoor et al. (2011) showed that RDM of *Anethum graveolens* L. decreased, significantly with decreasing water potential. Soltani and Galeshi (2002) believed that wheat seedling mass loss is due to decline of storage material in seeds and transfer them from cotyledons to embryonic axis. Saedi et al. (2007) reported that in some of wheat cultivars RDM increased with decreasing osmotic potential to -0.4 MPa afterward decreased. The RDM values of seeds at normal conditions (without water stress) subjected to priming with both of K sources were significantly higher than that of control. Furthermore, findings indicated that seed priming with K-nano chelate increased RDM, significantly as compared to that of control by about 63 %. The highest RDM was observed in seed primed with 4 % K-nano chelate at drought stress level of -0.3 MPa. Massarat et al. (2014) illustrated that seed priming of maize with distilled water and 1 % KNO_3 increased RDM significantly as compared to that of control.

3.4 Shoot fresh mass (SFM) and shoot dry mass (SDM)

Table 6 shows the effect of drought stress and seed priming with K treatments on SFM of corn. Results showed that increasing drought stress from Ψ of 0 to 1.2 MPa resulted in significant decreases in SFM of non-primed seeds. Also the same trend was obtained for the mean value of SFM over all of primed and non-primed seeds. So that, the mean value of SFM decreased significantly by about of 27, 60, 77, 92 and 97 % as compared to that of control at Ψ of -0.3, -0.6, -0.9, -1.2 and -1.5 MPa, respectively. Results showed that the influence of drought stress on SFM was more severe than on seminal root. Our results were in close agreement with the findings of some investigators like Keshavarz et al. (2013) who believed that at low drought stress, growth of root and shoot may stimulate.

Table 6: Effect of K priming treatments and drought stress (water potential) on shoot fresh and dry mass (mg) of corn seeds

Priming treatment† (%)	Water potential (MPa) 0	-0.3	-0.6	-9	-1.2	-1.5	Mean
			Shoot ' fresh mass (SFM)				
0	503 d††	265 i	110 q	120 o	22 x	20 y	173 D
NK2	585 c	627 b	260 j	130 n	60 t	40 v	284 A
NK4	690 a	393 h	140 m	100 r	40 v	10 z	229 C
SK2	230 k	115 p	60 t	80 s	50 u	0	89 E
SK4	465 e	415 g	420 f	150 l	30 w	0	247 B
Mean	495 A	363 B	198 C	116 D	40 E	14 F	
			Shoot ' dry mass (SDM)				
0	34 d	25 e	16 g	13 gh	8 ij	6 jk	17 C
NK2	26 e	51 a	27 e	10 m	10 h	6 jk	22 B
NK4	43 c	37 d	12 h	14 g	9 hi	5 k	20 B
SK2	8 ij	7 j	7 j	5 k	2 l	0	5 D
SK4	49 b	44 c	44 c	22 f	15 g	0	29 A
Mean	32 A	32.8 A	21 C	13 D	9 E	3 F	

† Treatments consisted of 0: control, NK2: 2 % potassium-nano-chelate, NK4: 4 % potassium-nano-chelate, SK2: 2 % potassium sulfate and SK4: 4 % potassium sulfate.

†† Means in each row or column followed by the same lowercase or capital letters are not significantly different ($p < 0.05$) by Tukey-Kramer's Multiple Range Test.

It has been reported that under drought stress conditions fresh mass of wheat (Range et al. 2001) and pearl millet (Kusaka et al. 2005) decreased. Sankar et al. (2007) showed that reduction of fresh mass under drought stress conditions might be due to the suppression of cell expansion and cell growth resulted from the low turgor pressure.

The SFM values of seeds at normal conditions (without water stress) subjected to priming with K-nano-chelate were significantly higher than that of control. But, seed priming with K-sulfate decreased SFM of non-water-stressed seeds as compared to that of control (non-primed seeds), significantly. However, results indicated that priming of seeds with 2 and 4 % K-nano chelate and 4 % potassium sulfate increased the mean value of SFM, significantly by about of 64, 32 and 43 % as compared to that of control, respectively. The highest SFM (690 mg) was obtained in seeds subjected to priming with 4 % K-nano chelate without drought stress, whereas, the lowest SFM (10 mg) was observed in seeds primed with 4 % K-nano chelate at -1.5 MPa water potential. Hopper et al. (1979) illustrated that seeds priming increases water absorption from growth medium, for this reason metabolic activity in seed during germination process begins much earlier than seminal root and shoot appearance. Masarat et al. (2014) reported that seed soaking of corn with distilled water and 1 % KNO_3 increased SFM significantly by about 26 and 21 %, respectively as compared to that of control.

Similar to those obtained for SFM, results showed that increasing drought stress from Ψ of 0 to 1.2 MPa resulted in significant decreases in SDM of non-primed seeds. Also the relatively same trend was obtained for the mean value of SDM over all of primed and non-primed seeds. In other words, results indicated that SDW decreased, significantly with reduction of water potential from -0.3 to -1.5 MPa. These reductions were 34, 59, 72 and 91 % as compared to that of control when seeds subjected to water potential of -0.6, -0.9, -1.2 and -1.5 MPa, respectively. Decreased plant biomass has been also reported by Pan et al. (2003) and Rodriguez et al. (2005) for wheat and *Pallenis maritime* (L.) Greuter, respectively. Bhatt and Srinivasarao (2005) reported that the reduction of dry mass may be due to the considerable decrease in plant growth, photosynthesis and canopy structure as indicated by leaf senescence during drought stress in *Abelmoschus esculentum* L.

The SDM values of seeds at normal conditions (without water stress) subjected to priming with high (4 %) levels of K-nano-chelate or K-sulfate were significantly higher than that of control or those of seeds primed with low level (2 %) of K. However, results indicated that that seed priming led to significant increase in the mean value of SDM as compared to that of control (except for 2 % K-sulfate). Our findings indicated that the adverse effect of drought stress on SDM of primed seed with the high level (4 %) of K-nano chelate and sulfate was less than that of non-primed seeds. The highest SDM was observed in seed primed with 2 % K-nano chelate at -0.3 MPa water potential. Similar results were found by Sivritepe et al. (1997) who reported an increase in melons seedling dry mass for seeds primed with NaCl. Elouaer and Hannachi (2012) revealed that reduction in germination parameters and seedling growth was more profound in unprimed seeds than that of primed seeds of safflower.

3.5 Shoot length (SL)

The effect of drought stress and seed priming with K-nano chelate and sulfate on SL of corn has been shown in Figure 1. Similar to those obtained for SFM and SDM, results indicated that increasing drought stress from Ψ of 0 to -1.2 MPa resulted in significant decreases in SL of non-primed seeds. In other words, results indicated that drought stress has significant inhibitory effect on SL. The shoot length of seedling decreased significantly by 15, 33, 62, 85 and 95 % at applied water potential of -0.3, -0.6, -0.9, -1.2 and -1.5 MPa, respectively. Ebadi et al. (2011) reported that the shoot length of *Matricaria recutita* L. was 6.94 and 2.58 cm in control and water potential of -0.6 Mpa, respectively. They also showed that at the lowest applied water potential germination did not occur.

Results indicated that the SL of germinated seeds at normal conditions (without water stress) increased significantly when seeds subjected to priming with K-nano-chelate. But, seed priming with K-sulfate had no significant effect on SL of germinated seeds as compared to that of control. Furthermore, findings revealed that seed priming with the highest level (4 %) of K-nano chelate and K-sulfate increased the mean values of SL over all of applied water potentials, significantly as compared to that of control. The highest shoot length was observed with application of 4 % K-nano chelate without any drought stress.

3.6 Seminal root length (SRL)

Results indicated the SRL of non-primed germinated seeds increased when low level of drought stress (Ψ of 0 to -0.9 MPa) were applied, whereas, applying high level of drought stress (Ψ of -1.2 and -1.5 MPa) decreased it significantly. However, the slightly different trend was obtained for the mean value of SRL. So that, applying water potential of -0.6, -0.9, -1.2 and -1.5 MPa decreased SRL, significantly by about of 33, 57, 81 and 91 % as compared to that of control, respectively (Figure 2). Khakshoor Moghadam et al. (2011) reported that with increasing drought stress from -0.4 MPa, SRL of *Anethum gravoelens* L. declined, significantly.

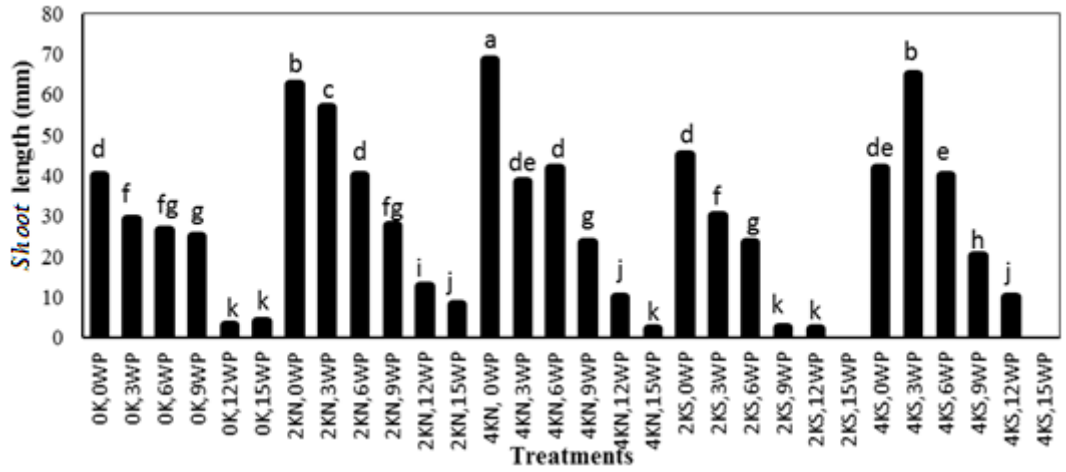

Figure 1: Effect of water potential (drought stress) and seed priming with potassium on shoot length of corn as compared to that of control, 0K, 0WP.Treatments consisted of drought stress (0WP, 0.3WP, 0.6WP, 0.9WP, 1.2WP and 1.5WP, shows drought stress of 0, 0.3, 0.6, 0.9, 1.2 and 1.5 MPa, respectively), potassium nano-chelate (0K, 2KN and 4KN shows seed priming with 0, 2 and 4 % of K-nano chelate) and potassium sulfate (0K, 2KS, and 4KS shows seed priming with 0, 2 and 4 % of K sulfate, respectively). Columns by the same lower letters are not statistically different at the probability level of 0.05 by Tukey-Kramer's Multiple Range Test.

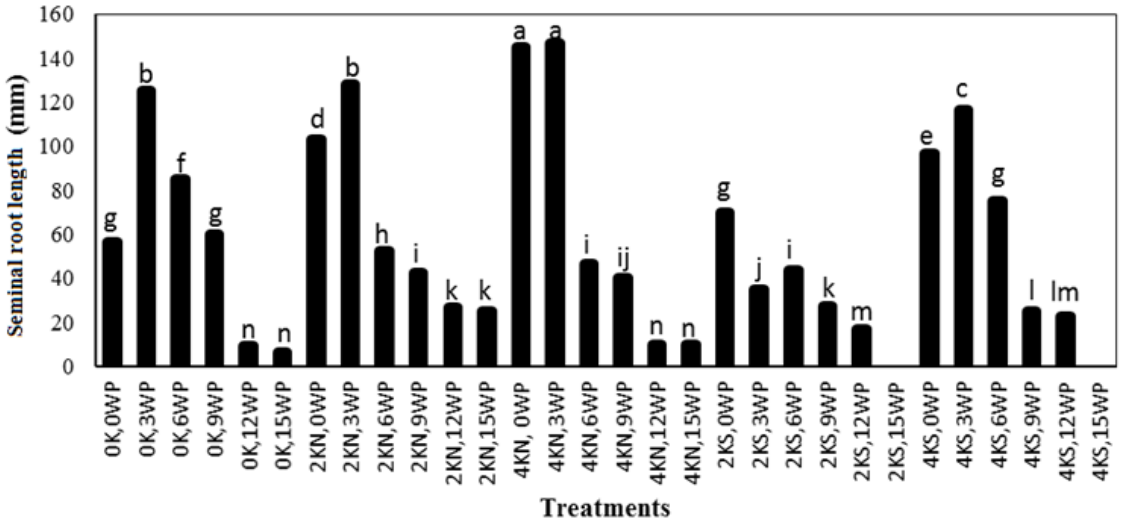

Figure 2: Effect of water potential (drought stress) and seed priming with potassium on seminal root length of corn as compared to that of control, 0K, 0WP.Treatments consisted of drought stress (0WP, 0.3WP, 0.6WP, 0.9WP, 1.2WP and 1.5WP, shows drought stress of 0, 0.3, 0.6, 0.9, 1.2 and 1.5 MPa, respectively), potassium nano-chelate (0K, 2KN and 4KN shows seed priming with 0, 2 and 4 % of K-nano chelate) and potassium sulfate (0K, 2KS, and 4KS shows seed priming with 0, 2 and 4 % of K sulfate, respectively). Columns by the same lower letters are not statistically different at the probability level of 0.05 by Tukey-Kramer's Multiple Range Test.

Results revealed that the SRL of germinated seeds at normal conditions (without water stress) increased significantly when seeds subjected to priming with K-nano-chelate or K-sulfate. Furthermore, findings indicated that seed priming with K-nano chelate and sulfate, increased the mean value of SRL over all of applied water potentials, significantly; whereas, high level of applied potassium sulfate (4 %) was not effective. The maximum value of SRL was observed in seeds primed with 4 % K-nano chelate without drought stress and at the lowest level of applied water potential (-0.3 MPa). Farooq et al. (2005) believed that seed priming due to earlier germination, resulted in drastic seedlings with more root and shoot length than that of the seedling of un-primed

seeds. Our results are in accordance to the finding of Basra et al. (2003) who reported that wheat shoot and root length increased in hydro-primed and matric-conditioned seeds for 12 or 24 h. This effect might be due to the higher embryo-cell wall extensibility.

3.7 Vigor index (VI)

Findings revealed that increasing drought stress from Ψ of -0.3 to -1.2 MPa led to significant decrease in VI of non-primed seeds. Also the same trend was obtained for the mean value of VI over all of primed and non-primed seeds. So that increasing drought stress from -0.3 to -1.5 MPa causes a significant decrease in VI of seeds (Figure 3). Results indicated that VI of seedling decreased significantly by 8, 37, 72 and 91 % in response to applied water potentials of -0.6, -0.9, -1.2 and -1.5 MPa, respectively. Van Gastel et al. (1996) illustrated that reduction of seed VI is probably due to decreasing water availability, which changes enzyme activity by inducing some problems in the transfer from endosperm reserves for the growth of embryonic axes and synthesis compounds of seed. Organ growth also depends on the speed of producing new cells and rapidly growing cells that is negatively influenced by drought stress. Both of processes are sensitive to cell swelling, but the sensitivity probably depends on tissue, species or stress intensity. So that when the seeds are exposed to drought, flexibility decreased in cells wall growing, that reduces cell expansion and consequently organs growth (Natale et al. 2010).

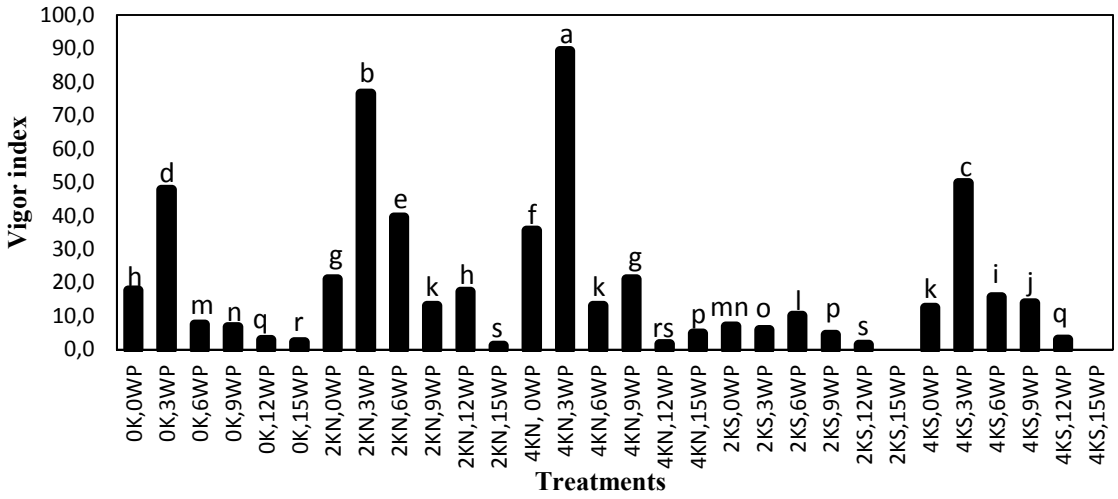

Figure 3: Effect of water potential (drought stress) and seed priming with potassium on vigor index of corn as compared to that of control, 0K, 0WP.Treatments consisted of drought stress (0WP, 0.3WP, 0.6WP, 0.9WP, 1.2WP and 1.5WP, shows drought stress of 0, 0.3, 0.6, 0.9, 1.2 and 1.5 MPa, respectively), potassium nano-chelate (0K, 2KN and 4KN shows seed priming with 0, 2 and 4 % of K-nano chelate) and potassium sulfate (0K, 2KS, and 4KS shows seed priming with 0, 2 and 4 % of K sulfate, respectively). Columns by the same lower letters are not statistically different at the probability level of 0.05 by Tukey-Kramer's Multiple Range Test.

The VI of germinated seeds at normal conditions (without water stress) increased significantly when seeds subjected to priming with K-nano-chelate, whereas this trait decreased significantly when subjected to priming with K- sulfate. Furthermore, our findings revealed that seed priming with the highest (4 %) level of K-nano chelate and K-sulfate increase the mean value of VI significantly compared to that of control. The maximum value of VI was observed with application of 4 % K-nano chelate at -0.3 MPa water potential. These observations are in accordance to the findings of Elouaer and Hannachi (2012) for safflower seeds. Their results showed that seed priming with NaCl and KCl increased VI compared to that of control. Nascimento and West (1998) illustrated that seed priming increased seed GP and VI due to supply mobilized food materials, activation and resynthesis of some enzymes and increased DNA and RNA synthesis.

4 CONCLUSION

Results showed that drought stress declined growth parameters of corn seedling. Seed priming with K-nano chelate and sulfate improved seedling traits and enhanced plant resistance to drought stress, obviously. Application of K-nano chelate for seed priming was more appropriate and efficient than K-sulfate. Our findings revealed that it is favorable to propose suitable seed-priming methods for different plant seeds to encounter the challenges of the environment.

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Agris category code: H60, h20, f30

Molecular identification of phytoplasmas associated with some weeds in West Azarbaijan province, Iran

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ABSTRACT

During field surveys in 2013 and 2014, about 14 weed plants showing phytoplasma diseases symptoms including yellowing and witches' broom were collected and tested by polymerase chain reaction (PCR) using universal primers for 16SrRNA starting by primer pairs P1/P7 in first round PCR followed by primer pair R16F2n/R16R2 in nested PCR. The detected phytoplasmas were characterized and differentiated through sequence analysis of PCR-amplified rDNA and virtual restriction fragment length polymorphism (RFLP). The phytoplasmas detected in symptomatic horseweed (*Erigeron canadensis* L.), common madder (*Rubia tinctorum* L.), Johnson grass (*Sorghum halepense* [L.] Pers.) and Sophora root (*Sophora alopecuroides* L.) were identified as members of the clover proliferation group (16SrVI group) by construction of phylogenetic trees. Further analysis by virtual RFLP classified the phytoplasmas of *Erigeron canadensis* L. and *Sorghum halepense* L. in subgroup 16SrVI-A and phytoplasmas of *Rubia tinctorum* L. and *Sophora alpecuriodes* L. in subgroup 16SrVI-D. This is the first report on the occurrence of phytoplasma diseases of weeds in west Azarbaijan, Iran.

Key words: common madder, Sophora root, Johnson grass, horseweed, nested-PCR, Urmia

IZVLEČEK

MOLEKULSKA DOLOČITEV FITOPLAZEM NAJDENIH V NEKATERIH PLEVELIH V PROVINCI ZAHODNI AZARBEJDŽAN, IRAN

Med pregledom polj, v letih 2013 in 2014, je 14 plevelnih vrst kazalo simptome okužbe s fitoplazmami kot so rumenenje in čarovniške metle. Nabrane vzorce smo analizirali z verižno reakcijo s polimerazo (PCR) z uporabo splošnih začetnih oligonukleotidov za 16SrRNA, začenši s pari začetnih oligonukleotidov P1/P7 v prvem krogu PCR analize, ki ji je sledila vgnezdena PCR anliza s parom začetnih oligonukleotidov R16F2n/R16R2. Odkrite fitoplazme so bile okarakterizirane in razlikovane s sekvenčno analizo PCR pomnožene rDNA in virtualnim polimorfizmom dolžin restrikcijskih fragmentov (RFLP). Fitoplazme, ki smo jih odkrili v simptomatični kanadski suholetnici (*Conyza canadensis* (L.) Cronq.), pravem brošču (*Rubia tinctorum* L.), divjem sirku (*Sorghum halepense* (L.) Pers.) in korenasti sofori (*Sophora alopecuroides* L.) so bile z izdelavo filogenetskega drevesa določene kot pripadnice skupine, ki povzroča proliferacijo detelje (16SrVI skupina). Nadaljnja analiza z virtualno klasifikacijo RFLP je fitoplazmi v kandski suholetnici in divjem sirku uvrstila v podskupino 16SrVI-A in fitoplazmi v pravem brošču in korenasti sofori v podskupino 16SrVI-D. To je prvo poročilo o pojavljanju od fitoplazem povzročenih bolezni na plevelih v zahodnem Azarbeidžanu, Iranu.

Ključne besede: pravi brošč, korenasta sophora, divji sirek, kanadska suholetnica, vgnezdena-PCR, Urmia

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1 INTRODUCTION

Phytoplasmas are a group of wall-less phloem-limited plant pathogenic bacteria belonging to Mollicutes which have been described relative recently (Lee *et al*., 1998). They are associated with diseases in several hundreds of plant species, including weeds (Marcone *et al*., 1997). Typical symptoms include virescence/phyllody, sterility of flowers, proliferation of axillary buds resulting in witches broom growth, abnormal internodes elongation and generalized stunting (Bertaccini *et al*., 1996; 2014). Phytoplasmas are introduced by insect vectors (mostly leafhoppers) during feeding activity into plant sieve tube elements, from which they spread systemically through the plants (Bertaccini *et al*., 2014). Currently, characterization and classification of phytoplasmas are based mainly on restriction fragment length polymorphism (RFLP) and sequence analysis of 16SrDNA or other less-conserved genes, whereas detection is done mainly by polymerase chain reaction (PCR) assay (Bertaccini *et al*., 2014; Lee *et al*., 2000; Seemuller *et al*., 1998). Weeds serve as both a reservoir for phytoplasma infection and as reproductive hosts for the vectors (Singh and Upadhyaya, 2013). Phytoplasmas are known to cause diseases in weeds including field bindweed (*Convolvulus arvensis* L.), prickly lettuce (*Lactuca serriola* L.), Johnson grass (*Sorghum halepense* (L.) Pers.), bermuda grass (*Cynodon dactylon* (L.) Pers.), horseweed (*Conyza canadensis* (L.) Cronq.) and some others were reported worldwide (Arocha-Rosete and Jones, 2010; Babaie *et al*., 2007; Chen *et al*., 2003; Li *et al*., 2013; Marcone *et al*., 1997; Salehi *et al*., 2006; Shubhrata, 2004; Thereza and Baross, 2002; Vali Sichani *et al*., 2014). However little is known about phytoplasma diseases of weeds in west Azarbaijan province of Iran. The aim of this study was to verify the presence of phytoplasma diseases in symptomatic weeds in West Azarbaijan province using PCR assay. The detected phytoplasmas were characterized and classified using sequence analysis of PCR-amplified 16SrDNA and virtual gel RFLP.

2 MATERIALS AND METHODS

2.1 Plant materials

Fourteen weed plants related to 4 plant species including Johnson grass (*Sorghum halepense* (L.) Pers.), Canadian horseweed (*Conyza canadensis*. (L.) Cronquist), Sophora root (*Sophora alopecuroides* L.) and common madder (*Rubia tinctorum* L.) showing symptoms typical of phytoplasmal infection were collected during 2013 and 2014 growing seasons (Table 1) from different regions of West Azarbaijan province including Urmia, Salmas, Khoy and Mahabad cities. Asymptomatic plants were also collected and used in molecular analysis as negative controls.

2.2 DNA Extraction

Total DNA was extracted from 0.25g of leaves and midribs according to the method described by Zhang *et al* (1998). Total DNA of healthy plants were extracted and used as negative controls.

2.3 PCR Analysis and Primer Pairs

The universal primer pair P1/P7 (Schneider *et al*., 1995) was employed in first round PCR to amplify a 1.8kbp fragment of 16SrDNA. A 30-fold dilution of the first round PCR product used as template for nested PCR using primer pair R16f2n/R16R2 which amplified an internal fragment of 1.2kbp from the 16SrDNA (Lee *et al*., 1993). The total volume of 20µl PCR reaction mixtures contained 20ng DNA, 0.2mM of each dNTP (Cinnagen, Iran), 1.6mM $MgCl_2$, 1U of *Taq* DNA polymerase (Cinnagen, Iran), 0.5µl of each primer pair (20pmol/µl) and 1X polymerase buffer. The reaction mixtures were subjected to 35 cycles at the following conditions: First round PCR (35 cycles): 1 min (3 min for the first cycle) for denaturation step at 94ºC, 1 min for annealing at 57ºC and 1.5 min (10 min for the last cycle) for primer extension step at 72ºC. Second round nested PCR (35 cycles): 2 min (5 min for the first cycle) for denaturation step at 94ºC, 1 min for annealing at 57ºC and 2 min (10 min for the last cycle) for primer extension step at 72ºC. The PCR

products were analyzed by electrophoresis in a 1 % agarose gel and stained with ethidium bromide. An ultraviolet (UV) transilluminator was used to visualize DNA band.

2.4 Sequencing and Phylogenetic Analysis

PCR products of nested PCR were sequenced directly. Sequencing was performed by Macrogen (South Korea) on both strands. Nucleotide sequence similarity, multiple alignment and phylogenetic tree construction using the neighbor-joining (NJ) method were done with MEGA5 software (Tamura *et al*., 2011) and subjected to bootstrap analysis using 500 replicates. The *Acholeplasma laidlawii* isolate was used as outgroup.

2.5 Virtual RFLP Analysis

Virtual RFLP analysis using iPhyclassifier (Zhao *et al*., 2009) was used to determine 16Sr group and subgroup affiliation of the detected phytoplasmas. RFLP profiles of detected phytoplasmas were compared to those of 16SrVI–subgroups A, B, C, D, E, F, H using *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I enzymes.

3 RESULTS AND DISCUSSION

During growing seasons of 2013 and 2014, fourteen weed samples with phytoplasma symptoms were collected from different parts of West Azarbaijan province, Iran. The symptoms varied with the host plant and the most characteristic symptoms were witches' broom, leaf malformation, little leaf and yellowing (the symptoms are summarized in Table 1 and some symptomatic plants were shown in Fig.1).

Table 1: Weeds showing phytoplasma-like symptoms

Common name	Scientific name	Plant family	symptoms	No of samples	Place of sampling	Date of collection	Latitude, Altitude and height above level sea of the city
Johnson grass	*Sorghum halepense* (L.) Pers.	Gramineae	Little leaf	2	Salmas	2 June 2014	38° N, 44°E, 1406 meters
Canadian horseweed	*Conyza canadensis* (L.)Cronq.	Asteraceae	Leaf malformation and witches' broom	6	Khoy	17 June 2013	38° N, 44°E, 1139 meters
Sophora root	*Sophora alopecuroides* L.	Fabaceae	Yellowing and little leaf	2	Abajaloo-Urmia	21 July 2014	37° N, 45°E, 1362 meters
Common madder	*Rubia tinctorum* L.	Rubiaceae	Little leaf	4	Mahabad	20 July 2013	36° N, 45°E, 1304 meters

Figure 1: Weeds with phytoplasma-like symptoms in West Azarbaijan province. A-Common madder showing little leaf, B- Johnson grass with little leaf symptoms, C- Sophora root with yellowing and little leaf symptoms, D-Canadian horseweed showing leaf malformation and witches' broom.

DNA fragments of approximately 1.8kbp and 1.25kbp were amplified using phytoplasma universal primer pairs, P1/P7 and R16f2n/R16R2 in first round and nested PCR, respectively from Johnson grass, Canadian horseweed, Sophora root and common madder. Neither by direct nor by nested PCR were DNA amplified form other weeds tested in this survey and from asymptomatic plants. The nucleotide sequences of the phytoplasmas detected in four plants (Johnson grass, Sophora root, Canadian horseweed and common madder) were deposited in GenBank (with accession numbers of KT807469, KT807470, KT807471 and KT807472, respectively). BLAST search showed that the 16SrDNA sequences of four detected phytoplasmas shared the highest homology (99 %) to members of the 16SrVI group '*Candidatus* Phytoplasma trifolii'. Computer-stimulated restriction analysis were carried out on R16f2n/R16R2 sequences from Johnson grass, Canadian horseweed, Sophora root and common madder together with 12 reference phytoplasmas and seven representative strains belonging to 16SrVI subgroups (A, B, C, D, E, F, H). Comparison of virtual gel plotted images revealed that RFLP patterns of common madder (Ruph) and Sophora root (Tph) were most similar to periwinkle little leaf, representative of 16SrVI-D subgroup and RFLP patterns of Canadian horseweed (Pph) and Johnson grass (Nph) were most similar to clover proliferation, representative of 16SrVI-A (Fig. 2). Phylogenetic analysis of sequences presented in this survey with 19 phytoplasmas from GenBank clustered phytoplasmas detected on Johnson grass, Canadian horseweed, Sophora root and common madder with phytoplasmas of 16SrVI group (Fig.3).

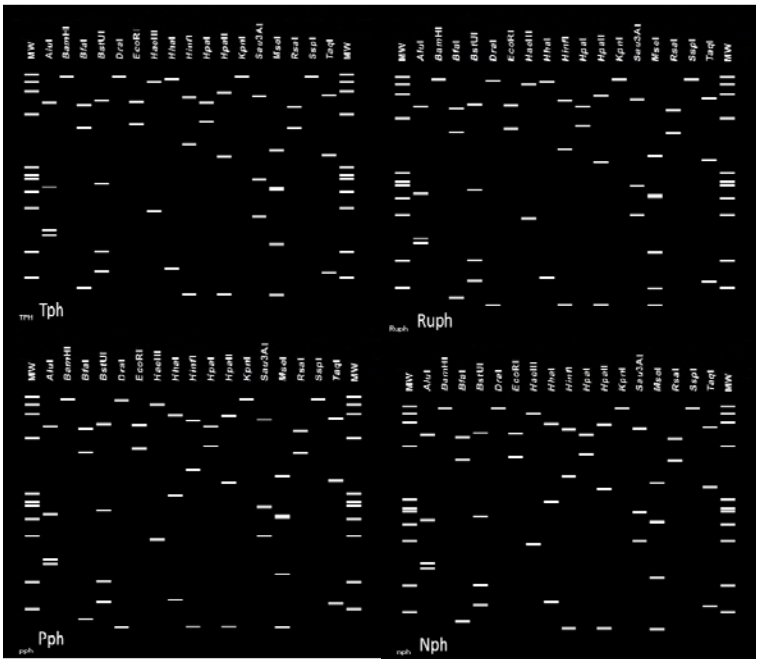

Figure 2: Virtual restriction fragment length polymorphism (RFLP) pattern of $R_{16}F_{2n}/R_{16}R_2$ PCR product sequence from Sophora root (Tph), common madder (Ruph), Canadian horseweed (Pph), and Johnson grass (Nph) phytoplasmas. Restriction sites for the 17 restriction enzymes were used in the simulated digestions: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I.

Two phytoplasmas which were previously reported from Canadian horseweed exhibiting yellowing and witches' broom symptoms were classified in 16SrI (SrI-A) (Lee *et al*., 2000) and 16SrVII groups (Thereza and Baross, 2002), respectively. This is the first report of little leaf symptom on Canadian horseweed that is classified in phytoplasma 16SrVI group. The phytoplasma which causes yellowing on Johnson grass, detected in our survey, was classified in 16SrVI group. Previously, on Johnson grass exhibiting yellowing Arocha-Rosete and Jones (2010) and Singh and Upadhyaya (2013) found phytoplasmas of 16SrXXIV-A and 16SrXII groups, respectively. Sophora root with little leaf and yellowing symptoms was classified in 16SrVI group in this study. Previous reports of phytoplasmas affecting *Sophora* species include a 16SrXII phytoplasma associated with *S. japonica* L (*Styphnolobium japonicum* (L.) Schott.) yellows in China (Duduk *et al*., 2010), '*Ca.* Phytoplasma ziziphi' in China associated with witches' broom and a 16SrI '*Ca.*Phytoplasma asteris' associated with Sophora root yellows (Yu *et al*., 2012; Chen *et al*., 2013). Recently Allahverdi *et al*. (2014) reported a 16SrXII phytoplasma association with *S. alopecuroides* from Firooz-koh (Tehran Province, Iran) with leaf yellowing, little leaf and stunting symptoms. The association of a phytoplasma belonging to 16SrVI was previously established in Sophora root (*Sophora alopecuroides* L.) exhibiting yellowing and witches' broom symptoms from China (Li *et al*., 2013). There is no data on common madder phytoplasma infection and to our knowledge it is the first report of phytoplasma (16SrVI group) infection of common madder.

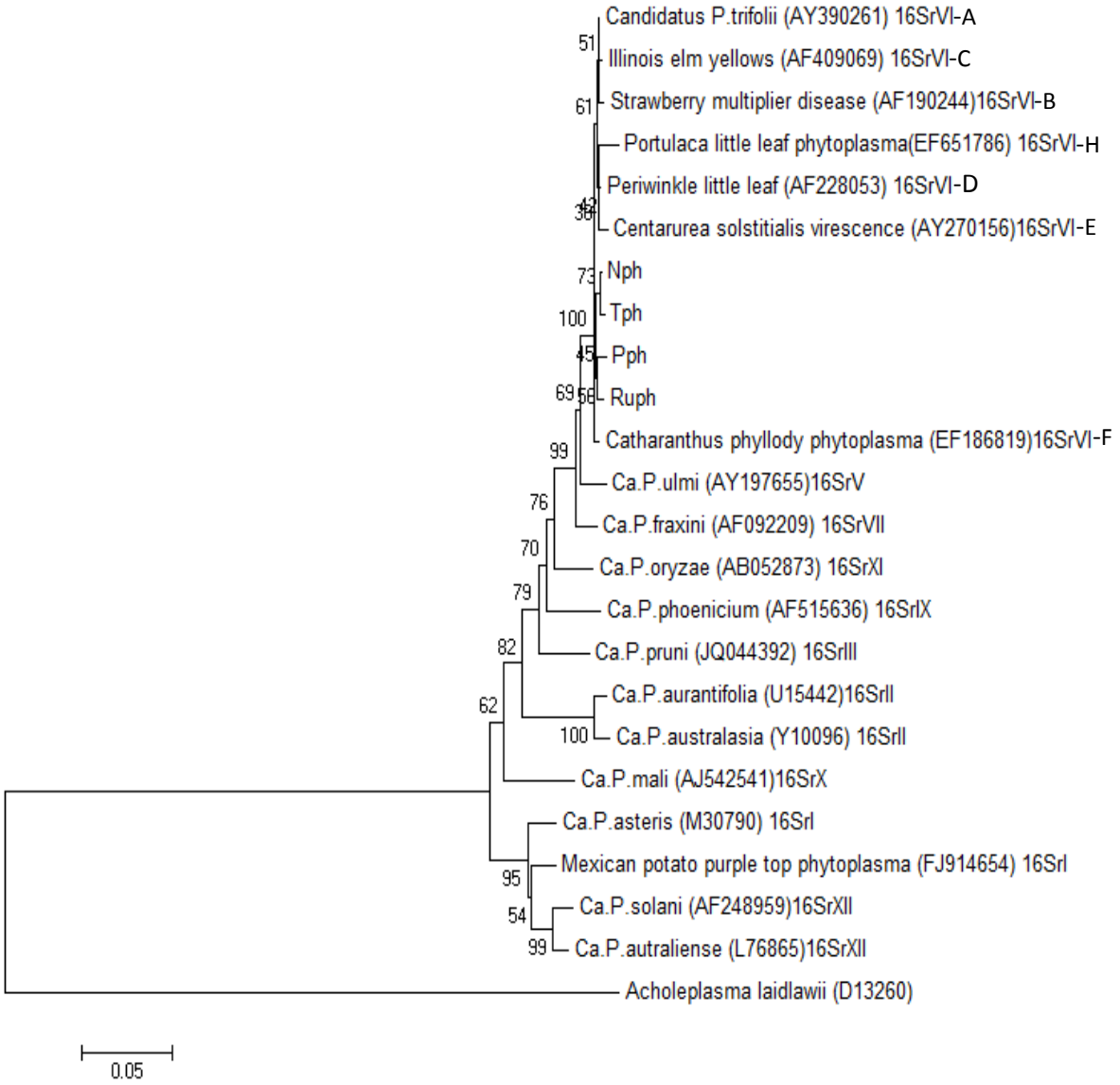

Figure 3: Phylogenetic tree constructed by the neighbor joining method of 16SrRNA gene sequences from 19 phytoplasma and phytoplasmas identified from Sophora root (Tph), common madder (Ruph), Canadian horseweed (Pph), and Johnson grass (Nph) and *Acholeplasma laidlawii* as outgroup. Numbers at the nodes are bootstrap values based on 500 repetitions. GenBank accession numbers for sequences are given in parentheses.

4 CONCLUSIONS

To date, phytoplasmas have been documented in more than 100 weed plant species. Phytoplasmas cause diseases on several weeds which result in serious threat as a source of alternative natural host for the spread of phytoplasma pathogen to other economically important plants, thereby creating a chance of causing severe losses. Detection of phytoplasma associated with diseases of weed crops is very important to check the possibility of further spread of their diseases to other plants. Results of this study can facilitate further works on ecology, epidemiology and diversity of phytoplasmas in Iran. This is the first report on the occurrence of phytoplasma diseases on weeds in West Azarbaijan province, Iran.

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DOI: 10.14720/aas.2016.107.1.14
Agrovoc descriptors: citrus; aphis spiraecola; aphidoidea; citrus reticulata, citrus sinensis, climatic change; population dynamics; algeria

Agris category code: H10, p40

Population dynamics of aphids (Aphididae) on orange (*Citrus sinensis* 'Thomson Navel') and mandarin (*Citrus reticulata* 'Blanco')

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ABSTRACT

Citrus fruits represent one of the most important fruit productions worldwide. However, they suffer from a numerous constraints. Aphids are among the causes of the decline in the production of citrus. In this study, the diversity of citrus aphids and their seasonal occurrence were explored on orange and mandarin, during 2012 and 2013, in an orchard located in Skikda province (Algeria). In total, six different aphid species were found during two years. The most common species was *Aphis spiraecola* Patch, 1914. Climatic conditions had an important role in the infestation level by aphids. There were changes of aphid dynamics between the two years of the investigation. No aphids was recorded in six months in 2012 (January, June, July, August, September and December) and in three months in 2013 (January, February and August). Besides, the number of identified aphid species increased from two to five. On the other hand, the orange trees seemed to be the most infested host species.

Key words: Algeria, citrus aphids, *Citrus reticulata*, *Citrus sinensis*, climate change, population dynamics

IZVLEČEK

POPULACIJSKA DINAMIKA LISTNIH UŠI (Aphididae) NA POMARANČEVCU (*Citrus sinensis* 'Thomson Navel') IN MANDARINOVCU (*Citrus reticulata* 'Blanco')

Plodovi citrusov so med najvažnejšimi v svetovni proizvodnji sadja, a je njihova proizvodnja omejena zaradi številnih omejitev. Listne uši so eden izmed vzrokov upada njihove proizvodnje. V tej raziskavi sta bila preučevana raznolikost in sezonsko pojavljanje listnih uši, ki se pojavljajo na citrusih v sadovnjaku pomarančevcev in mandarinovcev v provinci Skikda (Algeria), v letih 2012 in 2013. Celukupno smo v dveh letih raziskave našli šest različnih vrst listnih uši. Najbolj pogosta je bila vrsta *Aphis spiraecola* Patch, 1914. Pri okužbi z listnimi ušmi so imele pomembno vlogo klimatske razmere. Dinamika njihovega pojavljanja se je med obema letoma raziskave spreminjala. Listnih uši nismo zabeležili v obdobju šestih mesecev v letu 2012 in treh mesecev v letu in 2013. Poleg tega se je število vrst v tem obdobju povečalo iz dveh na pet. Pomarančevci so se izkazali kot najbolj okužen gostitelj.

Ključne besede: Alžirija, listen uši na citrusih, *Citrus reticulata*, *Citrus sinensis*, podnebne spremembe, populacijska dinamika

1 INTRODUCTION

Citrus is an important fruit crop worldwide (Altaha *et al*., 2012). The citrus are cultivated from about 15° N to 35° S, between sea level and 1000 m, and are susceptible to frost unless the tree is dormant (Hill, 2008). They are grown in more than 100 countries all over the world, mainly in tropical and subtropical areas, where favorable soil and climatic conditions prevail. Citrus fruits are marketed mainly as fresh fruit or as processed juice (Peña *et al.*, 2007). The citrus industry is one of the main components of Mediterranean agriculture, helping to guarantee incomes in underprivileged

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rural zones. Furthermore, citrus fruits provide the main source of vitamin C in the Mediterranean Basin, contributing to the general nutritional supply (Dambier *et al.*, 2011). In Algeria, the citriculture has a strategic importance because it is a source to supply fresh fruits (Biche, 2012). However, it suffers since a few years from a considerable decline of the production and the quality of fruits. Among the causes of this decline, the pests such as the aphids keep a predominant place (Boulfekhar-Ramdani, 1998). The latter are serious pests of many agricultural crops. Therefore, a good understanding of their population dynamics is vitally important for crop protection (Kindlmann and Dixon, 2010). Little quantitative data are available on the population dynamics of citrus aphids because of sampling difficulties (Lapchin *et al.*, 1994). Although aphids are dangerous pests, little is known about the aphid fauna of Algeria (Laamari *et al.*, 2010).

In this study, we established an inventory of the aphid species present on different citrus trees in the Northeastern Algerian region of Skikda, based on the prospection work carried out during two years (2012 and 2013). This constitute a step towards exploring the diversity of the Algerian aphid fauna on citrus and their seasonal occurrence, and comparing the aphids associated with different citrus species in order to elaborate an appropriate control plan against these pests, and thus contributing to increase the production of citrus fruits.

2 MATERIALS AND METHODS

The orchard used in this study is located in the northeast of Algeria (Emjez Djich: Skikda) (36° 42’ N, 6° 47’ E), planted with approximately 15-year-old citrus trees. The experimental area consisted of different citrus species and varieties, among them, we studied two species: ‘Thomson Navel’ sweet orange (*Citrus sinensis* L.) and ‘Carval Hal’ mandarin (*C. reticulata* Blanco), all grafted on Troyer citrange (*C. sinensis* (L.) Osb. × *Poncirus trifoliata* Raf.) rootstock. The trees were planted in rows with 5 m between rows. Intrarow-tree spacing was also 5 m. From January 2012 to December 2013, samples were taken every month.

Orange and mandarin trees were almost under the same management regime. There were no other crops under the trees. In general, the orchard was cultivated with low pesticide application, and weeding was executed mechanically.

In 2012, five young citrus shoots from each variety were randomly collected.

Lapchin *et al.* (1994) mentioned that sampling methods used to study the population dynamics of citrus aphids are generally based on counting infested shoots. Several authors (Yokomi and Tang, 1996; Kavallieratos *et al.*, 2002; Boukhris-Bouhachem, 2011) have used young citrus shoots to study citrus aphid in different countries (Puerto Rico, Greece and Tunisia respectively).

In 2013, four leaves from each tree, and four trees from each variety were sampled. Thus, totally 32 leaves were examined for each sampling date.

Fadamiro *et al.* (2008) and Yoldaş *et al.* (2011) also sampled leaves of citrus to study aphids in the United States and Turkey respectively.

On each sampling date, all the aphids (nymphs and adults regardless of species) and aphid mummies were counted on one shoot (in 2012) and on one leaf (in 2013). Individuals of dipteran predators were counted visually in the field only as larvae. Aphids were transferred in tubes to be conserved in ethanol and then identified in the laboratory using identification key of Stoetzel (1994) and those of Blackman and Eastop (2000).

Statistical analysis was done by using aphid densities obtained during study (24 sampling months). Data were analyzed by one-way ANOVA. All the statistical procedures were performed using SPSS for Windows 10.0.5 (SPSS, Inc.). Figures were drawn using Microsoft Excel 2007.

3 RESULTS

The aim of this work was to obtain knowledge on population dynamics of aphids on two citrus species in Algeria (Mediterranean region). Thus, samples (shoots or leaves) were taken every month during two years to assess and identify aphids.

Six species of aphids in total were found on two citrus varieties in this study (Tables 1 and 2) including *Aphis spiraecola, A. gossypii, A. nerii, A. craccivora, Myzus persicae* and *Macrosiphum euphorbiae.* We identified four species on orange and four on mandarin.

3.1 Aphids and population dynamics

Table 1: Occurrence of aphid species in the studied orchard during 2012
+ rare species; +++ dominant species

	'Thomson Navel' sweet orange	'Carval Hal' mandarin
Aphis spiraecola (Patch, 1914)	+++	+++
Aphis craccivora (Koch, 1854)	+	

Table 2: Occurrence of aphid species in the studied orchard during 2013
+ rare species; +++ dominant species

	'Thomson Navel' sweet orange	'Carval Hal' mandarin
Aphis spiraecola (Patch, 1914)	+++	+++
Aphis gossypii (Glover, 1877)	+	+
Myzus persicae (Sulzer, 1776)		+
Macrosiphum euphorbiae (Thomas, 1878)	+	
Aphis nerii (Boyer De Fonscolombe, 1841)		+

The infestation of orange and mandarin trees by aphids varied markedly among sampling dates (Figures 1 and 2). In 2012, densities of aphids decreased to zero in six months (January, June, July, August, September and December). However, no aphids was noted in 2013 in three months only (January, February and August). We remark also that the highest levels of infestation were concentrated in autumn and spring. About 35 % and 43 % of the total infestations was recorded on April and November 2012 respectively. In 2013, the biggest proportion of infestation was in April with about 36 % of the total infestation, and in September with approximately 42 %.

3.2 Difference of infestation between citrus species

During the first year of the study, ANOVA showed significant difference among the aphids that infested citrus species in April (231,6 aphids/shoot on orange 15,6 aphids/shoot on mandarin, $F = 17{,}469$, $p = 0.003$) and in November (240,8 and 65,8 aphids/shoot on orange and mandarin respectively, $F = 5{,}907$, $p = 0.041$). During the second year, ANOVA revealed significant differences of the infestation degree between the examined citrus species in June (11,06 aphids/leaf on orange and 0,75 aphid/leaf on mandarin, $F = 4{,}395$, $p = 0.045$), September (107,31 and 19,88 aphids/leaf on orange and mandarin respectively, $F = 5{,}280$, $p = 0.029$) and October (11,06 on orange and 0,75 on mandarin, $F = 8{,}901$, $p = 0.006$). No significant difference was recorded between aphids found on different citrus species during the other months in both years of the study.

'Thomson Navel' orange trees seemed to be the most infested cultivar with a peak of approximately 241 aphids/shoot observed on November 2012 (Figure 1), and 107 aphids/leaf noted on September 2013 (Figure 2). On the other hand, 'Carval Hal' mandarin appears the least infested with maximal value of about 66

aphids/shoot on November 2012 and 33 aphids/leaf on April 2013. Besides, no aphid was recorded on mandarin in twelve months among 24 sampling dates.

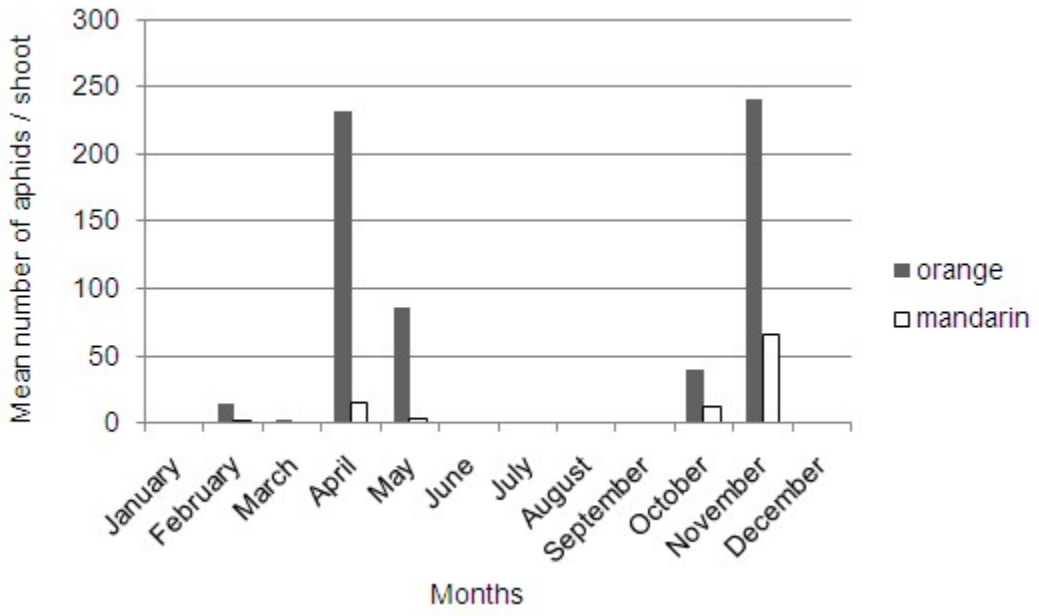

Figure 1: Seasonal abundances of aphids in the studied citrus orchard from January to December 2012. Values indicate mean number of aphids (nymphs + adults) of 5 shoots.

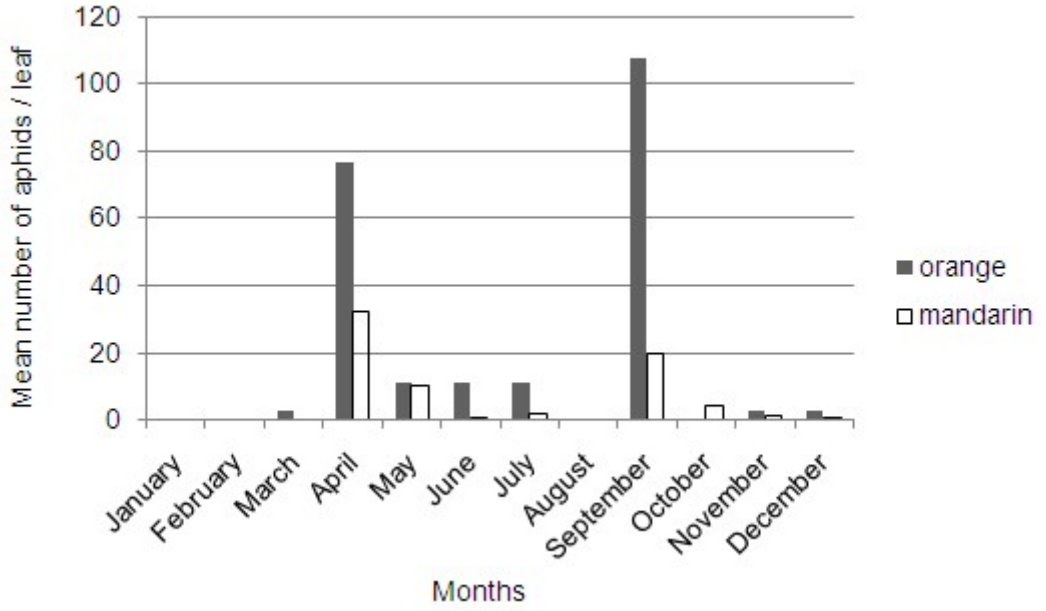

Figure 2: Seasonal abundances of aphids in the studied citrus orchard from January to December 2013. Values indicate mean number of aphids (nymphs + adults) of 16 leaves.

In general, on the two hosts, we noted a progressive increasing in the mean number of aphids until reaching a first peak in spring, and then decreasing until 0 in some months of summer. The infestation level increased once again to arrive to a second peak in the same year in autumn, and it reduced another time in winter.

3.3 Natural enemies of citrus aphids

Predators of citrus aphids found in this study were mainly larvae of syrphids. Its number was slightly higher than parasitoids, although it remains very limited (Tables 3 and 4). The distribution and abundance of predators varied among citrus species. The highest number was found on orange.

Concerning parasitoids, their number was much reduced. There were only two mummified individuals on mandarin (on February 2012) and three mummified aphids on orange (on May 2013).

Table 3: Number of predators of citrus aphids found on orange and mandarin trees in 2012. Values indicate total number of predators on 5 shoots.

	January	February	March	April	May	June	July	August	September	October	November	December
Orange	0	0	0	3	1	0	0	0	8	0	1	0
Mandarin	0	0	0	0	0	0	0	0	0	0	0	0

Table 4: Number of predators of citrus aphids found on orange and mandarin trees in 2013. Values indicate total number of predators on 16 leaves.

	January	February	March	April	May	June	July	August	September	October	November	December
Orange	0	0	0	2	5	0	0	0	0	0	0	0
Mandarin	0	0	0	0	0	0	0	0	2	2	0	0

4 DISCUSSION

4.1 Aphids and population dynamics

Six species of aphid was detected in this survey. There were five aphid species present as different degree vector of *Citrus tristeza virus* transmission: *Aphis spiraecola, A. gossypii, Myzus persicae, Toxoptera aurantii* Boyer de Fonscolombe, 1841 and *T. citricida* Kirkaldy, 1907. This latter is the most important, it is already present in certain countries of the Mediterranean region (Lebdi Grissa, 2010). However, in our study, it was not found.

We found four aphid species on 'Thomson Navel' sweet orange and four on 'Carvalhal' mandarin. Two among the identified aphids (*Aphis spiraecola* and *Aphis gossypii*) were present on the two citrus varieties. While *Aphis craccivora* and *Macrosiphum euphorbiae* were determined on orange only, *Myzus persicae* and *Aphis nerii* were observed on mandarin. Similarly, Ben Halima-Kamel and Ben Hamouda (2005) and Kavallieratos *et al.* (2007) mentioned differences between the species of aphids that infested orange, lemon, sour orange and clementine in Tunisia, and those attacking orange and mandarin in Greece respectively.

In this study, the aphids identified on orange were *Aphis spiraecola*, *A. gossypii, A. craccivora* and *Macrosiphum euphorbiae*. Four aphid species are found frequently on orange trees in the Mediterranean region, which are *Aphis spiraecola*, *A. gossypii, Toxoptera aurantii* and *Myzus persicae* (Loussert, 1989). Ben Halima-Kamel and Ben Hamouda (2005) found besides these species, *Aphis fabae* Scopoli, 1763 and *A. craccivora* Koch, 1854 on 'Maltaise' and 'Valencia' oranges in Tunisia. Nevertheless, Kamel (2010) identified only *A. gossypii* on both 'Baladi' and 'Navel' orange in Egypt; and Lopes *et al.* (2006) showed that seven species of aphids were found on orange in Portugal, among them *A. hederae* Kaltenbach, 1843, *A. solanella* Theobald, 1914 and *Anoecia haupti* Börner, 1950. Differences among these studies are due to environmental conditions and the effect of the host variety.

The most common species observed in this survey was *Aphis spiraecola*, and with lower importance *Aphis gossypii*. The hierarchy of the species is highly variable from country to country. *A. gossypii* is generally not the most abundant species (Lapchin *et al.*, 1994). Several studies showed that *A. spiraecola* and *A. gossypii* were among the most abundant species on citrus trees in Algeria (Franco *et al.*, 2006), in Morocco (Belati and Belabed, 2014), in Tunisia (Ben Halima-Kamel and Ben Hamouda, 2005; Lebda Grissa, 2010), in Italy (Yahiaoui *et al.*, 2009), in Spain (Marroquín *et al.*,

2004) and in the United States (Powel *et al.*, 2006; Fadamiro et *al.*, 2008). In contrast, *Toxoptera aurantii* and *T. citricida* were the major aphids of orange, mandarin, and other *Citrus* spp. in the southeast of Asia (Bayhan *et al.*, 2006).

On the other hand, the peak of infestation and number of auxiliaries were observed mainly in spring and in autumn. This coincides with moderate temperature and the production of new shoots appropriate for the reproduction of aphids and consequently the occurrence of predators and parasitoids. Many researches mentioned the peak of infestation by some aphids on citrus trees in spring (Saharaoui and Hemptinne, 2009; Kamel, 2010; Yoldaş *et al.*, 2011; Mostefaoui *et al.*, 2014). The within-year dynamics of aphids are largely determined by seasonal changes in host quality. Aphids do best when amino acids are actively translocated in the phloem. Thus on trees, the leaves are most suitable for aphids in spring and autumn (Kindlmann and Dixon, 2010).

The fluctuations of infestation during two years of study seem to be influenced by the changes of temperatures. Many authors mentioned the importance of temperature for the biology of aphids (Bayhan *et al.*, 2006; Dixon and Hopkins, 2010; Harrington and Clark, 2010; Gao *et al.*, 2013). Several generations follow each other during campaign in favorable conditions, elevated humidity and temperature between 20 and 25 °C; during big heats of summer, the infestations are less numerous (Bellabas, 2011). In addition, aphid reproduction and survival could be significantly reduced in summer if there are longer periods when temperature remains above the optimal threshold for aphid growth (Qureshi, 2010). Planet Earth has experienced many significant climatic changes. The increase in the concentration of greenhouse gases in the atmosphere induces an increase in temperature, which influences other climatic parameters (Ameixa, 2010). Benhamiche *et al.* (2014) cited some indicators of climate change in Algeria. Recent changes in climate, particularly warmer temperatures, have already begun to impact on biodiversity and ecosystems. Changes in species distributions, population sizes, the timing of reproduction and migration events, and in the frequency of pest and disease outbreaks have all been documented and linked to elevated temperatures (Bergant *et al.*, 2005; Roy and Majerus, 2010).

In this study, the number of identified aphid species increased from two in 2012 to five in 2013. Hullé *et al.* (2010) demonstrated that temperature changes had repercussions for aphid diversity and population dynamics. At a pan-European scale, the EXAMINE observation network has provided evidence for an increase in the number of aphid species present over the last 30 years and for earlier spring flights.

4.2 Difference of infestation between citrus species

A difference in infestation between the tested citrus species was noted, with the biggest number on orange. In the same way, Marroquín *et al.* (2004) found, in their study in Spain, that clementine was the most attacked host species, followed by lemon, sweet orange, grapefruit, and satsuma. Additionally, Kavallieratos *et al.* (2002) mentioned differences between the number of aphids that infested orange and tangerine trees in Greece.

Winged aphids visit many plants and the selection is realized based on many physical and chemical factors. This latter include stimulating or inhibiting substances such as essential nutrients (amino acids and sugars) which launch usually a behavior effect (Herrbach, 1985); and plant secondary metabolites that are involved in insect-host interactions mainly by chemical derived substances and volatile substances (Bhatia *et al.*, 2011).

4.3 Natural enemies of citrus aphids

Very limited number of predators and parasitoids were observed in this investigation. The predators found consisted of syrphid larvae. Some syrphids have larvae devour aphids (Sarthou and Speight, 2005; Biche, 2012). They feed on the aphid by piercing and sucking out the body contents, while holding the prey aloft (Sullivan, 2008).

Climate change can have diverse effects on natural enemies of pest species. The fitness of natural enemies can be altered in response to changes in herbivore quality and size induced by temperature and CO_2 effects on plants (Thomson *et al.*, 2010). Majority insect life history traits are linked to

temperature. All trophic levels stand to be affected by the increase in average global temperature: the herbivores, their natural enemies (parasitoids, predators and pathogens), and hyperparasitoids and tertiary predators (van Baaren *et al.*, 2010). Hotter, longer summers would imply extended periods of prey scarcity for aphid natural enemies and could further impede their ability to survive this difficult season (Qureshi, 2010).

In conclusion, this study showed differences between infestation of orange and mandarin trees by aphids in northeastern Algeria (Skikda). The 'Thomson Navel' orange was more infested than 'Carval Hal' mandarin. *Toxoptera citricida*, which is an effective vector of tristeza virus, was not identified; but four among the six aphid species identified are reported to transmit this quarantine virus. Comparing same periods of the two years of study, the degree of infestation changed. Thus, more concentration should be given to orange trees during all seasons, especially in spring and autumn, for best controlling aphid pests. On the other hand, a reduced level of parasitoids and predators were remarked, that cannot limit the proliferation of aphids. Therefore, new adapted auxiliaries should be researched and used, to contribute to the increasing of citrus production.

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Changes in the essential oil content and terpene composition of rosemary (*Rosmarinus officinalis* L.) by using plant biostimulants

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ABSTRACT

Plant biostimulants can stimulate the increase of growth, metabolism and the biosynthesis of metabolites in plants. This study investigated the changes of rosemary essential oil and its components composition under use of biostimulants for the possible reduction in use of chemical fertilizers. Treatments included biostimulants based on amino acids in four formulations, Aminolforte, Kadostim, Humiforte, and Fosnutren (each of them at 0.75 and 1.5 L ha^{-1}), and application of N.P.K fertilizer as a control treatment (by applied complete fertilizer at 100 kg per hectar with proportion of 15:8:15 percentage of N:P:K in the fertilizer). Results showed that the essential oil content and its components were significantly affected by biostimulants application. The maximum content of essential oil was obtained at 1.5 L ha^{-1} Humiforte and both concentrations of Aminolforte. While, the highest content of α-pinene, 1,8-cineole, and camphor as major components of rosemary essential oil were obtained at 1.5 L ha^{-1} Fosnutren. In addition, the maximum content of linalool, *z*-pinocamphone, bornyl acetate, and caryophyllene oxide were observed at 1.5 L ha^{-1} Fosnutren. Although, the highest content of myrcene and verbenone was obtained in the treatment with N.P.K fertilizer, but the maximum contents of β-pinene, camphene, borneol, and α-terpineol were related to the both concentrations of Aminolforte. We can conclude that biostimulants based on amino acids can be an effective alternative in reducing the use of chemical fertilizer and increasing the quantity and quality of rosemary essential oil.

Key words: amino acids, biostimulants, essential oil, monoterpenes hydrocarbon, oxygenated monoterpenes, *Rosmarinus officinalis* L.

IZVLEČEK

SPREMEMBE V VSEBNOSTI ETERIČNIH OLJ IN SESTAVI TERPENOV V ROŽMARINU (*Rosmarinus officinalis* L.) PO UPORABI RASTLINSKIH BIOSTIMULATORJEV

Rastlinski biostimulatorji lahko pospešijo rast, presnovo in biosintezo metabolitov v rastlinah. V raziskavi smo preučevali spremembe v sestavi eteričnih olj rožmarina po uporabi biostimulatorjev, ki so bili uporabljeni kot možnost zmanjšanja rabe mineralnih gnojil. Obravnavanja so obsegala biostimulatorje na osnovi aminokislin v štirih komercialnih pripravkih: Aminolforte, Kadostim, Humiforte in Fosnutren (vsakega od njih 0.75 in 1.5 L ha^{-1}). Kontrola je bila uporaba N.P.K gnojila (100 kg ha^{-1}, 15:8:15 N:P:K). Rezultati so pokazali, da je uporaba biostimulatorjev značilno vplivala na vsebnost eteričnih olj in njihovo sestavo. Največja vsebnost eteričnih olj je bila dosežena pri uporabi biostimulatorjev Humiforte in Aminolforte v koncentraciji 1.5 L ha^{-1}. Največja vsebnost α-pinena, 1,8-cineola in kamfora, kot glavnih sestavin eteričnega olja rožmarina, je bila dosežena pri uporabi biostimulatorja Fosnutren, v koncentraciji 1.5 L ha^{-1}. Tudi največja vsebnost linaloola, *z*-pinokamfona, bornil acetata in kariofilen oksida je bila dobljena pri isti koncentraciji biostimulatorja Fosnutren. Največja vsebnost mircena in verbenona je bila dobljena pri obravnavanju z N.P.K gnojili, toda največja vsebnost β-pinena, kamfena, borneola in α-terpineola je bila dobljena pri uporabi obeh koncentracij biostimulatorja Aminolforte. Zaključimo lahko, da so lahko biostimulatorji na osnovi amino kislin učinkovita alternativa pri zmanjšanju rabe mineralnih gnojil za povečanje količine in kakovosti rožmarinovega eteričnega olja.

Ključne besede: amino kisline, biostimulatorji, eterična olja, monoterpenski ogljikovodiki, oksigenirani monoterpeni, *Rosmarinus officinalis* L.

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1 INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is an evergreen branched, aromatic and medicinal plant that belongs to the Labiatae family. Rosemary has white-blue flowers and dark green small leaves. It grows along the north and south coasts of the Mediterranean sea and in the sub-Himalayan areas as wild type (AI-Sereiti et al., 1999). Rosemary essential oil is an almost colorless to pale yellow liquid with a characteristic and pleasant odor. Major components characterized for the essential oil are α-pinene, 1,8-cineole and camphor (Boutekedjiret et al., 2003). A systematic study identified about 38 compounds from the rosemary essential oil using gas chromatographymass spectrometry (GC/MS), and antimicrobial properties of the essential oil (mixture) were evaluated against 19 microbial strains (Bozin et al., 2007). Another comprehensive study identified 22 antioxidant compounds from 24 commercial rosemary extracts using high-pressure liquid chromatography with UV detector (HPLC-UV/MS), including polyphenolic acids such as vanillic acid, caffeic acid, and rosmarinic acid; phenolic diterpenes such as carnosic acid, carnosol, and rosmadial; and flavonoids such as genkwanin and cirsimaritin (Cuvelier et al., 1996).

Plant biostimulants include diverse substances and microorganisms that enhance plant growth. On the basis of the European Biostimulants Industry Council (EBIC), the following definition is proposed and each of its elements is justified: "Plant biostimulants are substances and materials, with the exception of nutrients and pesticides, which, when applied to plant, seeds or growing substrates in specific formulations, have the capacity to modify physiological processes of plants in a way that provides potential benefits to growth, development and/or stress response" (Du Jardin, 2012). In general, biostimulants are substances that stimulate plant metabolism and metabolic processes in order to improve plant growth (Starck, 2005). The base structure of biostimulants consists the mixture of various amino acids or their mixed with nutrition, hydrolyzed proteins, humic acid, seaweed extract and other ingredients (Gawronska et al., 2008; Thomas et al., 2009).

Some studies showed that primary metabolites such as sugars, amino acids, and carboxylic acids have important biological effects on plant physiology and their application on plants may change the secondary metabolite composition indirectly (Drew and Damon, 1977). Amino acids are involved in the synthesis of other organic compounds, such as proteins, amines, alkaloids, vitamins, enzymes, terpenoids (Ibrahim et al., 2010). Amino acids are crucial for stimulating cell growth, act as buffers, provide a source of carbon and energy and protect the cells from ammonia toxicity, with amid formation (Abdel Aziz et al., 2010).

Amino acids use can stimulate plant performance (Abdel-Mawgoud et al., 2011). Many studies have reported that foliar application of amino acids caused an increase in the growth and development of plants (Haj Seyed Hadi, 2010; Fawzy et al., 2012a; Fawzy et al., 2012b). Neeraja et al., (2005) found that amino acid treatment increased the number of flowers, fruit setting and fruit yield in tomato. Besides facts mentioned above, amino acids can be mixed with nutrients, protein hydrolyzate, triacontanol, humic acid, seaweed extract and brassinolides and they are formulated as a plant biostimulants. A study found that indicators of physiological, biochemical and yield parameters in tea bushes were improved by spraying with Humiforte, Aminolforte, Kadostim, and Fosnutren as biostimulants (base formulation of this biostimulants is consisted from different amino acids which were combined with organic matters) (Thomas et al., 2009). Kadostim caused the increase of germination, root formation, accelerated the formation of leaves and reduced the effects of stress (Anonymous, 2014).

Although plant biostimulants based on amino acids can influence the phytochemistry of plants, there is no research work done on the effect of plant biostimulants on the quantity and quality of rosemary essential oil in field. Therefore, this study aimed to evaluate the changes of terpenes content in rosemary essential oil by using plant biostimulants as a possibility to reduce the use of chemical fertilizers.

2 MATERIALS AND METHODS

2.1 Location and treatments

This study was performed at the experimental farm of Medicinal Plants Institute (MPI) of Academic Center for Education, Culture & Research (ACECR) located in Karaj region during 2013-2014 based on a randomized complete block design (RCBD) with 9 treatments and 3 replications. The geographical location of the station was 35° 54′ 17″ N and 50° 53′ 7″ E with about 1461 m (elevation) altitude above the mean sea level. The soil was loam-silt with 0.071 % N, 48.9 mg kg^{-1} phosphorous, 33.6 mg kg^{-1} potassium, EC 2.71 dS m^{-1}, and pH 8.3. Each experimental plot was 4 m long and 2.5 m wide, prepared after tillage operations. There was 1 m space between the plots and 3 meters between replications. Rosemary cuttings were planted in plots with a density of 20,000 plants per hectare. Other agronomic practices such as irrigation, pest and weed management were regularly designed according to the rosemary plant needs.

Treatments included plant biostimulants with four formulations of Aminolforte, Kadostim, Humiforte, and Fosnutren (each of them at 0.75 and 1.5 L ha^{-1}, respectivily), and application of N.P.K fertilizer as a control treatment (by application of complete fertilizer at 100 kg per hectar with proportion of 15:8:15 percentage of N:P:K in the fertilizer). The commercial formulations of biostimulants based on amino acids were supplied by Inagrosa Industries Agro Biological, Madrid of Spain (Anonymous, 2014). The details of the formulations are shown in Table 1. All treatments involved spraying in 4 times with 15 days intervals. The first spraying was 60 days after planting. To increase the absorption of solutions by plants, the foliar application of bio-stimulants was done in conditions without wind and rain and before sunrise when plant stomata are open.

Table 1: The formulation of biostimulants used in the experimental treatments

Biostimulants*	Formulation of compounds**
Aminolforte	3750 mg L^{-1} free amino acids, 2 % organic components, and 1.1 % total N (0.8 % urea N, and 0.3 % organic N)
Kadostim	3750 mg L^{-1} free amino acids, 2 % organic components, and 4.2 % total N (0.8 % ammonia N, 3.1 % nitric N, and 0.3 % organic N), and 6 % potassium (K_2O)
Humiforte	3750 mg L^{-1} free amino acids, 2 % organic components, and 6 % total N (1.4 % ammonia N, 3.7 % urea N, 0.5 % nitric N, 0.3 % and organic N), 5 % potassium (K_2O), and 3 % phosphorous (P_2O_5)
Fosnutren	3750 mg L^{-1} free amino acids, 2 % organic components, and 3.8 % total N (2.1 % ammonia N, 1.4 % nitric N, and 0.3 % organic N), and 6 % phosphorous (P_2O_5)

* Biostimulants supplied by Inagrosa Industries Agro Biological are compatible with the climate of Iran.
** Quantity and kind of free amino acids applied in the formulation of biostimulants in this experiment based on the percent of total amino acids are as follows: 11.2 % glycine, 5.1 % valine, 8.3 % proline, 13.2 % alanine, 4.4 % aspartic acid, 8.3 % arginine, 0.9 % glutamic acid, 5.1 % lysine, 16.4 % leucine, 4.4 % isoleucine, 5.1 % phenylalanine, 4.2 % methionine, 3.9 % serine, 0.3 % treonine, 0.3 % histidine, 1.5 % tyrosine, 0.9 % glutamine, 0.3 % cysteine, 0.4 % asparagine, and 0.4 % tryptophan.

2.2 Essential oils analysis

The aerial parts of the plants were harvested at the beginning of flowering stage. The harvested materials were air-dried in a shaded place at a convenient temperature and in an air-flow during 5 days. Samples were transferred to phytochemical analysis laboratory, for determine the percentage of essential oils according to the British pharmacopoeia method. Essential oils of the aerial parts were extracted by hydro-distillation method for 3 h, using Clevenger-type apparatus. The oils were dried over anhydrous sodium sulphate and kept on 4 °C until it was analyzed (Great Britain medicines commission, 1988).

The extracted essential oils were identified by gas chromatography (GC) and gas chromatographymass spectrometry (GC/MS) analysis. GC/MS analysis was carried out on an Agilent Instrument coupled with a 5973 Mass System equipped with flame ionization detector (FID) and a HP-5 capillary column (30 m × 0.25 mm; 0.25 µm film thicknesses). Temperature program included: oven temperature held for 3 minutes at 60 ºC and was enhanced to 220 ºC with 5 ºC per min rate. Then, enhancement of temperature was programmed up to 300 ºC with 15 ºC per min rate and this temperature held for 3 minutes. Other operating conditions included: carrier gas was *He* with a flow rate of 1 mL min^{-1}; injector and detector temperatures were 250 ºC, and split ratio, 1:50. Mass spectra were taken at 70 eV (Socaci et al., 2008). The components of the essential oils were identified by comparison of their mass spectra and retention indices with those published in the literature and presented in the MS computer library (Adams, 2007; Swigar and Silverstein, 1981). Each extraction was replicated three times and the compound percentages are the means of the three replicates.

2.3 Statistical analysis

All data were subjected to the statistical analysis (one-way ANOVA) based on a randomized complete block design (RCBD) with nine treatments and three replications by using the program of SAS software. The probabilities of significance were used to test for significance among treatments. The difference among treatments means were compared by Duncan's multiple range test at 5 % confidence interval. The obtained values were expressed as mean ± SD (standard deviation) from three replications (n = 3) of each treatment.

3 RESULTS AND DISCUSSION

Analysis of variance showed that the use of biostimulants had a significant ($p \leq 0.01$) effect on the essential oil content, *α*-pinene, camphene, *β*-pinene, myrcene, *p*-cymene, limonene, 1,8-cineole, linalool, camphor, borneol, pinocamphone, *α*-terpineol, bornyl acetate, caryophyllene and caryophyllene oxide contents. They had also a significant ($p \leq 0.05$) effect on the verbenone content. Application of various biostimulants had a significant ($p \leq 0.01$) effect on the aggregation of monoterpene hydrocarbon (MH) and oxygenated monoterpene (OM) (Table 2). Overall, all measured traits of this study have been significantly affected by the concentrations of biostimulants based on amino acids.

Mean comparison of data revealed that the content of rosemary essential oil was increased by the application of 1.5 L ha^{-1} Humiforte (1.4 %), and both concentrations of Aminolforte (1.3 %) compared to N.P.K treatment (1 %). Of course, there were no significant differences between the other treatments of biostimulants with N.P.K treatment. The highest content of *α*-pinene was obtained in the treatment of 1.5 L ha^{-1} Kadostim (27.37 %). While, the lowest content of it was showed in the treatment of 1.5 L ha^{-1} Fosnutren (21.74 %). Increasing the concentrations of Kadostim and Aminolforte caused to the improvement of *α*-pinene content. But, increasing the concentration of Fosnutren led to the decreasing of *α*-pinene content (Table 3).

Among the different biostimulants, the maximum content of camphene and *β*-pinene was caused by application of the Aminolforte treatment at 1.5 L ha^{-1} (5.93 and 1.78 %, respectively), but the minimum content of camphene and *β*-pinene was achieved when the Aminolforte treatment was applied at 0.75 L ha^{-1} (4.99 and 1.15 %, respectively). In contrast with N.P.K treatment, reduction of the myrcene content was observed with the use of various biostimulants. It is interesting that the increasing of Aminolforte and Kadostim concentrations had not significant effect on myrcene content, but its content by the increasing of Fosnutren and Humiforte concentrations was enhanced. The greatest and lowest content of *p*-cymene was detected at 1.5 L ha^{-1} Kadostim (1.33 %) and 0.75 L ha^{-1} Aminolforte (1.01 %), respectively (Table 3). In comparison with N.P.K treatment, using of the Aminolforte at 0.75 L ha^{-1} decreased the *p*-cymene content up to 15 percent, whiles the applied treatment of Aminolforte at 1.5 L ha^{-1} had not significant difference with N.P.K treatment. Also,

there was no significant difference between N.P.K treatment and Kadostim, Fosnutren, and Humiforte at 0.75 L ha^{-1} for the content of *p*-cymene. Moreover, increasing the concentration of Humiforte and Fosnutren caused to decreasing the content of *p*-cymene up to 6.7 and 13 percent, respectively. The content of *p*-cymene was also increased up to 11 percent at high concentration of Kadostim. The maximum content of limonene was obtained with the application of Kadostim at 1.5 L ha^{-1} which had not significant difference with N.P.K treatment. The lowest content of limonene was acquired at 1.5 L ha^{-1} Fosnutren. The content of limonene was increased with increasing the concentrations of various biostimulants, except Fosnutren. The application of Fosnutren at 1.5 L ha^{-1} improved the content of 1,8-cineol to 23 %. In contrast, it was reduced to 11 percent when used at 0.75 L ha^{-1} Aminolforte. The effect of other biostimulants application on 1,8-cineol content was similar to the treatment of N.P.K. The content of linalool increased with increasing the Fosnutren concentration, but it was decreased with increasing the Humiforte and Aminolforte concentration to 1.5 L ha^{-1}. Therefore, the highest content of linalool was related to application of Aminolforte and Fosnutren at 1.5 L ha^{-1} and the lowest content of it was recorded in the treatment of N.P.K and various concentrations of Kadostim biostimulants (Table 3). Among different biostimulants applied for rosemary, Fosnutren at 1.5 L ha^{-1} led to the high content of camphor that it was 33 percentages more than N.P.K treatments. Although, the camphor content in comparison with biostimulants had not a significant difference with the N.P.K. treatment. Increasing the concentration of each of the biostimulants had not a significant effect on the borneol content except the Aminolforte application. Thus, the highest and the lowest content of borneol was found in the concentration of Aminolforte at 0.75 L ha^{-1} (7.97 %) and 1.5 L ha^{-1} (5.5 %), respectively. The content of pinocamphone in each of the Aminolforte and Humiforte concentrations was alike to N.P.K treatment, whiles, the highest content of pinocamphone was obtained at 1.5 L ha^{-1} Fosnutren (1.46 %), and the lowest content of it was studied at 0.75 L ha^{-1} Fosnutren (1.21 %) and at 1.5 L ha^{-1} Kadostim (1.2 %). The content of α-terpineol was highest at 0.75 L ha^{-1} Aminolforte (2.01 %) and it was the lowest at 1.5 L ha^{-1} Kadostim (0.3 %). Though, the other biostimulants had not significant difference with N.P.K treatment. The maximum verbenone content was obtained with the application of N.P.K treatment (9.2 %), and the means of other biostimulants were less than N.P.K treatment. Therefore, the lowest content of verbenone was related at 1.5 L ha^{-1} of Kadostim (7.41 %) (Table 3). By the application of 1.5 L ha^{-1} Fosnutren, the content of bornyl acetate was reached to the maximum own value. The lowest content (4.09 %) of bornyl acetate was observed at 1.5 L ha^{-1} Kadostim that had not significant difference with N.P.K treatment (4.36 %). The highest content (1.94 %) of caryophyllene was observed at low concentration (0.75 L ha^{-1}) of Aminolforte, despite the fact that the lowest content (0.74 %) of caryophyllene was obtained at high concentration (1.5 L ha^{-1}) of Fosnutren. There was not significant effect on caryophyllene content when the concentrations of Kadostim and Fosnutren were increased, whereas the caryophyllene content was reduced with the enhancement of Aminolforte and Fosnutren concentration. The use of Fosnutren and Kadostim at 0.75 L ha^{-1} directed to the highest content of caryophyllene oxide, although, the lowest content was observed in N.P.K treatment (Table 3).

Table 2: Analysis of variance (ANOVA) for the biostimulants effect on essential oil components of rosemary

Source of variance (S.O.V)	D.f.	Mean squares										
		Essential oil	MH	MO	*α*-pinene	camphene	*β*-pinene	myrcene	*p*-cymene	limonene	1,8-cineole	linalool
Replication	2	0.054 ns	0.47 ^{ns}	5.39 **	0.001 ^{ns}	0.011 ^{ns}	0.016 ^{ns}	0.12 **	0.002 ^{ns}	0.086 ^{ns}	0.018 ^{ns}	0.073 **
Biostimulants	8	0.083 **	13.4 **	7.38 **	9.720 **	0.224 **	0.096 **	0.289 **	0.031 **	0.568 **	1.510 **	0.179 **
Erorr	16	0.019	0.98	0.41	0.846	0.018	0.006	0.009	0.002	0.037	0.055	0.006
CV	-	12.03	2.24	2.02	3.69	2.37	5.25	2.59	4.09	5.93	3.16	2.51

MH; Monoterpene Hydrocarbons, MO; Oxygenated Monoterpenes , and ns, * and **; showed non-significant and significant at the 5% and 1% probability, respectively.

Table 2: Continued.

Source of variance (S.O.V)	D.f.	Mean squares							
		camphor	borneol	pinocamphone	*α*-terpineol	verbenone	bornyl acetate	caryophyllene	caryophyllene oxide
Replication	2	0.458 *	0.423 *	0.001 ns	0.04 **	0.423 **	0.29 *	0.004 ns	0.011 *
Biostimulants	8	1.84 **	1.47 **	0.02 **	0.73 **	0.93 *	1.05 **	0.341 **	0.043 **
Erorr	16	0.096	0.070	0.002	0.006	0.024	0.051	0.018	0.002
CV	-	3.94	4.10	3.04	4.76	1.86	4.65	9.87	4.59

ns, * and **; showed non-significant and significant at the 5% and 1% probability, respectively.

Table 3: Means comparison* based on percentages for the biostimulants effect on essential oil components of rosemary

Biostimulants	Essential oil	*α*-pinene	camphene	*β*-pinene	myrcene	*p*-cymene	limonene	1,8-cineole	linalool
0.75 L ha^{-1} Aminolforte	1.3 ^{ab} ± 0.1	22.76 ^{de} ± 1.1	4.99 ^{d} ± 0.32	1.15 ^{e} ± 0.16	3.54 ^{de} ± 0.09	1.01 ^{e} ± 0.08	3.1 ^{bc} ± 0.065	6.66 ^{c} ± 0.4	3.37 ^{a} ± 0.16
1.5 L ha^{-1} Aminolforte	1.3 ^{ab} ± 0.2	26.56 ^{ab} ± 0.8	5.93 ^{a} ± 0.15	1.78 ^{a} ± 0.15	3.43 ^{e} ± 0.14	1.22 ^{b} ± 0.02	3.47 ^{ab} ± 0.12	7.16 ^{b} ± 0.15	2.63 ^{d} ± 0.21
0.75 L ha^{-1} Fosnutren	1 ^{c} ± 0.1	25.64 ^{abc} ± 0.34	5.7 ^{abc} ± 0.03	1.34 ^{d} ± 0.06	3.6 ^{de} ± 0.06	1.21 ^{b} ± 0.02	3 ^{c} ± 0.11	7.48 ^{b} ± 0.01	3.05 ^{bc} ± 0
1.5 L ha^{-1} Fosnutren	1.1 ^{bc} ± 0.2	21.74 ^{e} ± 1.61	5.72 ^{abc} ± 0.08	1.45 ^{bcd} ± 0.01	3.22 ^{f} ± 0.25	1.04 ^{de} ± 0.07	2.19 ^{d} ± 0.52	9.22 ^{a} ± 0.48	3.43 ^{a} ± 0.19
0.75 L ha^{-1} Humiforte	1.1 ^{bc} ± 0.2	24.11 ^{cd} ± 0.42	5.64 ^{bc} ± 0	1.38 ^{d} ± 0.045	4.02 ^{b} ± 0.15	1.24 ^{b} ± 0.03	3.43 ^{ab} ± 0.1	7.41 ^{b} ± 0.02	3.17 ^{b} ± 0.06
1.5 L ha^{-1} Humiforte	1.4 ^{a} ± 0.1	25.68 ^{abc} ± 0.36	5.87 ^{ab} ± 0.12	1.57 ^{bc} ± 0.05	3.62 ^{d} ± 0.05	1.12 ^{cd} ± 0.03	3.39 ^{ab} ± 0.08	7.28 ^{b} ± 0.09	3.01 ^{c} ± 0.02
0.75 L ha^{-1} Kadostim	1.1 ^{bc} ± 0.2	24.97 ^{bc} ± 0.01	5.66 ^{bc} ± 0.01	1.59 ^{b} ± 0.06	3.83 ^{c} ± 0.06	1.21 ^{b} ± 0.02	3.39 ^{ab} ± 0.08	7.08 ^{b} ± 0.19	2.97 ^{c} ± 0.04
1.5 L ha^{-1} Kadostim	0.9 ^{c} ±0.1	27.37 ^{a} ± 1.32	5.73 ^{abc} ± 0.05	1.43 ^{cd} ± 0.02	3.96 ^{bc} ± 0.12	1.33 ^{a} ± 0.08	3.63 ^{a} ± 0.2	7.33 ^{b} ± 0.06	2.93 ^{c} ± 0.06
N.P.K treatment	1 ^{c} ± 0.1	25.76 ^{abc} ± 0.4	5.5 ^{c} ± 0.07	1.54 ^{bc} ± 0.035	4.19 ^{a} ± 0.24	1.2 ^{bc} ± 0.01	3.47 ^{ab} ± 0.12	7.5 ^{b} ± 0.02	2.92 ^{c} ± 0.07

*Means with the same letters in each column indicate no significant difference between treatments according to Duncan's multiple range test at the 5 % level of probability. The obtained values were expressed as mean ± SD (standard deviation) from three replications (n = 3).

Table 3: Continued.

Biostimulants	camphor	borneol	pinocamphone	*α*-terpineol	verbenone	bornyl acetate	caryophyllene	caryophyllene oxide
0.75 L ha^{-1} Aminolforte	7.79 ^{b} ± 0.04	7.97 ^{a} ± 0.76	1.37 ^{b} ± 0.04	2.01 ^{a} ± 0.22	7.86 ^{d} ± 0.23	4.84 ^{bc} ± 0.003	1.94 ^{a} ± 0.3	1.06 ^{bc} ± 0.017
1.5 L ha^{-1} Aminolforte	7.35 ^{b} ± 0.26	5.5 ^{e} ± 0.47	1.32 ^{bc} ± 0.01	1.55 ^{d} ± 0.01	8.46 ^{bc} ± 0.075	4.84 ^{bc} ± 0.003	1.63 ^{b} ± 0.14	0.98 ^{cd} ± 0.023
0.75 L ha^{-1} Fosnutren	7.86 ^{b} ± 0.01	6.47 ^{bcd} ± 0.01	1.21 ^{d} ± 0.04	1.69 ^{bc} ± 0.06	8.74 ^{b} ± 0.22	4.5 ^{cd} ± 0.167	1.39 ^{bc} ± 0.02	1.19 ^{a} ± 0.082
1.5 L ha^{-1} Fosnutren	9.87 ^{a} ± 1	6.86 ^{b} ± 0.21	1.46 ^{a} ± 0.08	1.64 ^{cd} ± 0.04	8.53 ^{bc} ± 0.11	6.09 ^{a} ± 0.628	0.74 ^{e} ± 0.1	1.14 ^{ab} ± 0.057
0.75 L ha^{-1} Humiforte	7.91 ^{b} ± 0.02	6.71 ^{bc} ± 0.13	1.27 ^{cd} ± 0.01	1.81 ^{b} ± 0.12	8.48 ^{bc} ± 0.085	4.47 ^{cd} ± 0.182	1.08 ^{d} ± 0.13	0.92 ^{de} ± 0.053
1.5 L ha^{-1} Humiforte	7.86 ^{b} ± 0.01	6.3 ^{cd} ± 0.07	1.25 ^{cd} ± 0.02	1.71 ^{bc} ± 0.07	7.71 ^{d} ± 0.3	5.2 ^{b} ± 0.183	1.22 ^{cd} ± 0.06	0.96 ^{d} ± 0.033
0.75 L ha^{-1} Kadostim	7.42 ^{b} ± 0.23	6.25 ^{cd} ± 0.1	1.26 ^{cd} ± 0.02	1.73 ^{bc} ± 0.08	8.4 ^{c} ± 0.045	5.11 ^{b} ± 0.138	1.28 ^{cd} ± 0.03	1.17 ^{a} ± 0.072
1.5 L ha^{-1} Kadostim	7.43 ^{b} ± 0.22	6.01 ^{d} ± 0.22	1.2 ^{d} ± 0.05	0.3 ^{e} ± 0.04	7.41 ^{e} ± 0.45	4.09 ^{d} ± 0.372	1.47 ^{bc} ± 0.06	0.97 ^{d} ± 0.028
N.P.K treatment	7.4 ^{b} ± 0.24	5.97 ^{d} ± 0.24	1.32 ^{bc} ± 0.01	1.68 ^{bcd} ± 0.06	9.2 ^{a} ± 0.44	4.36 ^{d} ± 0.237	1.34 ^{c} ± 0	0.85 ^{e} ± 0.088

*Means with the same letters in each column indicate no significant difference between treatments according to Duncan's multiple range test at the 5% level of probability. The obtained values were expressed as mean ± SD (standard deviation) from three replications (n = 3).

With regard to the compound groups of essential oil, the maximum content of monoterpene hydrocarbons (MH) was obtained at 0.75 L ha^{-1} Kadostim (47.15 %) that it was identical with the monoterpene hydrocarbon content in N.P.K treatment (45.69 %) and Aminolforte at 1.5 L ha^{-1} (46.08 %). The lowest content of monoterpene hydrocarbons was observed at 1.5 L ha^{-1} Fosnutren (42.39 %) which it had no significant difference with Humiforte at 0.75 L ha^{-1} (43.8 %). Accumulation of monoterpene hydrocarbons was observed by increasing the Aminolforte and Kadostim concentration. However, increasing the Fosnutren concentration caused to reduce the accumulation of monoterpene hydrocarbon (Figure 1). In contrast, the content of oxygenated monoterpenes (OM) was reduced by increasing the concentration of Aminolforte, Humiforte and Kadostim. But the accumulation of oxygenated monoterpenes enhanced up by increasing the concentration of Fosnutren. Therefor, the greatest content of oxygenated monoterpenes was found at 1.5, and 0.75 L ha^{-1} for Fosnutren (33.98 %), and Aminolforte (33.47 %), respectivily. Also, the lowest content of oxygenated monoterpenes was attained at 1.5 L ha^{-1} Kadostim (28.91 %) (Figure 2).

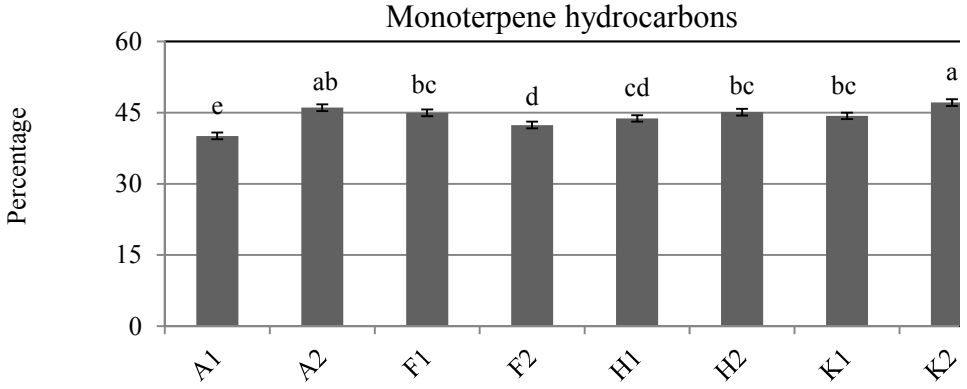

Figure 1: The effect of various biostimulants on monoterpenes hydrocarbon (MH %). Means with the same letter are not significantly different according Duncan's multiple range test ($p \leq 0.05$). Error bars represent standard errors (n = 3). A1: 0.75 L ha^{-1} Aminolforte, A2: 1.5 L ha^{-1} Aminolforte, F1: 0.75 L ha^{-1} Fosnutren, F2: 1.5 L ha^{-1} Fosnutren; H1: 0.75 L ha^{-1} Humiforte, H2: 1.5 L ha^{-1} Humiforte, K1: 0.75 L ha^{-1} Kadostim, K2: 1.5 L ha^{-1} Kadostim, and C: N.P.K treatment.

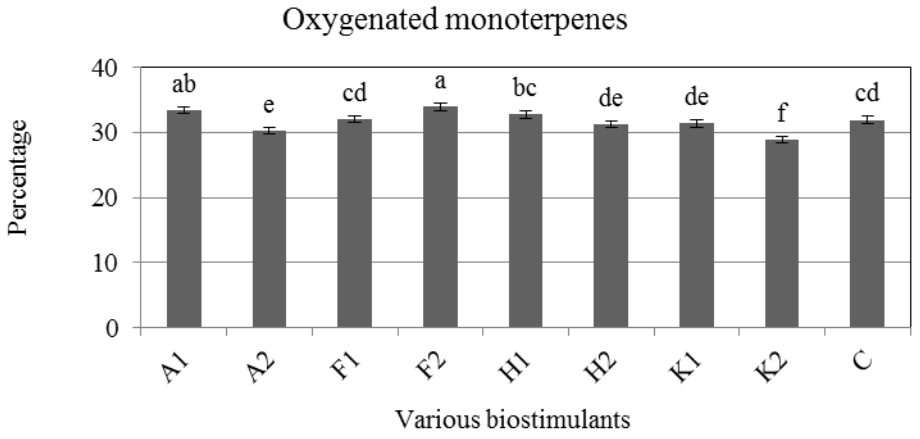

Figure 2: The effect of various biostimulants on oxygenated monoterpenes (OM %). Means with the same letter are not significantly different according Duncan's multiple range test ($p \leq 0.05$). Error bars represent standard errors (n = 3). A1: 0.75 L ha^{-1} Aminolforte, A2: 1.5 L ha^{-1} Aminolforte, F1: 0.75 L ha^{-1} Fosnutren, F2: 1.5 L ha^{-1} Fosnutren; H1: 0.75 L ha^{-1} Humiforte, H2: 1.5 L ha^{-1} Humiforte, K1: 0.75 L ha^{-1} Kadostim, K2: 1.5 L ha^{-1} Kadostim, and C: N.P.K treatment.

The results demonstrated that the application of various biostimulants increased the most components of rosemary essential oil in relationship to N.P.K treatment. The comparison of chemical composition in the essential oil of rosemary showed the significant differences in important components. The highest content of *α*-pinene, 1,8-cineole and camphor as major components of rosemary essential oil were obtained at 1.5 L ha^{-1} Fosnutren. In addition, the maximum content of linalool, *z*-pinocamphone, bornyl acetate and caryophyllene oxide were observed at 1.5 L ha^{-1} Fosnutren. Although the maximum content of myrcene and verbenone were acquired in N.P.K treatment. However, the maximum content of *β*-pinene, camphene, borneol, and *α*-terpineol was related to the both concentrations of Aminolforte.

This research reported that the quantity and quality of rosemary essential oil was changed by application of the plant biostimulants based on amino acids. These biostimulants which had various amino acids and other organic matters affected essential oil components of rosemary plant. Another study confirmed this finding (Neeraja et al., 2005; Toosi et al., 2014). Some authors reported that increased the soybean oil content was obtained by spraying with Kadostim (Toosi et al., 2014). Also, polyphenols and amino acid content in the tea plant by using of Aminolforte as bio-stimulant was increased (Thomas et al., 2009). Mehrabi et al., (2013) reported that use of Aminolforte, Kadostim, and Fosnutren in savory cultivation (*Satureja hortensis* L.) could increase the essential oil content. Maini (2006) summarized the early literature showing enhanced activity of NAD-dependent glutamate dehydrogenase, nitrate reductase and malate dehydrogenase in maize following application of Siapton (protein hydrolyzed from animal epithelial tissues). Also, biostimulants may through improvement of plant nutrients uptake, induction of phytohormones biosynthesis, biotic and abiotic stress reduction and enhancing of enzymes activity related to tricarboxylic acid cycle (TCA cycle) and nitrogen metabolism caused increassing essential oil components of the rosemary plant (Calvo et al., 2014).

Amino acids have a major role in protein structure formation and their presence is necessary for the proper function of metabolic and biological processes. For example, the asparagine and glutamine connect the two important metabolic cycles of the plants, the carbon and nitrogen cycles, and they have an influence both on sugars and proteins. The glycine is an amino acid that inhibits the photorespiration done by C_3 plants (Taiz and Zeiger, 2010). Also, in plants the amino acids decomposed and intermediate metabolites formed and afterward, these metabolites will transform into glucose or are oxidized in the citric acid cycle (Miflin, 1993). Unlike sugars and fatty acids, the extra amino acids are not stored or disposed, but they are used as energy compounds (Thomas et al., 2009). In plants, amine group of the additional amino acids can be converted to urea and will be used as a nitrogen source. The backbone of amino acids can be transformed to seven molecules including pyruvate, acetyl-CoA, ketoglutarate, succinyl-CoA, fumarate, oxaloacetate or an intermediate metabolite of urea cycle (Miflin, 1993). According to several studies, the foliar application of amino acids causes an improvement in plant growth and fruit yield in cucumber (El-Shabasi et al., 2005) and garlic (Fawzy et al., 2012). On the other hand, amino acids have important role in the biosynthesis of secondary metabolite and phytohormones. The methionine is the ethylene precursor and the tryptophan is responsible for regulation of auxin biosynthesis. Also, the glutamic acid is important for the synthesis of the auxin (Taiz and Zeiger, 2010).

4 CONCLUSION

The use of plant biostimulants to improve plant phytochemistry is one of the goals of ecological and sustainable agriculture. The maximum content of essential oil was obtained at 1.5 L ha^{-1} Humiforte, and both concentrations of Aminolforte. While, the highest content of α-pinene, 1,8-cineole, and camphor as major components of rosemary essential oil were found

at 1.5 L ha^{-1} Fosnutren. In addition, the maximum content of linalool, *z*-pinocamphone, bornyl acetate, and caryophyllene oxide were observed at 1.5 L ha^{-1} of Fosnutren. Therefore, application of the various biostimulants could increase the major components of rosemary essential oil in comparison with N.P.K treatment. According to the results, the use of amino acids in combination with other nutrients based on the formulation of plant biostimulants can help to improve the phytochemical traits of essential oil in rosemary at different stages of plant development. In general, the plant biostimulants as environmentally friendly products can be the effective alternative to reducing the use of chemical fertilizer and increasing the quantity and quality of rosemary essential oil.

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Soil suitability evaluation for crop selection using fuzzy sets methodology

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ABSTRACT

In this study appraisal of four different agricultural land evaluation methods including the so-called Storie method, square root method, maximum limitation method and fuzzy sets method, was done. The study was performed in Bastam region, located in Semnan province at the north east of Iran. Three crops including tomato, wheat and potato were assessed for the purpose of this research. Soil characteristics assessed were rooting depth, $CaCo_3$, organic carbon content, clay content, pH and slope gradient. Statistical analyses were done at significance levels of $\alpha = 0.1$ and $\alpha = 0.05$. Results of regression between land indices, calculated through the four methods, with observed yields of the crops, showed that the regression were significant in fuzzy sets method for all of the assessed crops at $p = 0.05$ but not significant in maximum limitation method for any of the crops. The Storie and square root methods also showed a significant correlation with wheat yield at $p = 0.1$. This study was a demonstrative test of fuzzy sets theory in land suitability evaluation for agricultural uses, which revealed that this methodology is the most correct method in given circumstances.

Key words: fuzzy sets, land evaluation, land use, soil classification, crop growth conditions, soil suitability, crop selection

IZVLEČEK

UPORABA METODOLOGIJE MEHKIH MNOŽIC PRI OCENI PRIMERNOSTI TAL ZA RAZLIČNE POLJŠČINE

V raziskavi so bile ocenjene štiri metode vrednotenja kmetijskih zemljišč vključujoč Storijevo metodo, metodo kvadratnega korena, metodo maksimalne omejitve in metodo mehkih množic. Raziskava je potekala na območju Bastama, v provinci Semnan, v severovzhodnem delu Irana. Primernost zemljišč je bila ovrednotena za tri poljščine, paradižnik, pšenico in krompir. Ocenjene so bile naslednje lastnosti tal: globina koreninjenja, vsebnosti $CaCO_3$, organskega ogljika in gline, pH in naklon zemljišča. Statistične analize so bile narejene pri stopnji značilnosti $\alpha = 0,1$ in $\alpha = 0.05$. Rezultati regresijske analize za odvisnost pridelkov izbranih polščin od indeksov primernosti zemljišč, izračunanih po zgoraj omenjenih metodah je pokazala, da je statistično značilna samo odvisnost pridelka od indeksa primernosti zemljišč dobljenega z metodo mehkih množic" Storijeva metoda in metoda kvadratnega korena sta pokazali značilno odvisnost prideleka pšenice od indeksa primernosti zemljišč pri $\alpha = 0,1$. Raziskava je bila demonstracijski preskus primernosti uporabe metode mehkih množic pri vrednotenju zemljišč v kmetijski rabi. Ugotovljeno je bilo, da je bila ta metodologija v danih okoliščinah najprimernejša.

Ključne besede: mehke množice, vrednotenje zemljišč, raba tal, klasifikacija tal, razmere za rast poljščin, primernost tal, izbira poljščin

1 INTRODUCTION

Classification of rural lands with regard to their suitability for a specific crop production is important, since different land units have different advantages and limitations. To allocate each land unit for optimal and sustainable production of a crop, land use planning should be implemented in

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every area. A reasonable land use plan is obtained through a precise and confident land evaluation method. The land evaluation results should present a pragmatic description of land units capabilities and restrictions. As every land unit has its own restrictions and advantages and each crop has its own requirements for growth, a precise land evaluation method is required for rational decision making on agricultural land use management and allocation of crops to different land units.

The soundness of the evaluation method should be investigated before using its results for decision making on land utilization. There are several methods of land evaluation such as parametric methods, maximum limitation method and fuzzy sets method. There are two methods called Storie (Storie, 1976) and square root (Khiddir, 1986) that both can be subsumed under the group of parametric methods. The core idea of parametric methods is based on combining (multiplying) numerical rates of several factors (soil characteristics) to obtain a total land index/rate in a given land unit for a given crop. Here, the soil characteristics are given a rate between 0 to 100 depending on their influence on a considered land use, then, they are combined in a few different ways to obtain a total rate for a specific land unit for a given crop/land use. A land unit with a higher land index/rate is more suitable for optimal utilization for a certain crop. In maximum limitation method relations among different soil characteristics is ignored and no combination of different characteristics is consider, instead the lowest rate among characteristics rates is considered as an index for a land unit for a specific utilization type.

In all of these classical methods, land suitability rating (classification) is implemented based on discretely defined classes of suitability and variability of characteristics values and their influence on crop growth. This is called Boolean logic, which states that membership to a set is only expressed by member (1) or non-member (0) and there is no values or degrees between this two categories. For example, if rooting depth is over 150 cm (which is suitable for a crop growth) is given the value of 1 (member to the suitable class) and if it is below 100 cm (which is not optimal for a crop growth) is considered as non-member and would be declared as unsuitable. This logic does not consider relative suitability for other values out of these rigid limits. However, gradual and continuous variability in characteristics values and their influence on crop growth has been clearly observed in soils. Therefore, applying this approach causes losing some information when reflexing the values within or out of the rigid limits, since variability in soil qualities and characteristics is rather continuous or fuzzy and not discrete.

Fuzzy mathematics was first presented by Zadeh (1965). In the fuzzy sets theory, elements membership to a set is defined by a degree of membership and variabilities are considered continuously. This would take into account a range of values between 0 to 1 as well. This can reflect the real situation in the nature by presenting the relative values within the rigid borders defined in the Boolean logic makes our understanding and surveys closer to the true conditions in the real world.

Fuzzy sets methodology in land evaluation has raised a lot of interest among researchers, since this method gives a more realistic output in comparison with the Boolean approach (Burrough, 1989; Tang et al., 1991; Burrough et al., 1992; Van Ranst et al., 1996 and Nisar Ahmed et al., 2000). McBratney and Odeh (1997) discussed the application of fuzzy sets in soil science. Torbert et al. (2008) discussed fuzzy modelling for soil quality assessment. Some recent studies on land evaluation by fuzzy methodology have been done by Rodrigo et al. (2005), Vliet (2013), Elaalem (2013) and Chang and Ko (2014). Fuzzy logic approach has also been used in different areas of soil science through different techniques (Malczewski, 2006; Jian-Hua et al., 2009; Reshmidevi et al., 2009; Yue-Ju et al., 2010; Gruijter et al., 2011; Kong et al., 2011 and Liu et al., 2013). Kalogirou (2002) has criticized fuzzy sets methodology; He concluded that further research is needed to confirm the prominence of fuzzy methodology in comparison with Boolean methods for land evaluation. Holistically, it seems to be a sound and useful methodology. No other significant studies have been done on assessing the different methods of agricultural land suitability evaluation. Comparative studies are rare among the recent decade publications and a confirmative research on land suitability evaluation frameworks

with special stress on soil significance in crop cultivation conditions is missing. Although the fuzzy methodology has been used in land evaluation studies with different purposes and different points of view, but testing the accuracy of this methodology and its comparison with other frameworks is needed.

The present research explores to find out the best methodology for soil suitability evaluation by comparing results of different methods and then classify agricultural lands of Bastam area based on intrinsic soil chemical and physical characteristics. Three crops including tomato (*Solanum lycopersicum* L. (*Lycopersicum esculentum* Mill.), common (bread) wheat (*Triticum aestivum* L.) and potato (*Solanum tuberosum* L.), which have been cultivated in the study area, were studied to test the methodologies overly.

2 MATERIALS AND METHODS

2.1 Study area description

This study was performed in Bastam region in Semnan province located on the north east of Iran. The study sites were located between coordinates 54° 39′ to 55° 20′ of east longitude and 36° 26′ to 36° 45′ of north latitude. The altitude was about 1600 m above the sea level. The area surface was about 53500 ha. Slope gradient varied from flat to 8 %. The physiography of studied land units is comprised of Gravelly Alluvio-Colluvial Fans, Piedmont Plateaux and Alluvial Plains. According to the bioclimatic map of the region (FAO and UNESCO, 1988), the study area climate is attenuated sub-desert climate. Mean annual precipitation is 154 mm and mean annual temperature is 14.6 °C, according to the 55-year mean data of Shahrood meteorological station located in the study area. Dominant crops of the area are wheat, maize, barley and potato. Some parts of the region are used as pasture and some of them are under fallow or set aside lands.

2.2 Soil sampling and analysis

In total, 104 soil profiles were investigated and among those, eleven representative profiles were selected. Therefore, 11 representative land mapping units, taxonomically classified to the family level, were separated (Fig 1). The procedure of taxonomic land classification was according to soil taxonomy manual of the United States Department of Agriculture (USDA, 2010). This classification is based on field surveys including morphological descriptions of soil profiles like leaching evidences, soil horizons positions and their depth, and chemical and physical analysis such as electrical conductivity, organic carbon, exchangeable sodium percentage, cation exchange capacity, carbonate content, texture, structure, etc. The geological and topographical base maps and aerial photos were used for eliciting basic information of the geology and geography of the study region and for help in delineation of land units. Some yearly and monthly climatic data for a 50 year period between 1955-2005 including temperature and precipitation rates were also used in taxonomic land classification when determining the soil moisture and thermal regimes according to the USDA Soil Taxonomy (2010). The taxonomic classification revealed that aridisols and entisols are dominant soil orders in the region. Soil moisture regimes were aridic and torric, and thermal regime was mesic. Geographical position of land mapping units (soil families) of the study area is shown in Fig. 1 and the measured site characteristics are presented in Table 1.

Samples were gathered from each horizon of the representative soil profiles independently and the laboratorial analyses were performed for each horizon separately. At last, one value is reported for each parameter in each soil profile as obtained from mean calculations of all horizons of a profile according to the procedure presented in Sys et al. (1993) which considers higher significance of surface layers for crop growth. The chemical and physical analyses were performed according to internationally accepted methods in the literature such as Carter and Gregorich (2008).

2.3 Physical land suitability evaluation

The physical land evaluation is regarded as a specific case of land evaluation. It is the assessment of land characteristics with regard to possible utilization types, which is important for maintaining long-term productivity of lands

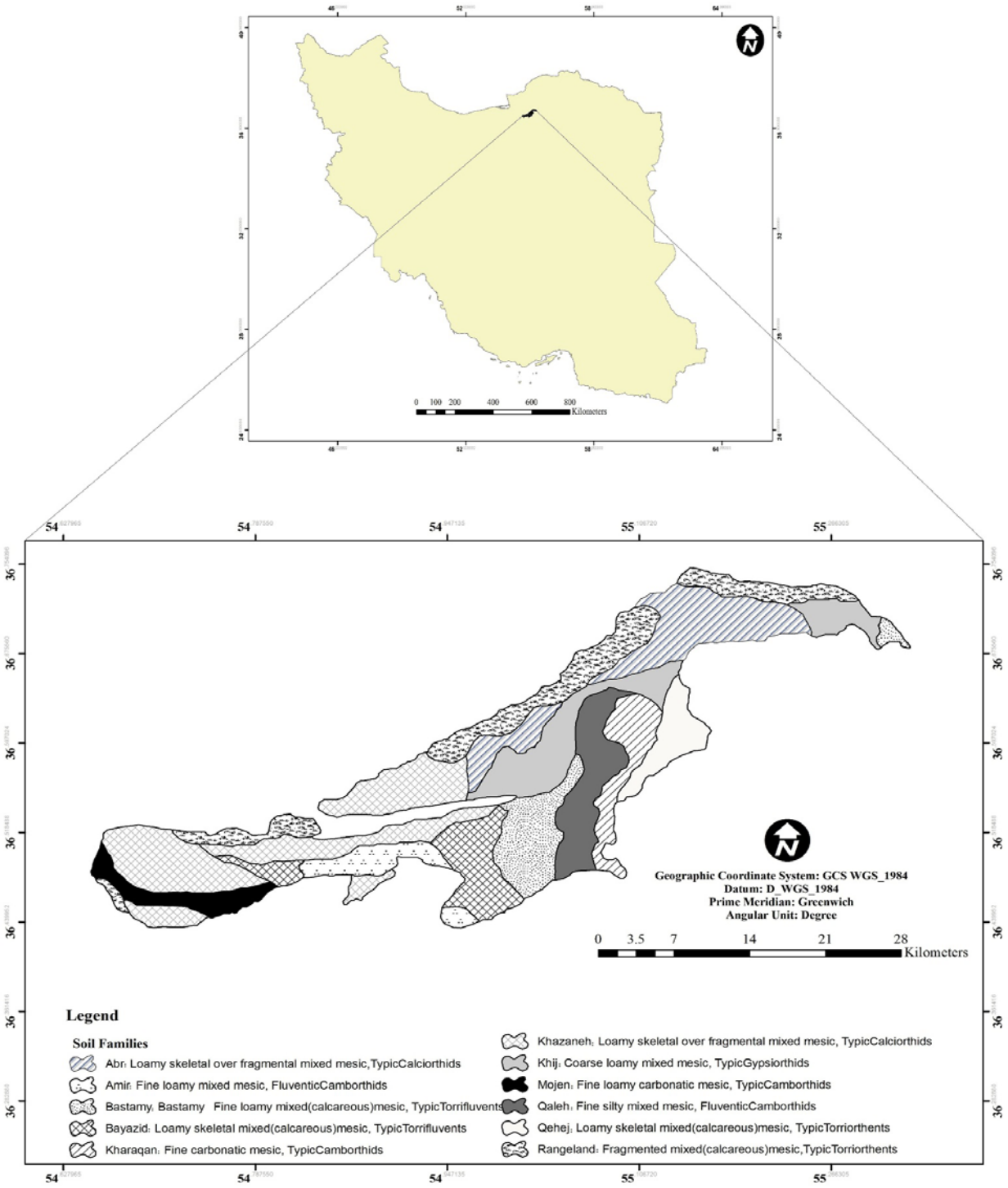
through optimal utilization (Sharififar et al., 2013). In agricultural land suitability assessments, each measured land characteristic value is compared with reference threshold limits of crop requirements in every land unit e.g. the reference tables presented by Sys et al. (1993). Then each characteristic is assigned a rate ranging from 0 to 100 through linear interpolation in reference intervals of each suitability class for each crop. Rates of the considered characteristics are then combined (through the four methods assessed in this research) to obtain a total rating, called land index, in every land unit for a given crop. The land index of a land unit is a score ranging from 0 to 100. A land unit is defined as an area of the soil surface, which has characteristics different from other areas and is separated from other areas with regard to soil taxonomic classification. In other words, a land unit is a taxonomically separated soil class and its bounds are determined according to the level of the soil taxonomic classification (Soil Survey Division Staff, 1993; USDA, 2010).

The characteristics considered for land suitability evaluation in this study are; rooting depth, soil clay content, $CaCO_3$ content, slope gradient of land units, pH and organic carbon content. These characteristics have significant influence on crop growth which have been confirmed by FAO (1976) and have been used and confirmed by other researchers such as Biox and Zinck (2008), Mendas and Delali (2012) and Sharififar et al. (2013). These characteristics have also been chosen for important soil suitability assessing and predictive crop yield models such as Almagra and Albero (De la Rosa et al, 1992, 2004, 2009; Shahbazi et al., 2008; Jafarzadeh et al., 2009; Shahbazi and Jafarzadeh, 2010; Sharififar, 2012). Some characteristics such as drainage class, salinity and climatic characteristics were not considered for the evaluation, since they have negligible differences among land units. The climatic parameters have not been considered for the evaluation, since they do not vary within land units significantly and their influences are approximately identical for all of the studied land units and therefor they are not applicable for land classification. All the studied crops are irrigated via underground water supplies. The criteria for land suitability evaluation have been discussed deeply by Messing et al. (2003). They have also confirmed the significant influence of some of the criteria used in this study for crop growth.

Table 1: Mean values of land characteristics of the study area

Land units	Clay content%[a)]	Carbonate content%	Rooting depth (cm)	pH	Slope%	Organic carbon%	Surface area (ha)
Abr	16.40	31.0	95	7.8	0-5	0.40	8125
Amir	29.75	23.7	145	7.9	0-2	0.33	1525
Bagh	5.00	5.5	22	7.9	5-8	0.24	7250
Bastamy	22.22	28.5	160	7.8	0-2	0.44	3975
Bayazid	7.11	14.7	95	8.0	2-5	0.38	3525
Kharaqan	42.40	55.8	125	7.8	0-2	0.60	3550
Khazaneh	18.00	24.0	40	7.9	5-8	0.35	9450
Khij	9.00	36.0	86	7.8	2-5	0.35	7225
Mojen	22.80	41.5	150	8.0	0-2	0.30	1725
Qaleh	36.18	30.2	150	8.0	0-2	0.80	4350
Qehej	11.00	20.0	140	8.0	2-5	0.09	2800

[a)]clay content as well as all other parameters values are mean values of the soil profile horizons (except slope and rooting depth),



according to the instructions in Sys et al. (1993).

Figure 1: Soils taxonomic classification and positions of the study area land units (by ArcGIS Software® ; Sharififar et al. (Sharififar@ut.ac.ir))**.**

2.4 Maximum limitation method

In this method, crop requirements are matched with soil characteristics and then suitability of a land unit for a specific crop is determined by the rate of the characteristic that has the lowest rate among all of the characteristics. This approach is based on the Liebig's Law of the Minimum (Brown, 1942), which in its generalized form states that growth is controlled not by the total amount of resources available, but by the scarcest resource (limiting factor). In other words, Liebig's law states that grow only occurs at the rate permitted by the most limiting, whichever factor it may be. In maximum limitation method, lowest rate of characteristics rates (the most limiting one) is reported as the land index in every land unit for each of the crops. The advantage of this method is that it does not consider the interactions and/or any relations among characteristics and ignores any proportionality between characteristics and no combination is carried out. Interactions among the characteristics is a fairly complicated issue and needs precise survey and expertise, thus, it can affect the total land index (rating) wrongly or correctly. When such interactions are omitted, a significant source of error is removed. Therefore, this method is simple and easily applied, as it does not need high expertise. It is simple and easy to use, but seems to have low precision, since it only takes one characteristic into account. For example, there may be only one characteristic with the rate of suitability (index) of e.g. 55 which does not show whether all other soil characteristics are also 55 in rating or higher than this rate. It has been discussed by Biox and Zinck (2008).

2.5 Square root method

The so-called square root method was presented by Khiddir (1986), which takes into account all the characteristics, but a higher impact is considered for the most limiting one (the least scored one) on crop growth. The following equation shows how the land index is calculated in the square root method.

$$I = (R_{min}) \times \sqrt{\frac{A}{100}} \times \sqrt{\frac{B}{100}} \times \ldots \quad (1)$$

Where:
I: index of the square root method
R_{min}: the minimum rated characteristic
A, B, …: criteria other than minimum rated criterion

A, B, etc. are the characteristics considered for assessment (rooting depth, organic carbon content, etc.) mentioned earlier. This method could also have inspired from the logic of Liebig's Law, but here all of the considered characteristics are taken into account and they are all combined by multiplication. In the equation, the most limiting characteristic (the one with the lowest rate) influences the final obtained land index (total rate for a land unit) more than others. When multiplied by other characteristics, the limiting factor that has the lowest rate, may affect the total land rating irrationally. Thus, it is square rooted in order to decrease its irrational influence on total land index and make it more balanced mathematically. The limiting factor may have a much lower rate and the outcome of the multiplication may be an abnormal number for decision making when compared to the reality in the nature.

2.6 Storie method

The Storie method was presented by Storie (1976). In this method, all the characteristics rates are multiplied by each other. There is no difference among their effectiveness on crop growth. The land index is calculated through the following equation:

$$SI = (A) \times (B/100) \times (C/100) \times \ldots \quad (2)$$

SI: Storie index
A, B, C: rates of the considered characteristics.

In this method, what is noticeable is the type of the equation, in other words, the in which characteristics are combined. That is the core idea for considering this method for evaluation, as it has been used by some researchers somewhat successfully and by some researchers it was reported as dissatisfactory and problematic in revealing real land capability or suitability (Tang, 1993; Van Ranst et al., 1999; Bazgir, 2000)

More explanations on the maximum limitation and parametric methods of land evaluation can be found in Sys et al. (1991a,b).

2.7 Fuzzy sets method

In this study, three fuzzy membership functions including S shaped, Z shaped and Kandel (a type of Gaussian) functions were used to give each of the land characteristics a degree of membership to each suitability classes ranging from 0 to 1. Zero is the least and one is the highest degree of membership of the measured characteristics to a reference suitability class. Four reference suitability classes including: S1: highly suitable (75-100), S2: suitable (50-75), S3: moderately suitable (25-50) and N: non-suitable (0-25), were defined for each of the land characteristics and for each crop separately. Separate functions are defined and modulated for each of the reference suitability classes of each characteristic. This three mathematical functions show the relation between a dependent variable (y) with an independent variable (x), in which the vertical axis (y) presents the relative degree of membership of a soil characteristic to a suitability class. For example, the less the land slope gradient, the better the ground is for cultivation operations, therefore, we use z-shaped function to assess that the less the x-axis value, the higher the membership degree for this characteristic in a given land unit for a given crop. S-shaped membership function was used for soil depth (rooting depth) and organic carbon content and Z-shaped function was used for $caco_3$ and slop gradient. Kandle function was used for clay content and pH. The nature of variability of these characteristics determines the type of function to be applied. Clay content and pH values do not vary in one way, in other words, we can not say that the higher the better or the lesser the better, but it depends on the type of the considered crop requirement for optimal growth. In some cases, a medium value or a range of values is suitable, which is best fitted and adapted with the type of variation/dependence in the Kandle function. The applied functions including S-shaped, Z-shaped and Kandel are as follows respectively:

Equation (3):

$$mf(x) = \begin{cases} 0, & x \in [-\infty, \alpha) \\ 2\left(\frac{x-\beta}{\gamma-\alpha}\right)^2, & x \in [\alpha, \beta) \\ 1 - 2\left[\frac{(x-\beta)}{(\gamma-\alpha)}\right]^2, & x \in [\beta, \gamma) \\ 1, & x \in [\gamma, +\infty) \end{cases}$$

Equation (4):

$$mf(x) = \begin{cases} 1, & x \in [-\infty, \alpha) \\ 1 - 2\left[\frac{(x-\beta)}{(\gamma-\alpha)}\right]^2, & x \in [\alpha, \beta) \\ 2\left(\frac{x-\beta}{\gamma-\alpha}\right)^2, & x \in [\beta, \gamma) \\ 0, & x \in [\gamma, +\infty) \end{cases}$$

Equations (3) and (4) represent increasing and decreasing fuzzy membership functions for land characteristic x respectively (e.g. rooting depth for increasing and slope gradient for decreasing). Where α and γ are lower and upper limits of reference threshold values (reference suitability classes which are determined based on specific crops requirements, (Sys et al. 1991)) of x characteristic and β is $(\alpha+\gamma)/2$.

Equation (5):

$$mf(x) = \begin{cases} \frac{1}{1 + \left[\frac{(x-b_1)}{d}\right]^2}, & x < b_1 \\ 1, & b_1 \leq x \leq b_2 \\ \frac{1}{1 + \left[\frac{(x-b_2)}{d}\right]^2}, & x > b_2 \end{cases}$$

In the Eq. (5), x is the value of measured characteristics, varying on a bilateral basis (e.g. soil pH having two poles of acidity and alkalinity), b_1 and b_2 are lower and upper limits of reference thresholds for characteristic x, m is $(b_1+b_2)/2$ and d is $(m-b_1)$. Figures 2 shows the graphs of functions S-shaped, Z-shaped and Kandel. The Kandel function is used to determine the membership degree of characteristics for S2 and S3 classes and the S-shaped and Z-shaped functions both are used for S1 and N classes depending on how the threshold values vary (increasingly or decreasingly).

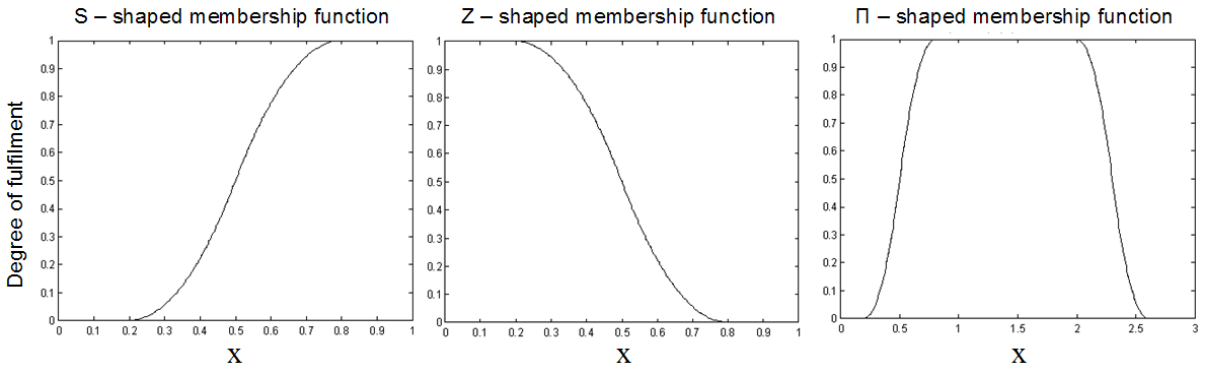


Figure 2: Fuzzy membership functions
S membership function for rooting depth and organic carbon content.
Z membership function for $CaCo_3$ and slope gradient.
Kandel membership function for clay content and pH.
In all three functions, x-axis is variability of a given soil characteristic and y-axis is degree of membership, which is defined in every suitability class separately.

After calculating the membership degree of all the characteristics to the four reference suitability classes (in every land unit for every crop), a so-called characteristics matrix is established for each land unit. Then, this matrix is combined with a so-called weights matrix. The weights matrix is comprised of relative weights of land characteristics with regard to their influences on crop growth. These weights are determined through expertise pairwise comparison of the characteristics by using analytical hierarchy process technique (Saaty, 1980, 2001). The rationale in which the weights were determined is the comparison of relative significance of intrinsic soil parameters influence on crop growth. The weights were not determined with regard to each crop condition, but with regard to the soil parameters relative importance through expert judgments. In the judgments, variations of the parameters within the study region and level of restriction they induce on crop growth with reference to the threshold values are also considered. For example, soil texture not only has a major influence on crop growth by controlling several other parameters like plant available water, nutrient retention, ventilation, etc., but also has a tangible variation and sample analyses revealed that it restricts several crops growth when compared with reference threshold limits. Therefore, this parameter was assigned the highest relative weight. The weights matrix is the same for assessment of all of the land units, but the characteristics matrix varies within different land units and different crop. The weight matrices calculated in this study are shown at the appendix of this paper. The following is an example of weights matrix and characteristics matrix in the land unit of Kharaqan for potato cultivation.

Characteristics matrix:

Weights matrix:

Clay%	Slope%	O.C%	Depth	pH	CaCO3%
0.235	0.155	0.105	0.175	0.135	0.195

	S1	S2	S3	N
Clay	0.14	0.81	0.09	0
Slope	1	0.31	0.2	0
O.C	0	0.5	1	0
Depth	1	0.02	0.01	0
PH	0.15	1	0.31	0
CaCO3	0	0.01	0.08	1

The weights matrix and characteristics matrix are combined using a fuzzy operator as follows:

$$E = W \circ R \quad (6)$$

Where, E is the suitability matrix (with one row and four columns), W and R are weights and characteristics matrices respectively and ($\circ$) is the

fuzzy operator that combines the two matrices via the following formula:

$$e_j = \min (a_1+a_2+\ldots..a_n, 1) \quad (7)$$

Where:

$$a_i = \max (0 , w_i+r_{ij}-1), i = 1, 2, \ldots..n. \quad (7\text{-}1)$$

e_j is the value of jth element in the suitability matrix, w_i and r_{ij} are given correspondent elements of weights and characteristics matrices respectively, and "min" and "max" signify the minimum and maximum value of the range inside the parenthesis respectively.

Afterwards, the matrix E is standardized in such a way that the summation of its elements is equal to one (e.g., dividing each element to the summation of all the elements). Then, the final land index is calculated through the following formula:

$$L_i = \sum (d_j \times A_j) \quad (8)$$

Where, L_i is land index in a specific land unit, d_j is standardized value of the jth element of the matrix E and A_j is mean value of the upper and lower limits of the jth reference suitability class. The reference suitability classes are divided to four classes including S1, S2, S3 and N, as defined for parameters previously. These total suitability classes determine a land unit suitability class with regard to cultivation of a specific crop. Detailed information on fuzzy mathematics can be found in the literature such as Wang (1997), and fuzzy functions can be searched through MATLAB® software.

To test the accuracy of each of these methods, the land indices obtained through each method are compared with observed yield of each land unit for each crop through a linear regression and a subsequent statistical significance t-student test. The observed yield data of the crops were mean values of several–year (about 5 years) cultivation, recorded in different land units at the Agriculture Organization of Bastam. The yield values were obtained by doing some corrections locally in different land units by direct interviews with some farmers of the region. They are usually recorded after each harvest event in different land units of the region at farm levels and are registered at the Bastam Agricultural Organization.

3 RESULTS

Chemical and physical analysis of soil samples shows that the major limitations of the region for agriculture are unsuitable soil texture (coarse and in some cases gravelly texture) for the determined crops and high amounts of carbonate content which is a restricting factor for growth of many crops. Results of land indices obtained by four evaluation methods for tomato, wheat and potato cultivation in each land unit are shown in Table 2, 3 and 4 respectively. Results of each evaluation method (land indices) were compared with observed yield in every land unit for the three crops mentioned. In The dependence of yield on four different land use indices is presented on Fig. 3, 4 and 5 for potato, tomato and wheat respectively. The Table 5 presents the statistical significance of this dependencis. The fuzzy sets indices explain the highest amount of yield variability for all three crops, so it was recognize as the best method among all. The yield data for the land units Bayazid and Khazaneh were missing, so the evaluation results for these land units are not presented in the paper. The land unit Bagh (named as rangeland in the maps) is used as a low return pasture and does not have capability of cultivation because of its very shallow rooting depth.

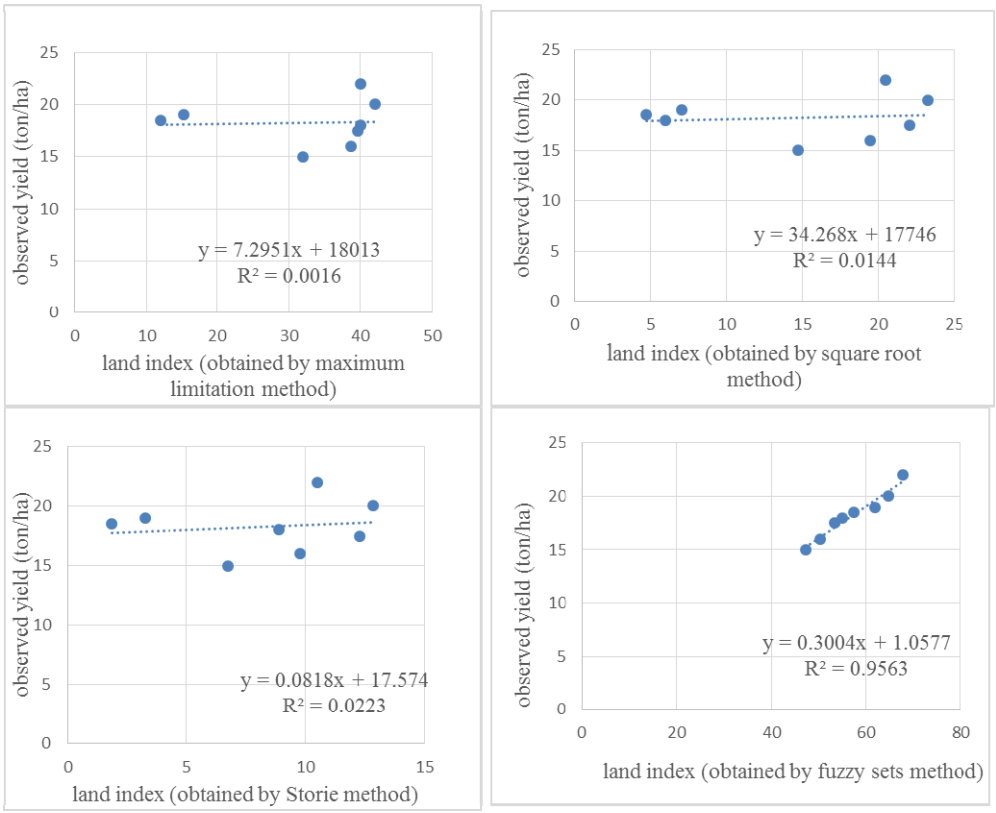


Figure 3: Regression results for land suitability evaluation of potato.

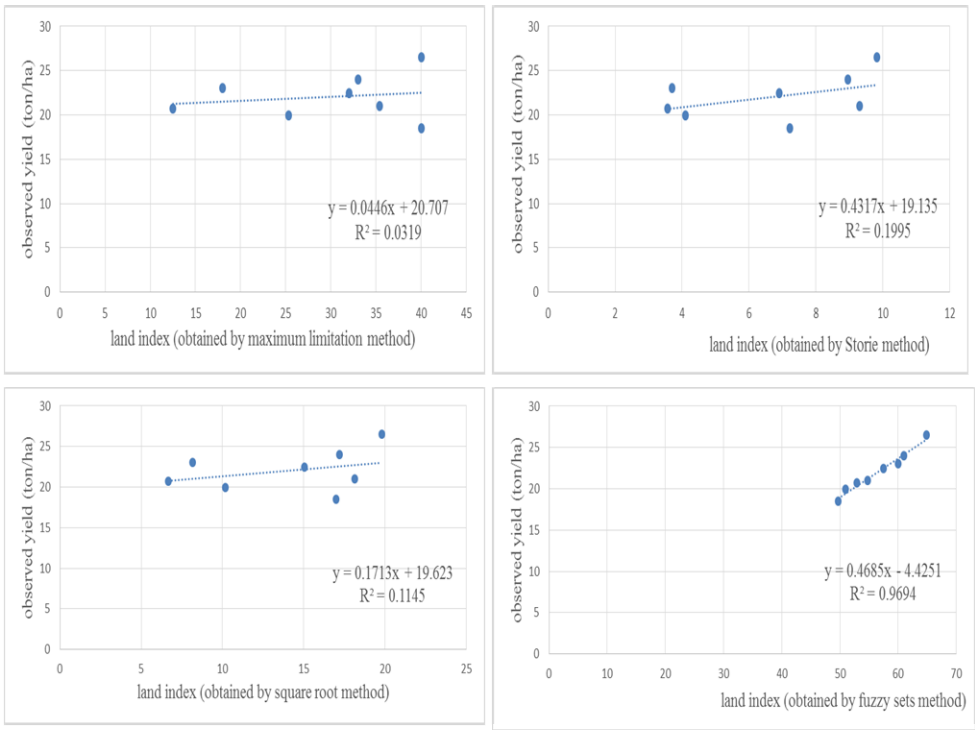


Figure 4: Regression results for land suitability evaluation of tomato.

Table 2: Land indices calculated by different methods and observed yields of potato

Land units	Maximum limitation	Storie	Square root	Fuzzy sets	Observed yield (ton/ha)
Abr	38.66	9.79	19.46	50.27	16.0
Amir	40.00	10.48	20.48	67.87	22.0
Bastamy	42.05	12.82	23.22	64.80	20.0
kharaqan	12.00	1.84	4.70	57.43	18.5
Khij	32.00	6.75	14.70	47.27	15.0
Mojen	15.33	3.25	7.06	61.89	19.0
Qaleh	39.66	12.28	22.07	53.30	17.5
Qehej	40.00	8.87	5.96	55.01	18.0

Table 3: Land indices calculated by different methods and observed yields of tomato

Land units	Maximum limitation	Storie	Square root	Fuzzy sets	Observed yield (ton/ha)
Abr	32.00	6.90	15.04	57.51	22.5
Amir	40.00	9.82	19.82	64.89	26.5
Bastamy	35.39	9.30	18.14	54.73	21.0
kharaqan	12.50	3.56	6.67	52.94	20.7
Khij	25.33	4.09	10.18	50.93	20.0
Mojen	18.00	3.70	8.16	59.99	23.0
Qaleh	33.00	8.96	17.20	60.97	24.0
Qehej	40.00	7.22	17.00	49.72	18.5

Table 4: Land indices calculated by different methods and observed yields of wheat

Land units	Maximum limitation	Storie	Square root	Fuzzy sets	Observed yield (ton/ha)
Abr	60.00	35.40	46.08	61.25	2.5
Amir	40.00	30.60	34.99	74.15	3.0
Bastamy	50.00	34.00	40.23	63.35	2.7
kharaqan	12.50	6.86	9.26	63.31	2.7
Khij	50.00	15.18	19.48	69.72	2.8
Mojen	40.00	14.02	23.68	59.74	2.0
Qaleh	75.00	45.12	58.17	78.25	4.0
Qehej	40.00	12.77	14.30	60.87	2.2

4 DISCUSSION

Although the crops yields are influenced by many factors other than soil, such as diseases and managerial measures, but they are used only as an indicator of each land unit potentiality, in order to compare capabilities of different land units. Moreover, the differences of farm management levels among land units of the study area, which could influence the yields, is negligible, as interviews were done with some farmers. Therefore, if we some scan approximations, we can be confident that the differences between different land units crop yields are purely due to different soil capabilities only.

The most suitable crop amongst the three crops, obtained by fuzzy sets method, was determined for cultivation in each land unit. Figure 6 shows the highest suitable crop for each land unit. The whole region is mainly suitable for wheat cultivation in comparison with the other two crops and the land unit Qaleh shows the highest suitability for wheat production. Tomato is not recommendable for

cultivation in the study area due to low suitability of soils of the region for production of this crop.

Other researchers have come to similar results such as Tang et al. (1992) that investigated the fuzzy method in comparison with other methods (maximum limitation and parametric methods) in China, and found that the fuzzy method has the highest correlation coefficient (0.96) when compared with observed yield, meanwhile this coefficient was lower for parametric and maximum limitation methods.

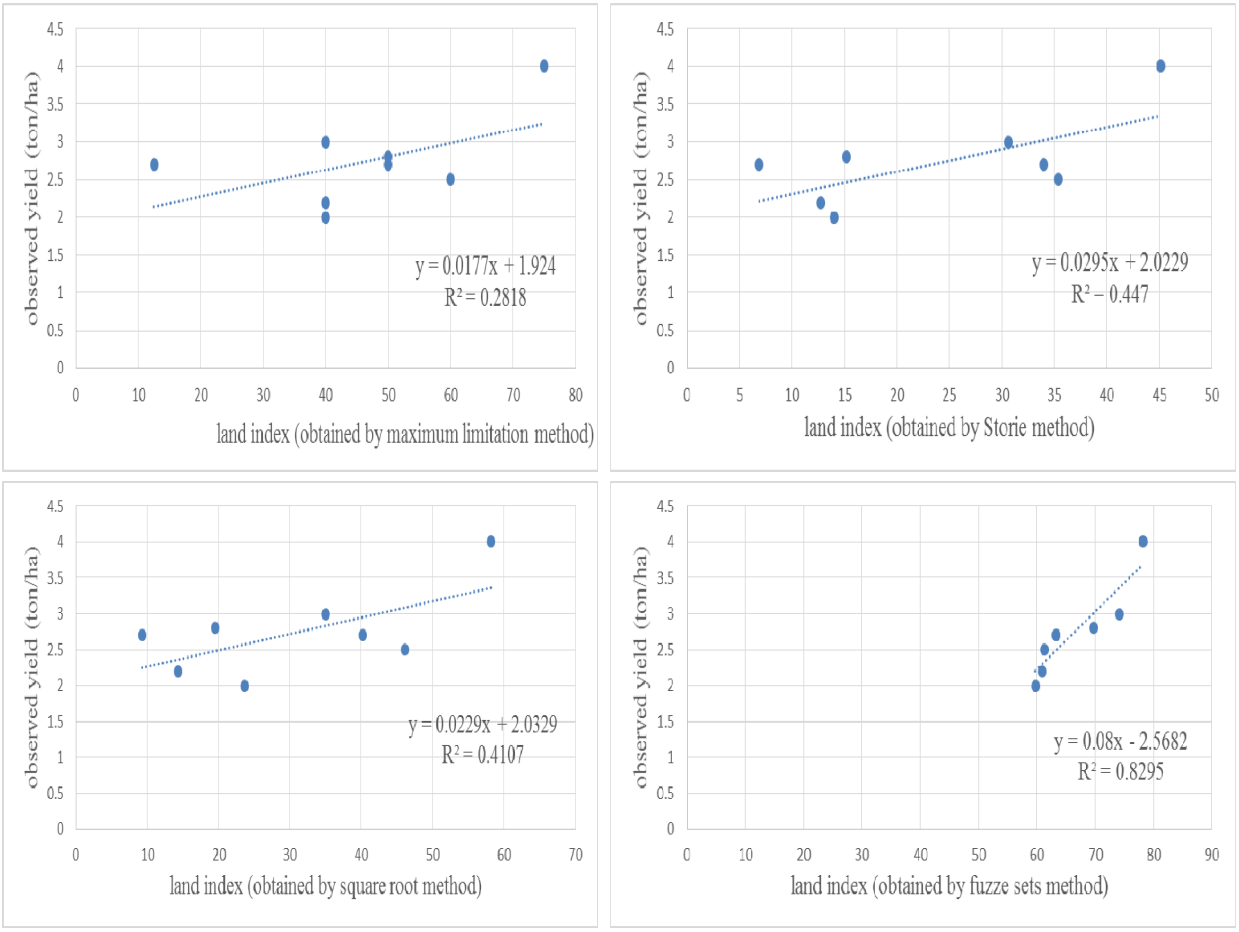


Figure 5: Regression results for land suitability evaluation of wheat

In Thailand, a similar study on rubber was done by Van Ranst et al. (1996). They also found the fuzzy sets method as the best method for land suitability classification. Likewise, the advantageous application of fuzzy methodology has been confirmed by Tang et al. (1997). They reported higher accuracy of fuzzy method in comparison with Boolean methods for land suitability evaluation of different crops. In another study, Van Ranst and Tang (1999) performed land suitability evaluation for corn cultivation using fuzzy and Boolean approaches. They compared land indices, calculated through different methods, with observed yield, and obtained correlation coefficient of 0.97 for the fuzzy approach and thereby reported the higher accuracy of fuzzy method compared with Boolean methods. Another study to compare parametric method with fuzzy method on irrigated wheat was done by Mohammadi and Givi (2002) in Iran. They compared the land indices with observed yield of the crop and obtained a correlation coefficient of 0.14 and 0.35 for parametric and fuzzy methods respectively. Corona et al. (2008) explained some benefits of fuzzy set application in land suitability assessment and performed a case study on land suitability by using fuzzy sets. They concluded that this methodology is quite useful in such projects. In a study in the

country where the study area is located, Keshavarzi et al. (2010) applied the fuzzy sets method for classification of lands for irrigated wheat production. They compared the wheat yield with land indices and obtained a correlation coefficient of 0.91, but they did not compare this method with any other methods. There are no other recent studies on comparing or testing the methods of land evaluation.

Despite the preeminence of the fuzzy method, it needs high amounts of calculations that makes it a demanding task. The typical difference between fuzzy method and other conventional methods is the procedure of combining the criteria (land characteristics) and allocation of different weights to the criteria in the fuzzy method. It is evident in soil specialists' point of view that different soil/land characteristics have different impacts on crop growth, for example, soil texture does not have identical influence on crop growth as pH, since the soil texture type controls several other soil qualities like amount of total soil water retention, plant available water, ventilation, water infiltration, etc. Therefore, assigning proportionate weights for different characteristics is necessary. Nevertheless, in the other three methods mentioned, all the characteristics influences on crop growth are considered evenly. That is a reason why their results are less reliable than fuzzy methos. Results of the Storie and square root methods regression were not significant for tomato and potato. The results of maximum limitation method regression with the crops yields were not significant for any of the crops in all of the land units. This could be because of not taking into account all of the characteristics that affect land capability/suitability for crop production. A conclusion can be elicited here that considering the effective characteristics and the way of combining them is important and affects the final calculation results significantly. The fuzzy sets methodology application in land evaluation is based on the assumption that the changes in soil properties and suitability classes of land units are not crisp but gradually changing within space. When we define the reference suitability classes limits as precise and crisp but not vague or fuzzy, we lose parts of the obtainable information in our analyses. Because soil parameters values vary continuously and naturally are not precisely separated. Therefore, using fuzzy approach in such analyses, which can reveal the intrinsic continuity and vagueness in land evaluation, seems to be a significantly more efficient methodology than traditional classical methods. This methodology has a high efficiency in showing slight but highly important differences in parameters variations, which greatly influences the results of land evaluation process.

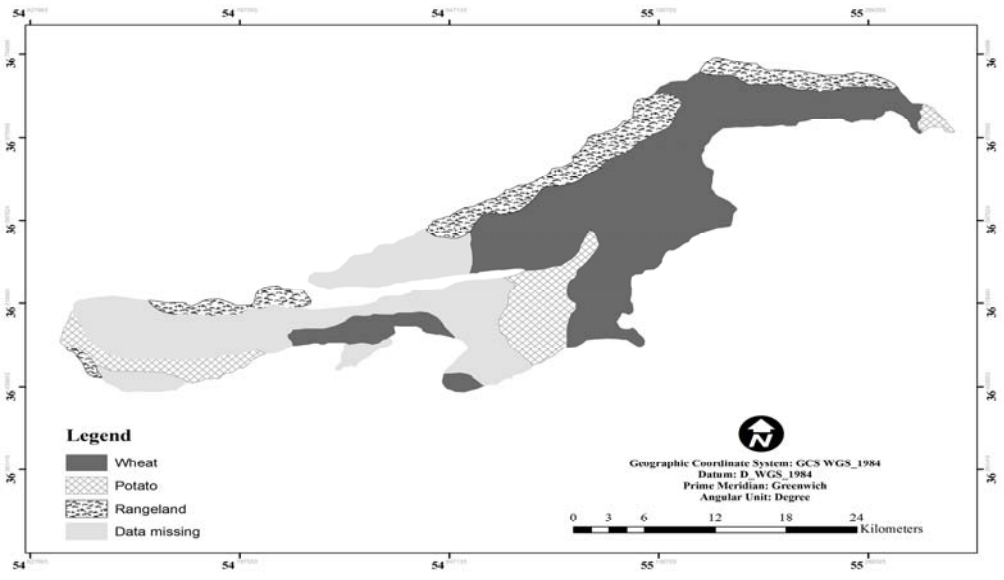


Figure 6: The most suitable crop in each land unit (land indices calculated through fuzzy sets method); [Sharififar et al., (Sharififar@ut.ac.ir)]

5 CONCLUSION

In the study area:

Fuzzy sets method regression results are significant at $p = 0.05$ and had the highest reliability in comparison with the other three methods.

Results of the Storie and square root methods correlations were only significant for wheat production at $p = 0.1$.

Regression analysis results of maximum limitation method with the crops yields were not significant.

This study was a demonstrative test of fuzzy sets theory in land suitability evaluation. Different techniques of land evaluation are used by researchers around the world, but among the basic frameworks of land evaluation, fuzzy methodology results are the nearest one to the real qualities of lands undoubtedly.

However, application of this methodology, in comparison with other classic methods, is rather a demanding task but it is sound and precise enough.

We suggest building a computerized model of the fuzzy procedure for easier implementation of land suitability assessments.

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Agrovoc descriptors: allelopathy; weeds; cultural weed control; medicago sativa; nitrogen fertilizers; sorghum, tobacco; nicotiana tabacum

Agris category code: H60, f04, f62

Effects of different nitrogen levels on phytotoxicity of some allelopathic crops

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ABSTRACT

Intensive usage of herbicides can result in the serious negative impacts on environment. Allelopathy by reducing seed germination and early seedling growth can play a fundamental role in suppressing weeds in crop fields. The effectiveness of allelochemicals is governed by different factors such as soil nutrient status, pH and microorganisms. Outdoor pot experiments were conducted at the Faculty of Agriculture and Natural Resources of Razi University, Kermanshah, Iran, in 2013, to evaluate the effects of different levels of N fertilizer (0, 150, 300 kg ha^{-1}) on the suppressing effects of alfalfa (*Medicago sativa* L.), sorghum (*Sorghum bicolor* L.), and tobacco (*Nicotiana tabacum* L.) plant materials on emergence and growth parameters of some weed species including Johnson grass (*Sorghum halepense* (L.) Pers.), barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) and redroot pigweed (*Amaranthus retroflexus* L.). Results indicated that adding plant materials of tobacco, sorghum, and alfalfa substantially reduced seed germination and early growth of the tested weeds. However, the weed species responded differently to the presence of the allelopathic plant materials. The use of N fertilizer had significant effects on the inhibitory potentials of the allelopathic plants. However, we didn't find consistent trends regarding the responses of the allelopathic crops to elevated N fertilizer levels in related to the traits under study.

Key words: alfalfa, allelopathy, nitrogen, sorghum, tobacco, weeds

IZVLEČEK

UČINKI RAZLIČNIH ODMERKOV DUŠIKA NA FITOTOKSIČNOST NEKATERIH ALELOPATIČNIH POLJŠČIN

Intenzivna raba herbicidov ima lahko resne negativne vplive na okolje. Z zmanjševanjem kalitve in začetne rasti lahko igra alelopatija pomembno vlogo pri zatiranju plevelov. Na učinkovitost alelokemikalij vplivajo številni dejavniki kot so količina hranil v tleh, pH in mikroorganizmi. Na Faculty of Agriculture and Natural Resources of Razi University, Kermanshah, Iran, je bil v letu 2013 izveden lončni poskus na prostem, z namenom ovrednotenja različnih odmerkov N gnojil (0, 150, 300 kg ha^{-1}) na zaviralni učinek lucerne (*Medicago sativa* L.), navadnega sirka (*Sorghum bicolor* L.), in navadnega tobaka (*Nicotiana tabacum* L.). Po dodatku zmletih nadzemnih delov teh poljščin so spremljali vznik in rastne parameter treh plevelov, divjega sirka (*Sorghum halepense* (L.) Pers.), navadne kostrebe (*Echinochloa crus-galli* (L.) Beauv.) in navadnega ščira (*Amaranthus retroflexus* L.). Rezultati so pokazali, da je dodatek navadnega tobaka, navadnega sirka in lucerne znatno zmanšal kalitev in zgodnjo rast testiranih treh plevelov. Testirani pleveli so se različno odzvali na dodatke alelopatskih rastlinskih ostankov. Uporaba dušičnih gnojil je imela značilen učinek na inhibitorni potencial alelopatskih rastlin, vendar niso uspeli ugotoviti konsistentnega trenda med odzivi plevelov na dodatke alelopatskih rastlin in povečanimi odmerki dušičnih gnojil.

Ključne besede: lucerna, navadni sirek, navadni tobak, alelopatija, dušikova gnojila, pleveli

1 INTRODUCTION

Based on the oldest definition by Molisch (1937), the adverse effect of one plant species on neighboring plants via releasing of toxic chemicals is called as allelopathy. Several plant species have shown allelopathic capability (Macias *et al*., 2004). Alfalfa (*Medicago sativa* L.), sorghum (*Sorghum*

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bicolor L.) and tobacco (*Nicotiana tabacum* L.) are extensively cultivated across the Iranian farmlands which have shown allelopathic potential in Petri dish trials (Nielsen *et al*., 1960; Klein and Miller, 1980; Jensen *et al*., 1981; Tesar, 1986; Hedge and Miller, 1990; Sène *et al*., 2000; Chon and Kim, 2004; Rehman *et al*., 2010). Alfalfa plant tissues and their aqueous extracts can inhibit the germination and seedling growth of a number weed species (Singh *et al*., 2003). Florentine *et al*. (2005) reported that the aqueous extract of tobacco shoots have inhibitory effects on growth and germination of some crops. According to Singh *et al*. (2003) all parts of sorghum release phytoinhibitors reducing the growth of grass and broadleaf weed species. Annually thousand tons of residues of these crops are incorporated into the agricultural soils which can be used as a useful alternative tool for weed control. By integrating of allelopathic plants to a weed management program, the need for herbicide to control weeds would diminish substantially (Dilipkumar et al, 2012).

In addition to reduce the cost of weed management, environment would not be affected by artificial herbicides. Unlike artificial herbicides, the use of allelopathy for weed suppression in agroecosystems is a complicated practice. A broad range of different factors can influence the allelopathic activity of allelochemicals, including soil and climatic conditions (Ponder *et al*., 1985; Blum, 1998; Kobayashi, 2004; Inderjit, 2005). Nevertheless, there is not a lot of works describing the inhibitory effects of allelopathic plant materials as influenced by soil factors. Physicochemical properties of soil as well as the soil organic matter, fertility and organisms have great impacts on adsorption, desorption, transport and the metabolism of allelochemicals in soil (Inderjit, 2001). Although, it has been demonstrated that seed germination of several weed species is stimulated by nitrogenous compounds like nitrate and ammonium (Adkins and Adkins 1994; Hartmann et al. 1997; Teasdale and Pillai, 2005) but the effects of this nutrient element on the potential of allelochemicals to reduce seed germination and growth of weeds under soil environment is not well known. Although, it has been well documented that allelopathy affects several biological processes in nitrogen cycle (Jobidon and Thibault, 1982; Weston and Putnam, 1985; Alsaadawi *et al*., 1986; Alsaadawi, 1988; Rice, 1992; Zwain et al, 1998). The purpose of this study was to evaluate the effects of different levels of nitrogen fertilizer on the inhibitory potentials of alfalfa, sorghum and tobacco on seed germination and early seedling growth of barnyard grass (*Echinochloa crus-galli* (L.) Beauv.), Johnson grass (*Sorghum halepense* (L.) Pers.) and redroot pigweed (*Amaranthus retroflexus* L.), some dominant weed species in many regions of Iran.

2 MATERIALS AND METHODS

This outdoor pot experiment was conducted at the Faculty of Agriculture and Natural Resources of Razi University, Kermanshah (34°18′51″N, 47°03′54″E; elevation 1350 m.), Iran during June to September of 2013. During the experiments, the mean temperature and relative humidity were 26.6 °C and 12.8 %, respectively. Ten seeds of Johnson grass, redroot pigweed and barnyard grass were sown in plastic pots with a diameter of 13 and a height of 19 cm containing 1.9 kg of field soil. Each pot was treated with powdered plant tissues including shoot and root of alfalfa, tobacco or sorghum. The allelopathic plant materials were prepared from the plants grown in the Research Field of Razi University and harvested just before flowering stage. After harvesting, the allelopathic plants were air-dried in the shade and then ground. The amount of powdered allelopathic plant tissues added to each pot was 16 g. This amount equalled with 11400 kg ha^{-1} and was the lowest effective concentration which was determined according to the results of our preliminary experiments (data not shown). Three levels of N fertilizer, 0, 2 and 4 g (as urea) were added to the pots which equalled with 0, 150 and 300 kg ha^{-1}, respectively. Three non-treated pots were also included as control. The trial was conducted as a factorial based on a completely randomized design with three replications and was repeated twice.

The number of emerged seedlings in each pot was recorded daily up to the seedling establishment was stabilized. Then the mean emergence rate

(MER) was calculated according to the following equation (Ellis and Roberts 1980):
MER= ∑n / ∑Dn
where *n* is the number of seedling emerged on day D and *D* is the number of days from the start of experiment.

Moreover, seedling emergence percentage was determined when the seedling establishment was stabilized.

Then weeds were thinned to five plants in each pot and were irrigated as needed throughout the experiment. Two weeks after weed emergence, leaf chlorophyll content (based on SPAD value) was measured on three randomly selected leaves of each plant by using a SPAD meter model (SPAD-502, Minolta, Japan), weekly. Other traits of the weed species including plant height, the number of tillers per plant, root length, the number of leaf per plant and shoot and root dry mass were determined at 35 days after the start of experiment. To determine shoot and root dry mass, these plant organs were dried at 70 °C for 48 h and then their mass was recorded. Obtained data were subjected to ANOVA and means were separated using Fisher's Protected LSD test at the 0.05 level of probability by using SAS software (SAS Institute 2003).

3 RESULTS

3.1 Barnyard grass

The results showed that alfalfa had the greatest reducing effect on barnyard grass seedling emergence under all three nitrogen levels with a positive response to increasing nitrogen fertilizer (Table 1), as the lowest seedling emergence caused by alfalfa occurred at the highest N level. Nitrogen application in a rate of 150 kg ha^{-1} decreased barnyard grass seedling emergence under tobacco and sorghum treatments but their reducing effects diminished when N level increased from 150 to 300 kg ha^{-1} (Table 1). The lowest barnyard grass root dry masst was recorded for sorghum treatment when N fertilizer was not applied (Table 1). In general, root dry mass responded positively to the increasing N level under tobacco and sorghum treatments, although this trait was not significantly influenced by N application when soil was treated by alfalfa plant material (Table 1).

Table 1: Barnyard grass emergence and root dry mass as influenced by different nitrogen levels and allelopathic treatments

Allelopathic treatment	Nitrogen level (kg h^{-1})	Seedling emergence (%)	Root dry masst (g $plant^{-1}$)
Control		83.33^{ab}(±1.923)	4.12^{a}(±0.092)
Tobacco	0	70.00^{bc}(±0.000)	0.71^{d}(±0.064)
	150	63.33^{cd}(±3.333)	1.87^{b}(±0.132)
	300	86.66^{a}(±3.333)	1.25^{c}(±0.063)
Alfalfa	0	46.66^{ef}(±3.333)	1.07^{cd}(±0.328)
	150	36.66^{fg}(±3.333)	1.07^{cd}(±0.302)
	300	30.00^{g}(±5.774)	1.06^{cd}(0.094)
Sorghum	0	53.33^{de}(±8.819)	0.89^{cd}(±0.103)
	150	43.33^{efg}(±3.333)	0.96cd(±0.269)
	300	46.66^{ef}(±6.667)	1.29^{c}(±0.142)
$LSD_{(0.05)}$		16.13	0.53

Means (± SE) within a column followed by the same letter are not significantly different at the 0.05 level of probability (LSD test).

3.2 Redroot pigweed

Redroot pigweed plant height was significantly increased in response to increasing N level under alfalfa and sorghum treatments, as the highest plant heights were recorded in the highest level of N fertilizer (Table 2). However, in the soil treated by tobacco plant material, increasing N level from 150 to 300 kg ha^{-1} led to a notable reduction in redroot pigweed plant height (Table 2). Under zero level of N in the soil treated by sorghum plant material, the greatest reduction in red root pigweed plant height was observed. In general, in the pots containing sorghum plant material, with increasing N concentration the inhibitory effect was reduced (Table 2). In the pots treated by alfalfa, increasing N level from 150 to 300 kg ha^{-1} increased redroot pigweed height compared to control. For tobacco plant material, with increasing N level from 150 to 300 kg ha^{-1}, redroot pigweed height decreased significantly. Addition of 300 kg ha^{-1} of N fertilizer to the soil treated with sorghum plant material produced the lowest number of leaves when compared with control (Table 2). As a general rule, in double-dose of N fertilizer, the reducing effects of alfalfa and tobacco on the number of redroot pigweed leaf were increased. The lowest values of root dry mass when compared to control were observed at the zero level of N under all allelopathic treatments. In the presence of tobacco plant material, with increasing N fertilizer, root dry mass was increased while for sorghum and alfalfa decreasing trends were observed (Table 2). The lowest redroot pigweed root length occurred in the pots treated by alfalfa plant materials under zero level of N fertilizer (Table 2). Increasing N level decreased the inhibitory effects of all allelopathic plant materials on root length, although in the soil treated by tobacco, redroot pigweed root length was significantly higher under zero level of N than that in the level of 150 kg ha^{-1} (Table 2). Nitrogen application in the soil treated by sorghum plant material led to the highest reduction in redroot pigweed emergence (Table 2). For tobacco treatment, the use of N fertilizer also significantly reduced seedling emergence compared to control, although there was no significant difference between the low and the high fertilizer levels in terms of their effects on this trait (Table 2). Alfalfa responded inversely to N application as its inhibitory effect decreased with increasing N fertilizer level (Table 2).

Table 2: Redroot pigweed emergence and plant traits as influenced by different nitrogen levels and allelopathic treatments

Allelopathic treatment	Nitrogen level (kg ha^{-1})	Plant height (cm)	Leaf number (no plant^{-1})	Root dry mass (g plant^{-1})	Root length (cm)	Seedling emergence (%)
Control		11.00^{bc}(±0.577)	72.33^{a}(±1.345)	0.78^{bcd}(±0.046)	25.16^{bc}(±1.155)	73.33^{a}(±1.923)
Tobacco	0	10.53^{cd}(±1.048)	27.00^{ef}(±3.512)	0.32^{e}(±0.078)	20.66^{de}(±0.028)	60.00^{b}(±0.000)
	150	12.50^{abc}(±1.323)	49.33^{b}(±0.882)	0.43^{de}(±0.297)	11.50^{f}(±1.041)	36.36^{cd}(±3.333)
	300	9.80^{cd}(±0.709)	41.33^{cd}(±2.028)	0.55^{cde}(±0.068)	22.00^{cd}(±2.517)	36.36^{cd}(±3.333)
Alfalfa	0	10.50^{cd}(±0.764)	47.33^{bc}(±3.180)	0.34^{e}(±0.037)	9.33^{f}(±0.882)	23.33^{ef}(±3.333)
	150	9.83^{cd}(±1.202)	52.00^{b}(±4.041)	1.24^{a}(±0.036)	11.33^{f}(±2.892)	30.00^{de}(±0.000)
	300	14.20^{a}(±0.473)	39.33^{d}(±2.186)	0.94^{ab}(±0.055)	23.33^{cd}(±0.667)	53.33^{b}(±3.333)
Sorghum	0	7.86^{d}(±0.948)	39.33^{d}(±2.333)	0.21^{e}(±0.006)	17.00^{e}(±0.577)	40.00^{c}(±0.000)
	150	10.10^{cd}(±0.379)	30.66^{e}(±1.202)	0.92^{abc}(±0.025)	29.00^{ab}(±0.577)	10.00^{g}(±0.000)
	300	13.56^{ab}(±1.260)	22.33^{f}(±1.453)	0.41^{de}(±0.024)	31.00^{a}(±1.528)	20.00^{f}(±5.774)
LSD $_{(0.05)}$		3.00	6.72	0.37	4.33	8.88

Means (± SE) within a column followed by the same letter are not significantly different at the 0.05 level of probability (LSD test).

3.3 Johnson grass

N fertilizer at the level of 150 kg ha^{-1} and in the presence of alfalfa plant material had the greatest inhibitory effect on Johnson grass seedling emergence (a 81 % reduction compared to control) (Table 3). Increasing N level from 0 to 150 kg ha^{-1} in the soil treated by tobacco or alfalfa decreased Johnson grass emergence but the inhibitory effects of these allelopathic crops were significantly reduced at the highest level of N fertilizer (300 kg ha^{-1}). However, increasing N level from 0 to 300 kg ha^{-1} consistently enhanced sorghum reducing effect on the weed seedling emergence (Table 3). Adding the 300 kg ha^{-1} of N fertilizer in the presence of sorghum plant material had the highest inhibitory effect on the number of leaf per plant followed by tobacco and alfalfa, respectively (Table 3). Johnson grass leaf number was not significantly influenced by different N fertilizer levels in the soil treated by tobacco plant material. However, in the presence of sorghum plant material, this trait showed a significant decreasing response to increasing N level from 150 to 300 kg ha^{-1} (Table 3). In the soil treated by alfalfa, the highest and the lowest reductions in the number of leaf occurred at the 0 and 150 kg ha^{-1} levels of N fertilizer, respectively (Table 3). For all three allelopathic plants, Johnson grass shoot dry mass was decreased in response to increasing soil nitrogen level (Table 3). However, the highest reductions occurred in the soil treated with sorghum plant material, as the lowest shoot dry mass was observed when pots treated by this allelopathic plant and received the highest level of N fertilizer (Table 3). Although, increasing N level in the soil containing tobacco plant material increased its reducing effect on shoot dry mass of Johnson grass but this was not significant from a statistical viewpoint (Table 3).

Table 3: Johnson grass emergence and plant traits as influenced by different nitrogen levels and allelopathic treatments

Allelopathic treatment	Nitrogen level (kg ha^{-1})	Seedling emergence (%)	Leaf number (no $plant^{-1}$)	Shoot dry mass (g $plant^{-1}$)
Control		70.00^{a}(±1.732)	33.66^{a}(±1.555)	2.53^{a}(±0.289)
Tobacco	0	26.66^{bcd}(±3.333)	16.00^{ef}(±1.000)	0.86^{cd}(±0.181)
	150	23.33^{bcde}(±3.333)	17.33^{de}(±1.764)	0.76^{de}(±0.019)
	300	33.33^{b}(±6.667)	16.66^{de}(±1.333)	0.84^{d}(±0.080)
Alfalfa	0	20.00^{cde}(±5.774)	19.66^{cd}(±1.453)	1.31^{b}(±0.009)
	150	13.00^{e}(±3.333)	24.33^{b}(±1.202)	1.23^{bc}(±0.066)
	300	33.33^{b}(±3.333)	21.66^{bc}(±1.764)	0.69^{de}(±0.104)
Sorghum	0	30.00^{bc}(±0.000)	17.66^{de}(±0.882)	1.34^{b}(±0.091)
	150	20.00^{cde}(±5.774)	16.66^{de}(±0.882)	0.79^{d}(±0.102)
	300	16.66^{de}(±3.333)	13.00^{f}(±0.577)	0.40^{e}(±0.009)
$LSD_{(0.05)}$		10.88	3.64	0.37

Means (± SE) within a column followed by the same letter are not significantly different at the 0.05 level of probability (LSD test).

4 DISCUSSION

As it is evident from the results, weed species and the traits under study responded variously to the presence of different allelopathic plant materials in soil. Different susceptibility of weed species to allelochemicals from several plant species has been reported, previously (Batish et al., 2002; Kobayashi, 2004). Differences in the responses of weed species to allelochemicals might be due to some weed characteristics including seed size (Liebman and Sundberg, 2006). The use of N fertilizer had significant effect on the suppressing ability of allelopathic plant materials on the

examined weed species. However, the weed species and the traits under study responded to different N fertilizer levels, inconsistently. According to Inderjit (2001) soil physical, chemical (e.g. inorganic ions) and biological properties can influence the effects of allelochemicals. Other workers have also demonstrated that soil properties can greatly affect allelopathy phenomena in various ways (Tongma et al., 2001; Hiradate et al., 2010). Different effects of N is probably due to its different influences on the microbes which decompose plant materials, as each one has its own specific microorganism capable of degrading its plant material (Kumar et al., 1993; Schmidt and Lipson, 2004; Inderjit, 2005; Xuan *et al*., 2005). The C/N ratio of the allelopathic crop materials is another important factor which can influence the rate of the release of allelochemicals into the soil and consequently their inhibitory effects on germination and seedling growth of weeds. A crop such as alfalfa with a low C/N ratio may be decomposed by soil microorganisms rapidly and releases its phythotoxins into the soil in a higher rate. Therefore, it can be expected that the crops with higher C/N ratios such as tobacco and sorghum show more positive responses to the elevated soil nitrogen concentration resulted from N fertilizer application when compared with alfalfa. However, in our study there were no consistent trends regarding the responses of the allelopathic plant materials to different N fertilizer levels in related to the traits under study. Increasing the emergence and seedling growth of some weeds in the allelopathic plant-treated soils in response to N application can be attributed to the stimulatory effects of this element on germination of some weed species (Adkins et al., 2002). In general, adding plant materials of tobacco, sorghum, and alfalfa substantially reduced seed germination and early growth of the three weed species under study. These weed species can seriously reduce crop production and their chemical control is somehow difficult and costly (Holm et al., 1991; Zimdahl, 2007). The most practical aspect of allelopathy as a tool to control weeds is its suppressing effect on seed germination of these unwanted plants. Because, it seems that allelochemicals may have lower inhibitory effects on weeds at the later stages of their life cycle.

5 CONCLUSION

In our study, the emergence of all weed species was notably reduced in the soils treated by the allelopathic plant materials. This reveals that allelopathy may be proposed as a promising tool in an integrated weed management (IWM) program and allelopathic plants can be included in crop rotations to reduce weed infestation in subsequent crops. However, their negative effects on germination and growth of subsequent crops should be considered, precisely. N fertilizer may have different effects on the weed suppressing ability of allelopathic crops in related to several factors including the nature of crop and weed species and soil physical, chemical and biological conditions such as microorganism communities. Certainly, further studies are needed to investigate these factors.

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Research on the involment of phenoloics in the defence of horticultural plants

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ABSTRACT

Phenolic compounds are not directly involved in the primary metabolism of plants but possess a number of important roles: (1) serving as attractants for pollinators and various animals, involved in the transfer of seeds, (2) plant protection from herbivores and against pathogen infection, (3) defining plant-plant relationships and the symbiosis between plants and microbes. The present review of our research work stresses the role of phenolic compounds in the defense mechanism against different fungi and bacteria. It has been established, that the content of phenolics is greatly affected by the infection with pathogenic organisms. Studies on several horticultural plants have demonstrated that the response to infection differs among the analyzed plant species. Generally, an increase of phenolic compounds can be expected in tissues near the infection site. The comparison of healthy and infected tissue reflects an increase of phenolics in infected tissues. Higher levels of all analyzed phenolic groups have been measured in the latter, with the exception of the anthocyanins. Based on the findings of many-year research studies, it can be concluded that phenolic compounds are involved in the plant defense mechanisms, but the response varies among species.

Key words: *Malus domestica* Borkh., fruits, vegetables, infection by fungy and bacteria, phenolics

IZVLEČEK

RAZISKOVANJE VKLJUČEVANJA FENOLOV V OBRAMBNE REAKCIJE HORTIKULTURNIH RASTLIN

Fenolne snovi so spojine, ki niso neposredno vključene v osnovni metabolizem rastlin, vendar imajo v njih številne druge pomembne vloge: (1) služijo kot atraktanti za opraševalce in druge živali, ki so vključene v prenos semen, (2) ščitijo rastline pred herbivori in pred okužbo z glivami in mikrobi, (3) sodelujejo pri kompeticiji rastlina-rastlina in simbiozi rastline z glivami in bakterijami. V pregledu naših raziskav z vidika vloge fenolnih snovi v obrambnem mehanizmu pred različnimi glivami in bakterijami lahko poudarimo, da so fenoli z gotovostjo ena od snovi, s katerimi se rastline odzivajo na okužbo s patogeni. Študije na različnih hortikulturnih rastlinah so pokazale, da odziv na okužbo ni enak pri različnih rastlinskih vrstah. Največkrat prihaja do povečanja koncentracij fenolnih snovi v okolici okužbe. Primerjava zdravega in okuženega tkiva v večini primerov kaže povečanje vsebnosti fenolov v okuženih tkivih pri vseh analiziranih skupinah fenolnih snovi, z izjemo skupine antocianov. Na podlagi povzetkov večletnih raziskav, ki so strnjene v delu, lahko podamo zaključek, da so fenolne snovi vključene v proces obrambe, vendar je odziv rastlin vrstno specifičen.

Ključne besede: *Malus domestica* Borkh., sadje, zelenjava, okužba z glivami in bakterijami, fenoli

1 INTRODUCTION

Plant phenolics are secondary metabolites that encompass several classes of structurally diverse products arising from the shikimate–phenylpropanoid pathways. Plants use phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and many other functions (Lattanzio et al., 2006). Polyphenols protect plants against adverse factors

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that threaten their survival in an unfavorable environment, such as drought, UV radiation, infection or physical damage. The presence of phenolic metabolites in plants is greatly influenced by environmental conditions and is also genetically controlled (Lattanzio et al., 2008). Therefore, they represent adaptive characters that have been subjected to natural selection during evolution (Lattanzio et al., 2006; Treutter, 2006; Russell et al., 2009).

Phenolic compounds possess antimicrobial properties against fungi, bacteria and viruses (Martini et al., 2009). As a result of microbial attack, phenolics may accumulate as inducible low-molecular-weight compounds, which can be post-inflectional or constitutive (Stack, 1997). Pre-formed antifungal phenolics belong to different classes like simple phenols, phenolic acids, flavonols and dihydrochalcones. If pre-formed antifungal phenolics are not sufficient to stop the development of the infection process, plant cells usually respond by increasing the level of antifungal phenols at the infection site or synthesize specific defense compounds (phytoalexins). In the first case, phenolics can rapidly accumulate upon attack, although they may already be present at low concentrations in the plant. In the second case, these compounds are already present in healthy tissues at concentrations high enough for defense, either as free compounds or in their conjugated forms, from which they are released after the attack (Strack, 1997). It has been shown that some hydroxycinnamic acids, flavanols (epicatechin, procyanidin B1, catechin) and dihydrochalcones may be involved in the defense mechanism of apple leaves against the scab fungus *Venturia inaequalis* (Cooke) G. Winter (Mikulič-Petkovšek et al., 2009; Slatnar et al., 2012).

In general, an increased level of phenols was observed in infected leaf tissues compared to healthy tissues (Mikulič-Petkovšek et al., 2011). Phenolic compounds slow down fungal growth, reacting with proteins and causing a loss of enzymatic function. Moreover, they restrict the viability of pathogens, and can be deposited inside the cell wall as an important first line of defense against fungal penetration and infection (Schwalb and Feucht, 1999).

The aim of this paper is to illustrate the response to biotic stress (specifically, fungal attack) in different horticultural plants. With knowledge of that response we could develop natural friendly cultivation methods and assist the plant to defend itself.

2 MATERIALS AND METHODS

The study was performed on different plants: apple (*Malus domestica* Borkh.), strawberry (*Fragaria x ananassa* Duch.), green bean (*Phaseolus vulgaris* L.), raspberry (*Rubus idaeus* L.), and walnut (*Juglans nigra* L.). Different parts of the tissue (fruits or canes in different developmental stages) infected with different causative agents (*Venturia inaequalis* (Cooke) G. Winter, *Xanthomonas arboricola* pv. *juglandis* (Pierce) Dye, *Didymella applanata* (Niessl) Sacc., *Leptosphaeria coniothyrium* (Fuckel) Sacc., *Colletotrichum nymphaeae* (Pass.) Aa, *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara) were sampled from plants growing in the University experimental orchard/field in Ljubljana (Mikulič-Petkovšek et al., 2013, 2014; Slatnar et al., 2010, 2012; Solar et al., 2012).

The selected tissue was differentiated to infected and healthy. Different numbers of replicates for each part of sampled tissue were performed for determination of phenolic compounds (Mikulič-Petkovšek et al., 2013, 2014; Slatnar et al., 2010, 2012; Solar et al., 2012). Samples were immediately shock-frozen in liquid nitrogen and stored at −80 °C until analysis of the samples.

Extraction with some modification was carried out as described by our previous reports (Mikulič-Petkovšek et al., 2013, 2014; Slatnar et al., 2010, 2012; Solar et al., 2012). The powdered samples were extracted with methanol containing 1 % 2,6-di-*tert*-butyl-4-methylphenol (BHT) and homogenized with the T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany). BHT was added to the samples to prevent oxidation during the extraction.

Samples were placed in a cooled ultrasonic bath (0 °C) for 1 h; then they were centrifuged and the supernatants were filtered through a Chromafil A0-45/25 polyamide filter produced by Macherey–Nagel (Düren, Germany). The high performance liquid chromatography (HPLC) analysis was performed on a Surveyor HPLC system with PDA detector (Thermo Finnigan, San Jose, CA, USA). The column was a Gemini C18 (150 × 4.6 mm, 3 μm; Phenomenex, CA, USA) operated at 25 °C.

The elution solvents, HPLC conditions and identification of phenolic compounds were the same as described by previous reports (Mikulič-Petkovšek et al., 2013, 2014; Slatnar et al., 2010, 2012; Solar et al., 2012). The concentrations of phenolic compounds were calculated from the peak areas of samples and the corresponding standards.

The data were analyzed using the Statgraphics Plus 4.0 (Manugistics, Inc., Rockville, MD, USA). Data from the chemical analyses were tested for any differences between treatments using one-way analysis of variance (ANOVA). The means and standard errors of the means (mean ± SE) are also reported.

3 RESULTS AND DISCUSSION

It has been determined that hydroxycinnamic acid (HCA) derivatives play a major role in plant resistance and exhibit a fungitoxic effect against different pathogens, because they inhibit the growth and sporulation of fungi (Sammi et al., 2009; Chardonnet et al., 2003). The highest content of HCA in apple peel was measured in the scab symptomatic spot, followed by the tissue around the spot (Table 1). This is probably a result of stress conditions in the plant, which are caused by the presence of *Venturia inaequalis*. Increased amounts of HCA were also reported by Mikulič-Petkovšek et al. (2009) and Leser and Treutter (2005) because of stress conditions. The plant response to artificial inoculation with the *Colletotrichum lindemuthianum* has been monitored in green bean pods and the content levels of HCA increased in both bordering and infected tissue (Table 1). The increase of all analyzed HCA after the *Xanthomonas arboricola* pv. *juglandis* (Xaj) infection (Table 2) may also be a consequence of stimulated PAL activity, as described in apple fruits infected with *V. inaequalis* by Slatnar et al. (2010). Significantly lower content (10–30 % decrease) of total hydroxycinnamic acid derivatives were detected in infected raspberry canes compared to healthy ones. Other research studies also confirmed a decrease of cinnamic acid derivatives after infection (Maurya et al., 2007; Rusjan et al., 2012), as observed in our study in raspberry canes.

The analysis of different bean pod parts (healthy, infected (*Colletotrichum lindemuthianum*) and bordering tissue) revealed a significant increase of total flavanols induced by the fungal infection (Table 1). In bordering tissue and lesion the content of flavanols was 3–10 folds higher compared to healthy tissue. Higher levels of individual procyanidin forms and epicatechin in infected strawberry fruit have also caused a 2.2−2.4-fold higher level of total flavanols compared to healthy fruit (Table 2). In green walnut husks higher accumulation has been observed in tissue infected with *Xaj* (Table 2). These results are in line with other studies (Mikulič-Petkovšek et al., 2011; Slatnar et al., 2012), which report a higher content of flavanols in plant tissue triggered by different pathogen infections. According to some studies, an increased synthesis and accumulation of flavanols in plant tissues could be linked to their higher resistance (Leser and Treutter, 2005). The role of catechin in the plants pathogen defense might be their interaction with proteins and inhibition of the enzymes secreted by pathogenic fungi (Bors and Michel, 2002). It has been suggested that a rapid accumulation of monomeric and polymeric flavanols at the infection site stops further dissemination of the pathogens (Treutter and Feucht 1990; Mayr et al., 1997).

Table 1: The content of different phenolic groups in healthy and infected French green bean pods and apple peel

	French green bean pods (µg/g DW)			apple peel (mg/kg FW)		
	healthy	lesion	boundary zone	healthy	spot	boundary zone
Hydroxycinnamic acids	203.7 ± 16.7 a*	379.9 ± 46.4 b	899.1 ± 81.97 c	196.4 ± 20.3 a*	349.0 ± 37.1 b	964.5 ± 38.2 c
Flavanols	1347.0 ± 159.4 a	4348.3 ± 659.5 a	14308.7 ± 1360.18 b	863.9 ± 58.8 a	2594.6 ± 98.0 c	1161.0 ± 102.3 b
Flavonols	377.5 ± 61.3 a	477.0 ± 86.2 a	1039.9 ± 101.56 b	3276.6 ± 456.0 a	2769.1 ± 91.8 a	2319.5 ± 100.0 a
Dihydrochalcones	25.2 ± 4.8 a	45.0 ± 5.1 a	179.5 ± 22.62 b	534.3 ± 62.2 a	1254.9 ± 60.4 b	831.6 ± 17.1 c

DW – dry weight; FW – fresh weight
*Different letters denote statistically significant differences among treatments for each individual phenolic group and plant.

Scab symptomatic spot contained more than twofold higher levels of total flavonols compared to healthy apple peel (Table 1). The infection with *C. lindemuthianum* caused an increase of total flavonols in bordering and infected tissue: a 1.3–3.3 fold higher content was measured compared to healthy bean tissue (Table 1). The results of our study indicate that infected strawberry fruit contained significantly higher levels of flavonols (Table 2). Similar to other analyzed phenols the infection with the *Xaj* bacteria caused an increased synthesis of flavonols (Table 2). A decrease of total quercetin glycosides has been measured in infected raspberry cane tissue, which could potentially occur at the expense of an increase in flavanol levels in infected tissue, as quercetin glycosides are precursors for the synthesis of mono- and poly-meric flavanols and are intensively consumed in the phenylpropanoid pathway. The accumulation of high flavonol amounts in the apple skin is probably related to the physiological function of UV protection but may also be related to pathogen defense (Dixon and Paiva, 1995). Shirley (1996) reported that flavonols play an essential role in plant defense against bacterial and viral pathogens, as they have been known to induce the virulence genes of *Agrobacterium tumefaciens* Smith & Townsend. Moreover, an increase in flavonoids due to the infection with pathogens has been reported in other fruit species (Mikulič-Petkovšek et al., 2008). In response to scab infection, cells surrounding the infection site accumulate flavonols, forming a barrier to the progression of the infection (Feuch and Treutter, 1998). Rusjan et al. (2012) on contrary reported that infection with bois noir phytoplasma caused a statistically significant decrease in flavonol content, probably due to formation of other compounds competing for the same substrate.

Our study on green bean pods indicated that the infected lesion and bordering tissue contained higher levels of total dihydrochalcones compared to healthy bean pod tissue (Table 1). Statistically higher content of dihydrochalcones has also been observed in the apple scab symptomatic spot and in the surrounding tissue compared to the healthy apple peel (Table 1). An involvement of dihydrochalcones in pathogen defense is frequently disputed because all apple cultivars generally contain high levels of phloridzin. However, differences in disease resistance could be determined by divergent local accumulation or by differences in the speed of the oxidation cascade (hypersensitive reaction) after pathogen attack rather than the actual amount of phloridzin present in the plant (Gosch et al., 2010). MacHardy (1996) and Hrazdina et al. (1997) reported that phloridzin produced in apple in response to *Venturia inaequalis*, accumulated around the infection sites and assumed that the corresponding aglycone phloretin, which is generated by plant glucosidases after pathogen attack, negatively affects the pathogen growth. The hypothesis that the aglycone rather than the 2'-*O*-glucoside is involved in the pathogen defense has further been confirmed by Slatnar *et al.* (2010) who determined a decrease of UDP-glucose: phloretin 2'-*O*-glycosyltransferase activity in the scab symptomatic spot compared to healthy tissue, which contrasted with the observed increase of other flavonoid enzymes.

Table 2: The content of different phenolic groups in healthy and infected raspberry canes, green walnut husks or strawberry fruit

	raspberry canes (mg/kg FW)	
	healthy	infected
Hydroxycinnamic acids	90.1 ± 4.1 b*	67.5 ± 3.3 a
Flavanols	8906.0 ± 635.6 a	15093.4 ± 760.9 b
Flavonols	735.4 ± 65.9 b	229.9 ± 34.4 a
Ellagic acid derivatives	362.7 ± 28.3 b	310.8 ± 12.3 a
	green walnut husks (mg/kg FW)	
	healthy	infected
Hydroxycinnamic acids	499.0 ± 4.1 a*	1912.5 ± 63.3 b
Flavanols	172.9 ± 15.6 a	2909.4 ± 150.9 b
Flavonols	192.2 ± 12.9 a	459.1 ± 34.4 b
	strawberry fruit (mg/kg FW)	
	healthy	infected
Ellagic acid derivatives	37.0 ± 2.9 a*	65.9 ± 4.5 b
Flavanols	559.9 ± 50.8 a	1237.2 ± 75.9 b
Flavonols	19.0 ± 0.2 a	34.9 ± 3.3 b
Anthocyanins	373.3 ± 27.5 b	308.1 ± 36.9 a

FW – fresh weight

*Different letters denote statistically significant differences between treatments for each individual phenolic group and plant.

Results indicate a significantly higher content of ellagic acid derivatives in healthy raspberry canes compared to the infected tissue (Table 2). Our results on strawberry fruit demonstrate that the infection causes an increase of ellagic acid derivatives (Table 2). Higher content of ellagic acid derivatives can be ascribed to modifications in the metabolic processes within the plant as several studies report increased accumulation of specific compounds as a defense mechanism to stress. With up-regulation of certain metabolic processes, the plant aims to stop or at least reduce the growth of the pathogen, and ellagic acid and its derivatives are known to possess antimicrobial activity (Zhou et al., 2007; Quare et al., 2012).

4 CONCLUSIONS

Although a large number of phenolics from different metabolic groups has been identified and quantified in various plants the resistance mechanisms cannot solely be explained by plant's phenolic profiles. Results support the hypothesis that phenolic compounds play an important role in host defence in infected tissue and that the mechanism of resistance may be influenced by responses linked to the host−pathogen interaction. However, it is obvious that the defense reaction is not enough to overcome the disease. In addition to polyphenols, other biochemical compounds are potentially involved in defense response. Additional studies may help clarify the precise mechanisms, processes, and important resistance indicators within the plants, which take place after the pathogen attack. Specific studies are of great importance to help minimize the negative effects of fungal diseases in fruit/vegetable production which cause large yield losses due to their fast spread, ineffective chemical treatments, and long withdrawal periods of fungicides.

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Production of vaccines for treatment of infectious diseases by transgenic plants

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ABSTRACT

Since the first pathogen antigen was expressed in transgenic plants with the aim of producing edible vaccine in early 1990s, transgenic plants have become a well-established expression system for production of alternative vaccines against various human and animal infectious diseases. The main focus of plant expression systems in the last five years has been on improving expression of well-studied antigens such as porcine reproductive and respiratory syndrome (PRRSV), bovine viral diarrhea disease virus (BVDV), footh and mouth disease virus (FMDV), hepatitis B surface antigen (HBsAg), rabies G protein, rotavirus, Newcastle disease virus (NDV), Norwalk virus capsid protein (NVCP), avian influenza virus H5N1, *Escherichia coli* heat-labile enterotoxin subunit B (LT-B), cholera toxin B (CT-B), human immunodeficiency virus (HIV), artherosclerosis, ebola and anthrax. Significant increases in expression have been obtained using improved expression vectors, different plant species and transformation methods.

Key words: transgenic plants; vaccine production; recombinant proteins; antibodies; infectious diseases; risk assessment

IZVLEČEK

ZDRAVLJENJE NALEZLJIVIH BOLEZNI S CEPIVI PRIDOBLJENIMI S TRANSGENIMI RASTLINAMI

Pridobivanje aktivnih sestavin za cepiva s transgenimi rastlinami se je začelo v devetdesetih letih prejšnjega stoletja. Od takrat se transgene rastline uveljavljajo kot alternativni ekspresijski sistem za sintezo aktivnih komponent za cepiva proti številnim humanim in živalskim nalezljivim boleznim. V zadnjih petih letih je bil glavni poudarek razvoja namenjen optimizaciji rastlinskih ekspresijskih sistemov za dobro proučene antigene, kot so virus prašičjega respiratornega in reproduktivnega sindroma, virus goveje virusne diareje, virus parkljevke, površinski antigen hepatitisa B, G protein virusa stekline, rotavirus, virus atipične kokošje kuge, plaščni protein Norwalk virusa, sev ptičje gripe H5N1, temperaturno nestabilna B podenota enterotoksina *Escherischia coli*, kolera toksin B, HIV, arterioskleroza, ebola in antraks. Uporaba optimiziranih transformacijskih metod in ekspresijskih vektorjev v kombinaciji s primerno izbrano rastlinsko vrsto je v večini naštetih primerov vodila do opaznega izboljšanja sinteze posameznih učinkovin.

Ključne besede: transgene rastline; pridobivanje cepiv; rekombinantni proteini; protitelesa; nalezljive bolezni; ocena tveganja

1 INTRODUCTION

Vaccination is the most effective known way of preventing outbreaks and spreading of viral and bacterial infections. Traditional vaccines contained attenuated or inactivated pathogens, however the development of molecular biology, genetics, medicine and biotechnology in past decades resulted in many novel vaccine types and production systems. During the last 20 years, transgenic plants have started gaining importance as a potential alternative host system for expressing high amounts of safe, cheap, effective easily applicable oral vaccines. Some of the main

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advantages of plant-made vaccines are the absence of pathogens commonly found in mammalian, bacterial and yeast production systems, easier storage and transport and ability of inducing both systemic and mucosal immune response thus granting bigger protection against pathogen challenge. The main focus of the initial research as well as the recent development has been expression of various antigens belonging to agents of viral and bacterial infectious diseases of humans, animals or both. Most of the expressed antigens have been successfully evaluated in animal and human models. Besides that quite a lot of research has been done in the field of expressing antigens related with autoimmune diseases, such as artherosclerosis, diabetes type 1, rheumatoid arthritis and multiple sclerosis. The following article aims to cover some of the recent advances in expressing pathogen antigens in transgenic plants, transformation methods and vectors.

2 VIRAL VACCINES

2.1 Porcine reproductive and respiratory syndrome

Porcine reproductive and respiratory syndrome (PRRS) is a highly transmissible disease of sows and piglets, which was first reported in the US in 1987 and in Europe in 1990 (Chen and Liu, 2011) and has since spread to become one of the most devastating infectious diseases of swine industry nowadays, causing major economic losses in swine herds worldwide. Its cause is the porcine reproductive and respiratory syndrome virus (PRRSV). Currently, modified-live and inactivated (killed-virus) PRRSV vaccines are being used, but due to safety risks (revertion back to virulence) and inability of protecting against heterologous PRRSV infections of the first ones and weak effectiveness of the second ones, there's a great demand for development of novel vaccines (Chia et al., 2010; Hu et al., 2012; Renukaradhya et al., 2015). Porcine reproductive and respiratory syndrome GP5 glycoprotein 5 (PRRSV GP5), one of the main target antigens, was first expressed in transgenic tobacco plants in 2010, with expression levels reaching app. 0.011 % total soluble protein (TSP), despite the addition of endoplasmic reticulum (ER) retention signal KDEL and cauliflower mosaic virus (CaMV 35S) promoter. However, oral immunization with fresh leaf tissue triggered both PRRSV-specific humoral and cellular immune response (saliva IgA and systemic IgG) in mice (Chia et al., 2010). With the aim of increasing the expression level of GP5, Chia et al. (2011) later fused GP5 with Escherichia coli heat-labile toxin subunit B (LT-B) and additional endoplasmic reticulum targeting HDEL sequence, expressed it in *Nicotiana tabacum* under the control of ubiquitin promoter 1 (UBQ1). The immunogenicity of the obtained fusion protein was evaluated in orally immunized pigs. Expression level in this case was 0.0155 % TSP. LT-B-GP5 fusion protein was able to induce GP5-specific PRRSV mucosal and systemic immunity after intranasal PRRSV challenge at slightly higher rate than GP5 alone. GP5 protein was also successfully expressed in transgenic potato tubers (yields 0.8 – 1.2 μg/g) and leaves (yields 2.5 – 4.7 μg/g) (Chen and Liu, 2011) and induced the production of PRRSV-specific intestine IgA and serum IgG when applied orally as crude tuber extracts. Chan et al. (2013) expressed GP5 in transgenic banana leaf tissue, driven by CaMV 35S promoter with 3'-flanking Mh-UBQ1 matrix-associated regions (MARs) and HDEL, with yields 0.021 – 0.037 % of TSP (154 – 257 ng/g of fresh banana leaves). That is 2-3 times higher than in transgenic tobacco (0.018 - 0.0155 % of TSP) (Chan et al, 2013), while the levels of neutralizing antibody (NA) titres in orally immunized pigs 3 weeks post infection (WPI) were similar to those obtained in pigs orally immunized with GP5 from transgenic tobacco- 1:4 - 1:16 (Chia et al., 2011). Immunized pigs developed PRRSV-specific humoral and cellular immune response. Hu et al. (2012) expressed PRRSV M protein driven by UBQ promoter in transgenic corn calli and obtained strong consecutive expression as well as induction of humoral, mucosal and cellular immune responses in orally immunized mice (30 mg of dried corn powder with adjuvants). The highest M protein yield was 5.1 μg/g of fresh and 86 μg/g of lyophilized callus. Recently, Piron et al. (2014) expressed a whole Eu-prototype LV PRRSV antigenic set in seeds of *Arabidopsis thaliana*, with or without transmembrane domains, with added affinity tags or stabilizing protein domains- Fc IgG

chain or green fluorescent protein (GFP), regulatory sequences of *Phaseolus vulgaris*, N-terminal fusion of *A. thaliana* 2S albumin seed storage signal peptide and C-terminal ER-retention signal HDEL. Even though all antigens were found to be correctly N-glycosylated, further studies will be needed to determine whether or not they have really been processed in ER. Fusion of stabilizing protein domains to truncated GP3- GP5-Tm positively affected their accumulation, the most of all GFP fusion, its addition to truncated GP3 and GP4 resulted in accumulation of 2.74 % and 2.36 %, respectively, which is way above the lowest economically feasible level. Full-length Gp4 and GP5 accumulated only at levels 0.08 and 0.1 % of TSP, respectively, while fusion of stabilizing protein domain increased it up to 2.36 % of TSP for GP3 and GP4. Fusion of pFc and mFc2a to truncated antigens increased the accumulation (from 0.05 % to 0.67 % and 1.59 %, respectively) while addition of pFc to GP5 decreased the accumulation (from 0.10 % to 0.01 % of TSP). Addition of Fc domain also allowed for single-step protein-A affinity chromatography with recovery of about 60 % (Piron et al., 2014). All 3 tested antigens (GFP-fused truncated GP3, mFc2a-fused truncated Gp4 and normal-size-mFc2a-fused GP4) induced high GP4-Tm-specific Ab titres, only GP4-Tm:mFc2a elicited NA. In pigs, there were no NA detected when immunized with pFC-fused truncated GP3, GP4 and GP5 and 5 weeks after the second immunization only 1 pig had high serum Ab titres. Recently, Uribe-Campero et al. (2015) co-expressed PRRSV GP5, M and N protein in *Nicotiana silvestris* leaf tissue. *Agrobacterium*-mediated transformation was performed. The proteins assembled in virus-like particles (VLPs) of expected size (15, 19 and 25 kDa). When applied to mice by intraperitoneal immunization, the VLPs were shown to be immunogenic in mice.

2.2 Bovine viral diarrhea virus

Bovine viral diarrhea disease is an important cause of economic loss in global bovine herds, caused by bovine viral diarrhea virus (BVDV). Currently, inactivated vaccines are being used, however the E2 protein has been deemed the main target in developing novel subunit vaccines. Dus Santos and Widgorovitz (2005) produced transgenic alfalfa plants containing the gene for BVDV E2 glycoprotein under the control of CaMV 35S promoter with TEV and KDEL signal sequences, transported into alfalfa in recombinant binary pBI121-TEV-Stag-E2-His-SEKDEL vector by *A. tumefaciens* transformation method. The approximate concentration of the recombinant protein expressed in 2 highest yielding plants was estimated between 0.05 and 0.5 mg/g TSP. In 2012, Nelson et al. expressed a truncated version of BVDV glycoprotein E2 (tE2), lacking the transmembrane domain with additional ER-retention signal KDEL, 2S2 *A. thaliana* plant secretory pathway signal, the Kozak sequence and a hexa-histidine tag, in transgenic tobacco plants (*N. tabacum*). pK7GW2 *Agrobacterium* binary vector was transported into leaf tissue by *Agrobacterium*-mediated transformation. The protein accumulated at levels app. 1.3 % TSP (20 µg/g) which is suitable for mass scale production and elicited an immune response comparable to that of commercial whole virus vaccine in subcutaneously immunized guinea pigs either with oily or aqueous adjuvant (86 % seroconversion with anti-BVDV Ab titres > 0.6 and 75 % seroconversion, respectively). When administered with aqueous adjuvant, recombinant tE2 elicited NA titers equal or higher than titers elicited by conventional vaccine. A year later, Aguirreburualde et al. (2013) expressed a truncated E2 protein, fused to APCH gene, under the control of Cassava striate mosaic virus (CsMV) promoter in recombinant binary plasmid pCs-APCHtE2, transformed into alfalfa petioles by *Agrobacterium*-mediated co-cultivation. APCH-tE2 was stably expressed in alfalfa leaves with level of 0.1 % of TSP, which is a significant increase as compared with the previous work. The final yield of 3.45 µg/ml of Ag permitted the formulation of an effective vaccine for cattle immunization. Parentally immunized guinea pigs developed NA titres > 2.4 and immunized cattle developed NA titres > 2 at the time of vaccination (t60). After challenge, 6 out of 8 bovines inoculated with 3 µg of alfalfa-produced APCH-tE2 showed complete virological protection after virus challenge. Aguirreburualde et al. (2013) suspect the APCH signal sequence directs the protein secretion to the apoplast but further investigations of this are needed. Average expression level of APCH-tE2 was 3 µg/ml of extract. From 50 g of transgene leaf tissue 80 µg of APCH-tE2 can be produced after processing,

which suffices for 27 doses of vaccine for cattle (1 dose = 3 μg of APCH-tE2).

2.3 Foot and mouth disease virus

Foot and mouth disease is a highly contagious disease of cloven-foot animals and a major cause of economical loss in livestock. There are 7 different serotypes of the foot and mouth disease virus (FMDV), infection with any serotype doesn't confer immunity against the others (Zhang et al., 2011). Currently used attenuated and conventionally inactivated vaccines are not completely safe neither effective so there is a great focus in developing novel vaccines. Experimental vaccines containing FMDV antigens from transgenic plants have been reported safer than conventional vaccines; they are also cheaper and more effective. The first experimental plant-made FMDV vaccine was produced by Carillo et al. (1998), FMDV viral protein 1 (VP1) from transgenic *A. thaliana* plants. Later on there was a lot of production of complete or fractionated VP1 with the aim of producing an effective, highly immunogenic, easy to use and cheap plant-made FMDV Ag vaccine. VP1 epitope 135 - 160 has been expressed in transgenic alfalfa (Widgorovitz et al., 1999; Dus Santos et al., 2002; Dus Santos and Widgorovitz, 2005), *A. thaliana* (Carillo et al., 1998; Dus Santos and Widgorovitz, 2005) and potato (Dus Santos and Widgorovitz, 2005), structural polyproteins- precursors for empty FMDV capsids, P1-3C and P1-2A3C have been expressed in transgenic alfalfa (Dus Santos and Widgorovitz, 2005; Dus Santos et al., 2005) and tomato (Pan et al., 2008), respectively, and two serotypes of the structural FMDV VP1 protein, O- and Asia 1-type have been expressed in transgenic forage maize (Zhang et al., 2011). When used as an experimental vaccine for either oral immunization by feeding fresh plant tissue or parental, intraperitoneal vaccination with transgenic plant extracts VP1 epitope induced FMDV-specific systemic immune response and gave protection against FMDV infection after experimental viral FMDV challenge (Carillo et al., 1998; Widgorovitz et al., 1999; Dus Santos et al., 2002), recombinant protein yields however were low, between 0.01 and 0.2 mg/g in transgenic alfalfa (Dus Santos and Widgorovitz, 2005), the highest level of expression of FMDV VP1 135-160 epitope expressed in alfalfa being 0.5 - 1 mg/g of TSP, which was 10 times higher than in the cases of Widgorovitz et al. (1999) and Carillo et al. (1998). No significant differences in transformation success, protein expression or immunogenicity were observed with regards of using different *A. tumefaciens* strains or host systems. Dus Santos and Wisgorovitz (2005) expressed FMDV VP1 P1-3C in transgenic alfalfa with expression level 0.01 - 0.2 mg/g of TSP when binary pRok plasmid and *A. tumefaciens* strain C58C1 were used and with expression levels of 0.005 - 0.01 mg/g of TSP in another case, in both cases the obtained recombinant peptide induced strong Ab and NA response as well as complete virological protection in vaccinated mice. Pan et al. (2008) expressed P1-2A3C FMDV VP1 polyprotein in tomato, its expression was lower than that obtained in FMDV-infected animal cells despite the use of KDEL, Kozak sequence and 35S promoter; however the immunogenicity in intramuscularly immunized guinea pigs was still very high, with Ab titres 21 days after the third vaccination 0.8 – 2 log10. A FMDV-specific memory cell response was generated as well. When Zhang et al. (2011) expressed FMDV serotypes O- and Asia 1-type in transgenic forage maize, 3 - 5 transgene copies of VP1 fusion protein were found inserted in genomes of transformed plants and the transgene was found to be passed on to offspring. Ag induced protective systemic immune response when fed to animals.

2.4 Hepatitis B

Hepatitis B is one of the most frequent contagious diseases. It is caused by a hepatitis B virus (HBV) and mainly damages liver, can lead to liver cirrhosis or hepatocellular carcinoma. HBV is a retrovirus, one of the smallest eukaryotic DNA viruses (Guan et al., 2013). Negative HBV strand has 4 ORFs, *C*, *P*, *S* and *X* gene encoding four major viral proteins: core Ag (HBcAg/HBeAg), HBV DNA polymerase (HBV DNA P), surface Ag (HBsAg) and X Ag (HBxAg), respectively (Guan et al., 2013). Since the first reported plant-made HB Ag in *A. tumefaciens* strain LBA4404-transformated tobacco by Mason et al. (1992) there has been a huge research on expressing major HBV viral proteins. Recombinant HBsAg has been expressed in lupine and lettuce (Kapusta et al., 1999), carrot suspension cells (Zhao et al., 2002), soybean (Smith et al., 2002), peanut (Chen et al., 2002), tomato (Ma et al., 2002; Carolina and

Francisco, 2004; Wang and Li, 2008; Srinivas et al., 2008), cherry tomatillo (Gao et al., 2003; Guan et al., 2010), potato (Shulga et al., 2004), banana (Sunil-Kumar et al., 2005), apple core and leaves and tomato (Lou et al., 2005), NT-1 cells of tobacco (Sunil-Kumar et al, 2007) and lettuce (Kostrzak et al., 2011). Chen et al. (2002) reported a high level expression of HBsAg in peanut, obtained by *Agrobacterium*-mediated transformation, with the yield of 2.42 µg HBsAg/g of fresh weight. Oral immunization was shown to have triggered anti-HBsAg response both in mice (Kapusta et al., 1999; Gao et al., 2003) and humans (Kapusta et al., 1999; Thanavala et al., 2005). Many of HBsAg expression systems are still undergoing the process of development and optimisation and new plant species are being tested as possible expression systems. The most recent experiments include cucumber (Unni and Soniya, 2010), sandalwood embryogenic cell suspension cultures (Shekhawat et al., 2010), maize (Hayden et al., 2012; Hayden et al., 2015; Shah et al., 2015) and lettuce (Pniewski et al., 2011), as well as the most recent progress in using potato as host system (Rukavtsova et al., 2015). Even though the first effective parental hepatitis B vaccine by yeast has been developed more than 20 years ago, the vaccine still isn't accessible, effective or is cost-prohibitive for a lot of world's population, especially for areas that need it the most. A big problem is also the storage of the vaccine as the adjuvanted purified Ag protein loses its immunogenicity when frozen, thawed or stored for a week at 45 °C and in not thermostable (Shah et al., 2015). An oral, low-cost thermostable plant-made subunit vaccine could help overcome those problems. In 2011, Pniewski et al. expressed HBsAg in glufosinat-resistant transgenic lettuce to use as a prototype oral vaccine suitable for human immunization. Total HBsAg level in lyophilized tissue was 5 mg/g, whereas the mean content of VLP-structuralized Ag was 11 µg/g and in tablets 5 or 2.3 µg/g. Obtained vaccine consisted of 100 ng VLP-assembled Ag dosage, produced in transgenic lettuce and mice were orally immunized with lyophilized tissue without additional exogenous adjuvants. Two immunizations with 60-day interval in between them resulted in both mucosal and peripheral humoral immune response against HBV, with titres above the nominal protective titres (10 mIU/ml). When converted into tablets and stored at room temperature, HBsAg content in lyophilized tissue was preserved for a year. Even though the lyophilisation reduced the HBsAg content for at least 90 %, the low dosage was still efficient. In 2010, Unni and Soniya expressed HBsAg in transgenic salad cucumber (*Cucumis sativus* cv. 'Swarnamukhi') plants as a part of trials to find new vaccine-producing plants suitable for raw human consumption. Using *Agrobacterium*-mediated transformation of *C. sativus* cotiledonary leaf section they produced transgenic *C. sativus* plants where HBsAg was successfully introduced and transcribed, with T-DNA being present as single copy in some of the plants and as multiple copy in others, the transgene insertion pattern that is typical for *Agrobacterium*-mediated transformation. Soluble proteins extracted from transformed plants showed a distinct band with molecular weight expected for the HBsAg gene. Protein expression levels were low, 0.006 % of TSP (11.5-50 ng HBsAg/mg TSP), which was attributed to the absence of signal peptides for increasing protein accumulation, such as ER SEKDEL.

Besides using different plants, Shekhawat et al. (2010) expressed HBsAg in embryogenic cells suspension cultures of sandalwood (*Santalum album*). The team used *Agrobacterium*-mediated transformation technique, a C-terminal ER retention signal was added to hepatitis HBsAg gene and eight different growth medium aditives were tested with the aim of improving the HBsAg expression in transgenic cells. Maximum HBsAg expression in untreated transformed cells was 11.09 µg/g of fresh mass, while cells treated with 30 mM trehalose reached 19.95 µg/g, addition of 0.4 % DMSO increased the expression by 1.36-fold, addition of gibberellic acid (1.45 µM) by 1.48-fold, doubled concentration of $CaCl_2$ in basic MS medium by 1.28-fold and 0.001 % Tween-20 by 1.34-fold. The biggest increase was obtained by addition of 54.88 mM mannitol, with the increase of 1.7-fold. Due to bigger genetic stability of sandalwood as opposed to previously investigated soybean suspension cells (Smith et al., 2002) this study proved promising for further investigations of stable HBsAg expression in suspension cells of sandalwood.

The first report on successful expression and immunogenicity of bioencapsulated HBsAg was done in 2012 by Hayden et al., in maize, as the first

commercially feasible oral subunit vaccine production for a major disease. HBsAg expression was driven by either polyubiquitin or the embryo-preferred globulin 1 promoter and the effect of different signal sequences on the HBsAg accumulation in grains was tested. Accumulation of the HBsAg was the highest when driven by embryo-preferred globulin 1 promoter, with the highest yielding seed accumulating over 0.25 % TSP. Germ enrichment and oil extraction from the seeds of plants produced by backcrossing of the 2 of the highest-yielding plants resulted in the total of 166 µg HBsAg/g of dry maize material, 20-fold higher than in the highest previously reported case. Orally immunized mice elicited both strong mucosal and systemic immune response. As the next step towards maize-produced oral HBV vaccine, Hayden et al. (2015) recently carried out a study to determine whether or not oral delivery of maize-produced HBsAg doses establish long-termn immunologic memory in mice over a one year time period. Using the plant material from the previous study, Hayden et al. (2015) produced hybrid HBsAg seeds which were then processed into supercritical fluid CO2 extraction (SFE)-defatted wafers. Serum IgG, serum IgA and fecal IgA responses were detected in mice intramuscularly immunized with commercial HBV vaccine Recombivax and fed with SFE-defatted wafers as well as in mice immunized with Recombivax and immunized with it afterwards. Storage and processing of wafers resulted in the final wafers containing 567 µg HBsAg/g, 3-fold more than in the beginning. Total serum IgG (mIU/ml) showed clear evidence of immunologic memory over one year of oral and parenteral administration, geometric mean titres (mIU/ml) were high both in the case of HBsAg- and Recombivax-treated mice. Obtained HBsAg levels in seeds and resulting wafers are the highest reported Ag concentrations in plants to date. In the same year, the biochemical and biophysical characteristics of SFE-defatted maize-derived HBsAg were evaluated (Shah et al., 2015). Expression of HBsAg in plants has been recently enhanced by Hayden et al (2014) by fusing HBsAg to barley alpha amylase signal sequence (BAAS) and preferentially expressing it in the germ fraction of the hybrid maize seed, which resulted in HBsAg expression at a level of 1023 µg/g, the highest reported HBsAg in plants up-to-date. Recently, Rukavtsova et al. (2015) tested the immunogenicity of marker-free transgenic potato-produced HBsAg in mice. The team developed a novel way of constructing a transgenic marker-free plant, inserting the HBsAg (driven by 35S CaMV promoter) into the marker-free pBM vector and performed *Agrobacterium*-mediated transformation. HBsAg level in the tubers of selected transgenic potato lines was up to 1 µg/g of wet weight. Existence of HBsAg multimers was confirmed by gel filtration. Anti-HBsAg levels in mice fed with transgenic potato tubers began increasing on day 36-50 after the 1st feeding and by day 70 the Ab content was up to 170 mIU/ml. After being experimentally challenged with recombinant yeast hepatitis B vaccine, that content rose up to 350 mIU/ml within 15-34 days after infection in orally immunized mice, staying high above the minimum protection level even 120 days after the start of the experiment (up to 100 mIU/ml). When having been given additional 3 doses of HBsAg (320 days after the start of the experiment), the HBsAg Ab levels in serum of previously immunized mice increased up to 185 mIU/ml, with animals preserving stable immunological memory of the infection. This was the first experiment using transgenic plants as a source of hepatitis B edible vaccine that lasted longer than 24-38 weeks, the way that was normal in similar experiments of other authors.

2.5 Rabies

Rabies is a globally widespread infectious disease affecting central nervous system in both animals and humans. It is caused by the Rabies virus. Despite the long research history it still remains incurable while vaccination is used as a mean of its prevention. Vaccines currently available on the market are obtained by propagating fixed Rabies virus strains in various animal cell cultures or chicken embryos (Starodubova et al., 2015). Despite being safe and efficacious, vaccines produced in such way can greatly vary between different producers, their efficiency can be significantly reduced by storage and transportation, are very costly, can sometimes cause adverse side effects and require 3 - 5 doses to provide sufficient immune protection (Rosales-Mendoza, 2015). In the last 20 years there has been a lot of research and progress in developing the fourth generation of rabies vaccines based on rabies glycoprotein (G protein) (Starodubova et al., 2015). Neutralizing Abs against G protein have been shown to be

capable of preventing rabies infection. The pioneering work in expressing G protein in transgenic plants was done in 1995 by McGarvey et al. by expressing an unmodified G protein gene under the control of the CaMV 35S promoter in *Agrobacterium*-transformated tomato leaves and fruits. Since then, G protein has been expressed on its own in plants such as tobacco (Modelska et al., 1997; Yusibov et al., 1997; Ashraf et al., 2005,), spinach (Modelska et al., 1997), carrot (Anaya et al., 2009) and maize (Loza-Rubio et al., 2012). A full-length rabies virus nucleoprotein has also been expressed in tomato (Perea Arango et al., 2008), cholera toxin subunit B (CT-B)-rabies glycoprotein fusion protein has been expressed in tobacco (Roy et al., 2010) and tobacco seeds (Tiwari et al., 2009), chimeric peptide containing rabies G protein and N protein determinants was expressed in tobacco and spinach (Yusibov et al., 2002) and a rabies glycoprotein- ricin toxin B chain fusion protein has been expressed in tomato hairy roots (Sigh et al., 2015). A similar expression approach that was used by Ashraf et al. (2005) to express a synthetic, codon-optimised G protein gene in tobacco for the first time. Native signal peptide was replaced by *N. tabacum* pathogenesis-related protein PR-S and an ER KDEL signal sequence was added to the C-terminus of the G protein gene. Selected nuclearly transformed plants expressed G protein at 0.38 % TSP in leaves. Intraperitoneal immunization of mice resulted in protective immunity against intracerebral challenge with live rabies virus. In 2012, Loza-Rubio et al. expressed G protein in maize seeds and evaluated its immunogenicity in polygastric species for the first time. As a transformation method biolistics was used. The average expression level in obtained transgenic plants was 25 µg/g of fresh tissue, equalizing about 1.4 % TSP, which is the same amount as was obtained in carrot by Anaya et al. (2009). When orally immunized with 2 mg of obtained G protein one single time, sheep (the main victims of rabies infections in Latin America) elicited anti-rabies neutralizing serum Ab and a protective immunity against rabies virus infection with a survival rate of 83 % was obtained, the same as the efficiency obtained by inactivated commercial vaccine (Loza-Rubio et al., 2012). Apart from G protein, a full-length rabies virus nucleoprotein (N protein) was transiently expressed in *Agrobacterium*-transformated tomato and *N. benthamiana* plants in 2008 for the first time (Perea Arango et al., 2008). N protein was expressed at levels ranging from 0.1 - 0.5 mg/g to 1 - 4 % TSP in tomato fruit and up to 4 mg/g and 45 % TSP in *N. benthamiana* leaves and it was found to have induced Ab production in both orally and intraperitoneally immunized mice, whereas only 50 % of the latter were protected against a peripheral viral challenge. Yusibov et al. (2002) expressed G and N protein in the form of a chimeric peptide together with tobacco virus mosaic proteins in tobacco and spinach. Parenterally immunized mice showed immunity to rabies virus and an anti-rabies immune response was elicited in human volunteers orally immunized by transgenic chimeric peptide from spinach. With the aim of making it more effective, rabies virus proteins have been expressed as fusion proteins with well-known potent mucosal adjuvants. Rabies-CT-B fusion protein has been expressed in tobacco (Tiwari et al., 2009; Roy et al., 2010). In the case of Roy et al. (2010) accumulation level was 0.4 % TSP, fusion protein formed pentameric protein which was biologically active in binding to the GM1ganglioside receptor. However its *in vivo* activity and immunogenicity have not been tested. Recently, Singh et al. (2015) expressed rabies virus glycoprotein fused to ricin toxin B chain in tomato hairy roots. Fusion gene construct contained N-terminal ER-trageting Calreticuline signal sequence from tobacco and C-terminal ER-retention signal SEKDEL and was under control of CaMV 35S double enhancer promoter. Its expression was between 1.4 and 8 µg/g of tissue, with the highest yield being 1.14 % TSP. The highest yielding tomato line, containing single copy of the transgene stably integrated into nuclear genome, was used in bench-top bioreactor for optimization of scale-up process. By optimizing the cultivation parameters over the span of 3 weeks, the team managed to increase the growth rate in the bioreactor 49.3-fold. Partially purified fusion protein was capable of inducing immune response in intra-mucosally immunized mice (Th2 lymphocite immune response) but due to the low titres of Abs protein quantity should be further optimized in order to obtain better responses both after the primal and booster dose. The use of hairy roots for production of vaccine antigens offers several advantages to their expression in whole plants: the ability to generate plants and store their germplasm long-term, the absence of toxic

compounds such as alkaloids, obtaining large quantities of protein in short time etc.

2.6 Rotavirus

Rotaviruses are the main cause of gastroenteritises in children and animal offspring, causing severe annual economic loses (especially) in cattle; over 500 000 annual deaths are estimated in humans, mainly in children living in developing countries in South Asia and sub-Saharan Africa due to insufficient hygienic conditions. Triple-layered rotavirus virions contain a genome of 11 dsRNA encoding 6 structural and 6 non-structural proteins. The capsid has 3 layers, named VP2, VP6 and VP7 (the outermost) with VP4 protein forming spikes on its surface. In the host intestine VP4 gets cleaved to VP5 and VP8* (Pera et al., 2015), especially the latter being highly immunogenic and playing a major role in rotavirus infectivity. The outer capsid layer, VP7, also contains several epitopes, important for viral activity. Therefore, VP7 and VP4 and its derivatives VP8* and VP5 were chosen as main candidate antigens for subunit vaccine development (Bergeron-Sandoval et al., 2011). Rotaviruses are divided into 7 groups (A-G). Group A contains 4 sub-groups based on the antigenic properties of VP6. Currently there are around 140 different genotypes of the human rotavirus described (genotype G, P or G/P). For human rotavirus there are currently two live vaccines available, monovalent vaccine based on the most common serotype G1P[8] and pentavalentG1-G4 P[8] vaccine. Due to their expensiveness making them unavailable to the majority of the population in the developing world and the recent emergence of several novel serotypes there is however a big demand for developing new and better rotavirus vaccines (Pera et al., 2015), an aim for which also transgenic plants have been utilized a lot during the recent years. Rotavirus capsid proteins have been expressed by various research groups: VP6 has been expressed in tomato (Chung et al., 2000; Chung et al., 2001), alfalfa (Dong et al., 2005), potato (Yu and Langridge, 2003), VP7 has been expressed in potato (Wu et al., 2003; Choi et al., 2005), VP8* in transplastomic tobacco plants (Lentz et al., 2011), bovine VP8* in potato (Matsumura et al., 2002) and *N. benthamiana* in recombinant form (Perez-Filgueira et al., 2004a). In addition, NSP4-CT-B fusion protein has been expressed in potato (Arakawa et al., 2001; Kim and Langridge, 2004), VP6-BSSV fusion protein in *Chenopodium amaranticolor* (Zhou et al., 2010) and VP2, VP6 and VP7 have been co-expressed in the form of VLPs in tobacco (Yang et al., 2011). These experiments proved that rotavirus coat proteins can be successfully expressed in various plant tissues where they accumulate to relatively high levels, as high as 0.28 % TSP of VP6 in tobacco (Dong et al., 2005) and 1.5 % TSP of rotavirus VLPs in tobacco (Yang et al., 2011). Obtained proteins were also capable of inducing both mucosal and systemic immune response in orally immunized mice as well as provide passive immunity against rotavirus challenge in suckling mice born to immunized mother: 60 % of pups in the case of VP6-BSSV from *C. amaranticolor* (Zhou et al., 2010), 85 % of pups in case of bovine VP8* from transplantomic tobacco (Lentz et al., 2011) and bovine VP8* from tobacco (Perez-Filgueira et al., 2004a). The most recent research includes transient expression of a single antigen and dimeric combinations of human VP7 and truncated VP4 fused with fljB flagelling subunit from *Salmonella typhimurium* in *N. benthamiana* (Bergeron-Sandoval et al., 2011) and a vaccine candidate against the newly emerged G9P[6] strain, produced by transient expression in *N. benthamiana* (Pera et al., 2015). Bergeron-Sandoval et al. (2011) expressed codon-optimized full-length VP7, truncated VP4 and dimeric combinations of VP7, VP4 and fljB flagellin fused to 5'UTR of tobacco etch virus which works as a transcription enhancer, in leaves of *N. benthamiana* with relatively high yields: 5 µg/g for truncated VP4, 4 µg/g for flagellin, 31.97 µg/g for VP7-flagellin dimer and 12.3 µg/g for VP4-flagellin dimer. In the case of VP7 and VP7-VP4 dimer there was mild leaf necrosis whereas all the other proteins were stably expressed. The amount of obtained VP4 was fit to the amount of VP8* previously obtained in *N. benthamiana* plants when TMV-based vector was used for transformation (Perez-Filgueira et al., 2004a). Expression of VP7 however was much lower than that previously reported in transgenic potato tubers (40 µg/g) (Li et al., 2006), probably due to differences between the plant systems used, but the system tested in this report provides much faster and efficient production of antigenic proteins than systems using stable transformation. As can be seen, it is possible that fusion of VP proteins with

flagellin results in much higher protein expression, which could be used in production of rotavirus vaccines. Mice, subcutaneously immunized by VP4 and VP7, generated weak immune responses against rotavirus while mice immunized by dimeric fusion proteins generated strong, anti-flagellin immune response. As flagellin is known to have protected mice against chemical, bacterial, viral and radiation challenge without adverse side effects and induces many different cytoprotectant activities, Bergeron-Sandoval et al. (2011) propose it as a promising candidate for vaccine adjuvant. Pera et al. (2015) in term successfully expressed VP2 and VP6 in the form of VLPs in *N. benthamiana* by transient *Agrobacterium*-mediated transformation. Because the expression of VP7 and VP4 was not successful, the team tried to produce more appropriately immunogenic particles and created 3 fusion proteins, adding VP8* to the VP6 and co-expressing it with VP2. The team tested the effect of different ODs, intracellular targeting and tomato spotted wilt virus silencing suppressor protein (NSs) on protein expression and yield; they also used the tomato spotted wilt virus suppressor protein in order to optimise the expression. VP6 was successfully expressed in cytoplasm (where the addition of NSs enhanced the accumulation), chloroplasts (where the NSs addition reduced the accumulation), and apoplast and ER (where the NSs addition increased the accumulation on day 3 out of 7, the day on which the expression was at the maximal level). The addition of sequences, targeting the VP6 to intracellular compartments, did not significantly increase its accumulation. VP2 was expressed in cytoplasm, ER, apoplast and chloroplasts only in addition of NSs, at significantly lower average level than VP6. VP7 and VP4 have not been successfully expressed at all. The expression of VP8*/VP6 was only successful when binary plant vector pEAQ-HT was used, but not when plasmids from pTRA family were used. The highly immunogenic epitope of VP8* was fused to either N- or C-terminal end of VP6 and successfully expressed in cytoplasm, whereas VP6/8*C was expressed faster and at higher levels than VP6/8*N. VP2 was co-expressed with all the proteins, expressed in cytoplasm, and was found to be expressed at the highest level when co-expressed with VP6 (Pera et al., 2015). The fusion proteins did not form VLPs but based on previous studies by other authors the team suspects they could still retain their high immunogenicity, which would have to be evaluated in animal experiments.

2.7 Newcastle disease virus

Newcastle disease virus (NDV) is the cause of an economically important avian diseases, heavily affecting global poultry industry. The main candidates for developing a vaccine against the disease are fusion protein (F) and hemagglutining-neuraminidase protein (HN) since they are both exposed on the surface of the virus and take part in the infection. HN protein has been expressed in *N. benthamiana* (Gomez et al., 2009; Lai et al., 2013), potato (Berinstein et al., 2005) and tobacco (Hahn et al., 2007), F protein has been expressed in transgenic rice (Yang et al., 2007), potato (Berinstein et al., 2005) and maize (Guerrero-Andale et al., 2006). These experiments proved that HN and F proteins can be successfully expressed in transgenic plants and that obtained proteins are immunogenic when delivered to either mice or chickens. Both maize-produced NDV F protein (Guerrero-Andrade et al., 2006) and tobacco-produced HN protein (Hahn et al., 2007) have been found to be immunogenic in orally immunized chickens; oral immunization with F protein gave 100 % protection of chickens against nasal challenge with NDV, while in the case of HN protein protection against nasal NDV challenge failed but the authors reported a big increase in anti-HN serum IgG levels 28 days after vaccination, increased by 2-fold in 40 % of examined chickens and 4-fold in 20 % of examined chickens (Hahn et al., 2007). HN and F proteins, co-expressed in potato, have been shown to elicit anti-NDV antibodies in intraperitoneally immunized mice and serum IgG at levels comparable to those elicited in mice fed with non-transformed plants soaked in NDV (Berinstein et al., 2005), while the intestinal fluids of those mice also showed considerable IgA and IgG levels. F protein, stably expressed under ubiquitin promoter or rice glutelin promoter in rice, elicited anti-NDV serum IgG in intraperitoneally immunized mice (Yang et al., 2007). From the point of view of expression level for each of the expressed protein it was noted that F protein was expressed higher in maize kernels than in rice (0.9 - 3 % TSP and 0.0025 - 0.0055 % TSP, respectively) (Guerrero-Andrade et al., 2006; Yang et al., 2007), while the expression of HN protein was higher when co-expressed with F protein in potato than when

expressed on its own in tobacco (0.3 - 0.6 µg/g total leaf protein and 0.069 % TSP, respectively) (Berinstein et al., 2005; Hahn et al., 2007). When transiently expressed in *N. benthamiana* as a pSPhnHN/KDEL, under the control of rubisco small subunit promoter, fused to its own KDEL ER retention signal, recombinant pSPhnHN/KDEL protein accumulated to the highest levels from all of the 5 tested constructs (Gomez et al., 2009). When potato was transformed with pSPhnHN/KDEL as described by Berinstein et al. (2005), the protein expression was as high as 3 µg/g of total leaf protein, which is 10-fold higher than levels obtained by expression of HN under the control of CaMV 35S promoter in potato (Berinstein et al., 2005). In 2006, US Department of Agriculture Center for Veterinary Biologics gave regulatory approval and a license to Dow Agro Sciences LLC for an injectable vaccine against NDV based on HN protein produced in tobacco suspension cell line. In a proof-of-concept study, two subsequent immunizations gave 90 % protection of birds against a lethal virus challenge (Gomez-Lim, 2014). This vaccine is the first fully-licensed plant-cell-produced vaccine for animals in the United States and the first plant-made product to be licensed by USDA's Animal and Plant Health Inspection Service (USDA …, 2006). The company has, however, shown no interest in commercialization of the vaccine up to date. With the aim of improving the HN expression in plant host system, Lai et al. (2013) recently expressed functional ectodomain of HN protein (eHN) from NDV strain AF2240 in *N. tabacum* BY-2 cells, which had been proven suitable for production of various recombinant proteins. The eHN translation was enhanced by 5'-UTR region of *N. tabacum* alcohol dehydrogenase gene driven by CaMV 35S promoter. An eHN cDNA was successfully integrated into plant cell genome and actively transcribed. For all 8 transformants showing the highest levels of eHN mRNA transcripts, immunoblot detected a protein band the size of 66 kDa, corresponding to the predicted eHN molecular weight. The eHN protein expression was stable and accounted up to 0.2 - 0.4 % TSP, significantly more than in previous studies (Hahn et al., 2009; Gomez et al., 2009) using strong constitutive promoter and translation enhancer sequence. Localization of eHN-GFP in BY-2 protoplasts showed predominant accumulation of the protein in cytosol. Mice, intraperitoneally immunized with 5 µg of purified plant-produced eHN in incomplete Freund adjuvant 4 times, elicited anti-NDV antibodies, immune response enhancing with each additional boost and reaching the peak by day 67 (Lai et al., 2013). Similar responses were obtained in mice immunized with full-length potato- and tobacco-produced HN (Berinstein et al., 2005; Hahn et al, 2007, respectively). These results proved that truncated HN protein, lacking the transmembrane domain, still maintains its biological and functional activity in murine model and is capable of inducing an immune response closely resembling that of the native protein, so it can serve as a promising candidate antigen for development of a NDV subunit plant-made vaccine.

2.8 Norwalk virus capsid protein

Norwalk virus is the cause of epidemic acute gastroenteritis in humans both in developed and developing countries. It spreads via water, food and human contact. 42 % of acute epidemic gastroenteritis in the United States is estimated to be caused by Norwalk virus (Tang and Page, 2013). Currently there is no Norwalk virus vaccine available. The main candidate antigen for its development is, however, Norwalk virus capsid protein (NVCP), which has been expressed in the form of virus-like particles (VLPs) in potato and tobacco (Mason et al., 1996), potato tubers (Tacket et al., 2000), tomato fruit (Huang et al., 2005), *Nicotiana benthamiana* (Santi et al., 2008; Huang et al., 2009; Souza et al., 2013) and lettuce (Lai et al., 2012). Immunogenicity of produced VLPs has been evaluated in mice, fed by gavage (Mason et al., 1996), human volunteers (Tacket et al., 2000) and orally immunized mice (Huang et al., 2005; Santi et al., 2008). Evaluated VLPs have been found capable of inducing sufficient antigen-specific systemic and mucosal immune response, even in the case when freeze-dried transgenic NVCP tomato fruit was used (Huang et al., 2005). Protein accumulation in transgenic tomato was much higher than in potato tubers, 26.7 µg/g in tomato fruit and 1.4 -3.6 µg/g in potato tubers (Huang et al., 2005; Tacket et al., 2000, respectively). The best expression of the NVCP was obtained when the first two Met residues of the N-terminus had been eliminated from the native NVCP (Huang et al., 2005). Santi et al. (2008) developed an efficient tobacco mosaic

virus- derived transient expression system and successfully expressed NVCP in leaves of *N. benthamiana* at levels 0.8 mg/g 12 days post-infection as a more rapid alternative to expressing NVCP in plants and found it to be immunogenic in orally immunized mice. The procedure of expressing NVCP VLPs has been further improved by Huang et al. (2009) who optimized geminivirus-derived DNA replicon vectors for expression of hepatitis B core antigen and NVCP in *N. benthamiana* leaves. Using bean yellow dwarf virus-derived vector and Rep/RepA-supplying vector for agroinfiltration of leaves and co-expressing the tomato bush stunt virus P19 protein as a mRNA stabilizator the team was able to greatly enhance the transgene expression and shorten the time needed for the final product, thereby producing as much as 0.34 mg/g NVCP VLPs in 5 days' time. The same system was tested in lettuce by Lai et al. (2012). NDCP was found correctly expressed in lettuce leaves, with the highest accumulation at 4 days post-infection and an average expression level of 0.2 mg/g. This is comparable to the results of Huang et al. (2009) and is the highest expression level of any non-chloroplast-derived vaccine component ever reported in lettuce plants (Lai et al., 2012). Recently, Souza et al. (2013) tried to optimize expression of norovirus-like particles, such as NVCP-VLPs, in plants, utilizing a binary vector and co-expression of PTGS suppressor to increase the target protein yield. In the series of experiments in *Agrobacterium*-transformed *N. benthamiana* leaves, the team first examined the effect of 4 post-transcriptional gene silencin (PTGS) suppressors on protein expression with GFP as a reporter in *N. benthamiana* co-infiltrated with PTGS and GFP, recording the observed amount of GFP. The most effective PTGS, 126 kDa Pepper mild virus protein, was then used for co-expression with major and minor capsid genes of NVCP (vp1 and vp2, respectively) with a 3'UTR. Obtained VLPs were purified by sucrose gradient centrifugation. While the previous studies all used so-called magnICON viral vectors and transient expression systems, this is the first trial of expressing inserts, longer than just vp1 in plant expression systems. Even though the team used *N. benthamiana* -optimized codons, the expression level remained the same as that of vp1 isolated from the virus itself. Tang and Page (2013) adapted the glucocorticoid-inducible expression system developed by Aoyama and Chua (1997) and Ouwerkerk et al. (2001), to express NVCP in *Agrobacterium*-transformated cell suspension cultures of tobacco, rice, slash pine and cotton, using dexamethasone (DEX) as an expression inducer. Transformation vector contained hygromycin phosphotranspherase, chimeric transcription factor GVG and NVCP gene. For each plant species, the effect of different dexamethasone concentrations (1.25, 2.5, 5, 10, 20 and 40 mg/l) on transgene expression have been tested. For all concentrations there was clear transgene expression 2 days after the addition of the inducer to the medium, and no expression of the transgene was found in plants with no inducer added. Maximum NVCP expression was obtained at 10 mg/g DEX; greater concentrations were deleterious to transgene expression.

2.9 HIV

Human immunodeficiency virus type 1 (HIV-1) is the cause of AIDS (acquired immunodeficiency syndrome), a deadly infectious global disease, affecting more than 34 million people worldwide (Lotter-Stark et al., 2012). The great genetic variety among HIV-1 subtypes, high mutation rate and biological properties of HIV-1 regulatory proteins by avoiding immune response (Cueno et al., 2010) significantly worsen the process of developing an efficient vaccine. Various HIV proteins have been expressed in plant systems so far: HIV-1 p24 in tobacco (Zhang et al., 2002; Perez-Filgueira et al., 2004b) and *A. thaliana* (Lindh et al., 2014), HIV 1 P55 Gag polyprotein in tobacco chloroplasts (Scotti et al., 2009) and tobacco plants both in full length and truncated form (Meyer et al., 2008), HIV-1 Tat in potato (Kim and Langridge, 2004a) and tomato (Cueno et al., 2010), HIV-1 Nef in tobacco (Marusic et al., 2007) and *N. benthamiana* (Lombardi et al., 2009; Circelli et al., 2010), HIV-1 CA capsid protein VLPs in *Lycium barbarum* (Du et al., 2004), HIV-1/HBV fusion protein has been expressed in tobacco (Greco et al., 2008; Guetard et al., 2008) and *A. thaliana* (Greco et al., 2008). Tat regulatory element of the simian-HIV-1 virus (SHIV 89.6 Tat) has also been expressed in potato (Kim and Langridge, 2004b), fused to cholera toxin subunit B. As an important early marker of HIV-1 infection p24 protein, a major component of the gag polyprotein, is a promising candidate for developing an oral vaccine used to offer immediate

protection of the gut-associated lymphoid tissue (GALT) against HIV-1 challenge. The first researches in expressing p24 in plants were done by Zhang et al. (2002), who stably expressed p24 gene cassette it in *Agrobacterium*-transformated tobacco with the average yield 3.5 mg/g leaf soluble protein and was proven antigenic by Western blot. Perez-Fligueira et al. (2004b) modified tobacco mosaic virus TMV-30B to express HIV-1 p24 in tobacco. The addition of 7 His residues to the C-terminal end of the transgene was used to transiently express HIV-1 p24 in tobacco leaves with yields as high as 2.5 mg of purified p24/25 g of leaf tissue, which would suffice for 20000 routine diagnostic tests for HIV-1 as carried out in accordance with the WHO standards. Rabbits, immunized with purified p24 diluted in buffer in the presence of Freund adjuvant, developed a specific anti-p24 humoral response following the second immunization and p24 was also proven functional when used for Western blot assay to confirm HIV-1 test results in a set of Zambian patients. So despite the denaturating conditions, IMAC columns, used to purify p24 expressed by TMV-p24-HISc modified vector, the protein retained its function and efficiency. In 2011, Gonzales-Rabade et al. reported a successful expression of HIV-1 p24 and HIV-1 p24-Nef fusion protein in the chloroplasts of transplastomic tobacco. Expression levels obtained in leaves were 4 - 40 % TSP. Oral immunization of mice by gavage, with CT-B as an adjuvant (ratio p24:CT-B 1:1, i.e. 10 µg of each), elicited strong p24-specific serum IgG responses, with IgG sub-clases found in sera of immunized mice after subcutaneous immunization indictating activation of humoral immune response as well. Recently, Lindh et al. (2014) carried out a series of experiments, using previously obtained transgenic carrots, expressing 90 ng/g FW, and high- and low-yielding *A. thaliana* (expressing 366 ng/g and 34 or 17 ng/g FW, respectively) were used for oral immunization of mice. Both plants were used in either fresh or freeze-dried form. 6/7 experiments contained fresh plant tissue and 1 freeze-dried, mice in 4/7 experiments were allowed to feed freely and 3 controlled by tube feeding. The effect of the plant tissue for all experiments was evaluated by ELISA, two weeks after the p24 intramuscular boost. In the case of carrot, high amounts of p24-specific IgG were found in sera of immunized mice 2 weeks after the boost. Immunization with freeze-dried *A. thaliana* by gastric tube did not result in detectable p24-specific Abs while immunization with fresh tissue gave results similar to those in the case of transgenic carrot. Interestingly, mice immunized with low-level p24 extract had higher response to the antigen boost while mice immunized with high-level p24 extract had much weaker immune response. When CT-B was added to the antigen, there was no serum immune response whatsoever. In the case of free feeding, anti-p24 IgG were observed in the serum of mice fed with low-level p24 *A. thaliana* after the p24 boost, while no anti-p24 IgG were detected in the serum of mice fed high-level p24 *A. thaliana*. This was the first dose-dependent HIV-1 p24 antigen delivery study using transgenic *Arabidopsis* (Lindh et al., 2014). Immunizations with fresh transgenic carrots (non-mandatory eating, free feeding, 720 ng p24/dose) induced anti-p24 IgG response in the serum, following the p24 boost, however the observed IgG levels were low. Fresh *A. thaliana* free feeding induced weak IgG response no matter which dose was delivered (28, 200, 620 ng or 2.2 µg p24/dose). Low-level p24 extract fed by tube (20 ng/dose) was more efficient than high-level extract (460 ng/dose), results supporting the threshold value of 20 ng of antigen as a minimum to induce detectable immune response, as described by Beyer et al. in 2007.

2.10 Avian influenza virus H5N1

Highly pathogenic avian influenza is a deadly disease that had initially been affecting only poultry, thus causing great economic losses, but has also been representing a serious risk for human health since the first outbreak of highly pathogenic influenza (H5N1) in humans in Hong Kong in 1997 (Guo et al., 2012) in Asian countries as well as in Europe and Africa. Development of safe, cost-effective and efficacious vaccines is thus needed to reduce the economic impact of the disease on agriculture and prevent possible future pandemic outbreaks. The cause of the disease is highly pathogenic influenza A virus subtype H5N1. The main targets for subunit vaccine development have been its principal surface antigen hemagglutinin (HA), which plays a major role in functional activity of the virus and is also a main target for neutralizing antibodies of the host, and the M2e viral peptide, the extracellular domain

of the viral protein M2, which is highly conserved between various influenza strains and therefore a promising candidate for a universal influenza vaccine. H5N1 HA has been expressed in *N. benthamiana* in full-length (Kalthoff et al., 2010) and in the form of VLPs (Landry et al., 2010), in *Lotus corniculatus* (Guo et al., 2012) and most recently, duckweed (Bertran et al., 2015). In addition, recombinant HA from A/Bar-headed Goose/Qinghai/1A/05 (clade 2.2) and A/Anhui/1/2005 (clade 2.3) have been expressed in *N. benthamiana* (Shoji et al., 2009). Kalthoff et al. (2010) were the first to evaluate the immunogenicity of plant-produced full-length HA in chicken. Using different magnICON vectors, HA was targeted either to cytosol, apoplast or chloroplasts and the apoplast-targeted HA was expressed at the highest level (0.3 g/kg of fresh leaf biomass), especially when tobacco calreticulin signal peptide was used. Chickens, intramuscullary immunized with vaccine containing HA with copolymer, catatonic lipid-DNA complex or Freund adjuvant, elicited strong neutralizing Ab responses against H5N1 and were protected against the lethal virus challenge. Landry et al. (2010) reported the ability of alum-adjuvanted H5N1 HA VLPs to induce cross-reactive anti-H5N1 antibodies in ferrets as well as good tolerance and immunogenicity of the experimental alum-adjuvanted vaccine in adult human volunteers in Phase I clinical trial. Immunization with HA did not trigger an immune response to plant-specific carbohydrate determinants neither were the known allergies to plant derivatives or presence of detectable anti-plant-specific glycans IgG levels lead to immune response against N-glycans present on the HA used in vaccine (Kalthoff et al., 2010). Recently, this founding was supported by the results of the research carried out by Ward et al. (2014), who evaluated the immune and possible allergic response to *N. benthamiana*-produced VLPs carrying HA from H5 or H1 influenza viruses, in 280/349 humans. Subjects were intramuscularly immunized with 1 (H1) or 2 (H5) doses of 5-45 µg HA/dose and monitored for 6 months. 34 % of them developed transient IgG and in some cases IgE against plant glyco-epitops but no hypersensitivity or allergy was observed. Guo et al. (2012) were the first to successfully express biologically functional H5N1 HA in *L. corniculatus*, the gene expression being driven by CaMV 35S promoter, using *Agrobacterium*-mediated transformation. Transformation frequency in successfully transformed cotyledon fragments was 58.8 % as determined by PCR or Southern blot analysis, while transformation of hypocotyl gave no results. Using pBI-MARS-HA plasmid resulted in HA expression with the maximum of 0.00786 % TSP, much higher than in the case of pBI-HA plasmid (0.00408 % TSP), which, considering the use of *L. corniculatus*-optimized codons, MARS and CaMV 35S promoter, is still very low. Recently, synthetic HA gene from H5N1 virus A/chicken/Indonesia/7/2003 (Indo/03) was expressed in duckweed. Its efficacy was first tested in birds immunized with 0.2 µg or 2.3 µg HA and challenged with 10^6 mean chicken embryo infectious doses (EID50) of homologous virus strain (Bertran et al., 2015) and in birds immunized with 0.9 µg or 2.2 µg HA challenged with 10^6 EID50 of heterologous H5N1 virus strains A/chicken/Vietnam/NCVD-421/2010 (VN/10) or A/chicken/West Java/PWT-WIJ/2006 (PWT/06) (Bertran et al., 2015). Almost all birds immunized with 0.2 or 2.3 µg of HA elicited anti-Indo/03 antibodies and were protected against homologous virus challenge, 100 % of birds immunized with either dosage of HA showed protection against VN/10 challenge while birds challenged with PWT/06 showed 50 % mortality when immunized with 0.9 µg HA and 30 % mortality when immunized with 2.2 µg HA. Only birds challenged with VN/10 developed humoral immune response against the challenge antigen. Shoji et al. (2009) expressed 2 strains of H5N1 in *N. benthamiana*. Sequences for each gene were optimized for plant expression using tobacco PR-1 signal peptide at the N-terminus and ER retention signal KDEL and poly-histidine tag at C-terminus and *Agrobacterium*-mediated transformation was performed. Subcutaneous immunization with purified antigen from both strains elicited hemagglutinin inhibition (HI) and virus neutralizing (VN) antibodies in mice, with HI titers > 1:40, which is the minimal titer consistent with protective immunity in humans. M2e has been expressed in tobacco (Tarasenko et al., 2013) and recently, in duckweed (Firsov et al., 2015) and also in a form of a hybrid protein M2eHBC, fused to hepatitis B core antigen (Ravin et al., 2012). Tarasenko et al. (2013) expressed synthetic, codon-optimized 22-, 30- or 43-amino acids long terminal fragments of the M2 protein, encoding 3 different variants of M2e peptide (M122, M130 and M143,

respectively, with M122 and M130 encoding truncated M2 protein variants), fused with N-terminus of β-glucuronidase, in *Agrobacterium*-transformed *N. tabacum*. Transformation by vectors containing M122 or M130 resulted in successful expression of both variants of M2e peptide, in the form of M122-β-glucuronidase and M130-β-glucuronidase fusion proteins. In plants transformed with vector containing M143 gene for non-truncated M2e, the protein was observed only in plants grown *in vitro* and disappeared when the plants were transported into greenhouse. M2eHBC hybrid protein has been transiently expressed in *N. benthamiana* using a recombinant potato X virus-based viral vector. M2eHBc accumulated in leaf tissue at amount 1 - 2 % TSP in the form of VLPs. Immunogenicity of the VLPs was evaluated in intraperitoneally immunized mice. Three-fold immunization induced high titers of serum IgG targeting both the synthetic polypeptide, used in immunization (M2eHBc polypeptide G19) and its corresponding peptide sequence from heterologous influenza virus strain. 90 % of the immunized mice was protected against the lethal influenza virus challenge. Recently, Firsov et al. (2015) successfully expressed M2e peptide in the form of M2e-β-glucuronidase fusion protein in *Agrobacterium*-transformed duckweed. The team expressed 30 amino acids long N-terminal fragment of M2 protein, containing 24 amino acid long fragment encoding M2e, optimized for expression in duckweed and fused upstream of the β-gucuronidase gene, under the control of CaMV 35S promoter. Stably transformed plants were obtained (nuclear transformation) with the highest yield of M2e-β-glucuronidase fusion protein being 1.89 and 1.96 % TSP (0.82 and 0.97 mg/g fresh weight, respectively, Firsov et al., 2015), accounting for app. 40 µg/g of fresh weight of M2e alone. The protein accumulated in cytoplasm. The expression levels were similar to those previously obtained in experiments using virus-based transient expression systems, which is especially attractive as it could make duckweed a promising candidate for large scale production of influenza vaccine with regards to being able to produce equal amounts of protein than transient expression system at lower cost.

3 BACTERIAL VACCINES

3.1 *Escherichia coli* heat-labile enterotoxin subunit B

Enterotoxigenic *Escherichia coli* (ETEC) is the leading cause of diarrhea in developing world, causing severe mortality and morbidity rates especially in children up to 5 years of age in the developing coutries. Enterotoxigenic strains of *E. coli* produce either the heat-stable toxin (ST), the heat-labile toxin (LT) or both. The structure and function of LT are highly similar to those of the cholera toxin. The B subunit of LT (LT-B) is non-toxic and works as a potent mucosal immunogen therefore it has been used as a target in developing candidate vaccines against ETEC-caused diarrhea as well as against cholera toxin, because the anti-toxin response caused by immunization with LT-B also extends to CT-B. Since the first LT-B subunit was expressed in potato and tobacco (Haq et al., 1995) many plant expression models have been developed and tested: tobacco leaf (Haq et al., 1995; Wang et al., 2001; Kang et al., 2006a; Chia et al., 2011), potato tubers (Mason et al., 1998; Tacket et al., 1998; Lauterslager et al., 2001), maize seeds (Streatfield et al., 2001; Chikwamba et al., 2002), tobacco chloroplasts (Kang et al., 2004a), tobacco mosaic virus infected *N. benthamiana* plants (Wagner et al., 2004), transgenic corn (Tacket et al., 2004), *Siberian ginseng* somatic embryos (Kang et al., 2006b), carrot (Rosales-Mendoza et al., 2007; 2008), soybean seed (Moravec et al., 2007), lettuce leaf (Kim et al., 2007; Martinez-Gonzales et al., 2011), *Peperomia pellucida* tissue culture (Loc et al., 2010), watercress (Loc et al., 2011a) and transgenic tomato fruit (Loc et al., 2011b; Loc et al., 2014). In general, these models proved that synthetic non-toxic LT-B can be produced in plants without losing its native antigenicity or immunogenicity. Obtained yields ranged between 0.3 and 3 % TSP, with the highest yield of 3.3 % TSP in tobacco (Kang et al., 2006a) and 3.7 % TSP in maize seed (Chikwamba et al., 2002) and the lowest reported yields of 0.001 % TSP in the first transformed tobacco leaf and potato (Haq et al., 1995), 0.05 % TSP in lettuce leaf (Martinez-Gonzales et al., 2011) and 0.095 % TSP in tobacco leaf (Wang et al., 2001). As in the case of other

plant-made vaccines it has been proven that the addition of protein targeting sequences such as ER SEKDEL and the use of plant-preferred codons contributes to higher expression and accumulation of LT-B in plant tissue and most of the research is focused on searching for plant species that would express a sufficient amount of LT-B and could deliver it to the patient in raw unprocessed form. LT-B expressed in lettuce leaves (Martinez-Gonzales et al., 2011) elicited both serum and intestinal Ab responses in orally immunized mice, even in the case of freeze-dried lettuce tissue, with elicited antibodies showing neutralizing activity against cholera toxin challenge. Mice immunized with 8 µg of LT-B were fully protected against CT in the same way as mice immunized with the pure yeast-produced LT-B. Lettuce-derived LT-B assembled into pentameric forms, similar to the watercress-derived LT-B (Loc et al., 2011b). LT-B expression in lettuce was deemed stable as expression levels in T1 generation were similar to those in T2. Plant-derived LT-B has been found to be immunogenic and protective in orally immunized mice models for a majority of abovementioned plant expression models. In addition to that, LT-B expressed in potato tubers (Tacket et al., 1998) and transgenic corn (Tacket et al., 2004) was also immunogenic, protective and well tolerated in orally immunized humans. LT-B expressed in corn seeds (Karaman et al., 2006) elicited both mucosal and systemic immune responses in both young and aged mice and boosting by oral administration or injection of LB-T dramatically increased IgA and IgG levels in aged mice, which could be an indicator of immunological memory assembly. When expressed in transgenic tobacco chloroplasts, the LT-B accumulation was app. 2.5 % TSP, app. 250-fold higher than in plants generated by nuclear transformation (Kang et al., 2004). In addition to being expressed independently, LT-B is often expressed as an adjuvant for co-administered antigens, one of such experiments was carried out by Chia et al. (2011), expressing a fusion protein of PRRSV GP5 and LT-B in transgenic tobacco and its immunogenicity was evaluated in orally immunized pigs. It was found that the GP5-LT-B-treated pigs developed PRRSV-specific Ab- and cell-mediated immune response, but their ratios were not significantly higher than those observed in GP5-immunized pigs. Loc et al. (2014) recently expressed LT-B in the fruits of *Agrobacterium*-transformated tomato. LT-B was expressed in the form of pentamers in the fruits in 2/5 transgenic tomato plants but thus obtained LT-B pentamers specifically bound to GM1 ganglioside, confirming their biological activity, which gives hope for tomato-produced LT-B as a suitable candidate for new-age subunit vaccine, however *in vivo* immunogenicity studies need to be performed.

3.2 Cholera toxin subunit B

Cholera is a highly epidemic diarrheal disease caused by the cholera toxin of enterotoxigenic *Vibrio cholerae* strain, affecting especially children in developing countries. The pentameric B subunit of the cholera toxin, CT-B, is structurally and functionally very similar to LT-B and has thus been the main target for developing plant-produced candidate vaccines against cholera as well as traveler's diarrhea. CT-B has been expressed in potato (Arakawa et al., 1997; He et al., 2007; Mikschofsky et al., 2009), tobacco (Daniell et al., 2001; Kang et al, 2004b, Kang et al., 2006c; Mishra et al, 2006; Mikschofsky et al., 2009; Tiwari et al., 2009; Rattanapisit et al., 2013), carrot (Kim et al., 2009), tomato leaves and fruit (Meena et al., 2002; Jiang et al., 2007; Loc et al., 2011b), lettuce (Huy et al., 2011), rice (Soh et al., 2015) and maize seeds (Karaman et al., 2012). As it is a potential mucosal and parenteral adjuvant and an effective carrier for chemically or genetically linked antigens, CT-B has often been expressed in the form of fusion proteins with antigens which do not induce sufficient immune response when orally applied on their own, much like LT-B. Examples of such use are CT-B- neutralizing epitope of the porcine epidemic diarrhea virus fusion protein (sCTB-sCOE) in lettuce (Huy et al., 2011), CT-B-rabies glycoprotein fusion protein expressed in tobacco seeds (Tiwari et al., 2009), CT-B- Vibrio cholera accessory colonization factor subunit A (ACFA) fusion in tomato (Sharma et al., 2008), fusion of simian-human immunodeficiency virus regulatory sequence and CT-B (Kim and Langridge, 2004b), simian immunodeficiency virus Gag p27 capside protein-CT-B fusion (Kim et al., 2004a) and CT-B- anthrax lethal factor fusion in potato leaf (Kim et al., 2004b), CT-B- human insulin B chain in tobacco (Li et al., 2006) and CT-B- rotavirus enterotoxin NSP4 fusion protein in potato tubers (Yu and Langridge, 2001). Expression levels of CT-B in most plant systems were 0.01 - 1 % TSP, with the lowest yield of

0.04 % TSP in tomato leaf and fruit (Jani et al., 2002) and the highest yield of 4 % TSP in tobacco leaf (Daniell et al., 2001). In the case of CT-B-fusion proteins, the expression levels were lower, ranging 0.002-0.2 % TSP, the lowest obtained expression being 0.0003 % FW in potato tubers (Yu and Langridge, 2001) and the highest expression of 0.4 % TSP in tobacco (Roy et al., 2010). In general, expression of synthetic CT-B with plant-optimized codons, additional protein targeting sequences and driven by powerful plant promotors, was much higher than expression of other CT-B forms. When CT-B was expressed under the control of 35S CaMV promoter, using plant-optimized codons and additional ER SEKDEL retention signal its expression increased 10-fold, from previously obtained 0.081 % TSP (Jiang et al., 2007) to 0.9 % TSP (Loc et al., 2011b). Some of the plant-produced CT-B have been evaluated for their immunogenicity and protection in mice models, applied either orally or by gavage and it has been found that CT-B was immunogenic in all cases and has also induced protection in the form of both mucosal and systemic immune response (Jian et al., 2007; Huy et al., 2011; Karaman et al., 2012) in most cases, while in the case of CT-B-NSP4 rotavirus fusion protein it induced passive immunity (Yu and Langridge, 2001). However it has to be noted that in most cases when CT-B was expressed in plant host systems, either on its own or as a part of fusion proteins, its immunogenicity was not evaluated. In 2012, Karaman et al. expressed a synthetic CT-B gene, driven by γ-zein promoter in transgenic maize seeds and evaluated its immunogenicity in orally immunized mice. The highest expression levels in T1 seeds was 0.0014 of total aqueous soluble protein (TASP) while it increased to 0.0197 % TASP in T2 seeds, suggesting that the CT-B expression could be enhanced through selective breeding to advanced generations. Obtained CT-B was found to be immunogenic in mice and induced both mucosal and systemic immune response as shown by fecal IgA and serum IgG against both CT-B and LT-B, however the effect of serum anti-CT-B IgG on LT-B is not as big as its effect on CT-B and both enterotoxins show significant cross-immunoreactivity (Soh et al., 2015). It was suggested that the combined application of CT-B and LT-B could be an efficient way of protection against both cholera and diarrhea (due to similar enterotoxin structure and mechanism of action). Soh et al. (2015) co-expressed recombinant LT-B and CT-B in rice grain for the first time. Both genes were driven by constitutive globulin promoter, an *Agrobacterium*-mediated transformation was performed and the obtained transgenic plants were grown to maturity to obtain 5 generations of transgenic seeds. Expression levels in the 5th generation of homozygous seeds as evaluated by ganglioside-dependent ELISA were 3.4 ng/ug of TSP for LT-B and 21.3 ng/ug of TSP for CT-B. Obtained CT-B and LT-B were present in their native pentameric form and capable of inducing both mucosal and systemic immune responses in orally and intraperitoneally immunized mice. When applied intraperitoneally, a dose of 100 µg rice-produced LT-B and CT-B induced LT-B and CT-B-specific IgG production with similar expression levels. When applied orally, rice-produced LT-B induced both mucosal and systemic IgA production. These findings suggest that mucosal and systemic immune response against both CT-B and LT-B is enhanced by co-administration of both antigens and strengthens the immune response in either orally or intraperitoneally immunized mice. This could be of help with developing edible vaccines against cholera and traveller's diarrhea in economically important crop species like rice and maize. CT-B and LT-B synergism could be further enhanced by breeding to advanced generations and the immunogenic and adjuvant abilities of the co-expressed fusion toxins in rice should be further evaluated in animal studies (Soh et al., 2015). Rattanapisit et al. (2013) have developed a method of rapid transient expression of CT-B in *N. benthamiana*, using geminiviral replicon system, *Agrobacterium*-mediated transformation and a plant-optimized CT-B sequence. The highest obtained CT-B expression level was app. 4 µg/g fresh weight ~ 0.14 % TSP as obtained on day 4, which was much higher than CT-B level transiently expressed in tobacco (Wang et al., 2001). Expressed CT-B showed biological activity for binding on the GM1-ganglioside in GM1-ELISA and could be thus a promising candidate for adjuvant for mucosal vaccines. The study suggests that geminiviral system could be efficiently used for high level expression of CT-B and further optimized, especially with regards to possible side effect of gene silencing.

4 OTHER RECENT VACCINES

4.1 Artherosclerosis

As an effort to develop immunotherapeutic treatments against artherosclerosis, different vaccination strategies have been proposed during the last 20 years. Most of these strategies were focused on targeting apolipoprotein B100 (ApoB100) and cholesterilester transferase protein (CETP), trying to elicit immune response capable of modulating either arterosclerosis-associated inflammatory reactions or other up-regulated physiological mechanisms leading to this medical condition. To date, several clinical trials on plant-based vaccines against artherosclerosis have been reported: CTEP-tetanus toxin fusion (rabbits), CETi-1 with tetanus toxin as an adjuvant (humans), RHSP65-CETP (rabbits), p45 with AlOH as an adjuvant (mice), CT-B-p210 (mice) (Salazar Gonzales and Rosales-Mendoza, 2013), with some kind of artheroprotection reported in all cases, mostly shown as IgG or IgA. With the aim of initiating the development of a plant-based artherosclerosis vaccine, Salazar-Gonzales et al. (2014) constructed a synthetic gene encoding a fusion protein consisting of CT-B, CETP and ApoB100 and expressed it in tobacco. Epitopes of both ApoB100 and CETP were fused at the C-terminal end of CT-B, creating CTB:p210:CETP under the control of CaMV 35S promoter. The total of 6 transgenic tobacco plants was obtained, all of them containing transgene. Chimeric CTB:p210:CETP was expressed in tobacco in correctly assembled pentameric form, at the highest yield of 10 µg/g of fresh leaf tissue as obtained from the lines with single copy transgene insert. GM1-ELISA and Western blot analysis proved that the protein retained the target antigenic determinants. Subcutaneous administration of the chimeric protein elicited humoral responses against CETP and ApoB100 epitopes as well as human serum proteins in mice. These findings evidenced for the first time that atherosclerosis-related epitopes can be expressed in plants retaining immunogenicity, which opens a new path in the field of molecular farming for the development of vaccines against atherosclerosis.

4.2 Ebola

In their recent work, Bhoo et al. (2011) used a geminiviral replicon system for production of Ebola immune complex (EIC) in *Nicotiana benthamiana*. Ebola glycoprotein GP1 was fused at the C-terminal end of the heavy chain of humanized 6D8 IgG monoclonal antibody, specifically targeting the linear epitope of GP1. When co-expressed via geminiviral vectors, heavy chain and 6D8 light chain resulted in assembled immunoglobulin. It was purified and its conformation was evaluated by CC1q binding assay, dynamic light scattering and size exclusion chromatography. When subcutaneously immunized, mice elicited anti-Ebola virus antibodies at levels comparable to those obtained with a GP1 VLP, which is promising for using plant-produced EIC as human vaccines. Recently, a combination of 3 anti-ebola antibodies, called ZMapp, has been expressed in transgenic tobacco by Californian company Mapp Biopharmaceuticals (ISIS …, 2014). ZMapp, previously tested only in monkeys, was administered to two Americans, infected in Africa, but in the case of the one that survived, it was not sure if that was due to ZMapp or was due to the blood transfusion received before ZMapp. So far no official report on production or function of ZMapp has been released. It is known, however, that the genes for humanized anti-ebola monoclonal antibodies are introduced to plant (tobacco) tissue via plant viral vectors by method of magnifection and that the final yield of mAb adds up to 1 % of the cytoplasmic protein of tobacco leaf.

4.3 Anthrax

Anthrax has recently gained importance due to its potential application as a bio-warfare agent, besides frequent natural outbreaks around the world, especially in Africa, Central Asia and South America. The first research towards producing a plant-based subunit oral vaccine against anthrax was done by Aziz et al. (2002), who expressed protective antigen (PA) in tobacco, transformed by *Agrobacterium*-mediated transformation method. The PA transgene was found successfully integrated into tobacco nuclear genome and the protein was expressed with a predicted molecular weight of 83 kDa. Cytotoxicity assay confirmed

the retention of its biological activity. CT-B-anthrax lethal factor fusion protein was expressed in transgenic potato (Kim et al., 2004b), assembled into oligomeric structures, resembling the native pentamers, its accumulation level being 0.00039 - 0.0018 % TSP. This proved the feasibility of edible plants, such as potato, for production of anthrax lethal protein and its theoretical delivery. Recently, Gornatala et al. (2014) expressed protective antigene (PA) in Indian mustard (*Agrobacterium*-mediated transformation) and in tobacco (plastid transformation), under the control of CaMV 35S promoter. Expression level was 0.3 - 0.8 % TSP in mustard and 2.5 - 4 % in tobacco. Macrophage lysis assay showed 23 - 81 % lysis for PA derived from mustard and 80 - 97 % lysis for PA from tobacco samples. Both oral and intraperitoneal application of either mustard- or tobacco-produced PA resulted in protective immune response with high serum PA-specific IgG or IgA titers in mice. Co-administration of CT-B as an adjuvant by gavage enhanced the response, even when antigen doses were as low as 5 or 10 µg. PA-specific mucosal immune response was observed in orally immunized mice.

5 CONCLUSION

During the last five years a lot of progress has been made in the field of plant-made vaccines. Novel transformation methods and expression vectors have been applied to plant species that had already proven themselves fit for expressing potential vaccine antigens, trying to enhance the expression of transgenes, protein accumulation and stability as well as facilitate the purification process. Obtained increases in accumulation and well-preserved biological function of produced antigens provide a promising basis for future development of plant-derived vaccines. Before such vaccines can be produced on industrial scale and broadly accepted, there is still a lot to be done in order to eliminate all possible risks they could pose towards environment and living creatures and gain public acceptance of their usage, however, a lot more research and development of the field can be expected in the following years.

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The impact of the period of sowing and fertilization on morphological characteristics and seed yield of garden poppy (*Papaver somniferum* L.)

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ABSTRACT

Garden poppy (*Papaver somniferum* L.) is a traditional crop that had already been cultivated in Slovenia in the past for the production of seed and oil. During its re-introduction in present time, numerous agro-technical dilemmas have been raised for the processes from sowing to harvesting and have to be studied in our present growing conditions. On the fields of the Ograjšek farm in Cerklje ob Krki two field experiments in complete randomized design had been sown in 2013 and 2014 to establish the influence of the sowing date and fertilization on morphological properties and crop yield of garden poppy seed of the Austrian variety 'Zeno 2002'. Results have shown that the average yield of seed in both trials (1643 kg/ha) is more than one time higher than the average crop yield recorded globally (700 kg/ha), proving how suitable growing conditions in the area of eastern Dolenjska are for the cultivation of poppy. Analysis of crop yield per plot showed there is no statistically significant interaction between the considered factors. The yield of poppy seed in the spring sowing term was 1742±77 kg/ha and in the autumn sowing was 1545±122 kg/ha. The difference is not statistically significant (p = 0.1845). Recorded yield per plant was higher for the autumn term but that was not transferred to the total crop yield due to poor overwintering and consequentially lower plant density at the time of harvest. Both sowing dates have confirmed that exposure to light and duration of growing period had an important impact on plants height – average height of plants sown in autumn was 139±1 cm, which is more than 60 cm higher than the average height of the plants sown in spring (p = 0.0000). A strong statistical dependence of the yield of seed per capsule on diameter of the capsule was proved (p = 0.0000). It can be concluded that when the capsule diameter increases by 10 mm, the seed yield increases for 2.1 g to 2.3 g with 95 % confidence. Dependence of the average seed yield on the number of lateral shoots per plant also proved to be statistically significant (p = 0.0000). Linear model was used for the comparison of the four lines showed that the lines representing poppy sown in autumn and spring are statistically different for the control and for the ENTEC; the slope was higher for the poppy sown in autumn. These trials have given the first practical advice for production of garden poppy in Slovenia and should be continued for further useful results.

Key words: garden poppy, *Papaver somniferum*, sowing date, fertilizing, morphological properties, seed yield

IZVLEČEK

VPLIV ROKA SETVE IN GNOJENJA NA MORFOLOŠKE LASTNOSTI IN PRIDELEK SEMENA VRTNEGA MAKA (*Papaver somniferum* L.)

Vrtni mak (*Papaver somniferum* L.) je tradicionalna poljščina, ki smo jo v preteklosti v Sloveniji pridelovali za seme in olje. Pri ponovnem uvajanju v pridelavo se od setve do žetve pojavljajo številne agrotehnične dileme, ki jih je treba preučiti tudi v naših rastnih razmerah. Na njivi kmetije Ograjšek v Cerkljah ob Krki smo v letih 2013 in 2014 posejali dva bločna poljska poskusa z namenom, da ugotovimo vpliv roka setve in gnojenja na morfološke lastnosti in pridelek semena vrtnega maka avstrijske sorte 'Zeno 2002'. Rezultati so pokazali, da je bil povprečen pridelek semena obeh poskusov (1643 kg/ha) več kot enkrat večji od povprečnega pridelka semena v svetu (700 kg/ha), kar nakazuje na ustreznost rastnih razmer za mak na območju vzhodne Dolenjske. Analiza pridelka na parcelo je pokazala, da interakcija med preučevanima dejavnikoma ni bila statistično značilna. Pridelek semena maka v spomladanski setvi (1742±77 kg/ha) je presegel pridelek jesenske setve (1545±122 kg/ha. Razlika med rokoma setve ni bila statistično značilna (p = 0.1845). Pridelek na rastlino je bil v jesenskem delu poskusa večji, kar pa se zaradi slabše prezimitve in posledično gostote rastlin ob spravilu ni izkazalo za skupni pridelek. Z rokoma setve smo potrdili vpliv osvetlitve in dolžine rastne dobe na višino rastlin - povprečna višina rastlin ozimne setve je bila 139±1 cm, kar je v povprečju več kot 60 cm višje od rastlin spomladanske setve, razlika v višini med rokoma setve je bila statistično značilna (p = 0.0000). Ugotovili smo močno statistično značilno odvisnost med pridelkom semena na glavico in premerom glavice (p = 0.0000), kar pomeni, da se ob povečanju premera glavice za 10 mm pridelek semen v njej pri 95 % zaupanju poveča od 2.1 do 2.3 g. Tudi odvisnost med povprečnim pridelkom semen in številom stranskih poganjkov na rastlino je bila statistično značilna (p = 0.0000). Linearni model za primerjavo štirih premic je pokazal, da se premici za mak posejan jeseni in spomladi pri kontroli in ENTEC statistično razlikujeta, naklon je večji pri maku sejanem jeseni. S pomočjo poskusov smo prišli do prvih praktičnih nasvetov pri pridelavi maka v Sloveniji, s čimer kaže nadaljevati.

Ključne besede: vrtni mak, *Papaver somniferum*, rok setve, gnojenje, morfološke lastnosti, pridelek semena

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1 INTRODUCTION

Poppy (*Papaver somniferum* L.) is a traditional Slovene crop that we want to re-introduce into production (Kocjan Ačko, 2015). Until the beginning of the 20th century it was mostly grown in the region of Prekmurje, on the slopes of Pohorje and in Koroška (Sadar, 1951). Similar to other traditional crops, poppy had disappeared from our fields and gardens with intensification and specialization of agricultural production. Poppy seeds that are used for the preparation of local deserts like »prekmurska gibanica« and »slovenska potica« with poppy seed filling were imported from Austria, Czech Republic and Hungary where they have managed to preserve the cultivation of poppy and improve the production with new varieties and modern agro-technical measures. Recent focus of EU agricultural policy on sustainable farming and local production has encouraged our farmers to include new and forgotten crops into the crop rotation. Company Panvita decided to sow garden poppy for the production of seed in 2007 and since then they cultivate around 20 hectares every year (personal info), on much smaller fields we can also see poppy on other fields in Prekmurje and elsewhere in Slovenia, mostly in the region of Dolenjska.

Poppy is an annual crop that grows in continental and sub-tropic climate. Different chemotypes have developed within the same somniferum *species;* each with higher or lower content of alkaloids in poppy milk (latex). Higher concentrations of alkaloids are often caused by higher growing temperatures and longer exposure to sunlight in sub-tropical conditions (Đorđevski and Klimov, 1986). In continental growing conditions that we have in Slovenia, poppy is produced for its seed with up to 50 % oil content which means that in our conditions poppy is an oil plant. Poppy production is controlled at international and also at national level for its possible misuse for the production of illicit drugs. In Slovenia production for seed and oil is allowed and poppy crops have to be registered at the Ministry of agriculture, forestry and food (Rules on requirements…, 2011). Poppy can be sown only after the Decision of the Ministry has been obtained; the Rules also provide for implementation of control measures during the growth period and at crop harvest to provide for complete traceability (Kocjan Ačko, 2015).

Poppy grows well in alluvial sandy-clayeous slightly alkaline (higher pH) soil that is rich in nutrients, especially calcium. During growing period it needs between 400 to 500 mm of rain; its highest demand for water is from the emergence phase until the beginning of flowering. Higher precipitation during the capsule maturing period can promote development of fungal infections and germination of seeds in mature capsules (Đorđevski in Klimov, 1986).

It is known that the varieties coming from northern areas of Middle and Western Europe have higher capability for overwintering (Bernáth and Németh, 1998). This means that we can sow the same variety in September as a winter crop (autumn sowing) or in early spring, when we start sowing spring crops (spring sowing). There are important morphological differences between the crops sown in autumn and those sown in spring due to different temperatures and duration of sunlight exposure in different pheno-phases of plants development and growth. The most obvious one is different height of plants that is much lower in crops sown in spring. Longer growth period of the autumn/winter crops proved as a positive factor in trials by Bernáth and Némethova (1998) giving drier mass of plants and higher seed yields (Bernáth in Németh, 1998). Slovenia is somewhere in between the two climates (Žnidarčič, 2012) and we can decide between both terms of sowing; therefore we can also expect for some crop loss caused by poor overwintering. In his book »Oljnice, korenovke, predivnice in hmelj« (Oilseeds, root vegetable, fibre crops and hops) Sadar (1951) indicates exclusively spring sowing for poppy, that has to be performed as soon as possible (beginning of March or as soon as the ground allows the use of sowing machine) to allow enough time for growth and avoid possible summer drought that can significantly reduce the crop of spring sown varieties (Németh, 1998).

Like for other crops, soil has to be analysed before sowing. Quantity of nutrients depends on soil type, climate and previously grown crop. Quantities of micro-nutrients can vary significantly, however average recommended quantity of fertilizers per one hectare of poppy crop is 140 to 160 kg of nitrogen, 70 to 110 kg phosphorus (P_2O_5) and 80 to

100 kg potassium (K_2O). All the nutrients are added with organic and mineral fertilizers (Đorđevski and Klimov, 1986). Relatively short growing period of spring poppy requires the use of easily accessible mineral fertilizers. Application of more than 80 kg of nitrogen per hectare can cause numerous negative effects like flattening of plants, higher incidence of pests and diseases (Ruminska, 1973, quoted after Németh, 1998). With less developed root system poppy has to be fertilised with smaller quantities of nitrogen several times. The highest requirement for nitrogen is in the period between the rosette phase and beginning of flowering, since that is the period of rapid growth and high green mass production (Bernáth and Németh, 1998).

The purpose of our research is to establish whether growing conditions in Krško basin (eastern Slovenia) are suitable for cultivation of poppy and to evaluate the influence of two different terms of sowing and three different nitrogen fertilizers on the yield of seed.

2 MATERIALS AND METHODS

2.1 Design of trials and description of variety

During the growth period of 2013/2014 two field trials with poppy crops have been conducted on the fields of Ograjšek family farm in Cerklje ob Krki. The first trial has been sown on September 14, 2013 (autumn sowing term) and the second one on March 22, 2014 (spring sowing term). Both trials were set in complete randomized blocks design with four treatments in three repetitions. In addition to one control plot with no fertilization the influence of fertilizers on yield of crop was tested with application of three different mineral fertilizers (NPK 15-15-15, KAN in ENTEC® (N-26)). Quantities of added fertilizers per plot corresponded to 60 kg N per hectare. Calculation of added quantities of mineral fertilizers is presented in Table 1. Size of individual plot was 6 m x 4.6 m, that is 27.6 m^2. To minimise the influence of plot edges at the time of harvest, we separately collected poppy capsules from the edge (40 cm wide) so that the real surface of one testing plot was 19.1 m^2 and data from this surface were used to calculate the yield of seed per hectare.

Table 1: Quantities of mineral fertilizers for addition of 60 kg N/ha

Type of mineral fertilizer	NPK 15-15-15 (kg)	ENTEC® (26 % N), (kg)	KAN (27 % N), (kg)
Quantity corresponding 60 kg N/ha	400	230.8	222.2
Quantity per plot (27.6 m^2)	1.1	0.64	0.62

For our trial the Austrian variety of 'Zeno 2002' was selected, one of the most popular and widely used varieties for poppy seed production in the area of Middle Europe, suitable also for autumn sowing (Backsaaten, 2014).

2.2 Sowing, cultivation and harvesting

Soil was treated classically with tillage and harrowing. Before sowing 500 kg/ha of NPK 5-20-30 was applied; sowing field was then fine treated with rotating harrow. Seed of 'Zeno 2002' poppy variety was sown with Amazone D7 seed drill to 12.5 cm row spacing with 2 kg of seed per hectare. The testing field was sown as one whole unit and testing plots were formed later when shoots started to grow and prolongate, that is just before fertilization. Crop was treated against weeds with Callisto 480 SC in the amount of 0.21 l/ha. Fertilization was done manually when rosettes with approximately 20 leaves had formed. Capsules were also collected manually; on every plot capsules were first cut off and then crushed. Seeds were cleaned; all green parts, capsule walls and other impurities were removed with sieve of 1.5 x 1.5 mm.

Before harvest 20 plants were randomly selected on every plot to measure the selected morphological parameters (plant height, number of lateral shoots, width and height of capsule, seed mass of one individual capsule). The number of

plants per m^2 was counted at the time of harvest (density) and weighted the total crop per plot.

2.3 Growing conditions in Cerklje ob Krki

Climate in Cerklje ob Krki is moderately continental with mean annual temperature around 10 °C. Mean July temperatures are around 20 °C and January average is around -1 °C. Annual quantity of precipitation in Cerklje is around 1000 mm.

Soil on the testing field is eutric brown soil and is very common in the plains of Slovenia. It originates from deposits of bigger rivers. By texture the soil is classified as sandy-clayeous (ICPVO, 2015). Soil pH was measured at 5.6, which is slightly too low in light of general guidelines for the preservation of soil fertility and also considering the growing requirements of poppy plants.

2.4 Statistical analysis

Results of the trial (yield of seed per testing plot, number of plants per m^2, plant height, number of lateral shoots, capsule diameter, yield of seed per individual capsule) were processed with analysis of variance for complete randomized block design for two-factor experiment. Treatments were determined in all combinations of the two tested factors: fertilising (NPK 15-15-15, KAN, ENTEC®) and control with no fertilising) and time of sowing (autumn, spring). The linear regression models were used to examine the dependence of average yield on the number of lateral shoots, yield of seed per capsule, capsule diameter, number of lateral shoots and number of plants per m^2, that is from the average density at harvest. All explanatory variables were centred for easier interpretation of the estimated regression parameters. Statistical analysis was performed with the R program (R Core Team, 2015).

3 RESULTS AND DISCUSSION

3.1 Yield of seed

Analysis of variance for crop yield per plot revealed no statistically significant interaction ($p = 0.87$) between the time of sowing and fertilisation, there was no statistically significant effects for fertilisation ($p = 0.61$) neither for time of sowing ($p = 0.24$). Table 2 shows the average yield across all four fertilisations for each sowing times separately. Average yield of poppy seed sown in autumn was recorded at 2.95±0.24 kg per plot, which is around 1544 kg/ha. Average yield of the spring sowing was 3.33±0.15 kg per plot or calculated to kg/ha - 1741 kg/ha.

Table 2: Average humidity of seed at harvest and yield of poppy seed (*Papaver somniferum* L.) for the autumn and spring sowing in Cerklje ob Krki in the years 2013/14.

Sowing term	Average humidity of seed at harvest (%)	Average yield of seed per plot at 8 -percent humidity (g)	Standard error (g)	Average yield of seed per hectare at 8-percent humidity (kg)	Standard error (kg)
Autumn	8.2	2950	235	1544.5	122.0
Spring	9.3	3326	146	1741.5	76.5

An important aim of the trial was to determine the difference between the same quantity of added nitrogen (60 kg/ha) through different mineral fertilizers and the control without fertilizing. Figure 1 shows there were no statistically significant differences between different treatments (NPK 15-15-15, KAN in ENTEC® (N-26) and control). Our results did not confirm our expectations raised from previous trial results published in the article by Bernáth and Némethova (1998).

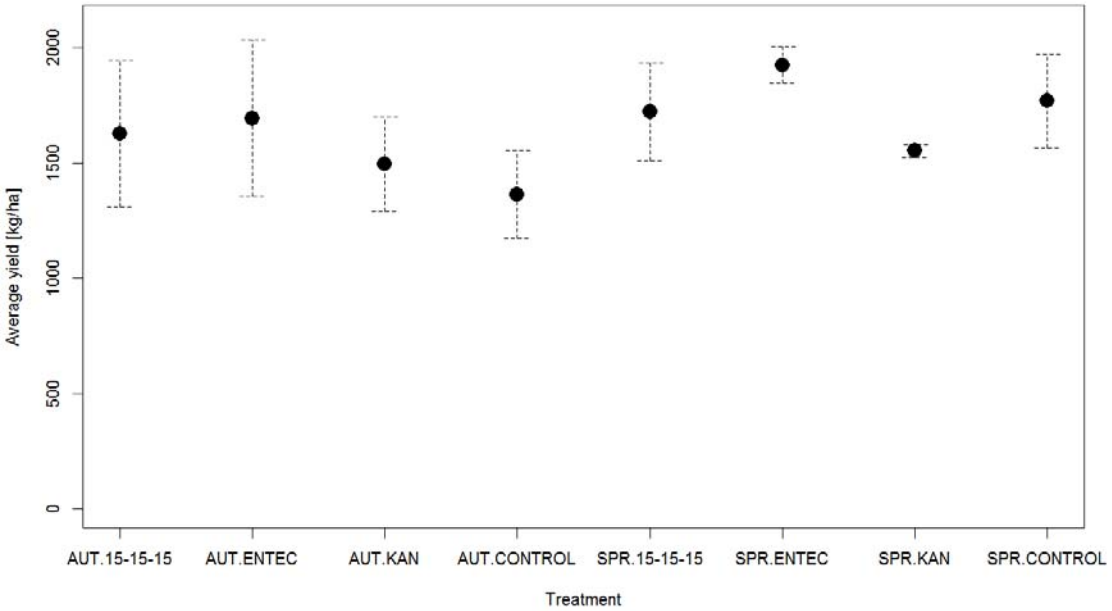

Figure 1: Average yield of poppy seed (*Papaver somniferum* L.) per hectare with 8-percent humidity per treatment for fertilisation in Cerklje ob Krki in the years 2013/14.

Results of previous trials (Laughlin, 1978) showed positive effect of fertilising with nitrogen on the yield of capsules. To expand our findings an analysis of nitrogen content in seeds and straw would be needed so that we could calculate the total nitrogen uptake.

With the analysis of seed yield per poppy plant according to the time of sowing and fertilizing, we were able to determine that the average yield of seed per individual plant was higher in the autumn sowing compared to the sowing in spring (p = 0.002) (figure 2). There was no statistically significant impact of fertilizers on average seed yield per poppy (p = 0.15).

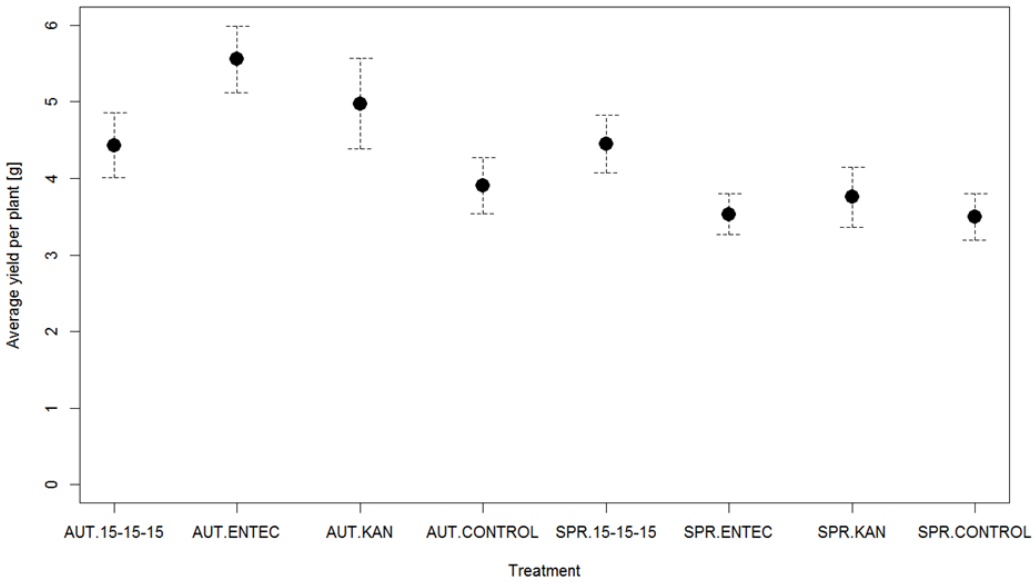

Figure 2: Average yield of poppy seed (*Papaver somniferum* L.) per plant with 8-percent humidity per treatment for fertilization in field trials in Cerklje ob Krki in the years 2013/14.

The recorded yield of poppy seed in both sowing terms was high, exceeding the average yields per hectare in major poppy producing countries in 2013 (FAOSTAT, 2013). Closer inspection of recorded yields for other poppy varieties cultivated around Europe (Németh, 1998) shows that higher yields are a common result on micro plots. Realised potential yield for different varieties is around 2 tons of seed per hectare. With recorded yields our trials confirmed that the conditions in Cerklje ob Krki are favourable for poppy production and allow high yields. When reviewing the literature, we found no research that would establish a difference in yields between the autumn and spring sowing for the same variety. However, research by Bernáth and Tétényi (1982) shows that in moderate climate conditions (southern parts of Hungary and Austria) that are comparable to our climate, we can apply autumn sowing but have to be prepared to assume the risk of poor overwintering of plants. In northern parts of Europe farmers are advised against the autumn sowing (Ruminska, 1973. quoted after Németh 1998). According to its breeder dr. Georg Dobos (Zeno projekte, 2015), the 'Zeno 2002' variety is the most commonly used poppy variety in Austria, suitable also for autumn sowing. Results of our trials however confirm the statements that overwintering of poppy plants can be lower than desired.

3.2 Crop density

The two sowing terms gave statistically different (p = 0.0000) results for the number of plants per m^2. We recorded higher density of plants harvested after spring sowing. Fertilisation had no statistically significant effect on plant density. The fact that during winter part of the crop failed might be the reason for such results, which according to Némethova (1998) is no surprise. Average density of both crops, the autumn and the spring crop (table 3) definitely exceed the optimum crop yield at the time of harvest that had been determined in previous trials abroad at the level of 300,000 to 400,000 plants per hectare (Földesi, 1992); this parameter still has to be established for the Slovenian conditions.

Table 3: Average number of poppy plants (*Papaver somniferum* L.) per surface with standard errors at the time of harvest in field trials in Cerklje ob Krki in the years 2013/14 per different fertilization treatment.

Time of sowing	Fertilizing	Average number of plants per m^2	Standard error	Number of plants per hectare
Autumn	NPK 15-15-15	56.3	3.92	563,333
	ENTEC	52.3	3.17	523,333
	KAN	55.3	2.84	553,333
	Control	50.0	3.61	500,000
	Average	53.6	1.64	535,000
Spring	NPK 15-15-15	66.0	2.31	660,000
	ENTEC	66.3	0.88	663,333
	KAN	67.3	0.67	673,333
	Control	64.7	2.97	646,667
	Average	66.1	0.88	660,833

3.3 Plant height

The most significant difference between the autumn and spring sowing was recorded for the average height of poppy plants ($p < 0.0001$) (table 4). The interaction between fertilisation and date of sawing was statistically significant (p = 0.002) while the average height of AUT control plants was statistically significant lower than fertilised plants. There is no statistically significant impact of fertilisation for the plants sawed in spring. Poppy plants from the autumn sowing were higher from the plants sown in sprig for almost 60 cm in average. Plants height is mostly influenced by different temperatures during the growth period and related duration of different phases of plant development. Different trials from the nineteen-eighties proved that higher temperatures in early

growing phases promote generative development and early flowering. Also Bérnath and Tétényi (1981) reached the same conclusions after the results of their trials on poppy showed that with rapid increase of temperature from 12 °C to 26 °C plants started flowering 10 to 15 days earlier and their final height remained for 10 to 15 cm lower.

Table 4: Average height of poppy plants (*Papaver somniferum* L.) at the time of harvest in field trials in Cerklje ob Krki in the years 2013/14 per different fertilization treatment. There is no statistically significant difference between averages denoted with the same letter at α =0.05.

Time of sowing	Fertilizing	Average plant height (cm)	Standard error (cm)
Autumn	NPK 15-15-15	140.4a	2.26
	ENTEC	142.0a	1.77
	KAN	140.9a	2.06
	Control	132.5b	1.95
	Average	139.0a	1.03
Spring	NPK 15-15-15	83.5c	1.13
	ENTEC	80.9c	1.11
	KAN	79.5c	1.23
	Control	82.3c	1.31
	Average	81.6c	0.60

3.4 Seed yield per capsule in dependence on capsule diameter

Statistical analysis showed statistically significant linear dependence of seed yield per capsule on diameter of the capsule (capsule size) (figure 3). The determination coefficient (R^2) is 76 %. For better interpretation of results capsule diameter was cantered; for the average capsule diameter the average seed yield per capsule equals 3 g (95 % interval of confidence is from 2.9 g to 3.2 g); with capsule diameter increasing for 10 mm, the seed yield in the capsule increases for 2.1 to 2.3 g with 95 % confidence.

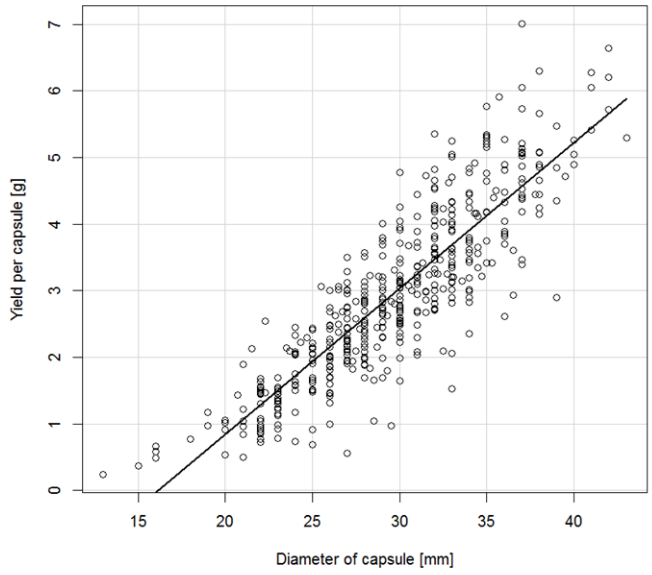

Figure 3: Dependence of poppy seed yield (*Papaver somniferum* L.) per capsule on the capsule diameter in Cerklje ob Krki in the years 2013/14.

3.5 Seed yield per plant in dependence on number of lateral shoots

Figure 4 shows that there is a statistically significant dependence of seed yield per plant on the number of lateral shoots on the poppy plant. Linear model that we used to compare the four lines showed that the two lines representing poppy from the autumn sowing and the spring sowing at CONTROL and ENTEC are statistically significantly different; the slope is bigger for the autumn poppy. Determination coefficient (R^2) of the model is 65 %.

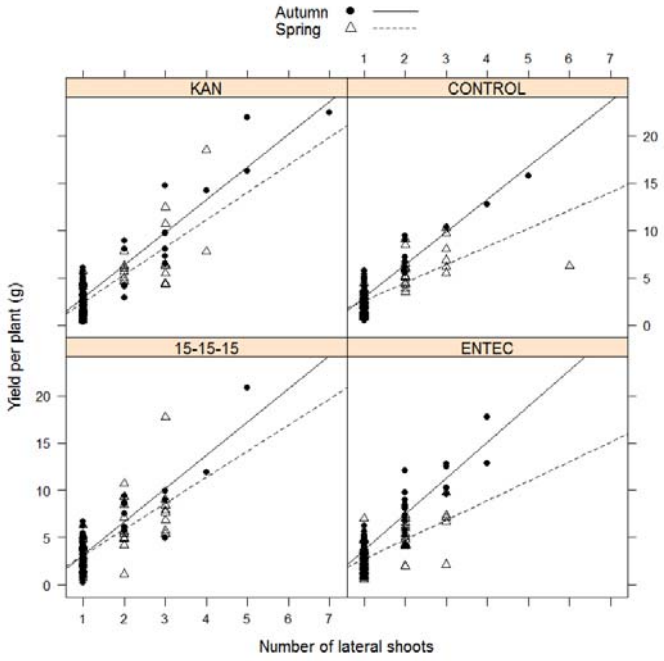

Figure 4: Dependence of the average poppy seed yield (*Papaver somniferum* L.) per plant on the number of lateral shoots in Cerklje ob Krki in the years 2013/14.

Considering the number of lateral shoots results of the model show that the average yield of poppy seed per plant for the autumn sowing is statistically significantly different between the two applied fertilizers - KAN and ENTEC. For the average number of lateral shoots the yield per plant for the autumn sowing reached 0.97 g more when fertilised with KAN than if fertilised with ENTEC (95 % interval of confidence for the difference is from 0.02 g to 1.95 g). For the average plot density of 59.79 plants per m^2, that translates to 580 kg per hectare higher yield of poppy fertilised with ENTEC.

With average number of lateral shoots (1.5 shoots per plant) the yield of the autumn sowing on the CONTROL plot was for 1.1 g lower than the spring sowing yield (95 % interval of confidence 0.06 to 2.05).

3.6 Correlation between number of lateral shoots and crop density

Correlation between the number of plants per m^2 (density) at the time of harvest and number of lateral shoots did not prove to be statistically significant (figure 5). Based on our results we can draw a conclusion that the number of lateral shoots on poppy plants should be as low as possible. We assumed that lower number of plants per m^2 would cause more branching, however we could not confirm that supposition with our analysis – even for the density that exceeded 60 plants per m^2 there was no statistically significant lower number of lateral shoots. To achieve higher number of plants at the same plant density, distribution of plants should be optimised. Plants growing at the mutual distance of 3 to 4 cm can form only one capsule (with no lateral shoots). Plants that grow at the distance of 10 to 20 cm form in principal 3 to 4 capsules, however the number of lateral shoots is

also influenced by climate and agro-technical conditions (Muchova et al., 1993).

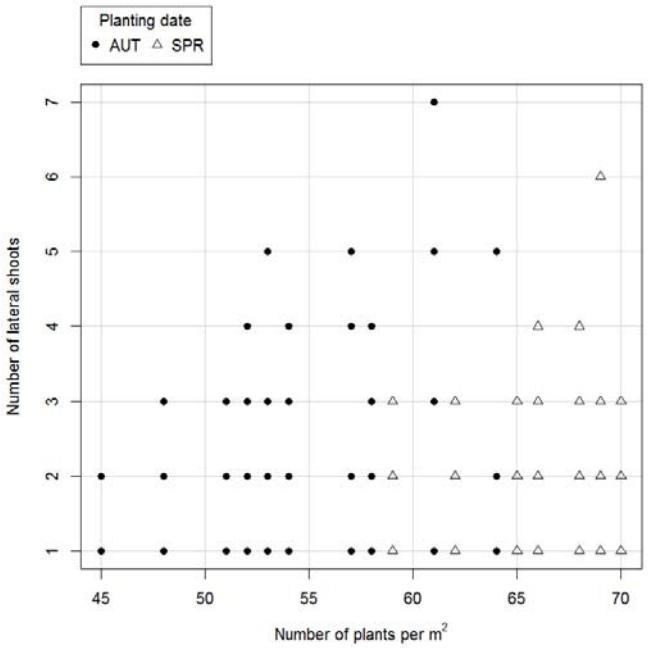

Figure 5: Correlation between number of lateral shoots on poppy plants (*Papaver somniferum* L.) and crop density in Cerklje ob Krki in the years 2013/14.

4 CONCLUSIONS

Our trials have shown that poppy is a plant that could be cultivated successfully in agricultural areas of eastern Slovenia with conditions similar to those in Cerklje ob Krki. The average yield of 1643 kg/ha from both trials was considerably above the world average. Considering the time of sowing, for the growing season of 2013/14 spring sowing proved to be the better choice; we should repeat the trial at least one more time to confirm the relevance of these results. We should definitely not disregard all the findings from the literature where higher yields are expected from the autumn sowing term. Autumn sowing also brings some other advantages (wider time frame for optimum sowing, avoiding the summer drought, early harvesting date of poppy also allows sowing of catch crops).

In case of fertilizing the results of our trials gave no clear and significant difference among applied fertilizers. Following recommendations in professional literature fertilizers should be applied several times during the growing period to increase the effect of fertilizing however in practice that would require more driving routes through the field like at the time of sowing. For more accurate findings and recommendations, multiannual trials should be performed that would also include analysis of nitrogen residue in seed and straw.

Within the two main aims of our research we have already reached the first practical advice for the production of poppy in eastern Slovenia. Particular challenge in cultivation of poppy is the sowing technique and the quantity of seed. Our results from measured parameters per individual plant show that in majority of cases plants from autumn sowing performed better, however that was not reflected in the total yield, mostly due to lower number of plants per m^2 at the time of harvest. Perhaps it would be suitable to use slightly higher quantity of seed for the autumn sowing and then perform manual thinning or machine combing of the crop if needed. Manual thinning takes a lot of time and costs a lot, so a product calculation should be considered first. We advise against autumn sowing in areas with strong winds since much higher plants with fragile stems and weak roots are prone to flattening and breaking that can subsequently cause a loss of crop and problems at

machine harvesting. Finally poppy is a crop that can be used to extend the rather narrow crop rotation on the majority of livestock and arable farms in south-eastern Slovenia, definitely the yield of seed and perhaps also the production of oil could become an additional income source on the farm.

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Priprava klimatskih podlag kot dodatnega kriterija za določanje območij z omejenimi možnostmi za kmetijsko dejavnost

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IZVLEČEK

Analizirali smo klimatske značilnosti Slovenije v obdobju 1981–2010, ki so predlagane za določitev območij z omejenimi možnostmi za kmetijsko dejavnost (OMD). Po navodilih Evropske komisije, ki jih je pripravil Joint Research Centre (JRC), je potrebno izračunati 30-letna povprečja za kriterij nizkih teperatur zraka (dolžina rastne dobe in vsote efektivnih temperatur zraka) in kriterij aridnega podnebja (indeks sušnosti *AI*). Dodatno smo izračune opravili tudi po metodi Agencije RS za okolje (ARSO), ki se pri določanju temperaturnih pragov nekoliko razlikuje od metode JRC. Po kriteriju nizkih temperatur zraka so pod pragom za OMD le hribovitejši predeli, najnižja uvrščena meteorološka postaja je v Ratečah. Glede na kriterij aridnega podnebja se nobeno območje v Sloveniji ne uvrsti med OMD, zato smo dodatno analizirali meteorološko vodno bilanco. V povprečju se je v obdobju 1981–2010 glede na obdobje 1971–2000 na večini lokacij zmanjšala. Vplivi podnebnih sprememb se kažejo v prisotnih trendih pri obravnavanih spremenljivkah, zato pri določanju OMD priporočamo izračune in upoštevanje trendov oziroma redne ponovitve analiz.

Ključne besede: območja z omejenimi možnostmi za kmetijsko dejavnost (OMD), rastna doba, vsota efektivnih temperatur zraka, kazalec sušnosti AI, meteorološka vodna bilanca

ABSTRACT

CALCULATION OF CLIMATE FACTORS AS AN ADDITIONAL CRITERIA TO DETERMINE AGRICULTURALLY LESS FAVOURED AREAS

Climate factors that are proposed to determine agriculturally less favoured areas (LFA) in Slovenia were analyzed for the period 1981–2010. Following the instructions of European Commission prepared by Joint Research Centre (JRC) 30-years averages of low air temperatures criteria (the vegetation period duration and sums of effective air temperatures) and aridity criteria (aridity index *AI*) have to be calculated. Calculations were additionally done using Slovenian Environment Agency (ARSO) method, which is slightly different when determining temperature thresholds. Only hilly areas are below the LFA low air temperatures threshold with the lowest located meteorological station in Rateče. According to aridity criteria no area in Slovenia is below the threshold, so meteorological water balance was also examined. Average water balance in the period 1981–2010 was in most of locations lower than in the period 1971–2000. Climate change impacts are already expressed as trend presence in time series of studied variables, so it is recommended to calculate trends and take them into account or to perform regular iterations of calculations.

Key words: agriculturally less favoured areas (LFA), vegetation period, sum of effective air temperatures, aridity index AI, meteorological water balance

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1 UVOD

Skupna kmetijska politika si z uvajanjem različnih ukrepov za različne vloge kmetijstva (npr. gospodarsko, prostorsko, ekološko, socialno) glede na Program razvoja podeželja RS za obdobje 2007–2013 (Program..., 2009) prizadeva za trajnostni razvoj kmetijstva. Del tega so tudi plačila za območja z omejenimi možnostmi za kmetijsko dejavnost (OMD), ki so namenjena ohranjanju proizvodnje in kulturne krajine na teh območjih in jih je v Sloveniji trenutno 86,3 % celotne površine. Slovenija je v obdobju 2007–2013 izvajala izravnalna plačila za OMD po treh skupinah kriterijev: za hribovsko gorska območja, območja s posebnimi omejitvami in druga območja. Do sedaj so bili upoštevani standardni omejitveni dejavniki in z njimi povezani kriteriji (nagib kmetijskih zemljišč, zemljiška in parcelna razdrobljenost, talne razmere, nadmorska višina) in regionalno specifični omejitveni dejavniki ter z njimi povezani kriteriji (kraško površje, poplave, močni vetrovi, erozija) (Program..., 2009).

V okviru Ciljnega raziskovalnega programa »Zagotovimo.si hrano za jutri« smo v projektu »Klimatske podlage kot dodatni kriterij za območja z omejenimi možnostmi za kmetijsko dejavnost (OMD)« analizirali spremenljivke, ki so predlagane za določitev tretje skupine, to je drugih območij z naravnimi omejitvami, in se navezujejo na njihove klimatske značilnosti. Pri tem smo sledili reformi drugih območij z OMD, ki poteka na ravni Evropske unije (EU) (Uredba EU, št. 1305/2013, člen 32). Metodologijo za določanje območij z OMD glede na biofizikalne kriterije so za Evropsko komisijo pripravili na Joint Research Centre (JRC) (Updated…, 2012). Naša analiza je zajemala dolžino rastne dobe, vsote efektivnih temperatur zraka za rastno dobo in sušni stres rastlin, pri čemer smo obvezani slediti metodam dela po JRC. Ker se v Sloveniji v splošni praksi na Agenciji RS za okolje (ARSO) uporabljajo nekoliko drugačne metode dela, smo izračune pripravili tudi po teh, kar Ministrstvu za kmetijstvo, gozdarstvo in prehrano nudi dodatno informacijo o obravnavanih spremenljivkah.

Klimatski kriteriji so določeni tako, da upoštevajo potrebno toploto za razvoj poljščin in da določijo morebitne sušne razmere. Kriterij nizkih temperatur zraka je določen na podlagi dolžine rastne dobe in vsote efektivnih temperatur zraka v rastni dobi. Pomemben je s kmetijskega vidika, saj nizke temperature zraka omejujejo rast in razvoj rastlin preko vpliva na njihovo zgradbo, razmnoževanje in pomembne fiziološke procese, kot sta na primer fotosinteza in olistanje. Nizke temperature zraka so definirane kot dejavnik, pri katerem je preživetje rastlin ali njihova produktivnost omejena, torej temperaturne razmere niso zadostne za njihov obstoj, optimalno rast in razvoj (Updated …, 2012). Kriterij aridnega podnebja pa je ob daljših obdobjih brez dežja še pomembnejši omejitveni dejavnik za rast in razvoj kmetijskih kultur.

Sušnik in Žust (2008) sta za potrebe vmesne diskusije na Evropskem svetu po naročilu tedanjega Ministrstva za kmetijstvo, gozdarstvo in prehrano leta 2007 pripravili izračune dolžine rastne dobe in vsote efektivnih temperatur zraka za rastno dobo za Slovenijo za določen nabor glavnih meteoroloških postaj za obdobje 1961–2007. Izkazalo se je, da dolžina rastne dobe v Sloveniji za večino nižinskih kmetijskih pridelovalnih območij ni omejevalni dejavnik glede na mejne vrednosti. Pri analizi vsot efektivnih temperatur zraka sta ugotovili, da so tudi območja v hribovitih predelih (med 600 in 1000 m nadmorske višine) nad mejnimi vrednostmi. Kot zaključek sta navedli, da so za naše razmere meje temperaturnih kriterijev preostre in zato neustrezne za slovenske razmere.

Kot glavni omejitveni dejavnik se v Sloveniji vedno pogosteje omenja sušnost (Sušnik, 2014). Na sušnejših območjih je kmetijstvo precej omejeno z možnostmi glede izbora poljščin in z manjšim pridelkom. Obstaja sicer veliko različnih kazalcev suše, vsak s svojimi prednostmi in omejitvami (Sušnik, 2014). Večinoma so zasnovani na osnovnih meteoroloških spremenljivkah: temperatura zraka, padavine in potencialna evapotranspiracija. Za izračun OMD mora biti kazalec suše preprost za izračunavanje, temeljiti pa mora na osnovnih meteoroloških meritvah. Pri suši v kmetijstvu so izrednega pomena tudi tla, ta pa so že ločeno vključena v obravnavo OMD, čeprav bi morali upoštevati interakcijo z meteorološkimi spremenljivkami. Kljub temu, da je Slovenija bogata z vodnimi viri,

vsi podatki kažejo, da zaradi pomanjkanja padavin ali njihove neugodne časovne razporeditve, suša predstavlja tveganje tudi pri nas. Slovenija porabi za odpravo posledic suše v kmetijstvu veliko sredstev in žal deluje na področju suš nekonsistentno in brez dobro definiranih kompetenc (Pogačar in sod., 2014).

2 METODE IN MATERIAL

2.1 Dolžina rastne dobe in vsote efektivnih temperatur zraka

Dolžina rastne dobe je po JRC (Updated…, 2012) določena kot število dni med (vključenim) spomladanskim in (izključenim) jesenskim pragom. Spomladanski prag oziroma začetek rastne dobe je določen s petim dnem, ko je v petih zaporednih dneh povprečna dnevna temperatura zraka nad 5 °C. Podobno je jesenski prag oziroma konec določen kot peti dan, ko je vsaj pet zaporednih dni povprečna dnevna temperatura zraka pod 5 °C. Vsote efektivnih temperatur zraka določimo tako, da v rastni dobi na podlagi dnevnih povprečnih temperatur zraka seštejemo presežke povprečne dnevne temperature zraka nad temperaturo praga 5 °C.

Predvsem zaradi zimskih otoplitev v jugozahodni Sloveniji ter zgodnje spomladanskih otoplitev drugod po nižinski Sloveniji, ki trajajo več kot pet dni, pa ARSO uporablja drugačno definicijo za doseganje temperaturnega praga. Spomladanski prag nastopi prvi dan vsaj 6 dni dolgega intervala s povprečno temperaturo zraka, večjo od temperature praga po koncu zadnjega vsaj 6 dni dolgega intervala s povprečno temperaturo zraka manjšo od temperature praga. Jesenski prag nastopi prvi dan prvega 6-dnevnega intervala s povprečno temperaturo zraka, manjšo od temperature praga (ARSO, 2014). Definiciji JRC in ARSO se torej razlikujeta za en dan v dolžini zahtevanega intervala in v dnevu, na katerega nastopi temperaturni prag (pri JRC je to zadnji dan intervala, pri ARSO pa prvi dan).

Izračune smo po obeh metodah izvedli za sedem meteoroloških postaj, ki so si različne po nadmorski višini ter zastopajo različne regije Slovenije (označene na sliki 1). Izračunane vrednosti smo primerjali z mejnimi vrednostmi za OMD po JRC (Updated…, 2012): vsota efektivnih temperatur zraka mora biti manjša od 1500 °C ali pa dolžina rastne dobe krajša od 180 dni. Pri kriterijih za OMD se dodatno upošteva medletno variabilnost meteoroloških razmer, zato je po navodilih JRC priporočeno uporabiti pristop 80 %/20 %. Ta predvideva, da se območje uvrsti med območja omejena z nizkimi temperaturami zraka, če so vsaj v 20 % let oziroma sedmih letih od obravnavanih 30 dolžine rastnih dob ali vsote efektivnih temperatur zraka pod določenim pragom.

2.2 Sušnost

V okviru evropske kmetijske politike in OMD je primanjkljaj vode v tleh definiran z letnim številom dni s primanjkljajem vode. Primankljaj vode v tleh pomeni, da količina padavin in razpoložljive vode v tleh v primerjavi s potencialno evapotranspiracijo ni zadostna, da bi rastline lahko napredovale s produkcijskim ciklom (Updated…, 2012). Za določitev OMD po JRC je izbran kazalec indeks sušnosti *AI* (Aridity Index), ki je določen kot razmerje med letno količino padavin (*RR*) in letno potencialno evapotranspiracijo (ET_p): $AI = RR/ET_p$ (Updated…, 2012). Za območja z OMD veljajo tista, pri katerih so vrednosti *AI* pod 0,5. Za primer smo izbrali dve glavni meteorološki postaji v Sloveniji, ki imata glede na padavinsko karto korigiranih padavin za dolgoletno obdobje 1971–2000 (Povprečna…, 2014) najmanj (Murska Sobota - Rakičan) in največ (Rateče) padavin. Za obe smo za obdobje 1981–2010 na letni ravni in ravni vegetacijskega obdobja (od začetka aprila do konca septembra) izračunali *AI*. Žal sistematičnih meritev potencialne evapotranspiracije v Sloveniji ni. Izjema so lizimetri, ki pa niso del merilne mreže ARSO (Zupanc in sod., 2012). Zato ima izbira dobre računske metode za oceno evapotranspiracije še poseben pomen. Priporočena je uporaba Penman-Monteithove metode (Allen in sod., 1998), ki je tudi za Slovenijo že večkrat preizkušena in je v stalni uporabi na ARSO.

Že pred začetkom projekta smo predvidevali, da za Slovenijo kazalec *AI* zelo verjetno ne bo pokazal sušnosti in se zato odločili za dodatno analizo

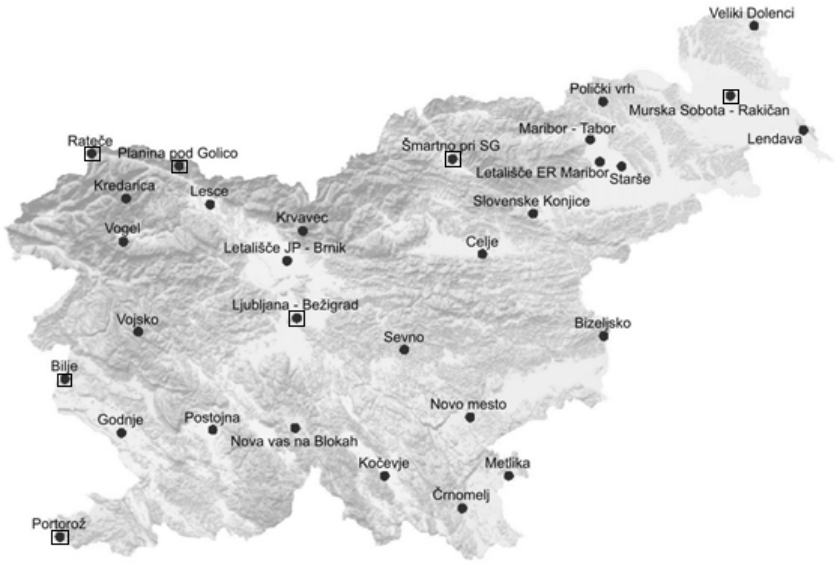
meteorološke vodne bilance. Ta se izračuna kot razlika med količino padavin in potencialno evapotranspiracijo na dnevni skali, obravnavali pa smo vsote meteorološke vodne bilance v vegetacijskem obdobju.

2.3 Statistična analiza

Osnovna statistična analiza je bila predpisana s strani JRC. Izračunali smo povprečne vrednosti obravnavanih spremenljivk v 30-letnem obdobju in medletno variabilnost, izraženo v številu let, ko je vrednost spremenljivke pod določenim pragom. Dodatno smo za izbranih sedem postaj pogledali časovne vrste spremenljivk, da bi dobili vpogled, ali obstaja trend ali jih lahko obravnavamo kot stacionarne. Izračunali in prikazali smo drseče sredine reda 10, s katerimi smo želeli preveriti, ali se v časovni vrsti kaže prisotnost trenda. V tem primeru se moramo namreč zavedati, da se povprečje v obravnavanem obdobju spreminja, kar pomeni, da so rezultati metod, ki temeljijo samo na povprečju, pristranski.

2.4 Podatki

Za izračun dolžine rastne dobe in vsote efektivnih temperatur zraka smo potrebovali povprečne dnevne temperature zraka, za izračune kazalca sušnosti in meteorološke vodne bilance pa dnevne izmerjene količine padavin in izračunane vrednosti dnevne potencialne evapotranspiracije. Za vse izračune in analize smo uporabljali podatke meteoroloških meritev ARSO (ARSO, 2014) za obdobje 1981–2010, za meteorološko vodno bilanco pa dodatno podatke o padavinah in potencialni evapotranspiraciji v obdobju 1971–2000 (z izjemo postaj Krvavec, Sevno, Maribor - letališče, Lesce, Metlika in Portorož, ki v tem obdobju nimajo dovolj dolgega niza podatkov).



Slika 1: Lokacije analiziranih meteoroloških postaj za izračune dolžine rastne dobe in vsot efektivnih temperatur zraka ter z izjemo Vojskega in Vogla tudi za izračune sušnosti. Označene (kvadratek) so postaje, na katerih smo opravili izračune po dveh metodah (JRC in ARSO).

Figure 1: Meteorological stations used for the calculations of vegetation period duration and sums of effective air temperatures and without Vojsko and Vogel also for the calculations of dryness. Calculations using both methods (JRC and ARSO) were done on marked stations (square).

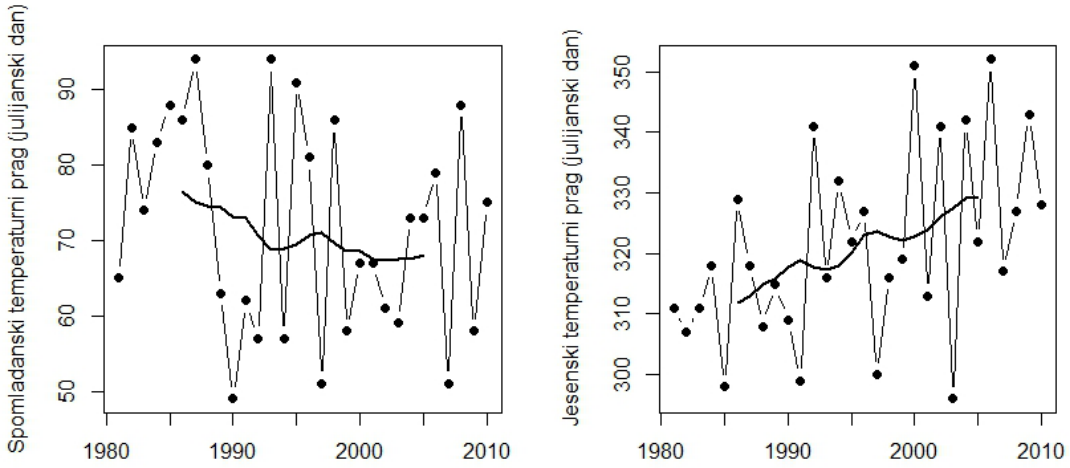
Velik del obdelave je bil namenjen pregledu podatkov, saj so za analize potrebni 30-letni nizi brez manjkajočih vrednosti za čim več meteoroloških postaj po Sloveniji. Za analizo dveh različnih izračunov dolžine rastne dobe smo izbrali sedem postaj (označene s kvadratkom na sliki 1): Planina pod Golico (956 m n.v.), Rateče - Planica (864 m n.v.), Bilje (55 m n.v.), Ljubljana - Bežigrad (299 m n.v.), Šmartno pri Slovenj Gradcu (444 m n.v.), Murska Sobota - Rakičan

(187 m n.v.) in Portorož - letališče (2 m n.v.), ki pa ima krajši niz podatkov (1989–2010) in je zato v preglednicah označen z *. Za izračune dolžine rastne dobe po JRC in vsot efektivnih temperatur zraka smo uporabili še dodatnih 23 postaj (slika 1), tudi višje ležeče (nad 1000 m nadmorske višine), kjer večinoma ni več kmetijskih površin, a so nam služile za vpogled v spreminjanje vrednosti obravnavanih spremenljivk z višino. Kredarico smo iz poročila nato izločili, ker leži v visokogorju. Za izračune meteorološke vodne bilance je bil uporabljen enak nabor postaj, le brez Vojskega in Vogla.

3 REZULTATI Z DISKUSIJO

3.1 Nastop spomladanskega in jesenskega temperaturnega praga

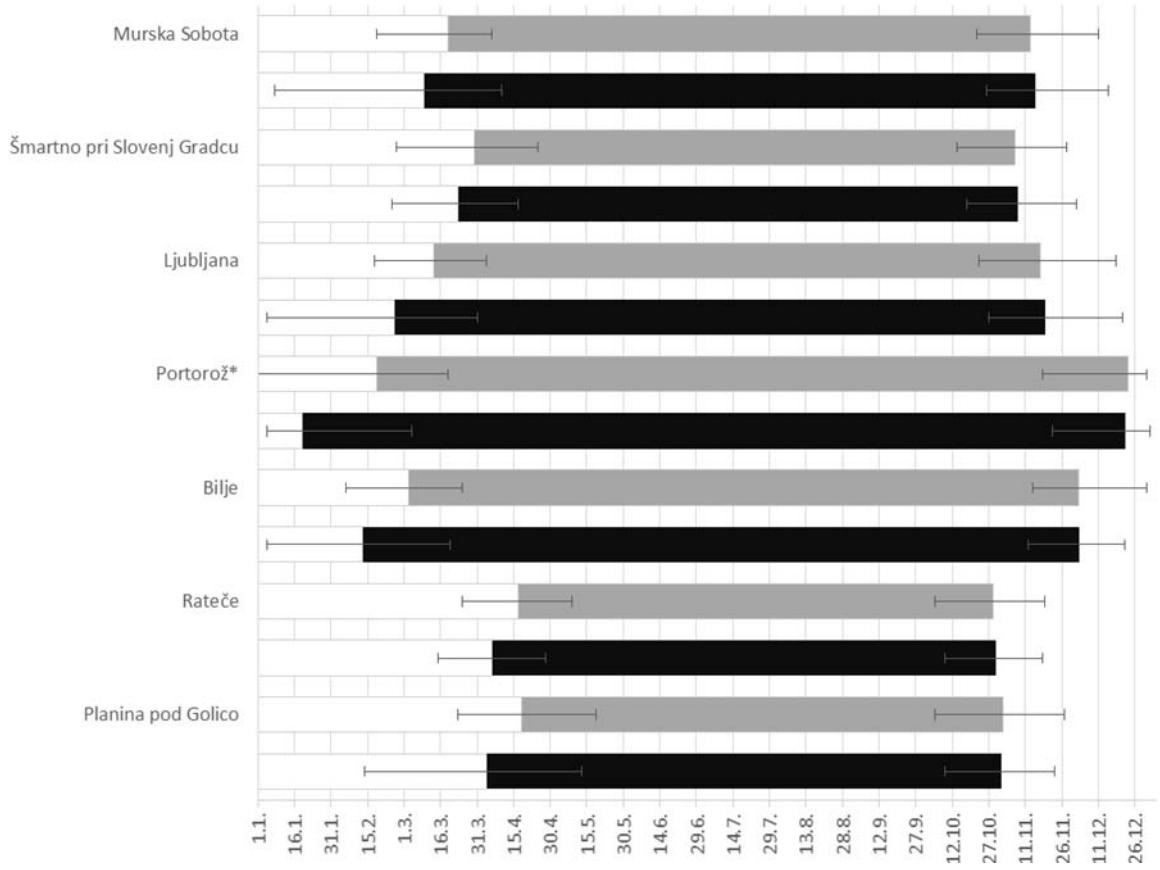
Časovni vrsti dneva nastopa spomladanskega in jesenskega temperaturnega praga z izračunanimi drsečimi sredinami reda 10 za Ljubljano (slika 2) odražata stanje na vseh sedmih izbranih postajah in po pričakovanjih tudi v veliki meri na vseh obravnavanih postajah. Drseče sredine reda 10 nakazujejo močno prisoten trend, kar pomeni, da 30-letno povprečje ne predstavlja dobrega opisa stanja, a mora biti vseeno uporabljeno kot del zahtevane metodologije.



Slika 2: Časovna vrsta (•-) ter drseče sredine reda 10 (črna črta) dneva nastopa spomladanskega (levo) in jesenskega temperaturnega praga (desno), izračunanega po metodi JRC, v Ljubljani za obdobje 1981–2010

Figure 2: Time series (•-) and 10th order (black line) moving averages of day of the occurence of spring (left) and autumn threshold (right), using JRC method for Ljubljana for the period 1981–2010

Za sedem izbranih postaj smo prikazali povprečni dan nastopa spomladanskega in jesenskega praga po obeh metodah z označenim variacijskim razmikom. Pri tem vmesno polje predstavlja povprečno dolžino rastne dobe (slika 3).



Slika 3: Povprečni dan nastopa in variacijski razmik spomladanskega in jesenskega praga (osenčena je povprečna dolžina rastne dobe) za izbrane postaje za obdobje 1981–2010 po metodi JRC (črno) in ARSO (sivo) (*krajši niz podatkov)

Figure 3: Average day of the occurence and variability range of spring and autumn threshold (average vegetation period duration is shaded) for chosen stations for the period 1981–2010 using JRC (black) and ARSO method (grey) (*shorter data set)

V povprečju je spomladanski prag po JRC najhitreje nastopil v Portorožu, 19. januarja, na začetku februarja v Biljah ter ob koncu februarja v Ljubljani, na začetku marca v Murski Soboti in ob koncu marca v Šmartnem pri Slovenj Gradcu ter v prvih dneh aprila v Ratečah in na Planini pod Golico. Spomladanski prag je v povprečju po JRC na vseh postajah nastopil prej kot po metodi ARSO. To lahko pripišemo januarskim otoplitvam v letih od 1981 do 1986, 1991, 1993, 1994, od 1996 do 1998, od 2003 do 2005 ter od 2008 do 2010 in februarski otoplitvi v letu 1987, ki jih metoda JRC ne izloči.

Predvsem je očitna razlika v nastopu najzgodnejšega spomladanskega praga, saj je po metodi JRC ta v Biljah in Ljubljani nastopil že 5. januarja, v Murski Soboti 8. januarja in na Planini pod Golico 14. februarja, po ARSO pa v istem vrstnem redu 6. februarja, 18. februarja, 19. februarja in 23. marca, torej so zamiki vsaj enomesečni. V Portorožu je po metodi ARSO najzgodnejši prag nastopil že 1. januarja, po JRC pa 5. januarja. Najkasneje je bil po metodi ARSO dosežen v letu 1992, 19. marca, a je bilo podobnih let še kar nekaj. V Ratečah in na Planini pod Golico pa je bil zaradi večje nadmorske višine prestop spomladanskega praga nekoliko zamaknjen, najbolj zgoden je bil v marcu, najkasnejši pa v maju. Prav posebno je bilo v Portorožu leto 1988, ko so bile povprečne dnevne temperature zraka z izjemo le nekaj dni vse leto nad 5 °C, tako da se je rastna doba po metodi ARSO teoretično začela 1. januarja, končala pa 31. decembra, praktično pa se je začela leta 1987 in nadaljevala v leto 1989.

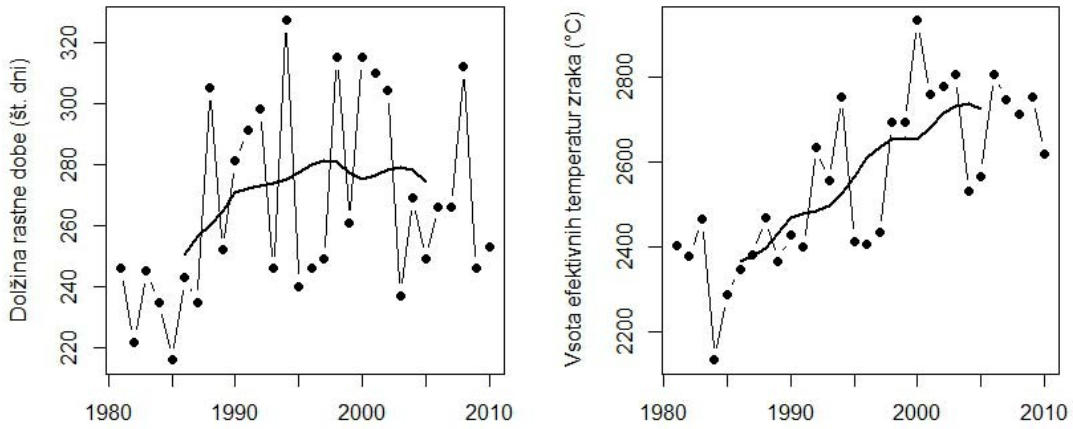
Jesenski prag je v povprečju nastopil po obeh metodah ob približno istem času. Najzgodneje je nastopil v celotnem obravnavanem obdobju po metodi JRC v Ratečah in na Planini pod Golico 9. oktobra (po ARSO 5. oktobra), v Šmartnem pri Slovenj Gradcu, Murski Soboti in Ljubljani v drugi polovici oktobra ter v Biljah 12. novembra (po ARSO 14. novembra) in v Portorožu šele 22. novembra (po ARSO 18. novembra). Najkasneje v celotnem obdobju pa je jesenski prag v Ljubljani, Biljah in Portorožu nastopil v drugi polovici decembra, v Šmartnem pri Slovenj Gradcu in Murski Soboti v prvi polovici decembra ter že v drugi polovici novembra v Ratečah in na Planini pod Golico.

Glede na že opaženo segrevanje ozračja, ki se odraža tudi pri padajočih drsečih sredinah spomladanskega in naraščajočih jesenskega temperaturnega praga, ter ob pričakovanem nadaljnjem segrevanju ozračja (Prihodnje spremembe ..., 2014), se bo pojav, da se rastna doba ne prekine, pogosteje dogajal ne samo na Primorskem, ampak tudi na drugih območjih. Podobno po modelskih napovedih ob koncu stoletja pričakujejo celo za južnejše predele Finske (Ruosteenoja in sod., 2010).

3.2 Dolžina rastne dobe in vsote efektivnih temperatur zraka

Pri prikazu časovne vrste dolžine rastne dobe (slika 4 levo) se je za izbrane postaje izkazalo, da prisotnost trenda ni tako očitna kot pri temperaturnih pragovih, po drugi strani pa se je za vsote efektivnih temperatur zraka (slika 4 desno) nazorno pokazala prisotnost naraščajočega trenda.

Predvsem za vsote efektivnih temperatur zraka velja enako kot za temperaturne pragove. Povprečje se zaradi trenda v obravnavanem obdobju spreminja. Kljub temu pa moramo za potrebe določanja OMD obravnavati povprečja, a hkrati opozarjamo, da se razmere spreminjajo in bi bilo zato potrebno metodologijo spremeniti.



Slika 4: Časovna vrsta (•-) ter drseče sredine reda 10 (črna črta) letne dolžine rastne dobe (levo) in vsote efektivnih temperatur zraka (desno), izračunanih po metodi JRC, v Ljubljani za obdobje 1981–2010

Figure 4: Time series (•-) and 10th order (black line) moving averages of vegetation period duration (left) and sum of effective air temperatures (right), using JRC method for Ljubljana for the period 1981–2010

Povprečne dolžine rastne dobe (preglednica 1) so po metodi ARSO od 195 dni v Ratečah do 307 dni v Portorožu, po metodi JRC pa od 208 dni v Ratečah do 338 dni v Portorožu. Razlika v metodologiji torej prispeva k precej različnim rezultatom. Standarne napake (preglednica 1) in koeficienti variacije so primerljivi, najbolj izstopata po metodi JRC Ljubljana z največjim in Portorož z najmanjšim koeficientom variacije.

Preglednica 1: Povprečne dolžine rastne dobe (povp) in standardne napake (se) za izbrane postaje za obdobje 1981–2010 po metodah JRC in ARSO
Table 1: Average vegetation period duration (povp) and standard errors (se) for chosen stations for the period 1981–2010 using JRC and ARSO method

	Planina pod Golico (št. dni)	Rateče (št. dni)	Bilje (št. dni)	Portorož* (št. dni)	Ljubljana (št. dni)	Šmartno pri Slovenj Gradcu (št. dni)	Murska Sobota (št. dni)
JRC-povp	211	208	293	338	266	229	251
JRC-se	3,6	2,8	5,5	3,1	5,8	3,5	4,6
ARSO-povp	198	195	276	307	249	222	239
ARSO-se	3,6	2,9	3,3	5,0	3,8	3,8	3,6

*krajši niz podatkov (*shorter data set)

Preglednica 2: Povprečne vsote efektivnih temperatur zraka nad pragom 5 °C (povp) in standardne napake (se) po metodah JRC in ARSO za izbrane postaje za obdobje 1981–2010
Table 2: Average sums of effective air temperatures over the threshold 5 °C (povp) and standard errors (se) for chosen stations for the period 1981–2010 using JRC and ARSO method

	Planina pod Golico (°C)	Rateče (°C)	Bilje (°C)	Portorož* (°C)	Ljubljana (°C)	Šmartno pri Slovenj Gradcu (°C)	Murska Sobota (°C)
JRC-povp	1444	1546	2885	3237	2554	2001	2402
JRC-se	21,6	22,1	40,0	27,8	35,1	24,1	31,8
ARSO-povp	1446	1546	2878	3218	2547	2002	2396
JRC-se	21,5	22,2	38,2	28,3	34,9	24,5	31,0

*krajši niz podatkov (*shorter data set)

Povprečna vsota efektivnih temperatur zraka nad pragom 5 °C (preglednica 2) je na Planini pod Golico (1444 °C oz. 1446 °C) in v Ratečah (1546 °C) pod pragom JRC, ki znaša 1500 °C. Na ostalih postajah so povprečne vsote nad to mejo, in sicer od 2001 °C (po JRC oz. 2002 °C po ARSO) v Šmartnem pri Slovenj Gradcu do 3237 °C (po JRC oz. 3218 °C po ARSO) v Portorožu. Razlike v povprečjih med metodama niso velike, standardne napake so povsem primerljive.

Za potrebe določanja OMD smo po metodi JRC za vse obravnavane postaje določili, kdaj je dosežen spomladanski in jesenski temperaturni prag, nato pa smo izračunali dolžino rastne dobe ter vsoto efektivnih povprečnih dnevnih temperatur zraka ter 30-letna povprečja teh vrednosti. Meja, ki je s strani Evropske komisije določena za vključitev v OMD, je presežena za povprečno dolžino rastne dobe le na Krvavcu, za povprečno vsoto efektivnih temperatur zraka pa na Krvavcu, Planini pod Golico, Vojskem in Voglu (preglednica 3). Vse te postaje ležijo nad 1000 m nadmorske višine.

Ker samo povprečje ne nudi zadostne informacije o razmerah, je variabilnost znotraj 30-letnega obdobja upoštevana tako, da se območje uvrsti v OMD tudi v primeru, ko je 7 od 30 let na postaji pod pragom 180 dni oziroma pod pragom 1500 °C (zadnja stolpca v preglednici 3). Po tem kriteriju se dodatno uvrsti v OMD območja le postaja Rateče.

Na evropski ravni so izračune za temperaturni kriterij za OMD za obdobje 1975–2004 objavili Eliasson in sod. (2010). Pokazali so, da večina Evrope z izjemo severa in izrazito gorskega sveta ne zadošča temperaturnemu kriteriju za OMD. Enako se je pokazalo tudi v naših izračunih za Slovenijo.

Preglednica 3: Povprečni nastop spomladanskega in jesenskega praga, povprečna dolžina rastne dobe in povprečna vsota efektivnih temperatur zraka za temperaturni prag 5 °C za obdobje 1981–2010 po metodi JRC. Krepko so označene postaje in vrednosti, ki so pod mejo 1500 °C, ki definira območje z OMD. Na desni je število let (od obravnavanih 30), v katerih je dolžina rastne dobe oz. vsota efektivnih temperatur zraka pod pragom za OMD. Ležeče so označene vrednosti večje od 7, kar je meja za uvrstitev med OMD.

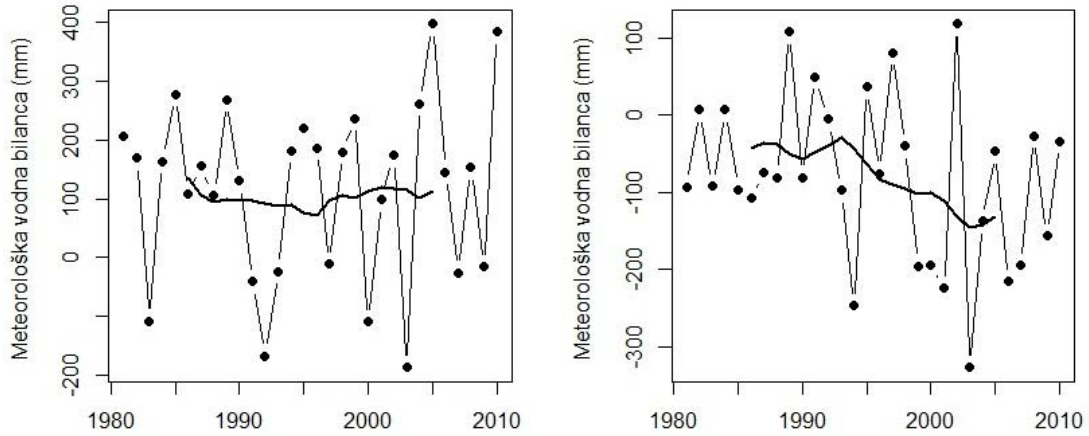
Table 3: Average occurence of spring and autumn threshold, average vegetation period duration and average sum of effective air temperatures with the threshold of 5 °C for the period 1981–2010 using JRC method. Values below the threshold 1500 °C for less favoured areas (LFA) are marked bold. On the right is the number of years (out of 30), in which is the vegetation period duration or the sum of effective air temperatures below the threshold for LFA. Values over 7, which is the threshold for LFA, are marked italic.

Postaja	Spomladanski prag 5 °C (datum)	Jesenski prag 5 °C (datum)	Dolžina rastne dobe (dni)	Vsota efektivnih temperatur zraka (°C)	Št. let pod pragom 180 dni	Št. let pod pragom 1500 °C
Krvavec	4.5.	7.10.	**156**	**761**	**25**	**30**
Brnik - letališče	18.3.	11.11.	238	2172	0	0
Planina pod Golico	4.4.	1.11.	211	**1443**	1	**20**
Rateče	6.4.	30.10.	208	1546	1	*12*
Vojsko	3.4.	31.10.	211	**1455**	2	**20**
Bilje	13.2.	3.12.	293	2885	0	0
Godnje	15.2.	27.11.	285	2582	0	0
Postojna	8.3.	13.11.	250	2025	0	0
Nova vas na Blokah	20.3.	5.11.	230	1718	1	1
Kočevje	10.3.	9.11.	244	2025	0	0
Ljubljana - Bežigrad	26.2.	19.11.	266	2554	0	0
Sevno	21.2.	13.11.	266	2182	0	0
Bizeljsko	2.3.	18.11.	260	2478	0	0
Novo mesto	21.2.	17.11.	269	2469	0	0
Črnomelj	27.2.	18.11.	264	2598	0	0
Celje	5.3.	16.11.	256	2367	0	0
Slovenske Konjice	16.2.	18.11.	275	2378	0	0
Starše	28.2.	16.11.	261	2450	0	0
Maribor - Tabor	2.3.	17.11.	260	2505	0	0
Maribor - letališče	6.3.	16.11.	255	2386	0	0
Šmartno pri Slovenj Gradcu	23.3.	8.11.	229	2001	0	0
Polički vrh	15.3.	12.11.	242	2230	0	0
Lendava	23.2.	16.11.	266	2504	0	0
Murska Sobota - Rakičan	9.3.	15.11.	251	2402	0	0
Veliki Dolenci	28.2.	15.11.	261	2378	0	0
Lesce	22.3.	11.11.	234	2012	0	0
Metlika	16.2.	18.11.	275	2580	0	0
Vogel	20.4.	20.10.	184	**981**	**12**	**28**
Portorož*	22.1.	20.12.	332	3205	0	0

*krajši niz podatkov (shorter data set)

3.3 Kazalec sušnosti in meteorološka vodna bilanca

Kazalec sušnosti *AI* za postajo Murska Sobota - Rakičan je na letni ravni 1,0, za vegetacijsko obdobje 0,8, za postajo Rateče pa na letni ravni 2,4, v vegetacijskem obdobju pa 1,6. Po priporočenem kazalcu sušnosti *AI* se torej noben del Slovenije ne uvršča pod določeni prag za OMD (0,5). Vrednosti so visoko nad pragom, zato lahko predpostavljamo, da je v vsej Sloveniji *AI* nad podano mejo in ga na ostalih postajah nismo analizirali.



Slika 5: Časovna vrsta (•-) ter drseče sredine reda 10 (črna črta) vegetacijske meteorološke vodne bilance v Ljubljani (levo) in Biljah (desno) za obdobje 1981–2010, izračunanih po metodi JRC

Figure 5: Time series (•-) and 10th order (black line) moving averages of meteorological water balance in vegetation period for Ljubljana (left) and Bilje (right) for the period 1981–2010, using JRC method

Vendar pa so izračuni že pred letom 2010 kazali na povečevanje vodnega primanjkljaja in s tem tudi števila sušnih dni v vegetacijskem obdobju (Pogačar in Kajfež-Bogataj, 2008). Kot prikazuje Sušnikova (2014), je predvsem po letu 2000 povprečna poletna (junij-avgust) meteorološka vodna bilanca (razlika med količino padavin in potencialno evapotranspiracijo) v več letih izrazito negativna, kar lahko predstavlja sušo na državni ravni. Zato smo v projektu, da bi bolje upoštevali dejansko stanje v Sloveniji, naredili tudi izračune meteorološke vodne bilance. Pri tem negativna vodna bilanca pomeni, da je v izbranem obdobju padlo manj padavin, kot pa je izhlapelo vode iz tal in referenčne rastline. Ne pomeni pa negativna vodna bilanca še nujno sušnega stanja, saj je pomembno, kako negativna je in koliko časa primanjkljaj traja. Tako tudi povprečna negativna vodna bilanca ne označuje suše.

Tudi za časovne vrste meteorološke vodne bilance v vegetacijskem obdobju nas je zanimalo, kakšen trend lahko ocenimo z drsečimi sredinami. Za izbranih sedem postaj se je pokazalo, da ne gre za izrazite trende kot pri temperaturnih pragovih in vsotah. V Ljubljani (slika 5 levo) se, na primer, trend ne kaže, v Biljah (slika 5 desno) se nakazuje negativni trend, v Šmartnem pri Slovenj Gradcu celo pozitivni. Zato moramo biti tudi pri obravnavi te spremenljivke previdni in upoštevati, da je potrebno izračune povprečij že čez nekaj let ponoviti.

Predstavljeni so rezultati meteorološke vodne bilance za vegetacijsko obdobje (preglednica 4). Povprečna meteorološka vodna bilanca je negativna na postajah (označene krepko v preglednici 4): Bizeljsko, Starše, Maribor - Tabor, Maribor - letališče, Lendava, Murska Sobota - Rakičan, Portorož. Mediana pa je negativna za Bilje in Metliko (prav tako označeni krepko). Od naštetih je najmanjša povprečna vrednost na postaji Portorož (-320 mm), sledijo Veliki Dolenci (-174 mm), Murska Sobota - Rakičan (-115 mm) in Lendava (-109 mm). Na teh štirih postajah (v preglednici 4 osenčene) je negativen še 75. percentil vodne bilance za obravnavano obdobje, kar pomeni, da je bila vodna bilanca vsaj v 75 odstotkih let negativna.

Velike povprečne vrednosti vodne bilance so pričakovano na višje ležečih postajah: Planina pod Golico (452 mm), Krvavec (364 mm) in Rateče (311 mm).

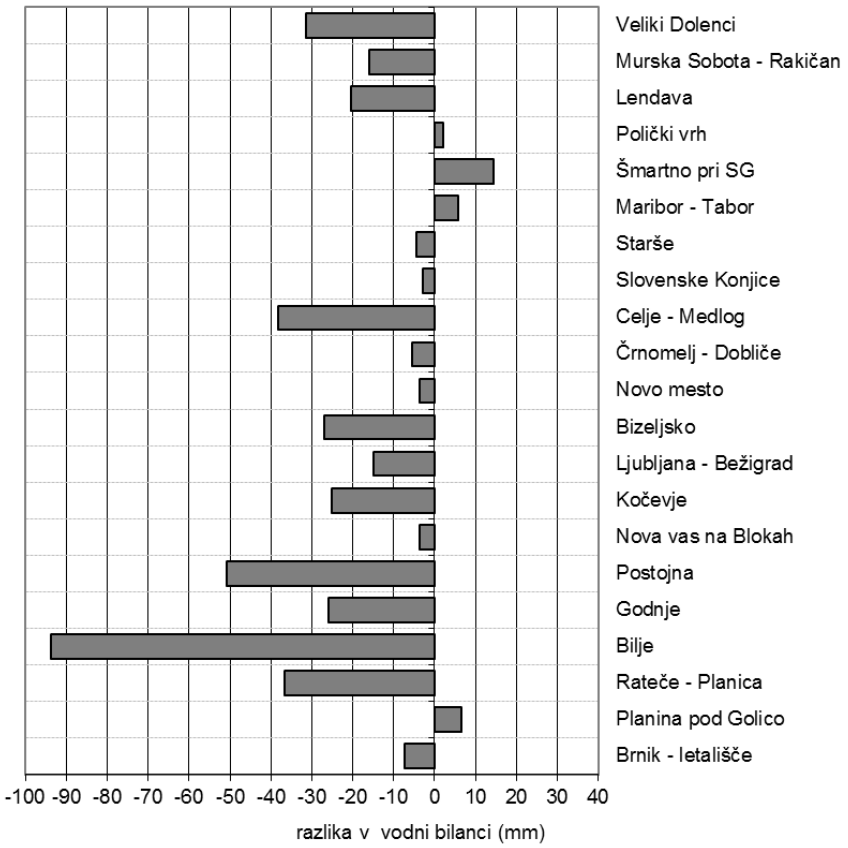
Preglednica 4: Meteorološka vodna bilanca (najmanjša in največja vrednost, 25., 50. in 75. percentil in povprečne vrednosti; v mm) ter delež analiziranih podatkov za obdobje 1981–2010. Krepko so označene postaje z negativnimi vrednostmi povprečja ali mediane; osenčene so postaje, pri katerih je negativna še vrednost vodne bilance pri 75. percentilu.

Table 4: Meteorological water balance (minimum and maximum, 25th, 50th and 75th percentile, and average values; in mm) and the proportion of analyzed data for the period 1981–2010. Negative values of the average or median are marked bold; negative values of 75th percentile are shaded.

Postaja	Min (mm)	25. percentil (mm)	Mediana (mm)	75. percentil (mm)	Maks (mm)	Povprečje (mm)	Delež analiziranih podatkov
Krvavec	-26,3	228,0	358,0	541,9	636,2	363,9	96 %
Brnik - letališče	-239,9	50,8	213,7	290,7	470,5	157,8	100 %
Planina pod Golico	158,1	360,0	445,0	544,9	778,4	452,0	99 %
Rateče	45,5	258,1	312,2	392,3	466,8	311,2	100 %
Bilje	-448,5	-89,1	**-11,0**	169,7	388,2	17,2	100 %
Godnje	-349,3	-25,2	79,4	199,6	512,6	86,9	100 %
Postojna	-233,0	39,8	226,2	307,4	452,4	171,3	100 %
Nova vas na Blokah	-109,6	145,3	269,5	348,4	560,2	244,1	100 %
Kočevje	-179,0	113,8	188,4	275,4	378,9	175,4	99 %
Ljubljana - Bežigrad	-187,7	-15,1	154,9	200,7	397,5	117,1	100 %
Sevno	-146,9	73,6	139,9	201,1	369,7	133,2	99 %
Bizeljsko	-410,1	-102,4	**-13,7**	82,6	315,5	**-35,0**	100 %
Novo mesto	-398,8	-22,5	27,8	129,0	416,9	47,2	100 %
Črnomelj	-274,6	-26,5	103,4	186,5	526,3	83,8	96 %
Celje	-385,0	22,1	60,9	112,8	326,0	46,0	100 %
Slovenske Konjice	-363,6	-40,4	70,8	110,9	294,5	26,2	99 %
Starše	-497,1	-70,9	**-26,1**	43,1	194,4	**-48,4**	100 %
Maribor - Tabor	-388,1	-82,5	**-32,8**	75,9	355,6	**-15,2**	100 %
Maribor - letališče	-403,4	-116,8	**-59,1**	32,5	234,7	**-67,2**	97 %
Šmartno pri SG	-84,9	110,4	152,3	270,4	364,4	169,2	100 %
Poličkі vrh	-237,3	-24,1	35,8	193,4	325,3	71,8	100 %
Lendava	-390,1	-165,3	**-104,4**	**-37,3**	130,6	**-108,9**	100 %
Murska Sobota - Rakičan	-416,1	-176,8	**-122,0**	**-34,2**	142,9	**-115,3**	100 %
Veliki Dolenci	-500,8	-235,9	**-154,8**	**-67,4**	109,8	**-174,0**	100 %
Lesce	-79,2	147,5	220,9	314,2	478,8	219,3	100 %
Metlika	-298,4	-91,6	**-1,0**	138,1	315,9	13,2	98 %
Portorož*	-574,9	-436,0	**-343,0**	**-257,4**	47,1	**-319,9**	62 %

*krajši niz podatkov (shorter data set)

Povprečje vegetacijske vodne bilance je bilo v obdobju 1981–2010 glede na obdobje 1971–2000 na skoraj vseh lokacijah manjše oziroma bolj negativno (slika 6). Najbolj negativna odstopanja so v Biljah, kar 94 mm, v Postojni 51 mm, v Celju 38 mm, v Ratečah 37 mm in v Velikih Dolencih 32 mm. Za rastline bolj ugodna pa je vodna bilanca postala v Šmartnem pri Slovenj Gradcu, Mariboru, na Poličkem vrhu in Planini pod Golico.



Slika 6: Odstopanja povprečne vegetacijske meteorološke vodne bilance za obdobje 1981–2010 glede na obdobje 1971–2000

Figure 6: Deviations of average vegetation meteorological water balance for the period 1981–2010 with regard to the period 1971–2000

4 SKLEPI

Metodi ARSO in JRC za izračun nastopa temperaturnih pragov se med seboj razlikujeta, kar se je pokazalo pri povprečnih dolžinah rastnih dob na sedmih izbranih postajah, razlike v povprečnih vsotah efektivnih temperatur zraka pa so bile zelo majhne. Ker je bila z metodo drsečih sredin prikazana prisotnost trenda v obravnavanih časovnih vrstah, nam povprečja ne dajo dovolj dobre informacije o vrednostih spremenljivk v 30-letnem obdobju, hkrati pa tudi niso primerna za dobro medsebojno primerjavo metod. Kljub temu morajo biti izračuni za Evropsko komisijo pripravljeni po metodi JRC, za določanje OMD moramo pripraviti povprečja za 30-letno obdobje, ne glede na trend. Pokazalo se je, da so po temperaturnem kriteriju pod pragom le vrednosti na meteoroloških postajah v hribovitem svetu, nad ali blizu 1000 m nadmorske višine, najnižja uvrščena meteorološka postaja je v Ratečah. Po kriteriju aridnega podnebja, določenim z indeksom *AI*, se Slovenija ne uvršča med OMD.

Pri odločitvah moramo torej upoštevati podnebne spremembe, predvsem naraščanje temperature zraka, ki lahko povzroči nove razmere v kmetijstvu. To se kaže že v sedanji analizi s prisotnostjo trendov. Z naraščanjem temperature zraka je povezano naraščanje števila vročih in toplih dni ter upadanje števila hladnih dni. Za fenološki razvoj rastlin je ključnega pomena, da je dovolj hladnih dni, preveliko število toplih ali celo vročih dni pa deluje stresno (Kajfež-Bogataj in sod., 2010). V zadnjih letih je bilo na kmetijskih rastlinah opažene veliko škode zaradi vročinskega stresa. Pri izračunih za OMD smo zato svetovali

izračune in upoštevanje trendov obravnavanih spremenljivk oziroma redne ponovitve izračunov.

V projektu smo po analizi podatkov in izračunih potrebnih spremenljivk nadaljevali s prostorsko interpolacijo izbranih osnovnih in izvedenih klimatskih spremenljivk. Ta lahko predstavlja problem, v kolikor so podatki dostopni za premajhno število meteoroloških postaj po Sloveniji. Upoštevati je potrebno tudi parametre, povezane z razgibanostjo reliefa v Sloveniji. Naše rezultate so na Kmetijskem inštitutu Slovenije vključili v točkovalni sistem za OMD. Za pomoč pri odločanju v primerih, kjer stanje ni povsem jasno, smo pri temperaturnem kriteriju dodatno analizirali pojav slane, število hladnih in ledenih dni ter nastop cvetenja domače češplje, pri kriteriju aridnega podnebja in meteorološki vodni bilanci pa vroče dni in vročinski stres, saj lahko predstavljajo dodatno obremenitev pri pridelavi kmetijskih kultur v Sloveniji.

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Oljna pogača navadnega ričkа (*Camelina sativa* (L.) Crantz) – neizkoriščeni vir fenolnih spojin

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IZVLEČEK

Delo zajema celovito študijo fenolnih spojin, njihovo zastopanost in identifikacijo v preostankih po stiskanju olja iz semen navadnega ričkа slovenskega porekla, to je v oljni pogači. Poleg tega poda rezultate določitve njihove učinkovitosti z metodami, ki vključujejo različne mehanizme antioksidativnega delovanja. Izkazalo se je, da se po stiskanju olja večina fenolnih spojin akumulira v pogači. Potrjena je bila prisotnost številnih antioksidantov: sinapina, 4-vinilgvajakola, 4-vinilkatehola, 4-vinilfenola, 4-vinilsiringola, elagne kisline, protokatehulne kisline, *p*-hidroksibenzojske kisline, sinapinske kisline, salicilne kisline, katehina, kvercetina in kvercetin glukozida. Izvlečki pogače so pokazali dobro redukcijsko moč in sposobnost lovljenja radikalov. Toplotna obdelava semen vpliva na količino prostih fenolnih spojin, topnih konjugatov in netopno vezanih fenolov, kot tudi na antioksidativno učinkovitost posameznih frakcij. Tudi primerjava z ostalimi oljnicami in sintetičnim antioksidantom opravičuje smotrnost uporabe ričkove pogače v živilski industriji.

Ključne besede: navadni riček (*Camelina sativa* (L.) Crantz), oljna pogača, antioksidativno delovanje, fenolne spojine

ABSTRACT

CAMELINA (*Camelina sativa* (L.) Crantz) OILCAKE – UNTAPPED RESOURCE OF PHENOLIC COMPOUNDS

The work includes a comprehensive study of phenolic compounds, their occurrence and identification in the residues after pressing of the oil from camelina seeds of Slovenian origin, i.e. oilcake. In addition, the efficiencies of antioxidant determinations using different methods according to different mechanisms are presented. These data demonstrate that almost all of the phenolic compounds in these seeds remain in the seed oilcake. The following antioxidants were confirmed: sinapine, 4-vinylphenol, 4-vinylguaiacol, 4-vinylsyringol, 4-vinylcatechol, ellagic acid, protocatechuic acid, *p*-hydroxybenzoic acid, sinapic acid, salicylic acid, catechin, quercetin and quercetin glucoside. The oilcake has high reducing power and radical scavenging activity. Heat treatment of seeds affects the amount of free, soluble and insoluble bound phenolic compounds as well as antioxidant capacity of individual fractions. Potential applications of camelina oilcake in the food industry are further justified by comparisons with other oilcakes and synthetic antioxidant.

Key words: Camelina (*Camelina sativa* (L.) Crantz), oilcake, antioxidant activity, phenolic compound

1 UVOD

Navadni riček (*Camelina sativa* (L.) Crantz), je oljnica, ki je na Koroškem znana z imenom toter (beseda izhaja iz nemškega izraza Dotter). Arheološke raziskave so potrdile, da so ga že v bronasti in železni dobi na območjih ob Severnem morju in ob Renu uporabljali za pripravo kašnatih jedi in kruha. Po sporadični gojitvi značilni za srednji vek, se je navadni riček v 20. stoletju razširil po deželah Evrope, kjer so iz semen pridobivali olje, proteinsko bogate oljne pogače pa so uporabljali za krmo živali (Zubr, 1997; Zubr, 2003a). Slovenija je ena redkih držav, kjer se je tradicionalna pridelava ričkа ohranila do danes, sejejo ga na Koroškem, medtem ko je drugod po

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Sloveniji skoraj neznan. Zaradi specifične prehranske vrednosti (velika vsebnost večkrat nenasičenih maščobnih kislin, prisotnost esencialnih in n-3 maščobnih kislin) se zanimanje za to alternativno oljnico v deželah Severne Evrope in Severne Amerike zadnja leta povečuje. Izsledki raziskav, ki so bile na olju in oljnih pogačah rička opravljene v Sloveniji (Abramovič in Abram, 2005; Abramovič in sod., 2007; Hrastar in sod., 2009; Hrastar in sod., 2011a; Hrastar in sod., 2011b; Hrastar in sod., 2013, Terpinc in sod., 2011a; Terpinc in sod., 2012a; Terpinc in sod., 2012b), opravičujejo potrebo po njegovi večji prepoznavnosti tudi na domačih tleh. Fenolne spojine, ki jih pridobimo iz semen, pogače in olja navadnega rička, bi se namreč lahko uporabile v živilski industriji za razvoj novih ter izboljšanje varnosti in kakovosti že obstoječih izdelkov. Posebno pozornost zasluži pogača, ki kot preostanek pri stiskanju olja predstavlja zanimiv in poceni naravni vir antioksidantov.

Navadni riček spada v družino križnic (Brassicaceae); ločimo ozimne in jare sorte (Zubr, 1997). Značilni so majhni rumeni cvetovi (približno 5 mm v premeru), plodovi hruškaste oblike vsebujejo več drobnih, rumeno rdečkastih semen, prevladuje samooprašitev (Schuster in Friedt, 1998). Semena vsebujejo med 35 in 45 ut. % olja. Viri navajajo, da je pridelek ričkovega olja na hektar do 907 litrov, kar je več kot pri sončnici in soji (Moser, 2010). Delež surovih proteinov v semenih navadnega rička je od 25 do 45 %, delež surovih vlaknin pa okoli 10 %. Kakovost semen je odvisna tako od sorte, kot tudi od rastnih razmer (Zubr, 2003b). Oljnica najbolje uspeva v zmernem podnebju, a dobro prenaša tudi sušo in nizke temperature. Ker je rastlina nezahtevna glede tal ter odporna na številne insekte, njeno gojenje ne zahteva visokih stroškov za gnojila in pesticide (Zubr, 2003b). Raziskave so potrdile, da je navadni riček primeren tudi za ekološko pridelavo (Kirkhus in sod, 2013).

Ričkovo ali totrovo olje je rumeno oranžne barve, značilnega vonja in okusa. Tradicionalno ga pridobivajo iz zmletih semen, ki jih pred stiskanjem zmešajo z vodo in toplotno obdelajo pri temperaturi do 90 °C. Zanimanje za ričkovo olje je posledica njegove specifične sestave. Vsebuje 7-14 % nasičenih, 26-41 % enkrat nenasičenih ter kar 46-64 % večkrat nenasičenih maščobnih kislin. Med nasičenimi maščobnimi kislinami prevladujeta palmitinska in stearinska kislina (Zubr, 1997). Uživanje ričkovega olja, kjer linolna kislina (LA; 18:2n – 6) predstavlja približno 15 %, α-linolenska kislina (ALA; 18:3n – 3) pa do 40 % vseh maščobnih kislin, pomembno prispeva k manjšemu razmerju med n-6 in n-3 maščobami v prehrani. Naslednja posebnost tega olja je relativno velika vsebnost gondojske kisline (20:1n-9) (Zubr in Matthäus, 2002). Olje navadnega rička je torej pomemben vir tako esencialnih nenasičenih (LA, ALA) kot n-3 maščobnih kislin (ALA) (Zubr in Matthäus, 2002).

Olje navadnega rička se je tradicionalno uporabljalo kot domače zdravilo. Kot splošno krepilno sredstvo so ga predpisovali pri težavah zaradi rane na želodcu in dvanajsterniku, prav tako pa tudi pri raznih vnetjih ter za oskrbo opeklin in ran (Rode, 2001). Svoje mesto je navadni riček našel celo v proizvodnji biogoriv, mil, barv in lakov (Zubr, 1997; Moser, 2010). Specifični dermatološki učinki večkrat nenasičenih maščobnih kislin pa omogočajo uporabo tega olja tudi v farmacevtski in kozmetični industriji kot sestavni del raznih olj, krem in losjonov (Schuster in Friedt, 1998; Zubr, 1997). Ričkovo olje se je ob uporabi zmerne temperature izkazalo kot primerno za kuhanje, pečenje, cvrenje, poskusno so ga uporabili tudi za izdelavo raznih namazov, solatnih prelivov, majonez, sladoleda (Zubr, 1997). Pri pripravi hrane, predvsem pa kot naravno ljudsko zdravilo ga nekateri domačini na Koroškem uporabljajo še danes.

Abramovič in Abram (2005) sta ugotavljali vpliv razmer skladiščenja na potek oksidacije ričkovega olja slovenskega izvora. Stopnjo primarnih oksidacijskih produktov sta spremljali s peroksidnim, stopnjo sekundarnih produktov oksidacije pa z anizidinskim številom. Izkazalo se je, da je ričkovo olje dokaj stabilno.

Naslednji poskus (Hrastar in sod., 2011a), ki je vključeval vzorce navadnega rička treh zaporednih sezon, je pokazal, da semena navadnega rička iz različnih lokacij Koroške vsebujejo 29 – 40 ut. % olja, medtem ko so avtorji v semenih potrdili tudi veliko vsebnost glukozinolatov, ki neugodno vplivajo na razvoj rakastih celic (Hayes in sod., 2008). Na podlagi dobljenih vrednosti za jodno in peroksidno število ter vsebnost prostih maščobnih

kislin, so avtorji zaključili, da se ričkovo olje lahko uporablja za najrazličnejše prehranske namene. Analizirano ričkovo olje je bilo nadalje izredno bogato z esencialno n-3 α-linolensko kislino (33,3 - 37,7 %) in γ –tokoferolom (532-798 mg/kg).

Glavni namen gojenja navadnega ričkа je olje. Pogača je preostanek semen po stiskanju olja. Ker je bogata s proteini, se uporablja za krmo domačih živali, predvsem perutnine (Zubr, 1997; Zubr, 2003b). V omejenih količinah se lahko dodaja krmi prašičev in prežvekovalcev; omejujoč dejavnik so prisotni glukozinolati (15-20 μmol/g pogače) (Schuster in Friedt, 1998; Zubr, 1997). Oljna pogača ima ugodno sestavo maščobnih kislin; izkazala se je kot pomemben vir vitaminov, mineralov in antioksidantov (Zubr, 2010; Matthäus, 2002).

Prispevek povzema rezultate raziskav, ki so bile opravljene na oljnih pogačah navadnega ričkа slovenskega porekla.

2 OLJNA POGAČA NAVADNEGA RIČKA – MOŽNOSTI APLIKACIJE NA PODLAGI DOSEDANJIH RAZISKAV

V okviru svoje raziskave so Terpinc in sod. (2012b) želeli ovrednotiti antioksidativni potencial pogače navadnega ričkа in pokazati, da je le-ta pomemben vir spojin z antioksidativno učinkovitostjo. Fenolne spojine so pridobili iz izbranih vzorcev s pomočjo ekstrakcije ob uporabi 80 % (v/v) metanola. Vsebnost skupnih fenolnih spojin so izrazili kot ekvivalent klorogenske kisline (KK) in ugotovili, da med stiskanjem olja večina fenolnih spojin preostane v pogači (1666 mg KK/100 g). Nekoliko manjše vrednosti, ki so jih določili v semenih, potrjujejo, da prisotnost vode in visoka temperatura med praženjem semen pospešita sprostitev fenolnih spojin iz vezanih oblik. S kvalitativno LC-MS analizo fenolnih spojin so določili zastopanosti le-teh v posameznih metanolnih izvlečkih pogače navadnega ričkа. Potrjena je bila prisotnost različnih pomembnih spojin.

Katehin in kvercetin sta v naravi precej razširjena flavonoida, ki se ponašata z antimikrobno, antikarcenogeno in antimutageno aktivnostjo (Rice-Evans in sod., 1996). O prisotnosti kvercetina v listih navadnega ričkа so pisali že Onyilagha in sod. (2003), medtem ko so o pomembnem antioksidativnem doprinosu flavonolov v pogači, zlasti kvercetin glikozidov, nekaj let kasneje poročali tudi Salminen in sod. (2006). Kot je pokazala analiza slovenskih vzorcev, se tako katehin, kot tudi kvercetin in kvercetin glukozid nahajajo v pogači, medtem ko je bilo v olju moč zaznati le manjše deleže katehina in kvercetina. Vsebnost slednjih dveh je bila v pogači večja kot v semenih, kar kaže na to, da je med praženjem prišlo do njune sprostitve iz konjugiranih in netopno vezanih oblik.

Med fenolnimi kislinami so avtorji (Terpinc in sod., 2012b) v izvlečkih pogače navadnega ričkа uspešno identificirali tri hidroksibenzojske (protokatehulna, *p*-hidroksibenzojska in salicilna kislina) in eno hidroksicimetno kislino (sinapinska kislina). Slednja je bila že pred leti razglašena za enega najpomembnejših antioksidantov pogače navadnega ričkа (Salminen in sod., 2006). Med fenolnimi kislinami se je v olje prenesla v manjši meri le sinapinska kislina, medtem ko je bila prisotnost salicilne in *p*-hidroksibenzojske kisline skorajda zanemarljiva, protokatehulna pa celo pod pragom detekcije. Količina slednje je v pogači glede na semena precej večja, tudi zastopanost *p*-hidroksibenzojske in salicilne kisline je bila večja v pogači kot v semenih, kar nakazuje, da so visoke temperature omogočile omenjenim spojinam sprostitev iz vezanih oblik. Naslednji pomembni antioksidant, ki so ga uspešno identificirali v izvlečkih omenjene oljnice slovenskega porekla, je elagna kislina, ki ima v svoji strukturi štiri hidroksilne skupine, kar ji vsekakor omogoča velik potencial za lovljenje prostih radikalov (Hayes in sod., 2009). Med stiskanjem se le v manjši meri prenese v olje, v večji meri pa ostane v pogači. Sinapin je ester sinapinske kisline in holina, njegova prisotnost v pogači navadnega ričkа pa je bila potrjena že pred leti (Matthäus in Zubr, 2000; Salminen in sod., 2006). V raziskavi slovenskih vzorcev so ugotovili, da je vsebnost sinapina v pogači in semenih praktično enaka, medtem ko v olje ne prehaja, kar je v skladu tudi z drugimi publikacijami (Matthäus in Zubr, 2000). Zadnjo

skupino uspešno identificiranih fenolnih spojin predstavljajo 4-vinil derivati hidroksicimetnih kislin (4-vinilgvajakol, 4-vinilkatehol, 4-vinilfenol, 4-vinilsiringol), ki se prav vsi ponašajo s pomembnimi antioksidativnimi lastnostmi (Terpinc in sod., 2011b).

Antioksidanti se razlikujejo tako po kemijskih kot fizikalnih lastnostih in delujejo na različne načine - kot reducirajoči dejavniki, lovilci prostih radikalov in/ali kelatorji kovin. V opravljeni raziskavi (Terpinc in sod., 2012b) se je izkazalo, da so fenolne spojine prisotne v izvlečku iz pogače slabši reducenti kot tiste v izvlečkih semen in olja, medtem ko so najuspešnejši lovilci radikalov prisotni v pogači. Metoda beljenja β-karotena se od ostalih analiznih metod razlikuje po tem, da reakcija med antioksidantom in radikalom poteka v sistemu emulzije linolne kisline v vodi. Ta metoda predstavlja enega bolj realnih medijev, saj so živila pogosto heterogeni koloidni sistemi. Kot najučinkovitejši se je izkazal izvleček iz olja. Glede na majhno število identificiranih spojin v olju ter na njihov majhen delež, Terpinc in sod. (2012b) predvidevajo, da so sodelovale zlasti neidentificirane in nekvantificirane fenolne spojine. Olje je namreč nepolaren medij in kot tak se vanj med stiskanjem semen prenesejo zlasti manj polarne spojine. Kot je splošno znano, se polarni antioksidanti zadržujejo v vodni fazi emulzije, zaradi česar so v oljni fazi slabše zastopani in tako posledično manj učinkoviti v zaščiti linolne kisline. Pravilnik o aditivih za živila (2010) omejuje uporabo sintetičnih antioksidantov, kot je butilirani hidroksitoluen (BHT), na 100 ppm. Kot se je izkazalo, so fenolne spojine v izvlečku iz olja izjemno učinkovite že pri precej manjši koncentraciji (30 ppm). Sposobnost vezave kovinskih ionov je naslednji način delovanja antioksidantov. Kot je znano, kovinski ioni katalizirajo verižno radikalsko reakcijo lipidne oksidacije, ki vodi v kvar živil (Andjelković in sod., 2006). Rezultati opravljene analize (Terpinc in sod., 2012b) so pokazali, da je za fenole iz pogače in semen značilna znatno manjša aktivnost kot jo ima izvleček iz olja s skoraj absolutno sposobnostjo keliranja kovinskih ionov.

Opravljena je bila tudi študija primerjave pogače navadnega rička z izvlečki pogač nekaterih drugih oljnic in sintetičnim antioksidantom BHT (Terpinc in sod., 2012a). Izsledki raziskave so pokazali, da vsebuje pogača navadnega rička več fenolov kot pogača oljne ogrščice (*Brassica napus* L. var. *napus*) in lanu (*Linum usitatissimum* L.), vendar manj kot bela gorjušica (*Sinapis alba* L.), katero je kot najbogatejši vir med naštetimi razglasil že Matthäus (2002). Nadalje so avtorji z metodo določitve redukcijske sposobnosti ugotovili, da so spojine, ki se nahajajo v pogači navadnega rička, boljši reducenti od tistih v beli gorjušici, medtem ko sta se oljna ogrščica in lan izkazala bolje. DPPH˙ test je pokazal, da je izvleček navadnega rička primerljiv z izvlečki nekaterih drugih oljnic in učinkovitejši od BHT. Rezultati določitve sposobnosti keliranja so pokazali, da imajo fenolne spojine ekstrahirane iz navadnega rička boljšo sposobnost vezave kovinskih ionov od izvlečkov oljne ogrščice in bele gorjušice, medtem ko se je kot najučinkovitejši izkazal izvleček iz pogače lanu. V sistemu emulgirane linolne kisline v vodi in β-karotena so se antioksidanti prisotni v izvlečku pogače navadnega rička med vsemi testiranimi oljnicami izkazali kot najučinkovitejši, boljši od navadnega rička je bil le nepolarni BHT. Med začetno fazo oksidacije nenasičenih maščobnih kislin pride v maščobnokislinski verigi do premestitve dvojne vezi in nastanka konjugiranega sistema, ki absorbira svetlobo pri 234 nm. Vpliv dodanih metanolnih izvlečkov iz pogač testiranih oljnic oz. BHT na tvorbo konjugiranih dienov (primarnih produktov oksidacije) in trienov (sekundarnih produktov oksidacije) so avtorji omenjene raziskave (Terpinc in sod., 2012a) spremljali v olju barvilnega rumenika, žafranike (*Carthamus tinctorius* L.), ki so ga inkubirali pri temperaturi 50 °C. Salminen in sod. (2006) so ugotovili, da je izvleček iz pogače navadnega rička učinkovit antioksidant tako v preprečevanju proteinske kot lipidne oksidacije. Rezultati raziskave slovenskih vzorcev so potrdili, da je nastanek konjugiranih dienov in trienov v olju ob prisotnosti izvlečka pogače navadnega rička omiljen v primerjavi s kontrolo (olje brez dodatka antioksidantov), primerljiv s preostalimi izvlečki, vendar obsežnejši v primerjavi z BHT. Pri tem je potrebno upoštevati, da je bila koncentracija dodanih izvlečkov znatno pod dovoljeno za sintetične antioksidante v živilski industriji. Polifenoli so resda glavna komponenta rastlin z antioksidativnimi lastnostmi, a vsekakor ne edina (vitamin C, vitamin E, karotenoidi, itd.) (Moure in sod., 2001). Še več, celo vzorci s podobno koncentracijo skupnih fenolnih spojin neredko

izražajo povsem različen antioksidativni potencial, ki ga med drugim lahko pripišemo tudi medsebojnemu delovanju antioksidantov ter vplivu medija na reaktivnost antioksidanta.

Fenolne spojine v rastlinski celici ne obstajajo le v prosti obliki (Huang in sod., 2009). T.i. netopno vezane fenolne spojine so sestavni del celične stene, kjer so običajno vezane na kompleksne ogljikove spojine kot sta lignin in arabinoksilan (Adom in Liu, 2002). Druge se nahajajo v vakuolah, kjer so povezane z različnimi sladkorji, alkoholi, organskimi kislinami in drugimi fenolnimi spojinami. To skupino pojmujemo topni konjugati (Liyana-Pathirana in Shahidi, 2006). Z ustrezno predpripravo semen (toplotna obdelava, mletje in razmastitev) in izbranim ekstrakcijskim postopkom (80 % metanol (v/v) ter alkalna hidroliza) lahko pridobimo posamezne frakcije fenolnih spojin. Izkazalo se je, da so fenolne spojine v razmaščenih zmletih semenih (v pogači) navadnega rička prisotne kot proste, kot topni konjugati in kot netopno vezane (Terpinc in sod., 2011a). Najmanjši delež so predstavljale ravno proste fenolne spojine. To pomeni, da se večina fenolnih spojin ne ekstrahira že s samim metanolom, ampak je za to potrebna hidroliza, ki omogoči ustrezno cepitev vezi. Raziskovalci so izvedli alkalno hidrolizo, ki je v primerjavi s kislinsko ali encimsko hidrolizo pogosteje uporabljena. Praženje pri višjih temperaturah je znatno povečalo vsebnost prostih fenolov. Temperatura je vplivala tudi na dobit topnih konjugatov, ki so bili v pogači toplotno netretiranih semen navadnega rička slabše zastopani od fenolov vezanih na celično steno. Tako je bilo mogoče ugotoviti, da je bilo za sprostitev slednjih in cepitve vezi v topnih konjugatih potrebno praženje pri 160 °C, medtem ko je prišlo do sprostitve topnih konjugatov kot takih že pri nižjih temperaturah. Hkrati je potekala tudi študija vpliva različnih načinov predpriprave semen na vsebnost skupnih flavonoidov, izraženih kot ekvivalent rutina. Dvig temperature praženja je pozitivno vplival na vsebnost prostih flavonoidov v pogači; optimalna temperatura je tako znašala 160 °C, medtem ko so bile za sprostitev netopno vezane frakcije primernejše nižje temperature (80 °C). Za cepitev vezi v tej frakciji pa temperatura nad 120 °C.

Izsledki proučevanja vpliva razmer predpriprave semen na vsebnost najbolj zastopanih fenolnih spojin v posameznih frakcijah so pokazali, da se skoraj ves sinapin nahaja v prosti obliki in sicer največ v pogači po praženju semen pri 160 °C. Večina protokatehulne kisline je vezana kot sestavni del celične stene, njena vsebnost v izvlečkih je naraščala s temperaturo toplotne obdelave. 4-vinilsiringol je bil med vsemi dekarboksilacijskimi produkti hidroksicimetnih kislin daleč najbolj zastopan, kot prost je dosegel maksimalno vrednost v izvlečku pogače po obdelavi pri 160°C. Prosta frakcija 4-vinilkatehola je s praženjem naraščala in v nasprotju s stanjem v toplotno neobdelanih semenih prevladala nad vezano obliko.

V okviru iste raziskave (Terpinc in sod., 2011a) so proučili tudi antioksidativne lastnosti fenolnih spojin, ki so prisotne kot proste, zaestrene ali netopno vezane. Rezultati so pokazali, da, neodvisno od toplotne obdelave, posedujejo največji redukcijski potencial tisti fenoli, ki so sestavni del celične stene, medtem ko so najmanj učinkoviti tisti, ki tvorijo topne konjugate. Ugotovili so, da dosežejo fenolne spojine, ki so prisotne kot proste oz. netopno vezane, maksimalno redukcijsko moč, če semena predhodno obdelamo pri 120 °C, medtem, ko se je za topne konjugate izkazalo, da so kot reducenti najučinkovitejši, če semen ne pražimo. Nadalje so ugotovili, da temperatura toplotne obdelave močno zaznamuje tudi sposobnost lovljenja DPPH$^{\cdot}$ radikalov, pri čemer je najboljšo učinkovitost pokazala frakcija prostih fenolov, kot optimalna pa se je izkazala temperatura praženja 120 °C. Kot so zapisali že Daglia in sod. (2000), je antioksidativna aktivnost odvisna od razgradnje obstoječih fenolnih spojin ter na drugi strani od uspešne tvorbe novih spojin z antioksidativno učinkovitostjo, predvsem produktov Maillardove reakcije, kadar so semena podvržena intenzivnejši toplotni obdelavi. V emulziji so se kot najslabši antioksidanti izkazale fenolne spojine, ki so prisotne kot topni konjugati, medtem ko so bili najučinkovitejši zoper alkilperoksilne radikale tisti fenoli, ki se v preiskovanem rastlinskem matriksu nahajajo v netopno vezani obliki. Nasprotno pa so se prosti fenoli izkazali za najučinkovitejše kelatorje. Frakcija topnih konjugatov v izvlečku iz pogače tako praženih kot nepraženih semen ni izkazala aktivnosti. Ker pa je praženje semen

pogosto neizogibno tako zaradi nastanka želene arome, barve, teksture, kot tudi povečane hranilne vrednosti, so avtorji kot sprejemljivo izbiro priporočili toplotno obdelavo pri 80 °C, saj je pri omenjenih pogojih obseg razpada kelatorjev najmanjši.

Slika 1: Oljna pogača navadnega ričkа (*Camelina sativa* (L.) Crantz)

3 ZAKLJUČKI

Tako določitev vsebnosti skupnih fenolov, kot tudi antioksidativni testi ter analize fenolnega profila potrjujejo, da se med stiskanjem olja iz semen večina fenolnih spojin ne prenese v olje, temveč preostane v pogači. V slednji je poleg 4-vinil derivatov in sinapina, potrjena prisotnost še drugih antioksidantov: elagne kisline, protokatehulne kisline, *p*-hidroksibenzojske kisline, sinapinske kisline, salicilne kisline, katehina, kvercetina in kvercetin glukozida. Slednje je dobrodošel podatek za živilsko industrijo, saj predstavlja pogača navadnega ričkа, ki je sicer stranski proizvod pri pridelavi omenjenega olja, izredno pomemben in poceni, a do sedaj neizkoriščen naravni vir antioksidantov. Še zlasti kot nadomestek za potencialno škodljive sintetične antioksidante. Različne razmere toplotne obdelave semen so močno vplivale tako na vsebnost posameznih fenolnih spojin v posameznih frakcijah kot tudi na antioksidativne značilnosti izvlečkov iz pogače. Toplotno labilnejše spojine so med praženjem razpadle, hkrati pa se je povečala vsebnost drugih; slednje predvsem kot posledica sprostitve flavonoidov in ostalih fenolnih spojin iz strukturnih materialov celice. Raziskave opravljene na slovenskih vzorcih navadnega ričkа so nadalje pokazale, da je izbira temperaturnega režima pri toplotni obdelavi semen odvisna od želenih antioksidativnih lastnosti izvlečkov iz pogače: 80°C za boljšo sposobnost keliranja kovinskih ionov in lovljenje prostih radikalov v emulgiranih sistemih, 120 °C pa v primeru potrebe po optimalni redukcijski sposobnosti in anti-radikalski aktivnosti v homogenem mediju.

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CONTENT ANALYSIS OF THE PAPERS IN THE ACTA AGRICULTURAE SLOVENICA

VSEBINSKA OBDELAVA PRISPEVKOV V ACTA AGRICULTURAE SLOVENICA let. 107 št. 1

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VSEBINSKO KAZALO PO SKUPINAH ZNANJA (PREDMETNIH KATEGORIJAH)

Recenzije knjig / Book review

Tla Slovenije s pedološko karto v merilu 1:250 000

Grčman, H., Vidic-Jaecks, N, Zupan, M., Lobnik, F., Jones, A., Montanarella, F. (ur.), 2015; European Union & University of Ljubljana, Luxemburg: Publication Office of European Union, EUR No 25212 EN, ISSN 1018-5593, ISNB 978-92-79-23063-9, Catalogue No LB-NA-25212-B7-C, doi:10.2788/88750

Konec preteklega leta, ki je minilo v znamenju svetovnega leta tal, je s sodelovanjem Centra za pedologijo in varstva okolja na Biotehniški fakulteti Univerze v Ljubljani in Evropske komisije, Raziskovalnega centra (JRC Ispra, Italy), izšla knjiga Tla Slovenije s pedološko karto v merilu 1:250 000. Knjiga podaja pregled tlotvornih dejavnikov, s poudarkom na matični podlagi, ter lastnosti in prostorsko zastopanost tal v Sloveniji. Pedološka karta je izrisana na 13 transparentnih listih, ki prekrivajo topografsko karto, kar omogoča dobro prostorsko orientacijo pri branju karte. Seznam pedokartografskih enot karte merila 1:250 000 je opremljen z razponom vrednosti pomembnejših talnih lastnosti, kot so tekstura, kislost tal in talno število ter s seznamom pedokartografskih enot izvorne pedološke karte merila 1:25 000. Knjiga podaja tudi kratko zgodovino pedološkega kartiranja v Sloveniji, opis klasifikacijskega sistema za tla, nastanek in zgradbo talnega informacijskega sistema ter podaja primere, kako lahko podatke pedološke karte koristno uporabimo; npr. pri prostorskem načrtovanju, ocenjevanju kmetijskih zemljišč z vidika ranljivosti za sušo ali spiranja nitratov v podtalnico idr.

Pedološke raziskave in pedološko kartiranje tal se je v Sloveniji začelo v šestdesetih letih prejšnjega stoletja, pod mentorstvom pokojnih profesorjev, začetnikov pedologije v Sloveniji, prof. dr. Bogdana Vovka, prof. dr. Albina Stritarja, prof. dr. Jožeta Sušina, prof. dr. Dušana Stepančiča in prof. dr. Marijana Ažnika, ki so vpeljali metode opisa in klasifikacije tal ter analitske postopke za ugotavljanje morfoloških, fizikalnih in kemijskih lastnosti tal. Poglobljen študij in v tujini pridobljene izkušnje so bile potrebne, da so znanje uspešno prenesli v slovenski prostor. Vendar je majhna skupina raziskovalcev uspela dokončati le posamezne tiskane liste pedološke karte do leta 1986. Posebno mesto v tem procesu pripada zaslužnemu profesorju dr. Francu Lobniku, ki je imel vizijo in pogum za dokončanje začetega dela in izdelavo digitalne pedološke karte, ki bi nudila informacijo o lastnostih tal za celotno ozemlje Slovenije. Zbral je ekipo pedologov in geologov, s katero se je lotil obsežnega dela ob finančni podpori Ministrstva za kmetijstvo gozdarstvo in prehrano Republike Slovenije. Da imamo sedaj na voljo informacijo o talnih lastnostih in prostorsko zastopanost tal za celotno Slovenijo, je bilo potrebno opraviti res obsežno delo: izvrtanih je bilo več 10 000 sond, izkopanih preko 2 000 profilov, opisanih 7 000 talnih horizontov in pridobljenih 70 000 analitskih podatkov, za kar so zaslužni terenski pedologi, predvsem specialist Jani Rupreht, Marjan Šporar, mag. Tomaž Prus, mag. Marko Zupan in sodelavci v pedološkem laboratoriju, Andreja Hodnik in drugi. Omeniti je potrebno še doc. dr. Natašo J. Vidic, ki je pomembno prispevala k razumevanju razvoja tal na različnih geoloških podlagah in doc. dr. Boruta Vrščaja, ki je imel ključno vlogo pri zasnovi in postavitvi talnega informacijskega sistema. Talni informacijski sistem, ki ga vzdržuje in posodablja Center za pedologijo in varstvo okolja na Biotehniški fakulteti, sedaj hrani vse pridobljene podatke, ki so na voljo za številne okoljske in kmetijske študije.

Prostorska heterogenost tal v Sloveniji je izjemno velika, kar je posledica razgibanega reliefa in prostorske variabilnosti geoloških podlag. Izvorna pedološka karta merila 1:25 000 je zajemala kar 1046 pedokartografskih enot, od katerih jih je bila večina sestavljenih iz več pedosistematskih enot. Da bi izbistrili pogled na slovenska tla in znanje približali širši javnosti, smo leta 2005 pedološko karto generalizirali za merilo 1:250 000; več kot 1800 pedokartografskih enot iz detajlne karte 1:25 000 smo združili v 63 kartografskih enot generalizirane karte, ki jo je izdelala prof. dr. Anka Lisec iz Fakultete za gradbeništvo in geodezijo.

Prostorska analiza je pokazala, da v Sloveniji prevladujejo kambična tla, ki prekrivajo 50 % ozemlja; distričnih rjavih tal je 20,7 %, evtričnih rjavih tal 17,4 % in rjavih pokarbonatnih tal 11,9 %. Druga najbolj razširjena skupina tal so humusno akumulativna tla, ki pokrivajo 31 % ozemlja; največ je rendzin, ki pokrivajo skoraj 29 % ozemlja in so najbolj razširjen talni tip v Sloveniji. Najdemo jih tako na apnencih in dolomitih, ki sta najbolj razširjeni kamnini v Sloveniji, kakor tudi na drugih karbonatnih sedimentnih kamninah; predvsem prodih in peskih ter fliših. Obrečnih tal je 5 %, oglejenih tal 4,4 %, psevdoglejev 3,7 % in izpranih tal 2,4 %. Mestoma se pojavljajo terra rosse (0,2 %), in šotna tla (0,2 %). Zabeležena so tudi majhna najdišča podzola (0,01 %). Zaradi plitvosti in skeletnosti tal ter drugih omejitvenih lastnostih, ki se pojavljajo na posameznih območjih (kislost, zastajanje vode), je povprečna boniteta tal v Sloveniji samo 41,5, v lestvici vrednotenja od 0 do 100. Samo 6 % tal ima boniteto večjo od 60. Slednje nam nalaga veliko skrb pri prostorskem načrtovanju, ki mora temeljiti na podatkih o lastnostih tal in stremeti k čim manjši izgubi rodovitnih tal.

Knjiga Tla Slovenije s pedološko karto 1:250 000 pomeni velik doprinos k širjenju znanja o tleh v Sloveniji in je zahvala vsem sodelavcem Centra za pedologijo in varstvo okolja Biotehniške fakultete, ki so svoje življenje posvetili raziskovanju tal in prenašanju znanja na nove generacije študentov, izmed katerih smo izšli sedanji profesorji, asistenti in raziskovalci.

Izzid v mednarodnem letu tal in dvojezična izdaja daje knjigi tudi mednarodni pomen. Leto 2015 je bilo na pobudo FAO proglašeno za mednarodno leto tal, ki postajajo eden od najbolj ogroženih naravnih virov. Ogrožajo jih številni degradacijski procesi; erozija in plazovi, zaslanjevanje, zbijanje, zmanjševanje organske snovi in biodiverzitete, onesnaževanje in pozidava. Slednja je tako v Sloveniji kot drugje v razvitem svetu izjemno pereč problem. V Sloveniji izgubimo dnevno 5 ha kmetijskih zemljišč, v Evropi 275 ha dnevno. Avtorji in uredniki iskreno upamo, da bo knjiga prispevala k zaščiti tal, ki podpirajo številne človekove aktivnosti in so temeljni vir za obstoj človeštva.

Izr. prof. dr. Helena Grčman

Predstojnica Centra za pedologijo in varstvo okolja

Soils of Slovenia with soil map 1:250 000

Grčman, H., Vidic-Jaecks, N, Zupan, M., Lobnik, F., Jones, A., Montanarella, F. (EDS.), 2015. European Union & University of Ljubljana, Luxemburg: Publication Office of European Union, EUR No 25212 EN, ISSN 1018-5593, ISNB 978-92-79-23063-9, Catalogue No LB-NA-25212-B7-C, doi:10.2788/88750

At the end of the year 2015 new book Soils of Slovenia with 1:250 000 scale soil map was published in collaboration of Centre for soil and Environmental Science at Biotechnical faculty, University of Ljubljana and European Commission, Joint Research Centre (Ispra, Italy).

The book features an overview of soil forming factors, soil properties and spatial distribution of soil types in Slovenia with special focus on parent material which is, beside topography, the most important soil forming factor in Slovenia. The soil map is printed on 13 transparent sheets which cover topographic maps so as to facilitate the reader's orientation. The list of pedocartographic units with ranges of important soil properties, such as soil texture, pH and production potential of the soil expressed as soil value are given. For each pedocartographic unit of new map the list of pedocartographic units of original soil map in scale 1:25 000 is given as well. The history of soil mapping in Slovenia, soil classification system and soil information system is described. Some examples, on how to use soil information data for spatial planning, evaluation of agricultural land, prediction of vulnerability to drought or leaching of nitrates into aquifers, are presented.

Soil research and mapping started in the early sixties with our first pedologists prof. dr. Bogdan Vovk, prof. dr. Albin Stritar, prof. dr. Jože Sušin, prof. dr. Dušan Stepančič and prof. dr. Marijan Ažnik. They introduced methods for soil description and classification along with procedures for morphological, physical and chemical analysis of soil. Being a small team, they only managed to print few sheets by 1986. In this context a special position belongs to professor emeritus, dr. Franc Lobnik due to his vision and courage to develop a comprehensive project – Soil mapping in the scale of 1: 25 000. It started in 1992 under financial support of Slovenian Ministry for agriculture, forestry and food production. He assembled a team of agronomists and geologists skilled in soil science and after ten years of intensive field and laboratory work, they gathered the information on soil properties covering the entire territory of Slovenia. Many thousands of soil probes, more than 2 000 soil profiles, 7 000 soil horizons and 70 000 soil analyses were processed. Specialists Jani Rupreht, Marjan Šporar, mag. Tomaž Prus, mag. Marko Zupan, Andreja Hodnik and many other experts and technicians from Soil laboratory deserve special mention. The knowledge of geologist doc. dr. Nataša J. Vidic was crucial for understanding soil development on different parent material. Parallel to field work, soil information system was also established by doc. dr. Borut Vrščaj. Soil information system is maintained and updated by the Centre for Soil and Environmental Science at the Biotechnical Faculty. Data are available for a number of environmental and agricultural studies.

As a consequence of diverse topography and parent material, the spatial heterogeneity of Slovenian soils is extremely high. The original Soil map in the scale of 1:25 000 includes more than 1800 pedocartographic units and most of them consist of two or three pedosystematic units. For a better overview of Slovenian soils, we decided to generalise the map to the scale 1:250 000. Pedocartographic units of the detailed soil map were grouped to 63 pedocartographic units of the generalised map. The generalisation was done in the year 2005 by prof. dr. Anka Lisec.

Spatial analyses revealed that cambic soils are predominant in Slovenia and cover 50 % of territory. There are 20.7 % of Dystric Cambisols, 17.4 % of Eutric Cambisols and 11.9 % of Chromic Cambisols. The second most frequent soil group is the group of humus accumulative soils (Leptosols) which covers 31 % of territory, the most common are Eutric Leptosols (i.e. Rendzinas), which cover almost 29 % of territory and represent the predominant soil systematic unit in Slovenia.

Rendzinas are found on limestone and dolomite, which are the most common rocks in Slovenia, and on other calcareous sediment rocks such as gravel, sandstone or flysch. Fluvisols cover 5 % of territory, Gleysols 4.4 %, Stagnosols 3.7 % and Luvisols 2.4 %. Rarely Rhodic Cambisols, known as "Terra rossa", (0.2 %) and Histosols (0.2 %) could be found. At some locations also Podzol (0.01 %) is found.

Shallow soils, with high spatial variability in depth; high surface stoniness and rockiness, low pH or hydromorphic properties are causes for low land rating in Slovenia. On the scale from 0 to 100, only 6 % of agricultural land has land rating greater than 60, average land rating is 41. Therefore, special attention on soils by spatial planning should be paid to minimize loss of good agricultural soil. Spatial planning should be based on data of soil properties.

We believe that generalized information about Slovenian soils is very important and will help Slovenians and other nations to understand heterogeneity of Slovenian soils and raise the awareness of how important and unique natural resource soils are. It is also an opportunity of thanks to all researchers from the Centre for Soil and Environmental Sciences, who have dedicated their lives to exploring the soil and transferring knowledge to new generations of students from whom emerged the current professors, assistants and researchers.

The book is of international importance; it is written in Slovenian and English language and was published during the International year of Soils. Year 2015 was dedicated to the soils on the initiative of Food and Agricultural organisation since soils are one of the most threatened natural resource. Degradation processes are soil erosion, landslides, organic matter and biodiversity decline, salinization, contamination, compaction and sealing. Soil sealing is the most pressing for fertile soils in Slovenia and other developed countries. Five hectares and 275 ha of agricultural land per day are sealed in Slovenia and Europe respectively. Authors and editors hope that the book Soils of Slovenia with soil map 1:250 000 will contribute to the protection of soils, which supported many human activities and are a fundamental resource for human survival.

Assoc. prof. Helena Grčman, PhD

Head of Centre for Soil and Environmental Science

NAVODILA AVTORJEM

(letniki z liho številko - rastlinska proizvodnja)

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