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Effect of source and sink limitation on yield and some agronomic characteristics in modern bread wheat cultivars under post anthesis water deficiency

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ABSTRACT

In order to examine the effect of source and sink limitation and post anthesis water deficiency stress in determining of grain yield potential in nine modern bread wheat cultivars in the west of Iran with arid and semi-arid weather that is one of the main centers of crop diversity in the world, a split plot-factorial experiment based on randomized complete block design with three replications was used in crop year 2010-2011. Three treatments includes: control, flag leaf removal and removal of half of each spike was applied in the field research campus of agriculture and natural resources of Razi University. Water deficiency stress was started at anthesis and continued till physiological maturity (withholding of irrigation). Water deficiency caused significant reduction in the grain yield and the 1000 grain weight and caused significant increase in the number of fertile spikelets per spike. Flag leaf removal (source limitation) treatments showed that flag leaf contribution in grain yield production during grain filling in control and post-anthesis water deficiency stress condition were 10.1% and 13.4% respectively. In both conditions removal of spikelets spike^{-1} (sink limitation) treatment had higher significant effect on fertility of spikelets, grains spike^{-1}, grain yield spike^{-1} and 1000 grain weight than flag leaf removal. Flag leaf removal treatment in some cultivars not only had no reduction effect on grain yield and 1000 grain weight but also increased them. These results may be due to an increase in photosynthesis rate of remaining leaves and/or increase in amount of carbohydrates remobilization that is stored in the stems. This phenomenon is called the compensatory effect. In both water regimes, there was a correlation between lower grain weight, no grains spike^{-1} and fertile spikelet spike^{-1} and lower yield potential of 'Chamran' cultivar. But, 'Zarin' and 'Pishgam' cultivars due to higher grain yield potential in post-anthesis under water deficiency stress and control, performed more studies, to advise farmers to cultivate them. There are probably better than any other cultivars that are common in these regions and sowing of them by farmers will be associated with less risk.

Key words: wheat, grain yield, water deficiency, source, sink, flag leaf, spike

IZVLEČEK

OMEJITVENI VPLIV VIRA IN PONORA NA PRIDELEK IN NEKATERE AGRONOMSKE LASTNOSTI NOVEJŠIH SORT KRUŠNE PŠENICE V RAZMERAH POMANJKANJA VODE PO ANTEZI

Za preučevanje omejitvenega učinka vira in ponora v razmerah pomankanja vode po antezi na potencial pridelka zrnja pri devetih novejših sortah krušne pšenice je bil v zahodnem Iranu, s sušnim in polsušnim podnebjem, na območju enega izmed glavnih centrov diverzitete kulurnih rastlin, izveden "split-plot" faktorski poskus, temelječ na naključno izbranih blokih v treh ponovitvah v pridelovalni sezoni 2010-2011. Tri obravnavanja so obsegala: kontrolo, odstranitev najvišjega lista ("zastavarja") in odstranitev polovice vsakega klasa na raziskovalnem polju Kampusa za agronomijo in naravne vire Razi univerze. Stres pomanjkanja vode je nastopil ob antezi s prekinitvijo namakanja in je trajal do fiziološke zrelosti. Pomanjkanje vode je povzročilo značilno zmanjšanje pridelka zrnja, zmanjšanje teže 1000 zrn in značilno povečanje števila fertilnih klaskov na klas. Odstranitev lista zastavarja (omejitev vira) je pokazala, da ta prispeva pridelku zrnja v obdobju polnjenja zrn v kontroli in v poanteznem stresu pomankanja vode 10.1 %, oziroma 13.4 %. V obeh razmerah je imela odstranitev klaskov v klasu (omejitev ponora) večji značilni vpliv na fertilnost klaskov, število zrn na klas, pridelek zrnja na klas in na težo 1000 zrn kot odstranitev lista zastavarja. Odstranitev lista zastavarja pri nekaterih sortah ne samo, da ni zmanjšala pridelka zrnja in teže 1000 zrn ampak ju je celo povečala. To bi lahko bilo posledica povečanja fotosinteze preostalih listov in /ali povečanja količine sproščenih ogljikovih hidratov iz zalog v steblu. Ta pojav se imenuje nadomestni učinek. V obeh vodnih režimih je bila korelacija med parametri kot so manjša teža zrnja, nič zrn na klas in fertilnimi klaski na klas z manjšim potencialom pridelka pri sorti 'Chamran'. Toda s sortama 'Zarin' in 'Pishgam', bi bilo zaradi njunega večjega potenciala v pridelku zrnja v razmerah sušnega stresa po cvetenju kot v kontroli, potrebno opraviti še več poskusov predenj bi svetovali kmetom njuno pridelovanje. Ti dve sta verjetno boljši kot katerakoli druga sorta, ki so pogoste v tem območju, in njuno sejanje bi kmetom povzročilo manjše tveganje glede na okoljske strese.

Ključne besede: pšenica, pridelek zrnja, pomanjkanje vode, vir, ponor, list zastavar, klas

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1 INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important food resources. This plant is cultivated in a wide range in agricultural land of the world (Royo et al., 2005). Approximately one third of the world population is using this plant as main food (Gallagher, 1984).

Different types of abiotic environmental stresses cause reduction in quantity and quality of wheat grain yield production (Jones, 2009). Among different types of abiotic environmental stresses, water stress is the most important factor in limiting wheat growth and grain yield formation (Ercoli et al., 2007). Due to the geographical situation, Iran's climate is Mediterranean and with respect to average participation (240 mm), is considered as arid and semi-dry regions of the world (Heidari-Sharifabad, 2008). Flowering and grain filling of wheat are the most sensitive stages to environmental stresses such as water stress (Winkel, 1989). Water stress in such areas often occurs during these periods. Under such conditions, providing of carbohydrates that are needed for grain filling to form the economical yield is very important. The most important factor in reducing grain yield in such areas is grain weight reduction (Saeidi et al., 2010).

Grain yield is a complex trait and influenced by many factors. So, to enhance grain yield production in wheat, determining factors should be identified (Acreche and Slafer, 2006). For breeders, determination of source and sink limitation in grain yield production of wheat is very important. So far, despite the importance of source and sink limitation in grain yield production of wheat, there has been little discussion about them especially in different bread wheat cultivars of Iran.

Leaves and spikes in wheat are two main photosynthetic tissues and have very important roles in grain filling and yield production (Birsin, 2005). Movement of photo-assimilates from sources (leaves, spikes and stems) to sinks (grains) are dependent on both source and sink strength (Fischer et al., 1977). Water stress during grain growth by creating of imbalance between source and sink strength caused reduction in grain yield. Wardlaw (1980) demonstrated that if photo-assimilates are not used in physiological sinks, photosynthetic production of photo-assimilates is reduced as a result of the feedback. Also Wardlaw (1980), Fischer et al. (1977) and Blum et al. (1988) concluded that, one way to increase grain yield in wheat is manipulation of sink (grain) capacity. In another major study, Miralles and Slafer (1995) found that in dwarf cultivars of wheat under water stress condition by reducing number of spikelets (artificial removing), the weight of remaining grains was increased. But this result was not found in long-legged cultivars. Increasing of grain weight and grain yield of wheat cultivars under removal of some spikelets in each spike was expressed in other reports (Calderini and Reynolds, 2000; Mahfoozi and Jasemi, 2010). In relation to source limitation, Zhu et al. (2004) found that in wheat cultivars, leaves defoliation at the early and the mid of tillering stage had no significant effect on grain yield production, but at the stage of tillering and at the jointing it significantly reduced it.

Whether, limiting factors in grain yield production at sink or source levels are dominant, is an issue that is still discussed (Cruz-Aguado et al., 1999). So that, some researchers have expressed that the grain yield of wheat is limited by the source (Duggan et al., 2000; Radmehr et al., 2004; Saeidi et al., 2012) or sink strength (Blum et al., 1983; Borra´s et al., 2004; Reynolds et al., 2007; Fischer, 2008), while some researchers have emphasized the concurrent limitations of source and sink (Aggarwal et al., 1986). These disagreements are probably because of differences in cultivars, environmental conditions and application time of treatments.

Due to occurrence of water deficiency stress after anthesis each year and reduction yield potential of different wheat cultivars in arid and semi-arid regions of world such as most of the areas in the western region of Iran, the objectives of this research are to determine the roles of source and sink limitations on formation of grain yield and its components in different improved wheat cultivars that are treated with water deficiency stress.

2 MATERIALS AND METHODS

The present study was conducted during 2010-2011 in the field research of Razi university in Kermanshah state in the west of Iran (47º 9′ E and 34º 21′ N), 1319 meters above sea level. The research was conducted on a field where the previous crop was a corn. The soil is a clay loam (36.1% clay, 30.7% silt) and the experiment was laid out in a split plot design arranged in a randomized complete blocks with three replications. Factors evaluated were moisture regimes (two levels), bread wheat cultivars (nine levels) and source/sink limitation (three levels). Moisture regimes as the main-plot factor included (1) irrigation in all stages of plant growth and (2) post-anthesis water deficiency with withholding of irrigation. Tested cultivars (subplot factor) were different improved bread cultivars: 'Bahar', 'Parsi', 'Pishtaz', 'Pishgam', 'Chamran', 'Zarin', 'Sivand', 'Marvdasht' and 'DN-11'. And also sink and source limitation treatments as sub-plot were considered. For the application of sink and source limitation during flowering in the middle rows of each plot for each cultivar 15 similar stems were selected and following three treatments were applied for five out of 15 stems: (1) control, (2) removing flag leaves (source limitation treatment) and (3) removing spikelets on one side of each spike using the forceps (sink limitation).

The investigated cultivars were chosen because of their contrasting grain yield productivity and the highest area under cultivation in the west of Iran. Also, post-anthesis water deficit occurr almost every year of in cultivated area in these regions. Date of anthesis was determined from middle rows in each plot when 50% of the spikes had extruded anthers (Ehdaie et al., 2006 a). Each plot included 54 rows 20 cm apart, 4 meters long, 4 and 3 meters distances were taken between test plots and replicates, respectively. Seeds were sown at a density of 400 seeds m^{-2} on 12th October. Based on soil analysis, nitrogenous fertilizer as urea ($CO(NH_2)_2$) was applied prior to planting, as topdressing at tillering stage and at flowering stage, 80 kg N/ha in each stage. At economical maturity, number of grain $spike^{-1}$, grain weight $spike^{-1}$, number of fertile and infertile spikelet $spike^{-1}$ and 1000 grain weight in each treatment in five spikes were calculated.

The Analysis of variance using MSTATC and SAS soft-wares was performed for each parameter measured or calculated. The means were compared using the least significant differences (LSD) test at level of 0.05 probability (Steel et al., 1997). Weather conditions during the crop season are presented in Table 1.

Table 1. Minimum and maximum of temperature and relative humidity also precipitation in the Kermanshah region in the west of Iran during 2010-2011.

Month	Average of temperature (°C)		Monthly total of precipitation (mm)	Average of relative humidity (%)	
	minimum	maximum		minimum	maximum
Oct.	10.6	30.3	1	13.2	46.4
Nov.	4.5	21.9	31	22.8	66.8
Dec.	-1.5	16.8	24	26.5	62.4
Jan.	-2.2	9.6	50	47.1	91.0
Feb.	-2.7	8.0	65	52.1	94.2
Mar.	0.6	15.4	21	28.1	82.0
Apr.	4.5	20.1	47	24.6	78.8
May.	9.5	23.6	128	33.6	87.4
Jun.	12.8	33.8	0	11.3	51.1
Jul.	17.1	38.5	0	6.6	32.1
Aug.	18.1	39.5	0	6	27.7
Sep.	13.8	34.6	0	7.8	32.0

3 RESULTS AND DISCUSSION

3.1 Cultivar evaluated in terms of yield and its components

According to the results of mean comparisons, the highest grain yield under both control and post anthesis water deficiency was observed for 'Zarin' and 'Pishgam' cultivars and the lowest for 'Chamran' cultivar (Table 2). Post anthesis water deficiency stress significantly reduced the grain yield (18%), the 1000 grain weight (20%) and significantly increased the number of fertile spikelet per spike (3%) in evaluated cultivars (Table 2). There are similarities between the results observed in this study and those described in literature such as: Shah and Paulsen (2003), Yang and Zhang (2006), Ehdaie et al. (2006 b) and Saeidi et al. (2010).

Significant reduction of 1000 grain weight in evaluated cultivars in response to post anthesis water deficiency stress as seen in this study, probably reflects the lack of an adequate supply of photo-assimilates that needed for grain filling during grain growth. This finding is in agreement with Ahmadi et al. (2009 a).

Different responses of 1000 grain weight of cultivars to post-anthesis water deficiency stress in this research showed that there was different sensitivity or resistance to post-anthesis water deficiency among cultivars. Greatest reduction in 1000 grain weight after exposure to post-anthesis water deficiency was seen in 'Zarin' and 'Marvdasht' (32.1 and 28.6 %) cultivars and lowest reduction was seen in 'Pishgam' and 'Chamran' (11.7 and 13.5%) cultivars (Table 5). Between control and stress conditions, in terms of the number of grains spike^{-1} there was no significant difference. This result is probably due to the fact that the potential of this component is formed before anthesis. These results are consistent with those of other studies such as Kobata et al. (1992), Araus et al. (2002), Shah and Paulsen (2003) and Tavakoli et al. (2009). In terms of the number of grain spike^{-1}, significant differences were observed between cultivars. 'Zarin' cultivar had the highest (59 grain spike^{-1}) and 'Chamran' cultivar had the lowest (36.4 grain spike^{-1}) value in both water regimes (Table 2).

Table 2: Mean comparisons of agronomic traits in wheat cultivars under well water and post-anthesis water deficiency stress.

Traits	Grain yield (g/spike)	1000 grain weight (g)	Grain spike^{-1}	Fertile spikelet	Non fertile spikelet
Irrigation					
Water	1.96 a	43.3 a	45.4 a	16.2 b	1.99 a
Stress	1.61 b	34.7 b	46.5 a	16.7 a	1.67 a
decrease (%)	-17.9	-19.9	2.53	2.84	-16.1
Cultivars					
Bahar	1.83 b	37.8 c	48.4 c	17.7 a	1.61 e
Parsi	1.56 ef	40.6 b	38.4 de	15.5 c	2.51 b
Pishtaz	1.67 cde	42.6 a	36.2 d	15.1 c	2.11 cd
Pishgam	2.20 a	41.2 ab	53.1 b	17.7 a	1.17 f
Chamran	1.48 f	40.8 ab	36.4 e	15.5 bc	3.18 a
Zarin	2.18 a	36.5 c	59.0 a	17.7 a	1.05 f
Sivand	1.62 de	41.5 ab	38.9 de	15.1 c	2.28 bc
Marvdasht	1.78 bc	32.6 d	54.1 b	17.4 a	0.62 g
DN-11	1.70 cd	37.1 c	45.9 c	16.2 b	1.86 de
Treatments					
Control	1.68 b	37.7 b	45.4 b	16.4 b	2.25 a
Remove flag leaf	1.49 c	36.1 c	41.9 c	15.1 c	1.73 b
Remove one side spike	2.16 a	43.2 a	50.6 a	17.8 a	1.49 c

Means in each column followed by at least one similar letter are not significantly different at 5% probability level, using LSD Test.

Under well-watered and post-anthesis water deficiency stress there were significant differences between cultivars in term of fertile spikelets per spike. Post-anthesis water deficiency significantly decreased fertile spikelet spike^{-1} (Table 2). In both control and post anthesis water deficiency stress 'Zarin', 'Marvdasht', 'Bahar' and 'Pishgam' cultivars had the highest and 'Sivand' and 'Pishtaz' cultivars had the lowest fertile spikelets per spike. Post-anthesis water deficiency had no significant effect on infertile spikelet spike^{-1} but in terms of this treat there were significant differences among of cultivars. In terms of numbers infertile spikelets 'Chamran' and 'Marvdasht' cultivars had the highest and the lowest values respectively (Table 2).

Despite lower grain yield of 'Chamran' cultivar than other cultivars in both conditions, water deficiency caused the lowest reduction in grain yield of this cultivar. So, using of this cultivar for physiological studies and finally transfers of its resistance traits to high-yield cultivars but sensitive to post-anthesis water stress can be useful. In both water regimes, there was a correlation between lower grain weight, no grains spike^{-1} and fertile spikelet spike^{-1} and lower yield potential of 'Chamran' cultivar (Table 5). But, 'Zarin' and 'Pishgam' cultivars due to higher grain yield potential in post-anthesis water deficiency stress and control, after more studies, to advise farmers to cultivate are probably better than any other cultivars that are common in these regions and sowing of them by farmers will be associated with less risk.

3.2 Flag leaf removal treatment

The results showed the flag leaf removal in the control and stress after anthesis conditions reduced grain yield per spike, 1000 grain weight, grain number per spike and number of spikelets was fertile and infertile (Table 2). Similary, a loss of yield caused by removal of the flag leaf has been reported by Biade and Baker (1991) and Radmehr et al. (2004). Reduced yield and 1000 grain weight removal in the flag leaf this suggests that important role in the flag leaf photosynthesis and grain filling. In this connection Cruz-Aguado et al. (1999) and Biade and Baker (1991) reports leaves, especially flag leaf as source material for production of photosynthetic and the most influential factors on the growth of the reservoir (seeds). The flag leaf removal in the control and stress after anthesis conditions reduce the yield spike, respectively 10.1 and 13.4%, 1000 grain weight 2.2 and 7.1%, number of grains per spike 8.4 and 7.2%, number of fertile spikelets 9.2 and 7.5% and number of non-fertile spikelets 23.8 and 22.2% toward control condition (Table 3). Reduce the number of grains per spike, 1000 grain weight and grain yield due to defoliation in other reports including Birsin (2005) and Alam et al. (2008). In this context Mohamadtaheri et al. (2010) in their research on cultivar of wheat were effect of defoliation on the number of grains per spike Significant but here was no significant effect on 1000 grain weight. Esmaielpur (2007) with no significant decrease in yield due to reduced power source the removal of leaves in the wheat.

Table 3: Variation in mean yield and its components in bread wheat cultivars as affected by the removal flag leaf and unremoval flag leaf treatments under well-water and water stress after anthesis conditions

Traits	Well water			Water stress after anthesis		
	Control	Remove the flag leaf	Changes of control (%)	Control	Remove the flag leaf	Changes of control (%)
Grain yield (g/spike)	1.87±0.10	1.69±0.09	10.1	1.50±0.06	1.30±0.06	13.4
1000 grain weight (g)	42.3±1.2	41.4±1.0	2.2	33.1±1.4	30.7±1.2	7.1
Grain spike^{-1}	44.8±3.0	41.0±2.5	8.4	46.0±2.7	42.7±2.1	7.2
Fertile spikelet	16.2±0.4	14.7±0.5	9.2	16.6±0.5	15.4±0.4	7.5
Non fertile spikelet	2.45±0.30	1.87±0.29	23.8	2.05±0.27	1.60±0.26	22.2

Data were means ± SE.

Results of the flag leaf removal treatments on grain yield per spike in control (no stress) conditions showed, in applying this treatment in 'Chamran' and 'Pishgam' cultivar grain yield was not reduced even increased the amount 4.8 and 3.7%, the cultivar were respectively but in the 'Pishtaz',

'Sivand' and 'DN-11' this treatment caused the greatest drop in yield (17.9, 17.3 and 17.3%, respectively) in moisture control conditions. Flag of the defoliation treatments in terms of stress after anthesis in the 'Chamran' cultivars (0.1%) minimum and 'Parsi' and 'Marvdasht' cultivars (25.3 and 24.1%) maximum yield loss created (Table 5). No reduction in grain yield due to removal of the flag leaf in the 'Chamran' and 'Pishtaz' show that likely in these cultivar, there is no resource constraints and perhaps remove of flag leaf in the cultivars stimulate the remobilization of this material stored stems the seed growing and or compensatory effect of other photosynthetic tissues including photosynthesis spike these conditions prevent a drop in yield has been.

1000 grain weight in the 'Pishgam' and 'Marvdasht' cultivars in remove flag leaf treatments showed a no decrease but an increase of about 4.2% and 'Zarin' cultivars the highest reduction (6.8%) in the control condition this trait of Allocated. In stress after anthesis conditions 'Bahar' cultivars with 2.6 % increased 1000 grain weight and 'Chamran' cultivars with 14 % decrease the different reaction conditions showed. Remove the flag leaf in control conditions respectively cause increase and reduce the number of grains per spike 'Chamran' cultivar (6.5%) and 'Marvdasht' (19.2%). Also stress after anthesis cause increased the number of grains per spike 'Pishtaz', 'Chamran' and 'DN-11' cultivars (2.9, 2.7 and 2.7%) and this feature reduces in 'Marvdasht' cultivar (18.6%). Remove the flag leaf in control conditions, increasing the number of fertile spikelet per spike 'Chamran' cultivar (5%) and reduce the number of fertile spikelets per spike 'Sivand' cultivar (20.3%). In terms of stress, removes the flag leaf to enhance and reduce the number of fertile spikelets per spike 'Pishtaz' cultivar (2.7%) and 'Marvdasht' cultivar (19.6%) (Table 5).

Failure to reduce some of the traits, especially grain yield and 1000 grain weight the number of cultivars, probably this is due to the number of leaves removed from the flag leaf (reducing photosynthetic resources) need source to the other leaves or part of the plant photosynthetic including spike photosynthesis is supplied (Junmin et al., 1999; Mohamadtaheri et al., 2010) and or perhaps photosynthetic material before the flowering period the plant is stored stems by remobilization to grain transferred and order to prevent loss of yield and seed weight (Noshin et al., 1996; Janmohammadi et al., 2010).

3.3 Treatment removal spikelet from one side of spike

Treatments artificial removal of spikelets per spike in the number cultivar a review increased 1000 grain weight, yield per spike, number grain and number of fertile spikelets and reduce the number of infertile spikelet per spike was treated (Table 2). In control conditions in the non-treated spikelets remaining grain weight, 1000 grain weight, number of grains per spike and number of fertile spikelets after removal of one side spike respectively 23, 9.1, 12.4 and 8.9% and in conditions of drought stress after anthesis 35.2, 21.8, 10.7 and 7.8% showed an increase (Table 4), the results with the results Mahfoozi and Jasemi (2010) increase in grain weight within 50% spikelet removal at the irrigation and drought stress of the growing season, was consistent. In other words, by removing one side of spikelet per spike compared with control, the average difference in grain weight per spike, 1000 grain weight, grain number per spike and spikelet fertility was significant, scilicet reducing the capacity of the reservoir (seed) traits is increased, therefore sink was not a limiting factor and the cultivar study limited supply of trained and transfer of resources.

Between cultivars study in terms of removing the seeds of a spike, yield spike cultivar 'Pishgam' maximum (35.6 %) and cultivar 'Sivand' and 'DN-11' minimum (Both 11.4%) increase showed in control conditions. In terms of stress after anthesis treatment to remove one side spike in all cultivars increased grain weight. In these circumstances the greatest increase 'Zarin' cultivar (60.5%) and Minimal increase to 'Parsi' and 'Chamran' (18.7 and 18.6%) (Table 5).

In the control condition highest increase in 1000 seed weight in the 'Pishgam' and 'Marvdasht' (19.9 and 15%) and the lowest cultivar 'DN-11' (0.4%) the control condition. In terms of stress after anthesis 'Marvdasht', 'Zarin', 'Bahar' and 'Pishtaz' cultivars most (33.8, 32.7, 31.7 and 31%, respectively) and the lowest 'Chamran' cultivar (1.3%) increase the 1000 grain weight.

Removing the grains of one side spike in the control condition the greatest increase in the number of seeds remaining in the spike cultivar 'Zarin' and 'Chamran' (18.9 and 16.6%) and the lowest increase in the number of grains per spike remaining 'Sivand' cultivar (5.8%) grains per spike was not treated to remove. In terms of stress after anthesis treated grains removed, cultivar 'Zarin' the highest increase in the number of seeds remaining in the spike (21.8%) and cultivar 'Parsi' the lowest.

Table 4: Variation in mean yield and its components in bread wheat cultivars as affected by the removal of spikelet from one side of spike and unremoval of spikelets under control water and water stress after anthesis conditions.

Traits	Well water			Water stress after anthesis		
	Control	Remove one side spike	Changes of control (%)	Control	Remove one side spike	Changes of control (%)
Grain yield (g/spike)	1.87±0.10	2.31±0.15	23.0	1.50±0.06	2.03±0.10	35.2
1000 grain weight (g)	42.3±1.2	46.2±1.3	9.1	33.1±1.4	40.2±1.3	21.8
Grain spike^{-1}	44.8±3.0	50.3±3.5	12.4	46.0±2.7	50.9±3.2	10.7
Fertile spikelet	16.2±04	17.7±05	8.9	16.6±0.5	18.0±0.5	7.8
Non fertile spikelet	2.45±0.30	1.64±0.26	-33.3	2.05±0.27	1.35±0.27	-34.4

Data were means ± SE.

Remove the seeds form one side spike the greatest reduction in the number of fertile spikelet per spike in the remaining 'Pishgam' and 'Chamran' cultivar (12.4%) and reduce the minimum in the cultivar 'Sivand' (4.1%). In terms stress cultivar 'Pishtaz' most (13.6%) and 'Marvdasht' minimum (5%) reduce the number of fertile spikelet at the showed a spike control.

Therefore, drought stress causes increasing resource limitation, the performance yield and 1000 grain weight. Other terminal drought stress additive effect on the resource constraints. Exacerbate resource constraints of drought stress during the reduced grain filling period (Koocheki et al., 2006), leaf senescence (Martinez et al., 2003; Gregersen and Holm, 2007) and reduction in leaf photosynthesis (Yang and Zhang, 2006).

According to the results obtained, likely resource constraints in modern bread wheat cultivar studied in the west region of Iran important factor in the potential of achieving high yield and to solve this problem should be followed cultivars with higher levels of green leaf and also leaf photosynthetic rate per unit area more in terms of the environment variable. Other ways of achieving cultivars storage materials with high photosynthetic in the stems before flowering and also it features high transfer material to the grain growing in terms of environment variable. The findings of Yang and Zhang (2006) and Yang et al. (2003) to achieve cultivars with high potential for remobilization in such environments is of best practices to sustain high performance.

Table 5: Mean comparison of interactions between irrigation regimes and treatments on agronomic traits in different improved wheat cultivars under post anthesis water deficiency.

Cultivars	Treatments	Grain yield (g/spike)				1000 grain weight (g)				Grain spike^{-1}				Fertile spikelet				Non fertile spikelet			
		water	Changes (%)	stress	Changes (%)	water	Changes (%)	stress	Changes (%)	water	Changes (%)	stress	Changes (%)	water	Changes (%)	stress	Changes (%)	water	Changes (%)	stress	Changes (%)
Bahar	control	1.84		1.57		40.7		30.5		45.2		51.2		17.0		18.1		2.3		1.8	
	Remove flag leaf	1.69	-8.5	1.40	-10.5	38.2	-6.1	31.3	2.6	44.2	-2.3	45.3	-11.6	16.4	-3.6	16.5	-8.6	1.8	-18.8	1.2	-30.2
	Remove one side spike	2.35	27.4	2.15	37.1	45.7	12.2	40.2	31.7	51.4	13.7	53.2	3.8	18.7	9.8	19.6	8.3	1.7	-26.5	0.9	-49.1
Parsi	control	1.61		1.45		43.3		35.5		37.2		40.5		15.3		15.5		3.2		2.3	
	Remove flag leaf	1.56	-3.2	1.08	-25.3	41.9	-3.2	31.1	-12.5	37.2	-0.1	34.8	-14.1	15.2	-0.2	13.8	-11.2	2.7	-13.6	2.0	-10.9
	Remove one side spike	1.98	22.7	1.72	18.7	49.4	14.1	42.5	19.9	40.1	7.8	40.5	0	16.4	7.1	16.5	6.4	2.4	-24.2	2.4	4.3
Pishtaz	control	1.77		1.37		46.0		35.8		38.3		38.1		15.0		14.6		3.0		2.4	
	Remove flag leaf	1.45	-17.9	1.23	-9.8	45.0	-2.3	31.6	-11.6	32.2	-15.9	39.2	2.9	12.5	-17	15.0	2.7	1.9	-36.6	2.0	-15.3
	Remove one side spike	2.22	25.7	2.04	48.9	50.5	9.7	46.9	31	44.0	14.8	43.4	14.1	16.7	11.1	16.6	13.6	1.7	-43.8	1.8	-26.9
Pishgam	control	2.15		1.95		41.6		36.8		52.0		52.6		16.8		17.7		1.8		1.3	
	Remove flag leaf	2.23	3.7	1.59	-18.4	43.4	4.2	31.6	-14	51.3	-1.3	50.1	-4.9	16.6	-0.8	17.3	-2.2	1.3	-29.8	1.1	-19.5
	Remove one side spike	2.91	35.6	2.39	22.5	49.9	19.9	43.8	19.2	58.3	12	54.5	3.6	18.8	12.4	19.2	8.7	0.8	-54.3	0.8	-40
Chamran	control	1.43		1.34		43.8		37.9		32.6		35.4		14.5		15.5		4.0		3.7	
	Remove flag leaf	1.50	4.8	1.34	-0.1	43.3	-1.3	37.0	-2.5	34.7	6.5	36.4	2.7	15.2	5	15.3	-1.2	3.5	-14	3.0	-19.7
	Remove one side spike	1.69	18.3	1.59	18.6	44.7	2	38.4	1.3	38.0	16.6	41.4	16.8	16.3	12.4	16.4	6	2.7	-33.9	2.2	-40.2
Zarin	control	2.41		1.61		41.9		28.4		57.5		56.3		18.0		18.2		1.8		1.4	
	Remove flag leaf	2.03	-15.7	1.44	-11.1	39.1	-6.8	27.8	-2.2	52.1	-9.4	51.3	-8.8	15.7	-12.8	15.5	-14.4	1.1	-36.3	0.8	-41.5
	Remove one side spike	3.01	24.9	2.59	60.5	44.0	5	37.7	32.7	68.3	18.9	68.5	21.8	19.8	10	19.3	6.4	0.8	-54.7	0.4	-71.4
Sivand	control	1.84		1.35		47.5		34.2		38.8		39.6		15.4		15.2		2.8		2.4	
	Remove flag leaf	1.52	-17.3	1.14	-16.1	45.0	-5.4	31.3	-8.5	33.8	-12.8	36.0	-8.9	13.3	-13.5	13.8	-9	2.2	-20.1	1.9	-23.4
	Remove one side spike	2.05	11.2	1.84	35.5	50.0	5.1	41.2	20.5	41.0	5.8	44.4	12.1	16.0	4.1	17.0	11.8	2.5	-11.9	1.9	-23.3
Marvdasht	control	2.00		1.40		35.4		25.3		56.5		55.2		17.8		18.6		1.0		1.0	
	Remove flag leaf	1.68	-15.9	1.06	-24.1	36.9	4.2	23.5	-7	45.6	-19.2	44.9	-18.6	14.3	-20.1	14.9	-19.6	0.6	-39.7	0.5	-50.4
	Remove one side spike	2.52	26	2.05	47.1	40.8	15	33.9	33.8	62.0	9.7	60.6	9.8	19.1	7.1	19.5	5	0.5	-50	0.2	-83.3
DN-11	control	1.82		1.49		40.6		33.1		44.9		45.0		16.4		16.5		2.3		2.1	
	Remove flag leaf	1.51	-17.3	1.44	-3.5	39.8	-1.9	31.1	-5.9	37.8	-15.7	46.3	2.9	13.6	-17.3	16.3	-1.1	1.7	-26.7	1.8	-13.7
	Remove one side spike	2.03	11.4	1.93	29.3	40.7	0.4	37.6	13.6	49.7	10.8	51.5	14.4	17.3	5.7	17.3	5.3	1.7	-23.5	1.6	-23.8
LSD (0.05)		0.48				7.74				4.52				0.48				0.53			
CV (%)		10.8				7.13				9.07				6.55				26.5			

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5 REFERENCES

Acreche, M.M., Slafer, G.A. 2006. Grain weight response to increases in number of grains in wheat in a Mediterranean area. Field Crops Research. 98(1): 52-59.

Aggarwal, P.K., Chaturvedi, Q.S., Singh, A.K., Sinha, S.K. 1986. Performance of wheat and triticale cultivars in a variable soil-water environment. II: Source-Sink relationships. Field Crops Research. 13: 317-330.

Ahmadi, A., Joudi, M., Tavakoli, A., Ranjbar, M. 2009 a. Investigation of yield and its related morphological traits responses in wheat genotypes under drought stress and irrigation conditions. Journal of Science and Technology of Agriculture and Natural Resources. 12(46): 155-166.

Alam, M.S., Rahman, A.H.M., Nesa, M.N., Khan, S.K., Siddquie, N.A. 2008. Effect of source and/or sink restriction on the grain yield in wheat. Journal of Applied Sciences Research. 4(3): 258-261.

Araus, L.A., Slafer, G.A., Reynolds, M.P., Royo, C. 2002. Plant breeding and drought in C_3 cereals: what should we breed for? Ann Bot. 89: 925-940.

Biade, S.F., Baker, R.J. 1991. Kernel weight response to source-sink changes in spring wheat. Crop Sciences. 31: 1117-1120.

Birsin, M.A. 2005. Effects of Removal of Some Photosynthetic Structures on Some Yield Components in Wheat. Tarim Bilimleri Dergisi. 11: 364-367.

Blum, A., Mayer, J., Golan, G. 1988. The effect of grain number per ear (sink size) on source activity and its water-relations in wheat. Journal of Experimental Botany. 39: 106-114.

Blum, A., Polarkova, H., Golan, G., Mayer, J. 1983. Chemical desiccation of wheat plants as simulators of post-anthesis stress: I. Effects on translocation and kernel growth. Field Crops Research. 6: 51-58.

Borra´s, L., Slafer, G.A., Otegui, M.E. 2004. Seed dry weight response to source-sink manipulations in wheat, maize and soybean: a quantitative reappraisal. Field Crops Research. 86: 131-146.

Calderini, D.F., Reynolds, M.P. 2000. Changes in grain weight as a consequence of de-graining treatments at pre and post anthesis in synthetic hexaploid lines of wheat (*Triticum durum* and *T. tauschii*). Australian Journal of Plant Physiology. 27: 183-191.

Cruz-Aguado, J.A., Reyes, F., Rodes, R., Perez, I., Dorado, M. 1999. Effect of source-to-sink ratio on partitioning of dry Matter and ^{14}C-photoassimilates in wheat during grain filling. Annuals of Botany. 83: 655-665.

Duggan, B.L., Domitruk, D.R., Fowler, D.B. 2000. Yield component variation in winter wheat grown under drought stress. Can. Plant Sciences. 80: 739-745.

Ehdaie, B., Alloush, G.A., Madore, M.A., Waines, J.G. 2006 a. Genotypic variation for stem reserves and mobilization in wheat: I. post anthesis changes in internode dry matter. Crop Sciences. 46:735-746.

Ehdaie, B., Alloush, G.A., Madore, M.A., Waines, J.G. 2006 b. Genotypic variation for stem reserves and mobilization in wheat: II. Post anthesis changes in internode water-soluble carbohydrate. Crop Sciences. 46: 2093-2103.

Ercoli, L., Lulli, L., Mariotti, M., Masoni, A., Arduini, I. 2007. Post anthesis dry matter and nitrogen dynamics in durum wheat as affected by nitrogen supply and soil water availability. European Journal of Agronomy. 28: 138-147.

Esmaielpur, M. 2007. Response of tow wheat cultivars to source size modification: interaction of cultivars and plant density under water stress and non-stress condition. M. Sc. thesis, College of Agriculture, University of Tehran, Karaj.

Fischer, R.A. 2008. The importance of grain or kernel number in wheat: A reply to Sinclair and Jamieson. Field Crops Research. 105: 15-21.

Fischer, R.A., Aquilar, I., Laing, D.R. 1977. Post-anthesis sink size in high yielding dwarf wheat, yield response to grain number. Australian Journal of Agricultural Research. 28: 165-175.

Gallaghe, E.J. 1984. Cereal Production. Butterworths. 354 pp.

Gregersen, P.L., Holm, P.H. 2007. Transcriptome analysis of senescence in the flag leaf of wheat. Plant Biotechnology. 5: 192-206.

Heidari-Sharifabad, H. 2008. Drought mitigation strategies for the agriculture sector. The 10th Iranian Congress of Crop Sci. 18-20 Aug. 2008, SPII, Karaj, Iran.

Janmohammadi, M., Ahmadi, A., pustyni, K. 2010. The effect of reducing leaf area and nitrogen on wheat flag leaf stomatal characteristics and performance under irrigation. Journal of Crop Production. 3(4): 177-194.

Jones, M.G. 2009. Using resources from the model plant Arabidopsis thaliana to understand effects of abiotic stress. Salinity Water Stress. 44: 129-132.

Junmin, J., Hua-Guo, H., Xu, H., Ji-Chun, D., Jiang, J.M., Hua, G.H., Xu, H.J., Ji, C.D. 1999. Effects of different treatments on dry matter production after heading and

grain yield in wheat. Acta Agriculture Shanghai. 15(1): 83-86.

Kobata, T., Palta, J.A., Turner, N.C. 1992. Rate of development of post anthesis water deficits and grain filling of spring wheat. Crop Sciences. 32: 1238-1242.

Koocheki, A.R., Yazdansepas, A., Nikkhah, H.R. 2006. Effects of terminal drought on grain yield and some morphological traits in wheat (*Triticum aestivum L.*) genotypes. Iranian Journal of Agriculture Sciences. 8: 14-29.

Mahfoozi, S., Jasemi, S. 2010. Study of the possibility of increasing grain yield by increasing grain weight in winter and facultative wheat genotypes with manipulating sink capacity. Iranian Journal of Crop Sciences. 12(1): 76-84.

Martinez, D.E., Luquez, V.M., Bartoli, C.G., Guiamét, J.J. 2003. Persistence of photosynthetic components and photochemical efficiency in ears of water-stressed wheat (*Triticum aestivum*). Physiology Plant. 119: 1-7.

Miralles, D.J., Slafer, G.A. 1995. Yield, biomass and yield components in dwarf, semi-dwarf and tall isogonic lines of spring wheat under recommended and late sowing dates. Plant Breeding. 114: 392-396.

Mohamadtaheri, M. 2008. Determine the critical level of resource constraints in modern and ancient commercial varieties of wheat, modified for cold regions, and warm temperate. MS Thesis of Agriculture, Department of Agriculture, Tehran University. Iran.

Mohamadtaheri, M., Ahmadi, A., pustyni, K. 2010. Old and new varieties of wheat response temperate, warm andcold cuts power supply to Iran. Iranian Journal of Crop Sciences. 41(2): 271-280.

Noshin, B., Hac, I.U., Shap, P. 1996. Source reduction and comparative sink enhancement effects on remobilization of assimilates during seed filling of old and new wheat varieties. Rachis. 15: 20-23.

Radmehr, M., Lotf-Ali, G., Aeyneh, A., Naderi, A. 2004. A study on source-sink relationship of wheat genotypes under favorable and terminal heat stress conditions in Khuzestan. Iranian Journal of Crop Sciences. 6(2): 101-114.

Reynolds, M., Calderini, D., Condon, A., Vargas, M. 2007. Association of source/sink traits with yield, biomass and radiation use efficiency among random sister lines from three wheat crosses in a high yield environment. Journal of Agricultural Science. 145: 3-16.

Royo, C., Miloudi, M.M., Fonze, N.D., Arraus, J.L., Pfeiffer, W.H., Slafer, G.A. 2005. Durum wheat breeding current approaches and future strategies. Vol 1. Editors: Food product press.

Saeidi, M., Moradi, F., Ahmadi, A., Spehri, R., Najafian, G., Shabani, A. 2010. The effects of terminal water stress on physiological characteristics and sink-source relations in two bread wheat (*Triticum aestivum L.*) cultivars. Iranian Journal of Crop Sciences. 12(4): 392-408.

Saeidi, M., Moradi, F., Jalali-Honarmand, S. 2012. The effect of post anthesis source limitation treatments on wheat cultivars under water deficit. Australian Journal of Crop Science. 6(7): 1179-1187.

Shah, N.H., Paulsen, G.M. 2003. Interaction of drought and high temperature on photosynthesis and grain-filling of wheat. Plant and Soil. 257: 219-226.

Steel, R.G.D., Torrie, J.H., Dickey, D.A. 1997. Principles and procedures of statistics. 3rd ed. McGraw-Hill, New York.

Tavakoli, A., Ahmadi, A., Alizade, H. 2009. Some aspects of physiological performance of sensitive and tolerant cultivars of wheat under drought stress conditions after pollination. Iranian Journal of Crop Sciences. 40(1): 197-211.

Wardlaw, F. 1980. Translocation and source-sink relationships. In (eds) The biology of crop productivity. Academic press (New York): 297-333.

Winkel, A. 1989. Breeding for drought tolerance in cereals. Vortage-Fur-Pflanzenzvchtuny. 16: 368-375.

Yang, J., Zhang, J. 2006. Grain filling of cereals under soil drying. New Phytologist. 169: 223-236.

Yang, J., Zhang, J., Wang, Z., Liu, L., Zhu, Q. 2003. Post-anthesis water deficits enhance grain filling in two-line hybrid rice. Crop Science. 43: 2099-2108.

Zhu, G.X., Midmore, D.J., Radford, B.J., Yule, D.F. 2004. Effect of timing of defoliation on wheat (*Triticum aestivum*) in central Queensland. Field Crops Research. 88: 211-226.

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Effect of nitrogen rate on seed yield, protein and oil content of two canola (*Brassica napus* L.) cultivars

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ABSTRACT

A field experiment was conducted at Rice Research Station, Tonekabon, Iran, to determine the effect of N rate on seed yield, protein and oil content of two canola (*Brassica napus* L.) cultivars. Two canola cultivars ('Hayola-308' and 'RGS-003') and five N rates (0, 50, 100, 150, and 200 kg ha^{-1}), organized into a randomized complete block design with a factorial treatment arrangement and three blocks, were applied to plot areas. Results showed that N rate effect was significant ($P < 0.01$) for seed yield, protein content and yield, and oil yield but not for oil content. On the other hand, cultivar had only significant ($P < 0.01$) effect on seed protein and oil content. Moreover, the interaction between N rate and cultivar was significant at $P < 0.01$ for seed, protein and oil yield, illustrating that cultivars showed different responses to N rates for these traits. In general, the quadratic equation provided a good description of the relationship between seed, protein and oil yield and nitrogen rate. For 'Hayola-308', seed, protein and oil yield increased significantly as N application rate increased from 0 to 150 kg ha^{-1}, but thereafter remained constant. In contrast, for 'RGS-003', seed, protein and oil yield increased significantly as N application rate increased from 0 to 200 kg ha^{-1}. Therefore, at the highest N application rate (200 kg ha^{-1}), 'RGS-003' produced greater seed, protein and oil yield than 'Hayola-308'. Averaged across N application rate, the seed protein content of RGS-003 was significantly ($P < 0.01$) higher than that of 'Hayola-308', while the opposite result was observed for seed oil content. This study demonstrated the differential response of two canola cultivars to N rate in terms of seed, protein and oil yield.

Key words: canola, nitrogen rate, oil, protein

IZVLEČEK

VPLIV GNOJENJA Z DUŠIKOM NA PRIDELEK SEMEN, VSEBNOST BELJAKOVIN IN OLJA PRI DVEH SORTAH OLJNE OGRŠČICE (*Brassica napus* L.)

Za določanje vpliva različnega gnojenja z dušikom na pridelek semen in vsebnost beljakovin in olja v dveh sortah oljne ogrščice (*Brassica napus* L.) je bil izveden poljski poskus na Rice Research Station, Tonekabon, Iran. Dve sorti oljne ogrščice ('Hayola-308' in 'RGS-003') sta bili posejani v petih obravnavanjih z dušikom (0, 50, 100, 150, in 200 kg ha^{-1}) v naključnem bločnem poskusu s faktorsko obravnavo v treh blokih. Rezultati so pokazali, da je gnojenje z N statistično značilno ($P < 0.01$) vplivalo na pridelek semen, vsebnost beljakovin in pridelek olja, ne pa na vsebnost olja. Po drugi strani sta imeli sorti statistično značilen vpliv ($P < 0.01$) samo na vsebnost beljakovin in olja v semenu. Še več, interakcija med obravnavanji z N in sortami je bila statistično značilna ($P < 0.01$) za pridelek semen, beljakovin in olja, kar kaže na različen odziv sort v teh znakih na gnojenje z dušikom. V splošnem je kvadratna enačba dobro opisala razmerja med pridelkom semen, beljakovin in olja z gnojenjem z dušikom. Pri sorti 'Hayola-308' je pridelek semen, beljakovin in olja statistično značilno naraščal pri uporabi od 0 do 150 kg N ha^{-1}, potem je ostal konstanten. Nasprotno, se je pri sorti 'RGS-003' pridelek semen, beljakovin in olja značilno povečeval od 0 do 200 kg N ha^{-1}. Sorta 'RGS-003' je pri obravnavanju z največjo količino dušika (200 kg N ha^{-1}) dala večji pridelek semen, beljakovin in olja kot sorta 'Hayola-308'. Povprečno je bila pri vseh obravnavanjih z dušikom vsebnost beljakovin značilno večja pri sorti 'RGS-003'($P < 0.01$) kot pri sorti 'Hayola-308', obratni so rezultati za vsebnost olja. Raziskava je pokazala različen odziv pridelka semen, beljakovin in olja dveh sort oljne ogrščice na gnojenje z dušikom.

Ključne besede: oljna ogrščica, gnojenje z dušikom, pridelek, semena, olje, beljakovine

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1 INTRODUCTION

Canola (*Brassica napus* L.) is a member of the mustard family that is grown for the production of animal feed and vegetable oil for human consumption. Canola oil has the lowest levels of saturated fat compared to some other vegetable oils. Although canola is a summer crop in the temperate and cool areas of the world, it is mainly grown in the northern Iran as a winter crop in rotation with rice. In this area, canola should be planted in mid October to early November and harvested in mid-late May to achieve the highest yields.

Nitrogen (N) is an essential nutrient for plant growth and is a key limiting factor in agro-ecosystems. Nitrogen is a constituent of amino acids, which are required to synthesize proteins and other related compounds. It plays a role in almost all plant metabolic processes. Nitrogen is a part of chlorophyll, the green pigment of the plant that is responsible for photosynthesis. Plant growth and developmental aspects such as seed germination, leaf development (Walch-Liu et al., 2000), flower and fruit development (Stitt et al., 2000), root architecture (Zhang and Forde, 1998) can be affected by the amount of N supplied to plants. N fertilizer mainly increases canola leaf area index, leaf duration (Wright et al. 1988), growth rates, number of flowering branches, plant height, number of flowers, number and weight of siliquae and seed yield (Grant and Bailey, 1993).

It has been frequently reported that N fertilizer increased seed yield of canola and winter oilseed rape (Taylor et al. 1991; Asare and Scarisbrick, 1995; Hocking et al., 1997; Brennan et al., 2000; Jackson, 2000; Cheema et al., 2001; Hocking and Stapper, 2001). Rathke et al. (2005) reported that application of N fertilizer increased the seed yield of winter oilseed rape. Nevertheless, some researchers documented a stagnation or reduction in seed yield at high N- rates. At the same time, N fertilization generally increases the protein content of canola seed and meal. In contrast, N fertilization usually has little effect on canola seed oil content (Brennan et al. 2000) or may significantly decrease it, especially at higher rates (Cheema et al., 2001). Moreover, it has been reported that canola cultivars had different response to N fertilizer (Svecnjak and Rengel, 2006). Therefore, this study was conducted to study seed yield, protein and oil content response of two canola cultivars to N fertilizer application.

2 MATERIALS AND METHODS

A field experiment was conducted at Rice Research Station, Tonekabon (36^{o} 51' N, 50^{o} 46' E; -20 m above sea level), northern Iran, from early November 2011 through late May 2012. Soil properties were 2.71% organic matter content, 33% clay, 42% silt, 25% sand and 6.9 pH.

Two canola (*Brassica napus* L.) cultivars ('Hayola-308' and 'RGS-003') and five N rates (0, 50, 100, 150, and 200 kg ha^{-1}), organized into a randomized complete block design with a factorial treatment arrangement and three blocks, were applied to plot areas. Plot size was 3 m × 3 m, with a row spacing of 30 cm and a plant spacing of 5 cm between plants. Planting date for both canola cultivars was 21 October 2011.

After the harvest of rice, the soil was disked (cross-disking) in the autumn to a depth of 15–20 cm consistent with local practices in north of Iran. Half of nitrogen fertilizer (applied as urea) was incorporated into the top 5 cm of soil two weeks before sowing time and remaining half nitrogen was top dressed in two split doses at stem elongation and flowering stages. Moreover, triple super phosphate and potassium sulfate were applied to provide 50 kg P_2O_5 ha^{-1} and 75 kg K_2O ha^{-1} at each plot, respectively, and incorporated before sowing. Weeds were controlled by trifluralin (2.5 l ha^{-1}) application before seed sowing and after this by manual removal if necessary.

At maturity stage, to determine seed yield, seeds were collected from 2 m^2 in each plot and subsequently was adjusted to 9% moisture content. N content in seed was determined by the Kjeldahl method and then protein content in seed was

calculated by multiplying the N content in seed by 6.25 (Williams et al., 1998). Protein yield was calculated by multiplying seed yield by protein content. Oil content was determined by nuclear-magnetic resonance as described by Robertson and Morrison (1974). Oil yield was calculated by multiplying seed yield by oil content.

Statistical analysis of the data was done by standard analysis of variance (ANOVA) using the SAS software package version 9.1.3 (SAS Institute, 2004). For cultivar factor, where the F-ratios were found to be significant, treatment means were compared by fisher's protected LSD at the 5% level. For N rate factor, where the F-ratios were found to be significant, quadratic regressions with standard error of the mean were used to describe the relationship between N application rate and dependent variables such as grain yield, protein content and yield, and oil yield.

3 RESULTS AND DISCUSSION

3.1 Seed yield

The main effect of N rate was significant ($P < 0.01$) for canola seed yield, but the main effect of cultivar was not significant. Nevertheless, the interaction between cultivar and N rate was significant at $P < 0.01$ level (Table 1), illustrating that cultivars showed different responses to N rates for seed yield. For both cultivars, a positive quadratic equation expressed the relationship between N application rate and canola seed yield (Figure 1). However, for 'Hayola-308', seed yield increased rapidly as N application rate increased from 0 to 150 kg ha^{-1}, but did not significantly increase at higher N rate. In contrast, for 'RGS-003', seed yield increased significantly as N application rate increased from 0 to 200 kg ha^{-1}. Therefore, at the highest N application rate, 'RGS-003' produced greater seed yield than 'Hayola-308' (Figure 1). This result is consistent with data of Qayyum et al. (1998) who reported that canola grain yield increased significantly when N rate was increased from 0 to 180 kg ha^{-1}. Moreover, Cheema et al. (2001) reported that the seed yield of canola increased as N application rate increased from 0 to 90 kg ha^{-1}, while at the highest N application rate (120 kg ha^{-1}), canola seed yield was significantly reduced.

Table 1: Mean squares of ANOVA for seed yield (Y), seed protein content, protein yield, seed oil content, and oil yield as affected by cultivar, and N rate.

Source	df	Seed yield	Seed protein content	Protein yield	Seed oil content	Oil yield
R	2	71158 ^{ns}	2.58^{ns}	9251^{ns}	1.56 ^{ns}	5443 ^{ns}
Nitrogen (N)	4	6356476**	3.70 **	444005 **	3.75 ^{ns}	1023596**
Cultivar (C)	1	17376 ^{ns}	13.12**	11029 ^{ns}	20.68**	1887 ^{ns}
N×C	4	200138 **	0.30 ^{ns}	12625 **	0.001^{ns}	29298 **
Error	18	53076	0. 18	2840	1.53	6502

*, ** represent significance at 0.05 and 0.01probability level, respectively.
ns represents no significant difference

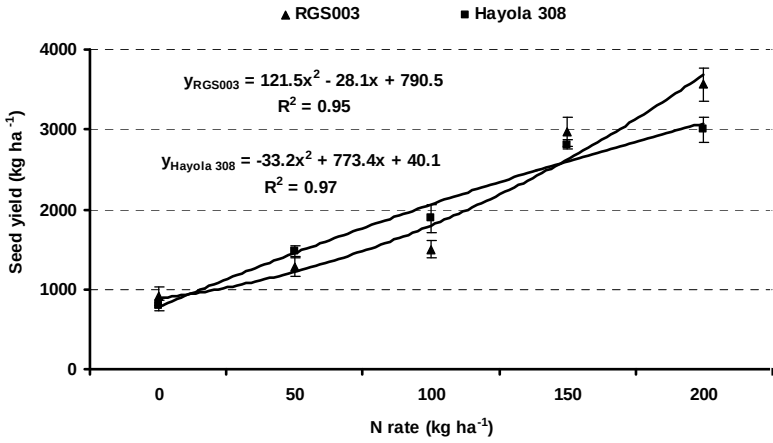

Figure 1: Effect of N rate on seed yield of two canola cultivars ('RGS-003' and 'Hayola-308')

3.2 Protein content

Analysis of variance showed that protein content varied significantly ($P < 0.01$) because of cultivar and N rate, but these factors did not interact significantly (Table 1). Averaged across N application rate (Table 2), the seed protein content of 'RGS-003' (25.5%) was significantly ($P < 0.01$) higher than that of 'Hayola-308' (24.1%). Regardless of canola cultivar, seed protein content followed a positive quadratic relationship as N rate increased from 0 to 200 kg ha^{-1}. The highest protein content of 25.8% was recorded when 200 kg N ha^{-1} was applied, this value being significantly higher than the values recorded in other N rates except 150 and 100 kg N ha^{-1}, where the difference was statistically non-significant (Figure 2). Between the N rates of 0 to 200 kg N ha^{-1}, seed protein content increased by 2.1% when averaged across canola cultivar. These results are confirmed by those reported by Kutcher et al. (2005), Asghar et al. (2003) and Saleem et al. (2001) who concluded that increasing nitrogen fertilizer rate had a significant positive effect on the protein content of canola seed. As nitrogen is the major constituent of protein, increases in N fertilizer application frequently lead to an increase in protein content (Brennan and Bolland, 2007 a, b; Malhi and Gill, 2007).

Table 2: Seed yield, protein content, protein yield, oil content, and oil yield of two canola cultivars when averaged across N rate.

Trait / Cultivar	Seed yield (kg ha^{-1})	Protein content (%)	Protein yield (kg ha^{-1})	Oil content (%)	Oil yield (kg ha^{-1})
'RGS-003'	2043	25.5	526	41.02	828
'Hayola-308'	1995	24.1	488	42.68	845
LSD (0.05)	221	0.3	52	0.95	77

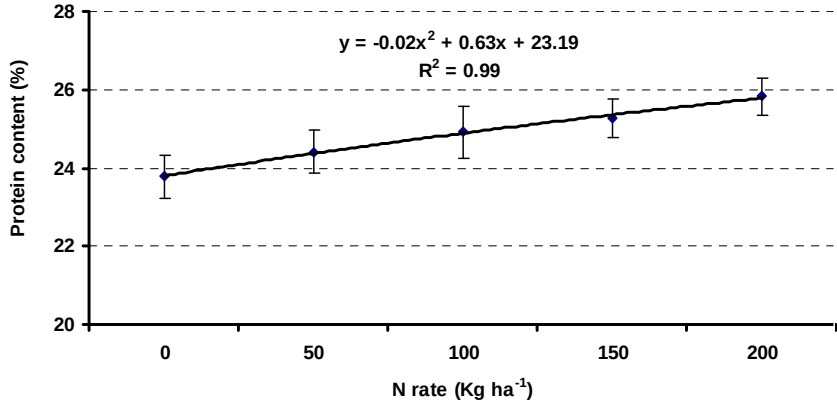

Figure 2: Seed protein content of canola as influenced by N application rate, averaged over cultivars.

3.3 Protein yield

The rate of N fertilizer application significantly ($P < 0.01$) affected protein yield (Table 1). Despite observed differences in seed protein contents between 'RGS-003' and 'Hayola-308', the cultivars had similar protein yield (Table 2) as variations in protein content were offset by differences in seed yield. Moreover, effect of N rate × cultivar interaction was significant at $P < 0.01$ level (Table 1), indicating different response of canola cultivars in protein yield to N application rate. For 'RGS-003' and 'Hayola-308', quadratic equations ($Y = 31.4\ X^2 + 1.5\ X + 184.9$, $R^2 = 0.96$ and $Y = -3.3\ X^2 + 196.5\ X + 16.0$, $R^2 = 0.97$, respectively) provided a good description of the relationship between protein yield and nitrogen rate. As shown in figure 3, at lower N rate (0, 50, and 100 kg ha^{-1}), there was no significant difference between 'RGS-003' and 'Hayola-308' for protein yield, but at higher N rate, 'RGS-003' produced higher protein yield than 'Hayola-308' (Figure 3).

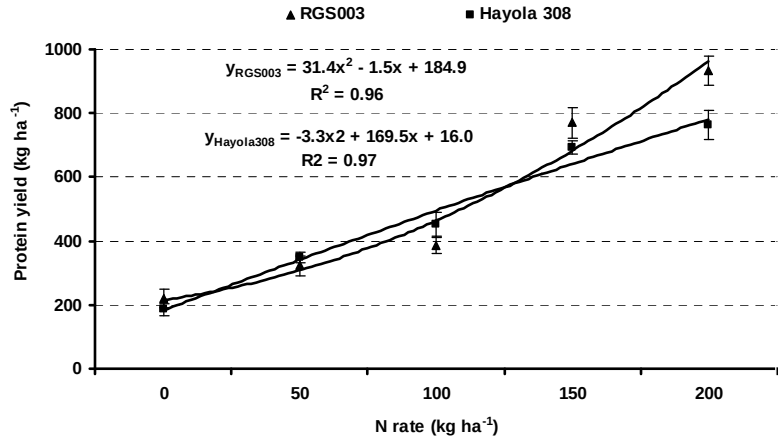

Figure 3: Effect of N rate on protein yield of two canola cultivars ('RGS-003' and 'Hayola-308').

3.4 Oil content

N rate had no significant effect on oil content, but the effect of cultivar was significant ($P < 0.01$). The interaction between N rate and cultivar was not significant at $P < 0.01$ level, illustrating that cultivars showed similar responses to N rates for oil content (Table 1). Seed oil content ranged from 42.8% in the unfertilized plot to 40.9% in plot which received the highest N rate, although these differences were not statistically significant (data not shown). Similar result was reported by Dreccer et al. (2000) for winter oilseed rape. In contrast, it

has been reported that seed oil content in canola (Jan et al., 2002; Saleem et al., 2001; Cheema et al., 2001; Hocking et al., 1997; Taylor et al., 1991) and winter oilseed rape (Rathke et al., 2005) reduced significantly as N application rate increased. Moreover, Cheema et al. (2001) reported that the highest oil content was recorded in unfertilized winter oilseed rape while the lowest one appeared at high N- supply. Seed oil content of 'Hayola-308' was significantly ($P < 0.01$) higher than that of 'RGS-003' (Table 2).

3.5 Oil yield

Although N rate had no significant effect on oil content, oil yield was significantly ($P < 0.01$) affected by N rate (Table 1). This was due to higher seed production at higher N application rate. Moreover, oil yield did not significantly affect by cultivar (Tables 1 & 2). On the other hand, the interaction between N rate and cultivar was significant for oil yield, indicating varietal differences of oil yield response to N application rate. The relationship between N rate and oil yield was well fitted by a quadratic curve for both cultivars. For 'Hayola-308', oil yield increased significantly as N application rate increased from 0 to 150 g ha^{-1}, whereas there was only a small rise in oil yield from 150 to 200 kg N ha^{-1} (Figure 4). In contrast, for 'RGS-003', oil yield increased significantly as N application rate increased from 0 to 200 kg ha^{-1}. At the N rate of 50 and 100 kg ha^{-1}, 'Hayola-308' produced greater oil yield compared to 'RGS-003', but at the highest N rate, oil yield of 'RGS-003' was significantly ($P < 0.01$) higher than that of 'Hayola-308'. Rathke and Schuster (2001) reported that seed oil yield of canola remained constant when N application rate increased from 160 to 240 kg ha^{-1}. Moreover, Cheema et al. (2001) declared that increasing the rate of N fertilizer application up to 90 kg ha^{-1} significantly increased oil yield, but thereafter oil yield was significantly reduced.

Canola grain yield was positively correlated with seed protein content, seed protein yield, and seed oil yield at $P < 0.01$ level, but negatively correlated with seed oil content at $P < 0.01$ level (Table 3). Moreover, there was a significant ($P < 0.01$) negative correlation between oil content and protein content. Protein and oil are two main components of canola seed. Increases in protein content in response to N fertilizer application normally result in a corresponding decrease in oil content (Ahmad et al., 1999; Brennan et al., 2000; Prithchard et al., 2000; Brennan and Bolland, 2007 a, b; Malhi and Gill, 2007). Many researchers reported that N fertilizer application enhanced the protein content at the expense of oil content (Andersen et al., 1996; Rathke et al., 2005). In general, high protein content is correlated with low oil content and vice versa (Asare and Scarisbrick, 1995; Andersen et al., 1996). Higher oil content would be beneficial for oil extracting industry, however low protein will decrease the quality of feed to be used for livestock.

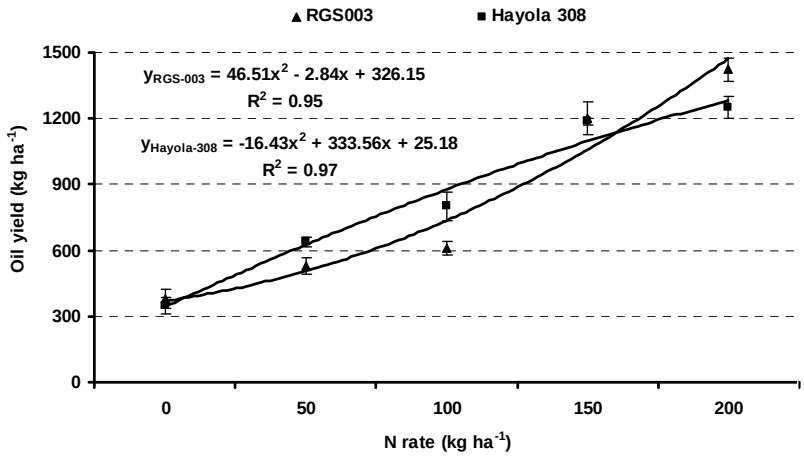

Figure 4: Effect of N rate on oil yield of two canola cultivars ('RGS-003' and 'Hayola-308').

Table 3: Correlation coefficients for measurements of canola as influenced by N rate and cultivar.

Parameter	Seed yield	Protein content	Protein yield	Oil content
Protein content	0.55 **			
Protein yield	0.99 **	0.60 **		
Oil content	-0.56 **	-0.64 **	-0.59 **	
Oil yield	0.99 **	0.52 **	0.99 **	-0.51 **

** Significant at the 0.01 probability levels

4 CONCLUSION

This experiment documented that N fertilizer had significant ($P < 0.01$) positive effect on seed, protein and oil yield of canola cultivars, although seed, protein and oil yield responses of two canola cultivars to N rate were different. For 'Hayola-308', seed, protein and oil yield increased significantly as N application rate increased from 0 to 150 kg ha^{-1}, but thereafter remained constant. In contrast, for 'RGS-003', seed, protein and oil yield increased significantly as N application rate increased from 0 to 200 kg ha^{-1}. Averaged across N application rate, the seed protein content of 'RGS-003' was significantly higher than that of 'Hayola-308'. This study demonstrated the differential response of canola cultivars to N rate in terms of seed, protein and oil yield.

5 REFERENCES

Ahmad A., Abraham G., Abdin M.Z. 1999. Physiological investigation of the impact of nitrogen and sulphur application on seed and oil yield of rapeseed (*Brassica campestris* L.) and mustard (*Brassica juncea* L. Czern. and Coss.) genotypes. Journal of Agronomy and Crop Science, 183:19–25.

Andersen M.N., Heidman T., Plauborg F. 1996. The effects of drought and nitrogen on light interception, growth and yield of winter oilseed rape. Acta Agriculturae Scandinavica. B: Soil and Plant Science, 46: 55–67.

Asare E., Scarisbrick D.H. 1995. Rate of nitrogen and sulphur fertilizers on yield, yield components and seed quality of oilseed rape (*Brassica napus* L.). Field Crops Research, 44: 41–46.

Asghar A.M., Kashif M.M., Asghar M., Saleem M.F. 2003. Effect of different irrigation and nitrogen levels on the seed and oil yield of canola (*Brassica napus* L.). Pakistan Journal of Agricultural Science, 40(3–4):137-139.

Brennan R.F., Bolland M.D. 2007a. Effect of fertiliser phosphorus and nitrogen on the concentrations of oil and protein in grain and the grain yield of canola (*Brassica napus* L.) grown in south-western Australia. Australian Journal of Experimental Agriculture, 47: 984–991.

Brennan R.F., Bolland M.D. 2007b. Influence of potassium and nitrogen fertiliser on yield, oil and protein concentration of canola (*Brassica napus* L.) grain harvested in south-western Australia. Australian Journal of Experimental Agriculture, 47: 976–983.

Brennan R.F., Mason M.G.,Walton G.H. 2000. Effect of nitrogen fertilizer on the concentrations of oil and protein in Canola (*Brassica napus*) seed. Journal of Plant Nutrition 23 (3): 339–348.

Cheema M.A., Malik M.A., Hussain A., Shah S.H., Basra S.M.A. 2001. Effects of Time and Rate of Nitrogen and Phosphorus Application on the Growth and the Seed and Oil Yields of Canola (*Brassica napus* L.). Journal of Agronomy and Crop Science, 186: 103–110.

Dreccer M.F., Schapendonk A.H.C.M., Slafer G.A., Rabbinge R. 2000. Comparative response of wheat and oilseed rape to nitrogen supply: absorption and utilisation efficiency of radiation and nitrogen during the reproductive stages determining yield. Plant and Soil, 220:189–205.

Grant C.A., Bailey L.D. 1993. Fertility management in canola production. Canadian Journal of Plant Science, 73: 651–670.

Hocking P.J., Stapper M., 2001. Effect of sowing time and nitrogen fertilizer on canola and wheat, and

nitrogen fertilizer on Indian mustard. I. Dry matter production, grain yield, and yield components. Australian Journal of Agriculture Research, 52: 623–634.

Hocking P.J., Kirkegaard J.A., Angus J.F., Gibson A.H. and Koetz E.A. 1997. Comparison of canola, Indian mustard and Linola in two contrasting environments. I. Effects of nitrogen fertilizer on dry-matter production, seed yield and seed quality. Field Crops Research, 49:107–125.

Jackson G.D. 2000. Effects of nitrogen and sulfur on canola yield and nutrient uptake. Agronomy Journal, 92:644–649.

Jan A., Khan N., Khan N., Khan I.A., Khattak B. 2002. Chemical Composition of Canola as Affected by Nitrogen and Sulphur. Asian Journal of Plant Sciences, 1: 519–521.

Kutcher H.R., Malhi S.S., Gill K.S. 2005. Topography and management of nitrogen and fungicide affects diseases and productivity of canola. Agronomy Journal, 97:533–541.

Malhi S.S., Gill K.S. 2007. Interactive effects of N and S fertilizers on canola yield, seed quality, and uptake of S and N. Canadian Journal of Plant Science, 87:211–222.

Prithchard F.M., Eagles A., Norton R.M., Salisbury P.A., Nicolas M. 2000. Environmental effects on seed composition of Victorian canola. Australian Journal of Experimental Agriculture, 40: 679–85

Qayyum S.M., Kakar A.A., Naz M.A. 1998. Influence of nitrogen levels on the growth and yield of rape (*Brassica napus* L.). Sarhad Journal of Agriculture, 15: 263–268.

Rathke G.W., Christen O., Diepenbrock W. 2005. Effects of nitrogen source and rate on productivity and quality of winter oilseed rape (*Brassica napus L.*) grown in different crop rotations. Field Crops Research, 94 (2–3), 103–113.

Rathke G.W., Schuster C. 2001. Yield and quality of winter oilseed rape related to nitrogen supply. In: Horst, W.J., et al. (Eds.), Plant nutrition: Food Security and Sustainability of Agro-Ecosystems through Basic and Applied Research. Kluwer Academic Publishers, Dordrecht, pp. 798–799.

Robertson J.A., Morrison W.H. 1974. Analysis of oil content of sunflower seed by wide line NMR. Journal of the American Oil Chemists Society, 56: 911–964.

Saleem M., Cheema M.A., Malik M.A. 2001. Agro-economic assessment of canola planted under different levels of nitrogen and row spacing. International Journal of Agriculture and Biology. 3(1): 27–30.

SAS. 2004. SAS Institute, version 9.1.3. Cary, NC, USA

Stitt M., Muller C., Matt P., Gibon Y., Carillo P., Morcuende R., Scheible W.R., Krapp A. 2002. Steps towards an integrated view of nitrogen metabolism. Journal of Experimental Botany, 53:959–970.

Svecnjak Z., Rengel Z. 2006. Canola cultivars differ in nitrogen utilization efficiency at vegetative stage. Field Crops Research 97: 221–226

Taylor A.J, Smith C. J., Wilson J.B. 1991. Effect of irrigation and nitrogen fertilizer on yield, oil content, nitrogen accumulation and water use efficiency of canola (*Brassica napus* L). Fertilizer Research, 29: 249–260.

Walch-Liu P., Neumann G., Bangerth F., Engels C. 2000. Rapid effects of nitrogen form on leaf morphogenesis in tobacco. Journal of Experimental Botany, 51:227–237.

Williams P., Sobering D., Antoniszyn J. 1998. Protein testing methods. p. 37–47. In: Wheat protein production and marketing. Fowler D.B., Geddes W.E., Johnston A.M., and Preston K.R. (ed.). Univ. Ext. Press, Univ. of Saskatchewan, Saskatoon, SK, Canada.

Wright G.C., Smith C.J., Woodroofe M.R. 1988. The effect of irrigation and nitrogen fertilizer on rapeseed (*Brassica napus*) production in south eastern Australia. Growth and seed yield. Irrigation Science, 9: 1–13.

Zhang H., Forde B.G. 1998. An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science, 279:407–409.

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Sheep wool and leather waste as fertilizers in organic production of asparagus (*Asparagus officinalis* L.)

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ABSTRACT

Sheep's wool and leather shavings tanned without chromium (III) salts would be suitable for fertilization in organic farming, where is the lack of easily accessible fertilizer nitrogen. This hypothesis was tested in a two-year field experiment growing asparagus at Rogelj organic farm in Kranj (Slovenia). The block designed experiment with three replicates comprised fertilization treatments with sheep's wool (W), leather shavings (L), cattle manure (FYM) and unfertilized (Ø). Doses of fertilizers were relevant to 0 (Ø), 140 (W1, L1), 280 (W2, L2, FYM) and 560 kg (W3, L3) N/ha. Fertilizers were dosed the first year before the start of the vegetation. Within the next year we followed their subsequent effect. The highest soil mineral N was found in the W2, which produced also the highest asparagus yield (non-significant) in the first year. On contrary, NO_3-N content in the asparagus crop was small what reflects the good synchrony of N mineralization and consumption of N at W2. Treatments W and L released significantly more N in the next year than the same dose of nitrogen from FYM. The experiment showed that mainly sheep wool represents a quality alternative organic fertilizer.

Key words: horticulture, asparagus, leather waste, sheep wool, organic fertilizers, nitrogen

IZVLEČEK

OVČJA VOLNA IN OSTRUŽKI USNJA KOT GNOJILI V EKOLOŠKI PRIDELAVI ŠPARGLJA (*Asparagus officinalis* L.)

Ovčja volna in ostružki usnja strojenega brez kromovih (III) soli bi bili lahko primerni za gnojenje v ekološkem kmetijstvu, kjer primanjkuje gnojil z lahko dostopnim dušikom. To hipotezo smo preverjali v dvoletnem poljskem poskusu z vzgojo špargljev na ekološki kmetiji Rogelj v Kranju. V bločnem poskusu smo v treh ponovitvah obravnavali gnojenje z ovčjo volno (W), ostružki usnja (L), govejim hlevskim gnojem (FYM) ter negnojeno (Ø). Odmerki gnojil so ustrezali 0 (Ø), 140 (W1, L1), 280 (W2, L2, FYM) in 560 kg (W3, L3) N/ha. Gnojila smo odmerili prvo leto pred začetkom vegetacije, v naslednjem letu pa spremljali njihov naknadni učinek. V tleh je bilo največ mineralnega dušika pri W2, tudi pridelek je bil največji, vsebnost NO_3-N v pridelku špargljev pa je bila majhna, kar kaže na dobro sinhronost mineralizacije in porabe N. Obravnavanji W in L sta imeli tudi v naslednjem letu značilno večje sproščanje N kot enak odmerek dušika iz FYM. Poskus je pokazal, da predvsem ovčja volna predstavlja kakovostno alternativno organsko gnojilo.

Ključne besede: hortikultura, špargelj, odpadki usnja, ovčja volna, organska gnojila, dušik

1 INTRODUCTION

Organic farmers face many constraints, one of which is prohibition of the use of synthetic fertilizers. The result is often a lack of fertilizer with easily accessible nitrogen. Organic fertilizers contain a lot of organic nitrogen, but it is not mineralized fast enough to meet the needs of the plants during critical periods (Pang and Letey, 2000). Therefore, the need for fertilizer to fill this gap and at the same time being allowable and economically accessible for organic farming is high.

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Typical by-products of leather such as leather scraps, and waste sheep's wool are now mostly deposited in landfills, and nutrients they contain can no longer be exploited. More environmentally friendly alternative is to use them as fertilizers. These by-products are richer in organic N (over 5%) and C (30-50%) than manure and compost (Baker, 1991). Sheep wool hydrolyzate improves growing conditions, by increasing contents of total N, C, and P in the soil (Govi et al., 1998). Applied hydrolysed wool also improved emergence and plant growth (Nustorova et al., 2005). The addition of unwashed and cut sheep wool showed similar positive results on mangold and basil (Zheljazkov et al., 2009).

For normal tanning, the chromium (III) salts are used. Thereof a potential risk of oxidation and forming of the carcinogenic chromium (VI) are becoming increasingly problematic in the leather industry (Blackman and Kildegaard, 2003). The same applies to dyes containing heavy metals. In addition to chromium, these are often cobalt, nickel, copper, etc. One ton of salted skin produces about 200 kg of leather and about 600 kg of waste (Cabeza et al., 1998). With the cessation of the use of chrome tanning salts and heavy metal dyes open up other options, such as the use of waste as fertilizer in agriculture, especially in organic farming.

Two types of animal by-products originated of animals raised according to approved organic agriculture rules were tested in a field trial, the organically produced sheep's wool and leather shavings. In a previous pot experiment, carried out by the Centre for Soil and Environmental Science at the Biotechnical Faculty in Ljubljana, they had been proven to be a good source of nitrogen (Hodnik et al, 2008). Due to the technology used in the processing of hides and skins (processing with organic tannins) they do not contain heavy metals or synthetic additives and as such are suitable for use in organic farming.

In this paper the results of a field experiment using these two residues as fertilizers for the production of asparagus are presented. Our hypotheses were that the sheep's wool and leather shavings are a good source of nitrogen in the soil. The yield of asparagus should be higher and nitrate contents in the young asparagus shoots lower compared to standard fertilization with farmyard manure.

2 MATERIALS AND METHODS

Field conditions and experiment design

The field experiment took place in 2008 and 2009 at 3 years old green asparagus plantation in town Kranj on the properties of the family farm Rogelj, which is engaged in organic production of plants and animals for the last several years.

Soil at the experimental site is /eutric brown soil on gravel and sand (Eutric Cambisols). The texture of the soil is loam. The plough layer contains about 20% of skeleton (sand and gravel > 2 mm in diameter), but with a depth its content increases rapidly. Horizons they appear in the following order: Ap (0 - 25 cm), AB (20 - 40 cm) and BC (40 - 60 cm). Soil in the Ap horizon contains about 7.5% organic matter, pH of the soil is 6.9. The content of "plant-available" (ammonium-lactate extractable) P_2O_5 ranges from 51 to 67.5 mg/100g soil and K_2O content from 55 to 61.2 mg/100g soil which is extremely very high, and indicates intensive fertilization with mineral fertilizers prior to switch to organic farming. The soil is airy, structurally stable and well drained without waterlogging. Capacity of soil to retain water is relatively good, but because of the shallowness of the soil and the proportion of the skeleton in the overall profile is very limited. The effective field capacity is approximately 100 mm (Mihelic, 2004).

The average temperature in 2008 and 2009 was higher than the long-term average. The year 2009 had the average temperature of 11.7 °C. Exceptionally warm were April, May and August. The year 2008 was well provided with precipitation (Ljubljana airport - 1592 mm). Rainfall was in 2009 a little above average (1431 mm). After dry winter, the spring and summer were quite wet. In July and August storms provided enough water for good plant growth (ARSO ..., 2010).

In a randomized field experiment with three blocks in three replicates we compared the effects of fertilizers on the growth of green asparagus. Land was relatively homogeneous. In each block the plots were fertilized either with farm-yard manure (FYM), different doses of sheep's wool (W) and leather (L), and unfertilised control plot (Ø). Each plot measured 35 m^2 (5 m x 7 m) (Tab. 1).

Table 1. Experimental treatments, application rates of fertilizers and corresponding N

Treatment	Fertilizer	Application rate (t/ha)	Applied N (kg N/ha)
Ø	Unfertilized control	–	0
FYM	Farmyard manure	56.00	280
W1	Sheep wool	1.00	140
W2	Sheep wool	2.00	280
W3	Sheep wool	4.00	560
L1	Leather shavings	1.61	140
L2	Leather shavings	3.22	280
L3	Leather shavings	6.44	560

Fertilization was carried out on 15 March 2008. Doses of sheep wool and leather shavings were sprinkled evenly by hand; manure was scattered as evenly as possible with pitchforks.

After fertilization the soil was tilled with a rotary harrow. Before the start of the asparagus shoots sprouting out of the ground field was again processed by a comb. During the year and at the end of the growth of asparagus again rotary harrow was used to prevent the growth of weeds between the rows of asparagus.

Soil was sampled at a depth of 0-25 cm with a grooved probe. Sample dates are indicated in tab. 2. The samples were put in a paper bag, and then put in a drying chamber at a temperature of 40 °C for 24 hours. Dried samples were crushed in a mill and screened through 2 mm mesh.

Table 2: Dates of plots sampling

Date of sampling	Comment
07/03/2008	before fertilization, 3 samples – 1 composite sample for each block
04/20/2008	samples collected at the beginning of the asparagus sprouts harvest
12/06/2008	after the termination of the collection of asparagus sprouts
12/01/2008	out of growing season
19/03/2009	just before a start of the asparagus growing season
14/05/2009	the time of maximum growth

Asparagus in 2008 was collected in two periods of 5 days, and in 2009, in one, 4-day period. Thus, we get an average yield in each plot. Every day we pick up all the stems longer than 20 cm. The asparagus sprouts were weighed and counted. Plant samples of each run were cut, dried at 40 °C for 24 hours, grinded in a coffee grinder and stored at room temperature in a dark for further analysis.

Soil and plant analyses

Nitrate nitrogen in plant and soil samples, ammonium nitrogen and total soluble nitrogen in air-dry soils was determined using calcium chloride as extracting solution and measured by spectrophotometer (Perkin Elmer, Lambda 2) (SIST ISO 1425). pH of soil was determined according to SIST ISO 10390.

"Plant available" phosphorus and potassium was extracted with ammonium-lactate solution

according to a modified method of the Austrian Standard (ÖNORM L 1087). Phosphorus was determined by spectrophotometry (Perkin Elmer, Lambda 2), and potassium by flame photometry (FLAPO 40).

The total N content in dry soil and plant samples was measured after incineration at 900 °C using TCD detector (Thermal Conductivity Detector) on CNS elemental analyser VarioMAX of Elementar Company (ISO 10694, 1995 and ISO 13878, 1995).

Soil organic matter was measured by the oxidation of chromium in sulphuric acid according to standard SIST ISO 14235.

N-uptake

Withdrawal of N to the crop was calculated by measurements of total N in plant samples multiplied by the quantity of harvested shoots of each treatment. Withdrawal throughout the season was calculated so that we have assumed that the harvesting season lasted for 60 days, yield considerations were converted into yield per hectare per season.

Statistical analysis

For statistical analysis we used the program Statgraphics plus 4: analysis of variance (ANOVA) and LSD test (statistically significant differences). The method used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0. We graphically present data using Microsoft Excel.

3 RESULTS AND DISCUSSION

Nitrate nitrogen in the soil

The average amount of nitrate (over all measurement dates) was the highest at W2 (2.29 mg/100 g soil) and lowest in the control treatment Ø (1.39 mg/100 g of soil). In all treatments, fertilized with leather or wool, the amount of NO_3-N was higher than at the Ø, and in most cases, also higher than at the treatment fertilized with FYM (1.61 mg/100 g of soil). Analysis of variance showed a statistically significant difference between treatments ($p = 0.00$) at 95% confidence level (Fig. 1).

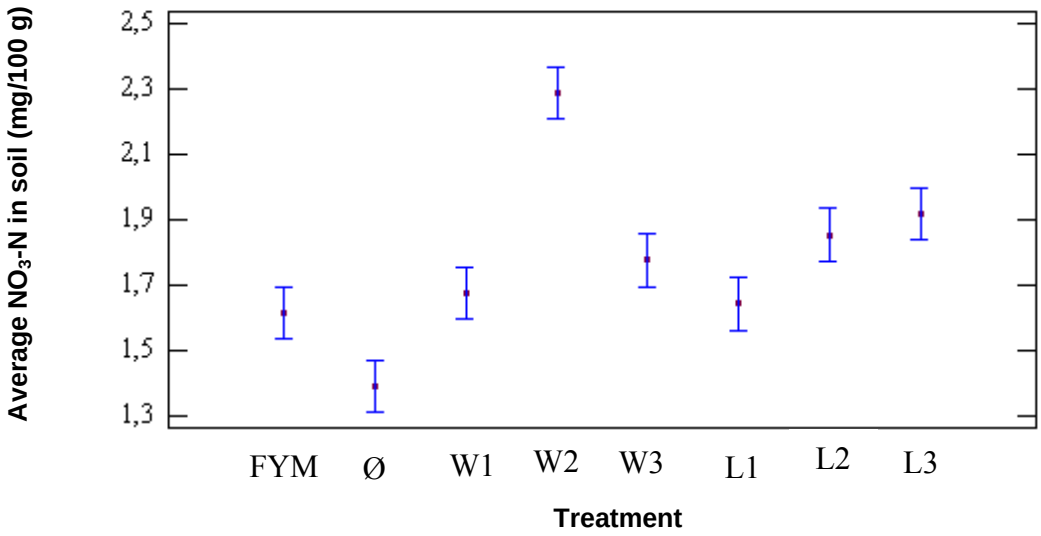

Figure 1: NO_3-N content in soil (In the figure are presented averages and least significant difference at 5.0% risk; LSD = 0.16)

It was interesting to observe the dynamics of NO_3-N in the soil during the experiment. One month after fertilization which was done on 15 March 2008 there was evidence of a nitrification in W2 and L2 in comparison to FYM which released only slightly more nitrate-N compared to non-fertilized control. The concentrations of nitrate-N were very low in FYM and Ø in the mid June 2008, whereas a contrast situation was with W2 and L2. Especially in W2 the NO_3-N was very high (3.84 mg/100 g = ca. 125 kg NO_3-N/ha in the upper 25 cm of soil) at that time. For a normal growth of field crops (e.g. maize) around 2.0 to 2.5 mg NO_3-N/100 g soil is recommended in the technological guidelines for integrated crop production. Above this level side-dressing of maize is generally not needed (Tehnološka navodila…, 2010). This is indication of intensive mineralization and nitrification of organic-N from wool and slightly less intensive from leather chips. Higher nitrate value prevailed at W and L during the entire growing season. On average, the difference was 0.9 mg NO_3-N/100 g soil or ca. 30 kg/ha. Towards the end of the year (late autumn), the nitrate from W and L has reached the same level as the non-fertilized soil. Mineralization and nitrification again started more intensively in these treatments the next spring at the end of March. This is an indication of their subsequent N fertilization effect (fig. 2).

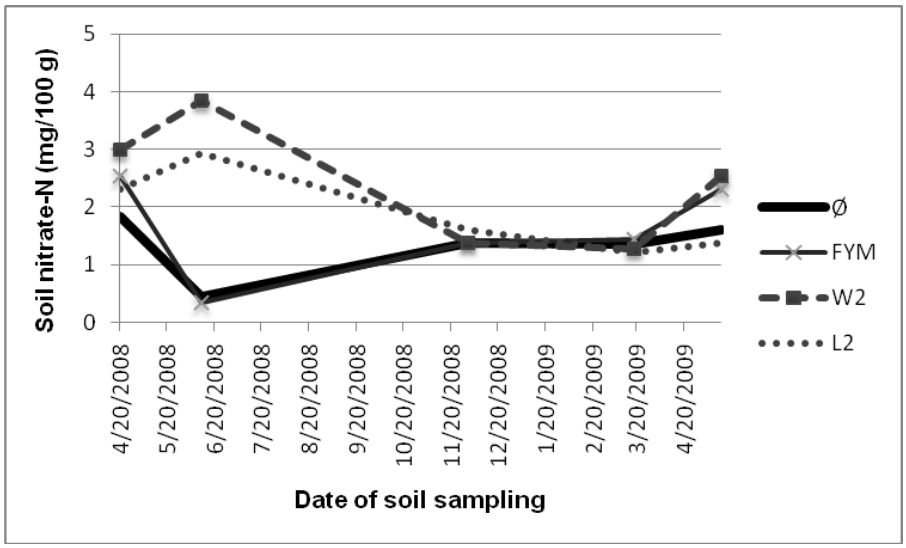

Figure 2: Average soil nitrate-N dynamics during the span of the field experiment.
Treatments W1, W3, L1, and L3 are not presented due to clarity of the figure, but their values are in-between those which are presented.

Ammonium nitrogen in the soil

Ammonium nitrogen in the soil is a product of mineralization of organic-N. Fast mineralizable organic matter could produce higher amounts of ammonium in the soil. Normally, the ammonium-N content in the soil is from 0.3 – 2.0 mg/100 g (Mihelič, 2004). In our experiment the NH4-N values were always within this range, so there was no accumulation of ammonium in the soil. Apparently, the ammonium produced was consecutively nitrified. The highest average value of NH_4-N was at FYM (1.29 mg/100 g soil) and L3 (1.27 mg/100 g of soil). The lowest average value of NH_4-N had L1 (1.07 mg/100 g soil) and W2 (1.28 mg/100 g of soil). Low ammonium-N in W2 is in contrast to high nitrate-N values of this treatment during the growing season. Obviously, ammonium-N was concomitantly transformed into nitrate form.

C/N ratio in the soil

Soil C/N ratios of fertilized treatments taken 1 month after fertilization were higher compared to the unfertilized control treatment, where C/N level did not change. This may mean that the added organic fertilizers contain a higher C/N ratio as the

soil organic matter. In the experiment with the hydrolysed sheep wool Nustorova et al. (2005) have also found that the C/N ratio increased with increasing doses of sheep's wool. This was also reflected by an increased mineralization of hydrolysate by microorganisms in the soil.

Nitrate nitrogen in vegetation samples

The plant samples had the lowest average value of NO_3-N in W2 (70 mg/kg) and the maximum at L3 (117 mg/kg). The observed differences between treatments were statistically significant (Fig. 3). It is interesting to note that W2 had the lowest nitrate content in plants when the same treatment exhibit the highest NO_3-N content in the soil. We can deduce that the relatively high soil nitrate content was not too high for asparagus crop which was able to metabolize the consumed nitrate. Only the highest addition of leather waste (L3) caused a significantly the highest nitrate content in the plant, but also this was not extremely high. Vegetables such as asparagus or onions, including tomatoes, had the lowest concentrations (normally less than 100 mg/kg) (Shalaby, 2004).

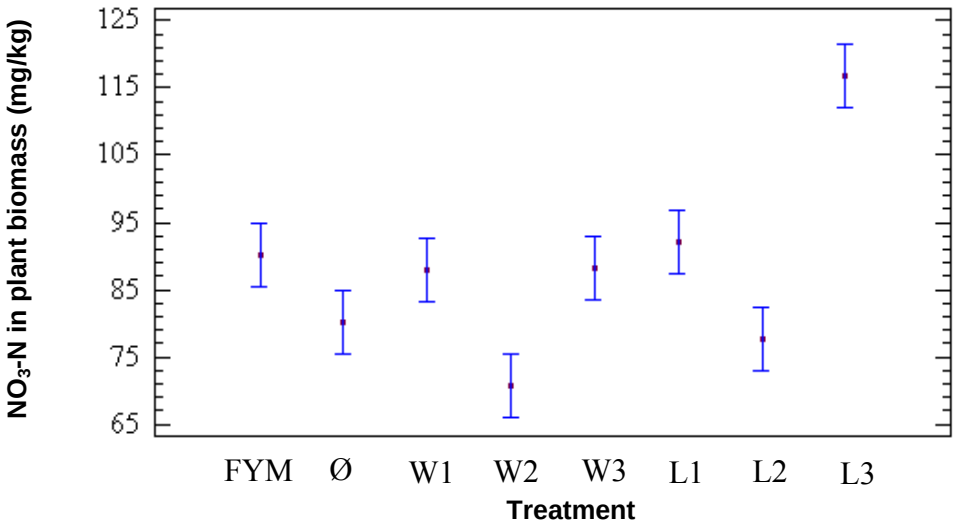

Figure 3: Nitrate-N in biomass of harvested asparagus (in the figure are presented averages and least significant difference at 5.0% risk; LSD = 0.16)

Asparagus yields

When comparing the yields between the different treatments and blocks there were no major differences. The maximum average yield of the all asparagus collections summed together for the 2008 and 2009 was achieved on a plot of L2 (5.68 kg), and by was 19% higher than the control treatment. The treatment of W2 (5.43 kg) was approximately 13% higher than the control. The highest application rate of leather shavings (L3) gave even 4% less than the controls (mean 4.56 kg). The differences were however not significant (Fig 4, 5).

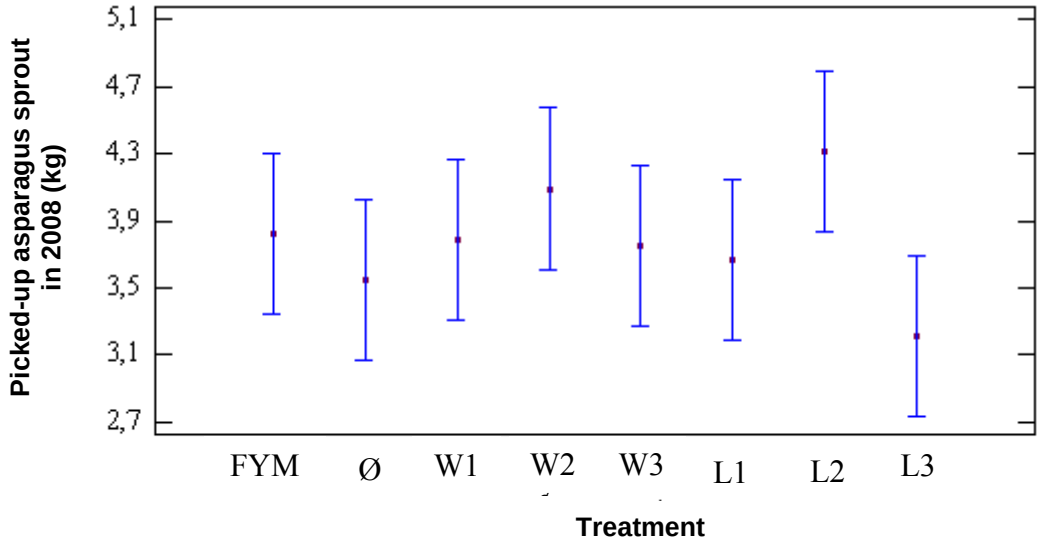

Figure 4: The cumulative yields of asparagus in 2008 (in the figure are presented averages and least significant difference at 5.0% risk; LSD = 1.1)

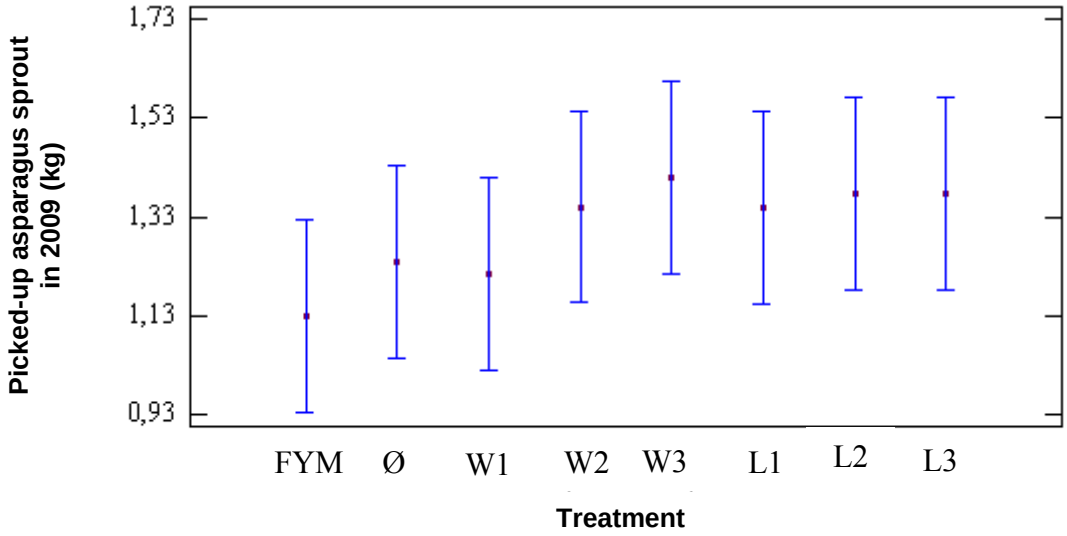

Figure 5: The cumulative yields of asparagus in 2009 (in the figure are presented averages and least significant difference at 5.0% risk; LSD = 0.4)

The entire asparagus harvest season lasted for 60 days. The average yields of the entire harvest season in 2008 were from 5.5 t/ha (L3) to 7.4 t/ha (L2), and in 2009 from 4.8 t/ha (G) to 6.0 t/ha (W3). There were no significant differences in yields among treatments. Even the non-fertilized control produced yield on the same level as the fertilized ones. At maximum dose - 560 kg N/ha (treatment W3 and L3) there was a slight depression of the yield. The same was observed in the pot experiment of Hodnik et al., (2008) with the highest doses of sheep wool (tab. 7).

Table 7: Asparagus yield

	Asparagus yield (kg/ha)		N-uptake (kg/ha)	
Treatment/Year	**2008** ($LSD_{\alpha=0.05}$ = 1649 kg/ha)	**2009** ($LSD_{\alpha=0.05}$ = 1667 kg/ha)	**2008** ($LSD_{\alpha=0.05}$ = 6.0 kg/ha)	**2009** ($LSD_{\alpha=0.05}$ = 7.7 kg/ha)
Ø	6069	5307	23,8	22,0
FYM	6543	4843	24,9	20,1
W1	6491	5185	23,9	22,2
W2	7005	5778	26,3	23,1
W3	6420	6029	23,9	25,1
L1	6284	5771	21,6	25,6
L2	7386	5894	26,0	24,1
L3	5504	5900	19,0	24,7

Thus we negate the hypothesis of asparagus crop response to the amount of added fertilizer. Small, insignificant differences could be due to the fact that the plants did not respond to specific doses of fertilizer. Even in a pot experiment with asparagus (Shalaby, 2004), different amounts of added nitrogen did not affect the yield. In the Guidelines for expert based fertilization (Mihelič et al., 2010) 40 kg/ha is proposed as a minimal level of soil mineral nitrogen (SMN) and 110 kg SMN/ha as target value for fertilization to achieve 5 t/ha of asparagus yield. The level of SMN in our experiment after fertilization in 2008 during the most intense asparagus growing period was from 50 kg/ha at the control, 90 kg/ha at FYM, and 160 kg SMN/ha at W2 in the upper 25 cm of soil. The SMN content of the other treatments was in between these.

Failure to crop response could also be due to over-fertilization with organic and mineral fertilizers in the past, because the values of the nutrients measured in the soil at the beginning of the experiment were extremely high.

Measurements of ^{15}N in asparagus revealed that spring asparagus shoots obtained N mainly by remobilisation of N from the rhizome and roots where it is stored during the dormancy of the plants (Ledgarden et al., 1994). The same experiment also showed that the plants from the soil take N mostly in the summer, and 90% of plant assimilated N at harvested is transferred and stored again into the rhizome and roots in the autumn, from where it is used for the growth of shoots the next season.

4 CONCLUSION

Sheep's wool and leather shavings are a good source of nitrogen in the soil. The highest levels of nitrate in the soil were at the treatments W2 (sheep wool; application rate 2 t/ha; soil nitrate level up to 4 mg NO_3-N/100 g) and L2 (leather shavings; application rate 3.22 t/ha; soil nitrate level up to 3 mg NO_3-N/100 g). Plants however did not respond to fertilization as we expected. Levels of potassium and phosphorus in the soil and humus are suggesting over-fertilisation of the asparagus field before the experiment. In the second year the treatments fertilized with sheep's wool and leather shavings produced a greater amount of asparagus shoots as the unfertilised control and treatment fertilized with manure, however yields were not significantly different from the unfertilized control. Sheep wool (W2) produced the highest content of soil NO_3-N; however the content of nitrate-N in the plants was the smallest. W2 also produced high yields of asparagus shoots, which all meant that this treatment represented the best fit between fertilization and the nitrogen needs of the crop.

5 REFERENCES

ARSO - Agencija Republike Slovenije za okolje. Meteorološki letopisi. http://www.arso.gov.si/vreme/podnebje/meteoroloski%20letopis/meteoroloski_letopisi.htm (10. avg. 2010)

Baker R.A. 1991. Organic Substances and Sediments in Water: Humics and soils. Chelsea, Lewis Publishers: 408 str. http://www.google.com/books?hl=sl&lr=&id=ESaXI8JoCcAC&oi=fnd&pg=PA351&dq=related:NzYa3ExI3JEJ:scholar.google.com/&ots=yUlXXRRVpM&sig=hrk4Br-udL4wJhsZF3qbnGP3Pgs#v=onepage&q&f=false (15. jun. 2010)

Blackman A., Kildegaard A. 2003. Clean technological change in developing country industrial clusters: Mexican leather tanning. Discussion paper 03 - 12. Washington D.C., Resources for the Future. http://ageconsearch.umn.edu/bitstream/10545/1/dp030012.pdf (15. jul. 2010)

Cabeza L.F., Taylor M.M., DiMaio G.L., Brown E.M., Marmer W.N., Carrio R., Celma, P.J., Cot, J. 1998. Processing of leather waste: pilot scale studies on chrome shavings. Isolation of potentially valuable protein products and chromium. Waste Management, 18, 3: 211 - 218

Govi M., Ciavatta C., Sitti L., Gessa C. 1998. Influence of organic fertilisers on soil organic matter : a laboratory study. 16th World Congress of Soil Science. http://natres.psu.ac.th/Link/SoilCongress/bdd/symp40/974-r.pdf (5. avg. 2010)

Hodnik, A., Mihelič, R., Zupan, M., Šijanec, V., Ilc, R., Gogič, S., Mohorovič, B. 2008. Možnosti uporabe stranskih produktov iz biološke proizvodnje usnja v IUV - primernost za rabo v kmetijstvu oziroma hortikulturi. Ljubljana, Biotehniška fakulteta, Oddelek za agronomijo, Center za pedologijo in varstvo okolja: 33 str.

Ledgard S. F., Douglas J. A., Sprosen M. S., Follett J. M., 1994. Uptake and redistribution of 15N within an established asparagus crop after application of 15N-labelled nitrogen fertilizer. Annals of Botany, 73: 169 - 173

Mihelič R. 2004. Influence of farmyard manure fertilization to maize (Zea mais L.) on net-nitrogen-mineralization, dynamics of soluble nitrogen fractions in the soil and nitrogen losses from shallow soils under the conditions of the humid climate of central Slovenia. Doktorska disertacija. Aachen, Shaker Verlag: 191 str.

Mihelič, R. 2007. Pomen organske snovi v kmetijskih tleh ter humusna bilanca na njivah v Sloveniji - Significance of soil organic matter in agricultural soil and humus balance in arable fields of Slovenia. V: Knapič, Matej (ur.). Strategija varovanja tal v Sloveniji: zbornik referatov Konference ob svetovnem dnevu tal 5. decembra 2007. Ljubljana: Pedološko društvo Slovenije, 2007, str. 259-260

Mihelič, R, Čop, J., Jakše, M., Štampar, F., Majer, D., Tojnko, S., Vršič, S. 2010. Smernice za strokovno utemeljeno gnojenje (= Guidelines for expert based fertilization). Ljubljana: Ministrstvo za kmetijstvo, gozdarstvo in prehrano, 182 p., ilustr. ISBN 978-961-6761-09-3. http://www.mkgp.gov.si/fileadmin/mkgp.gov.si/pageuploads/PRP/smernice09_skupaj.pdf.

Nustorova M., Braikova D., Gousterova A., Vasileva-Tonkova E., Nedkov P. 2005. Chemical, microbiological and plant analysis of soil fertilized with alkaline hydrolysate of sheep's wool waste. World Journal of Microbiology & Biotechnology, 22, 4: 383 - 390

ÖNORM L 1087. Chemiche Bodenuntersuchungen: Bestimmung von pflantzenverfugbarem. Phosphat und Kalium nach der Calcium-Acetat-Lactat (CAL) – Metode. 1993: 8 str.

Pang X.P., Letey J. 2000. Organic farming: Challenge of timing nitrogen availability to crop nitrogen requirements. Soil Science Society of America Journal, 64, 1: 247 - 253

Shalaby T.A.E.W. 2004. Genetical and nutritional influences on the spear quality of white asparagus (Asparagus officinalis L.). Disertacija. Gemeinsamen Naturwissenschaftlichen Fakultät der Technischen Universität Carolo-Wilhelminazu Braunschweig: 110 str.

SIST ISO 11277. Kakovost tal – Ugotavljanje velikostne porazdelitve delcev v mineralnih tleh - sedimentacijska metoda (modificirano po Janytzki). 1998: 45 str.

SIST ISO 10390. Kakovost tal - Ugotavljanje pH. 1996: 5 str.

SIST ISO 11265. Kakovost tal - Ugotavljanje specifične električne prevodnosti. 1996: 2 str.

SIST ISO 14255. Soil quality -- Determination of nitrate nitrogen, ammonium nitrogen and total soluble nitrogen in air-dry soils using calcium chloride solution as extractant. 1998: 12 str.

SIST ISO 14235. Kakovost tal - Določanje organskega ogljika z oksidacijo v kromžvepleni kislini (modificirano po Walkley - Black). 1999: 5 str.

Tehnološka navodila za integrirano pridelavo poljščin. 2010. Ministrstvo za kmetijstvo, gozdarstvo in prehrano RS. http://www.mkgp.gov.si/fileadmin/mkgp.gov.si/pageuploads/saSSo/2008_Sektor_za_sonaravno_kmetijstvo/2010/IP_poljščine-TN_2010.pdf (25. avg. 2010)

Zheljazkov V.D., Stratton G.W., Pincock J., Butler S., Jeliazkova E.A., Nedkov N.K., Gerard P.D. 2009. Wool-waste as organic nutrient source for container-grown plants. Waste Management, 29, 7: 2160 - 2164

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Cultivar and growth phases – the factors affecting antioxidant activity of buckwheat (*Fagopyrum esculentum* Moench.)

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ABSTRACT

The aim of this study was to assess the influence of cultivar and growth phase on the antioxidant activity (AOA) changes in common buckwheat (*Fagopyrum esculentum* Moench), as well as the its distribution in different plant parts. During 4 growth phases (GP) (buds formation - I, beginning of flowering - II, full flowering - III, full maturity - IV) stems, leaves, flowers, seeds were collected sequentially from 6 buckwheat cultivars – 'Pyra', 'Spacinska', 'Kasho', 'Jana C1', 'Hrusowska', 'Emka'. The highest values of AOA were measured in flowers (GP III) in 'Jana C1' (93.17%) and the lowest value in stems (GP I) in 'Spacinska' (46.09%). The highest increase of AOA was observed in GP IV in stems in 'Pyra'. Differences were compared for statistical significance at the level $P < 0.05$.

Key words: buckwheat, cultivar, growth phase, plant part, antioxidant activity

IZVLEČEK

SORTA IN RAZVOJNE FAZE RASTLINE KOT DEJAVNIKI VPLIVA NA ANTIOKSIDATIVNO AKTIVNOST NAVADNE AJDE (*Fagopyrum esculentum* Moench.)

Namen te raziskave je bil oceniti vpliv sorte in razvojnih faz navadne ajde (*Fagopyrum esculentum* Moench) na antioksidativno aktivnost različnih organov rastline. V štirih razvojnih fazah (GP; tvorba popkov-I, začetek cvetenja-II, polno cvetenje- III, polna zrelost-IV) smo vzorčili stebla, liste, cvetove in semena pri šestih sortah navadne ajde ('Pyra', 'Spacinska', 'Kasho', 'Jana C1', 'Hrusowska', 'Emka'). Največja antioksidativna aktivnost (AOA) je bila izmerjena v cvetovih pri sorti 'Jana C1' (GP III, 93.17 %) in najmanjša v steblih pri sorti 'Spacinska' (GP I; 6.09%). Največje povečanje AOA je bilo izmerjeno v steblih pri sorti 'Pyra' v razvojni fazi GP IV. Statično ovrednotenje razlik je bilo opravljeno na ravni $P < 0.05$.

Ključne besede: navadna ajda, sorta, razvojne faze, organi rastline, antioksidativna aktivnost

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1 INTRODUCTION

Buckwheat was one of the basic components of diet of our ancestors. In 17th - 19th century, was very popular in western countries, which was later replaced by wheat (Cawoy et al., 2009). Buckwheat currently serves as an alternative crop, replacing rice or potatoes, and is used as animal feed, pharmaceutical, and honey plant (Holasova et al., 2002; Christa and Soral-Śmietana, 2008; Tang et al., 2009). Agricultural value is attributed mainly to 9 varieties of common buckwheat (*Fagopyrum esculentum* Moench.) which is used more frequently and tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertner) which is grown mainly in the mountain areas.

Almost all parts of buckwheat are the source of many health-benefit components: vitamins, with a balanced amino acid composition, proteins (rich in arginine and lysine), microelements (Cu: 4.29 μg g^{-1}, Mn: 10.20 μg g^{-1}, Fe: 25.14 μg g^{-1}, Zn: 17.89 μg g^{-1}) and macroelements (K, Ca, Mg) (low content of N and high content of K is desirable to reduce the risk of certain diseases of people in developed world. The buckwheat flour contains 12.61% dry matter (DM) of proteins and 1.74% DM of total minerals (Krupa-Kozak et al., 2011). Buckwheat is also the important source of elements and phenolic compounds, which contribute to the antioxidant effect of buckwheat on the human organism.

Phenolic compounds in buckwheat include phenolic acids and flavonoids. In buckwheat, the content of ferulic acid and hydroxycinnamic acid is low. Bran-aleurone fraction of buckwheat contains bound syrignic, *p*-hydroxybenzoic, vanillic and *p*-coumaric acids. Zadernowski et al. (1992) have identified 20 and 14 phenolic acids in buckwheat groats and hulls, respectively. Of these, *p*-coumaric, vanillic, *p*-hydroxybenzoic and caffeic acids are the predominant phenolic acids in groats (4.6, 1.7, 1.7 and 1.3 mg 100 g^{-1} respectively); *p*-coumaric, vanillic, sinapic and gentisic acids are the major phenolic acids in the hulls (3.6, 1.65, 1.4 and 1.1 mg 100g^{-1} respectively) (Shahidi and Naczk, 2004).

A larger proportion of phenolic compounds in buckwheat are flavonoids. Although the flavonoids, in general, possess ideal structure for antioxidant activity, the differences in chemical structures of different flavonoids would affect their antioxidant activities. The synergism among the antioxidants in the mixture made the antioxidant activity, not only dependent on the concentration of antioxidant, but also on the structure and interaction among the antioxidants (Sun and Ho, 2005; Liu et al., 2008). The antioxidant activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule; this will be strengthened by steric hindrance. The antioxidant effect of phenolic compounds was declare by authors (Ismail et al., 2004; Prakash et al., 2007; Faller and Fialho, 2009), while the antioxidant activity was found to be significantly correlated to the polyphenolic content, with a correlation coefficient of 0.624 ($P < 0.01$, $n = 17$) (Ikeda et al., 2001).

Although phenolic compounds and some of their derivatives are very efficient in preventing autooxidation, only a few phenolic compounds are currently allowed as food antioxidants. The major considerations for acceptability of such antioxidants are their activity and potential toxicity and /or carcinogenicity. The approved phenolic antioxidants have been extensively studied, but the toxicology of their degradation products still is not clear (Shahidi and Naczk, 2004).

The presented work is a part of a broader topics dealing with polyphenolic compounds with antioxidant effects in selected pseudocereals. One of the aims of which is discussed in this section is to study the influence of buckwheat cultivar on changes in antioxidant activity in different parts of the plant during its growth.

2 MATERIAL AND METHODS

2.1 Plant material

In the experimental work we investigated changes of antioxidant activity:
- in six cultivars of common buckwheat (*Fagopyrum esculentum* Moench.) ('Pyra', 'Spacinska', 'Kasho', 'Jana C1', 'Hrusowska', 'Emka'),
- during the four growth phases (GP I: making of buds, GP II: beginning of flowering, GP III: full flowering, GP IV: full maturity),
- in different parts of the plant (stem, leaf, flower, seed),
where:
- stems and leaves were collected at all four growth phases,
- flowers were collected in GP II and GP III,
- seeds were collected in GP III and GP IV.

The buckwheat cultivars were grown on land Plant Production Research Center in Piešťany.

2.2 Antioxidant activity (AOA)

AOA in different parts of the plant was determined using method based on radical reaction of 2.2-diphenyl-1-picrylhydrazyl (DPPH) according Brand-Williams et al. (1995). Absorbance was measured at 515.5 nm using a Shimadzu spectrophotometer UV-VIS 1800 at 23 °C and % inhibition DPPH indicating how is monitored component able to remove radical DPPH at the time calculated from the formula:

$$\% \text{ inh. DPPH} = [(A_{t0} - A_{t10})/A_{t10}].100;$$

where: A_{t0} - absorbance at time t = 0 min (solution DPPH)
A_{t10} - absorbance at time t = 10 min.

2.3 Statistical analysis

All analysis were run in quadruplicate. In the work the statistical program Statgraphics (multifactorial analysis of variance, LSD-test contrasts, $P < 0.05$) was used.

3 RESULTS AND DISCUSSION

Based on the results obtained from the analysis of six cultivars of buckwheat (*Fagopyrum esculentum* M.) it can be concluded that the AOA in GP I was highest in the stems 'Jana C1' and lowest one in 'Spacinska'. In contrast, in this cultivar the highest AOA in leaves was determined (Tab. 1).

Table 1: Antioxidant activity (%) determined in different parts of buckwheat plant during four growth phases

	'Pyra'				'Spacinska'				'Emka'			
GP	S	L	F	A	S	L	F	A	S	L	F	A
I	54.94	67.11	-	-	46.09	86.97	-	-	62.20	69.53	-	-
II	59.22	83.95	89.55	-	47.50	88.65	88.79	-	65.06	78.26	89.44	-
III	63.30	88.34	91.16	77.37	51.35	88.72	90.94	85.51	69.79	81.43	92.38	68.39
IV	84.17	89.66	-	87.47	60.64	89.83	-	87.33	88.36	89.25	-	88.86
IV/I	1.53	1.34			1.32	1.03			1.42	1.28		
	'Jana C1'				'Hrusowska'				'Kasho'			
GP	S	L	F	A	S	L	F	A	S	L	F	A
I	68.29	79.93	-	-	52.54	74.20	-	-	53.66	79.98	-	-
II	71.64	81.55	89.19	-	59.03	78.00	90.34	-	69.42	82.85	90.73	-
III	75.95	89.11	93.17	75.17	62.26	87.98	91.73	88.88	70.27	90.12	92.01	88.40
IV	80.82	91.29	-	88.94	66.73	88.94	-	90.47	76.71	91.55		89.21
IV/I	1.18	1.14			1.27	1.20			1.43	1.14		

Highly statistically significant differences AOA (*P*-value<0.01) among plant parts in GP I were confirmed; AOA significant difference (*P*-value<0.05) between 'Jana C1' and other cvs. were also confirmed, there are no significant differences in AOA among cultivars 'Pyra', 'Spacinska', 'Emka', 'Hrusowska' and 'Kasho' (Tab. 2).

Table 2: Analysis of Variance for AOA (GP I)

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A:cultivar	803.616	5	160.723	3.23	0.0150
B:plant part	4423.87	1	4423.87	88.89	0.0000
RESIDUAL	2040.45	41	49.7672		
TOTAL (CORRECTED)	7267.94	47			

In GP II in all investigated buckwheat cultivars AOA was increased in plant parts in order: stems < leaves < flowers, the AOA differences among plant parts are highly significant (*P*-value < 0.01, Tab. 3). There is no statistically significant difference (*P*-value > 0.05, Tab. 3) in AOA among cultivars in GP II.

Table 3: Analysis of Variance for AOA (GP II)

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A:cultivar	374.164	5	74.8327	1.76	0.1348
B:plant part	9856.36	2	4928.18	115.61	0.0000
RESIDUAL	2728.2	64	42.6282		
TOTAL (CORRECTED)	12958.7	71			

From the point of view of antioxidant activity of most utilised parts of buckwheat plant the GP III is the most important growth phase, providing stems, leaves, flowers and seeds. In this phase the highest AOA was determined in flowers across cultivars increased in the order: 'Spacinska' < 'Pyra' < 'Hrusowska' < 'Kasho' < 'Emka' < 'Jana C1' (Tab. 1). Highly significant differences of AOA among all parts of the plant (*P*-value < 0.01, Tab. 4) as well as among cultivars 'Emka', 'Jana C1', 'Emka', 'Kasho', 'Kasho', 'Pyra', 'Kasho' and 'Spacinska' (*P*-value < 0.05; Tab. 4) were confirmed.

Table 4: Analysis of Variance for AOA (GP III)

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A:cultivar	616.519	5	123.304	2.50	0.0368
B:plant part	9664.57	3	3221.52	65.21	0.0000
RESIDUAL	4297.91	87	49.4012		
TOTAL (CORRECTED)	14579.0	95			

In the GP IV we analysed stems, leaves and seeds of buckwheat. In all plant parts the maximum of AOA in this growth phase (Tab. 1) was determined. Highly statistically significant differences in AOA (P-value < 0.01, Tab. 5) among the parts of plants as well as among the cultivars (*P*-value < 0.05) 'Emka' and 'Hrusowska', 'Emka' and 'Spacinska', 'Hrusowska' and 'Jana C1', 'Hrusowska' and 'Pyra', 'Jana C1' and 'Spacinska', 'Kasho' and

'Spacinska', 'Pyra' and 'Spacinska' (Tab. 5) were confirmed.

Table 5: Analysis of Variance for AOA (GP IV)

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A:cultivar	784.37	5	156.874	4.60	0.0012
B:plant part	2795.51	2	1397.75	41.02	0.0000
RESIDUAL	2180.77	64	34.0746		
TOTAL (CORRECTED)	5760.65	71			

Holasova et al. (2002) compared the AOA values in whole buckwheat seeds, dehulled buckwheat seeds, buckwheat straws, leaves and hulls. The leaves proved a higher than triple antioxidant activity compared with seeds, whereas the straws and hulls had a lower antioxidant activity than seeds. The above findings correspond to our results, when the highest AOA values were determined in all cultivars except 'Hrusowska' in flowers and leaves, then in seeds and stems (Tab. 1). Gorinstein et al. (2007) determined the AOA in different cereals and pseudocereals including buckwheat. The values of AOA determined by DPPH radical scavenging method in seeds are comparable to our results, ranging between 80.0 ± 7.0%. Brindzová et al. (2009) evaluated the AOA using DPPH test in fifteen cultivars of cereals and nine cultivars of pseudocereals and confirmed statistically significant differences ($P \leq 0.05$) between the investigated cultivars.

In common buckwheat, the polyphenolics (rutin, quercetin, cyanidin and others) in the groats might be an important factor that determines their colour properties. On the other hand, buckwheat has an abundance of polyphenolic compounds (flavonoids, catechins, vitamin P), which have a yellow colour (Ikeda et al., 2001).

The colour of peel is one of the cultivar sign of buckwheat. The relationship between the hull colour and antioxidant activity of the flour was analysed by Fujita et al. (2004) and they found, that the hull colour would not be consider to be useful estimating the antioxidant activity of the flour. The authors suggested to judge antioxidant effects of buckwheat by flour colour and not by the colour of peel. Sedej et al. (2010) presented, that strong antioxidant activity of buckwheat flour extracts might be attributed to the presence of polyphenols, especially rutin, as the main antioxidative component in buckwheat.

The largest increase in antioxidant activity in parts of buckwheat during different growth phases was found in 'Pyra'. AOA determined in *stems* in GP IV (AOA_{IV}) was 1.53 multiple higher than that in GP I (AOA_I) and about 32.97% higher than that in GP III (AOA_{III}) (Tab. 1). Even when evaluating this dependence the impact of cultivar was confirmed, e.g.: the biggest difference in AOA between the first and the second growth phase (Fig. 1) was determined in 'Kasho'. 'Pyra' the largest dynamics in AOA between GP I and GP IV in buckwheat leaves (AOA_{IV}/AOA_I = 1.34), as well as the largest increase between GP I and GP II (Δ = 25.9%) was observed. In 'Spacinska' was not even 1 % difference in AOA (Fig. 2) between GP II and GP III confirmed.

In flowers and *seeds*, which were collected only during two growth buckwheat phases, the determined AOA values were increased in most cultivars (Δ = 4.46% in flowers of 'Jana C1' and Δ = 29.93% in seeds of 'Emka') (Fig. 3, 4).

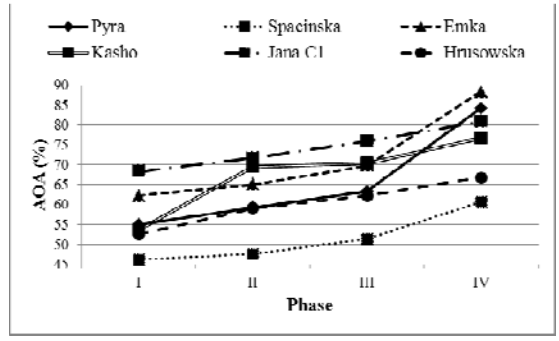

Figure 1: Dynamics of AOA (%) in stems during growth phases I – IV

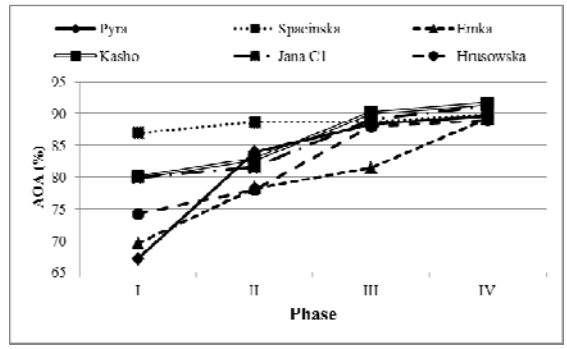

Figure 2: Dynamics of AOA (%) in leaves during growth phases I - IV

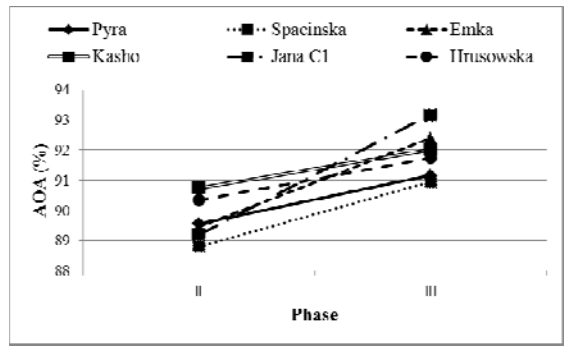

Figure 3: Dynamics of AOA (%) in flowers during growth phases II – III

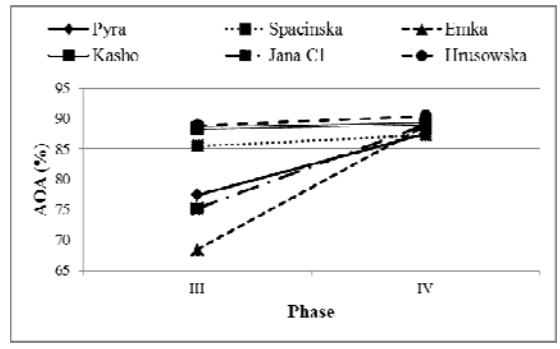

Figure 4: Dynamics of AOA (%) in seeds during growth phases III - IV

In all investigated cultivars highly statistically significant differences in AOA values between studied buckwheat plant parts (*P*-value < 0.01) (Tab. 6) were confirmed. With exception of 'Spacinska' (P-value < 0.05) there are also statistically high significant differences in AOA in all buckwheat cultivars between growth phases (*P*-value < 0.01) (Tab. 7).

Table 6: Multiple Range Tests for AOA by plant part (Method: 95.0 percent LSD)

	Pyra	Spacinska	Emka	Kasho	Jana C1	Hrusowska
plant part	HG	HG	HG	HG	HG	HG
stems	X	X	X	X	X	X
seeds	X	X	X	X	X	X
leaves	X	X X	X	X X	X	X
flowers	X	X	X	X	X	X

HG – Homogeneous Groups

Table 7: Multiple Range Tests for AOA by growth phase (Method: 95.0 percent LSD)

	Pyra	Spacinska	Emka	Kasho	Jana C1	Hrusowska
plant part	HG	HG	HG	HG	HG	HG
stems	X	X	X	X	X	X
seeds	X	X	X	X	X	X
leaves	X	X X	X	X X	X	X
flowers	X	X	X	X	X	X

HG – Homogeneous Groups

4 CONCLUSION

In six cultivars of common buckwheat we monitored changes in antioxidant activity, depending on the growth phase, as well as on the part of buckwheat plant. We have confirmed statistically significant differences in AOA among cultivars during plant development as well as among cultivars in different parts of the plant. Flowers harvested in GP III showed the highest AOA and measured values ranged from 90.94% (cv. Spacinska) to 93.17% ('Jana' C1). Seeds are the most frequently used buckwheat part plant in the food industry, which are used e.g. for the production of flour and meal. In GP IV (full maturity) the highest average AOA value was determined in seeds of 'Hrusowska' (90.47%) followed by 'Kasho' (89.21%), 'Jana C1' (88.94%), 'Emka' (88.86%), 'Pyra' (87.47%) and 'Spacinska' (87.33%).

Although buckwheat does not belong to the majority of agricultural crops, its use in the food industry has great perspectives. In addition, it contains a large number of bioactive substances, is a source of antioxidants, with a positive effect on the human organism. The use of buckwheat in food production - and not just seeds, but also other parts of the plant - can improve the nutritional value of foods, or to replace the synthetic antioxidants used as food additives by antioxidants from natural sources.

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6 REFERENCES

Brand-Williams W., Cuvelier M.E., Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. Lebensmittel – Wissenschaft and Technologie, 28: 25-30

Brindzová L., Zalibera M., Šimon P., Čertík M., Takácsová M., Mikulaková A., Mikušová L., Rapta P. 2009. Screening of cereal varieties for antioxidant and radical scavenging properties applying various spectroscopic and thermoanalytical methods. International Journal of Food Science and Technology, 44: 784-791

Cawoy V., Ledent J.F., Kinet J.M., Jacquemart A.L. 2009. Floral Biology of common buckwheat (*Fagopyrum esculentum* Moench). The European Journal of Plant Science and Biotechnology, 3: 1-9

Christa K., Soral-Śmietana M. 2008. Buckwheat grains and buckwheat products – nutritional and prophylactic value of their components – a review. Czech Journal of Food Science, 26: 153-162

Faller A.L.K., Fialho E. 2009. The antioxidant capacity and polyphenol content of organic and conventional retail vegetables after domestic cooking. Food Research International, 42: 210-215

Fujita K., Inoue N., Hagiwara S., Yang Z., Kato M., Hagiwara M. 2004. Relationship between antioxidant activity and flour and hull color in Tartary buckwheat. Fagopyrum, 21: 51-57

Gorinstein S., Vargas O.J.M., Jaramillo N.O., Salas I.A., Ayala A.L.M., Arancibia-Avila P., Toledo F., Katrich E., Trakhtenberg S. 2007. The total polyphenols and the antioxidant potentials of some selected cereals and pseudocereals. Eur Food Technol, 225: 321-328

Holasova M., Fiedlerova M., Smrcinova H., Orsak M., Lachman J., Vavreinova S. 2002. Buckwheat – the source of antioxidant activity in functional foods. Food Research International, 35: 207-211

Ikeda K., Arai R., Mori K., Tuogo M., Kreft I., Yasumoto K. 2001. Characterization of buckwheat groats by mechanical and chemical analyses. Fagopyrum, 18: 37-43

Ismail A., Marjan Z.M., Foong Ch.W. 2004. Total antioxidant activity and phenolic content in selected vegetables. Food Chemistry, 87: 581-586

Krupa-Kozak U., Wronkowska M., Soral-Śmietana M. 2011. Effect of Buckwheat Flour on Microelements and Proteins Contents in Gluten-Free Bread. Czech Journal of Food Sciences, 29: 103-108

Liu C.L., Chen Y.S., Yang J.H., Chiang B.H. 2008. Antioxidant activity of tartary (*Fagopyrum tataricum* (L.) Gaertn.) and common (*Fagopyrum esculentum* Moench) buckwheat sprouts. Journal of Agricultural and Food Chemistry, 56: 173-178

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

Sedej I.J., Sakač M.B., Mišan A.Č., Mandić. 2010. Antioxidant activity of wheat and buckwheat flours. Matica Srpska Proceedings for Natural Sciences, 118: 59-68

Sun T., Ho Ch-T. 2005. Antioxidant activities of buckwheat extracts. Food Chemistry, 90: 743-749

Shahidi F, Naczk M. 2004. Phenolics in Food and Nutraceuticals. CRC Press, Boca Raton, USA: 30-31

Tang Ch.H., Peng J., Zhen D.W., Chen Z. 2009. Physicochemical and antioxidant properties of buckwheat (*Fagopyrum esculentum* Moench) protein hydrolysates. Food Chemistry, 119: 672-678

Zadernowski R., Pierzynowska-Korniak G., Ciepielewska D., Fornal L. 1992. Chemical characteristics and biological functions of phenolic acids of buckwheat and lentil seeds. Fagopyrum, 12: 27-35

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A decade of research in mofette areas has given us new insights into adaptation of soil microorganisms to abiotic stress

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ABSTRACT

Natural CO_2 springs (mofettes) represent extreme ecosystems with severe exhalations of ambient temperature geological CO_2, inducing long-term soil hypoxia. In this paper an overview of mofette research in the fields of microbial ecology and biodiversity in presented, with a focus on the studies describing the impact of the changed soil gas regime on communities of arbuscular mycorrhizal fungi, archaea and bacteria. Along with the fast development of new, high-throughput molecular techniques driving the field of molecular ecology, mofettes enable new insights into the importance of the abiotic environmental factors in regulating soil biodiversity, and the community structure of these functionally important microbial groups.

Key words: natural CO_2 springs, hypoxia, abiotic environmental factors, carbon capture and storage – CCS, soil ecology, archaea, bacteria, Glomeromycota

IZVLEČEK

DESETLETJE RAZISKAV NA OBMOČJIH MOFET NAM JE OMOGOČILO NOVE VPOGLEDE V ADAPTACIJO MIKROORGANIZMOV NA ABIOTSKI STRES

Naravni izviri CO_2 ali mofete predstavljajo ekstremen ekosistem, kjer zaradi izhajanja geološkega plina v tleh prihaja do dologoročnega pojava hipoksije. V preglednem članku so predstavljene raziskave z območij mofet s področja mikrobne ekologije, ki opisujejo vplive spremememenjenih koncentracij talnih plinov na združbe arbuskularnih mikoriznih gliv, arhej in bakterij. Skupaj s hitrim razvojem novih molekulskih pristopov, predvsem novih generacij visokozmogljivega paralelnega sekvenciranja, ki poganjajo področje molekularne ekologije, mofete omogočajo raziskovanje vpliva abiotskih dejavnikov okolja na biodiverziteto in strukturo združb teh funkcionalno pomembnih skupin talnih mikrobov.

Ključne besede: naravni izviri CO_2, hipoksija, abiotski dejavniki okolja, zajemanje in skladiščenje CO_2, ekologija tal, arheje, bakterije, Glomeromycota

1 INTRODUCTION

Natural CO_2 springs, or mofettes, are extreme ecosystems where ambient temperature geological CO_2 reaches the surface, resulting in a severe and relatively constant change in concentrations of soil gases. CO_2 vents are present in areas with tectonic activities in many locations worldwide (Pfanz et al., 2004), while in Slovenia they are in the north-eastern part of the country close to Gornja Radgona. Several CO_2 vents in this area represent Stavešinci mofette system where a soil gas regime has been well described, both spatially and temporally (Vodnik et al., 2006, 2009). In addition, also other soil parameters (e.g. soil chemistry, soil water content) (Vodnik et al., 2006, Vodnik et al., 2009) and plant eco-physiological responses have been well characterized in more than ten scientific

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papers (e.g. Kaligarič, 2001, Vodnik et al., 2002a, 2002b, Pfanz et al., 2004, Maček et al., 2005, Pfanz et al., 2007). An important but often neglected feature in practically all mofette sites is the CO_2 induced soil hypoxia (reduced O_2 concentration) that affects all the present biota in this ecosystem (Maček et al., 2005, Maček et al., 2011, Šibanc et al., under review). Hypoxia is a common but usually transient abiotic stress factor that is also present in many other terrestrial ecosystems, e.g. flooded or compacted soils (Perata et al., 2011). Mofette systems, however, enable new insights into microbial responses and adaptations to long-term changes in the soil abiotic environment. This represents a new research direction, driven by the rapid development of the new molecular tools progressively used in research of molecular and microbial ecology.

In this paper we present an overview of the mofette research performed over the last decade with a focus on the studies describing the impact of the changed soil gas regime on soil microorganisms, their communities, and biodiversity. This includes several groups of organisms, focusing mainly on the arbuscular mycorrhizal fungi, bacteria and archaea.

Figure 1: A meadow within the Stavešinci mofette area (NE Slovenia) where different groups of soil microorganisms (fungi, bacteria and archaea) have been studied. A decreased growth of vegetation can be seen in the centre of the CO_2 vents (in front, left side) with the highest concentrations of geological CO_2 in the soil.

Slika 1: Travišče znotraj območja mofet v Stavešincih (SV Slovenija), kjer so potekale obstoječe raziskave različnih skupin talnih mikroorganizmov (gliv, bakterij in arhej). V središču vrelcev CO_2 (levo spredaj) je vidna slabša rast vegetacije na mestih, kjer je izpostavitev geološkemu CO_2 v tleh največja.

2 WHY MOFETTE RESEARCH MATTERS?

In the beginning of the 1990s the first reports about the possibilities of using mofettes in environmental and biological studies were published using Italian CO_2 springs (e.g. Miglietta et al., 1993, Raschi et al., 1997). Following the initial use – primarily for research of the vegetation and plant above-ground responses to elevated, atmospheric CO_2 concentrations in the range of those predicted by climate change models (e.g. Raschi et al., 1997) – a second feature, the importance of high soil CO_2 concentrations and CO_2 induced hypoxia in mofette soils and its impact on soil biota, was observed (Maček et al., 2005). Mofettes were consistently shown to be very specific ecosystems with extremely high concentrations of CO_2 present in the soil air and high CO_2 efflux from soil surface (Vodnik et al., 2006, 2009). This is also one of the reasons why, in the last few years, the focus of

mofette research has shifted to the use of different mofette sites as model ecosystems for studies of plant and soil microbial responses to potential CO_2 leakage from underground carbon capture and storage (CCS) systems (Lal, 2008, Krüger et al., 2011, Noble et al., 2012, Frerichs et al., 2013). CCS is the process of capturing CO_2 from large point sources and depositing it underground. It is proposed as one of the possible measures for storing waste CO_2. Thus, in the 20 years of mofette research, the focus of the studies in different fields of applied sciences has moved from the initial studies of plant ecophysiological responses to elevated CO_2 in the atmosphere as a long-term natural analogue to other above ground fumigation systems (e.g. FACE – Free Air Carbon dioxide Enrichment experiments), to measuring plant (Maček et al., 2005) and microbial responses (e.g. Maček et al., 2009, Videmšek et al., 2009, Krüger et al., 2011, Maček et al., 2011, Frerichs et al., 2013, Šibanc et al., under review) to high soil CO_2 concentrations and CO_2 induced hypoxia. Only recently, the first reports on soil fauna responses to CO_2 induced soil hypoxia were also published, with a description of the new Collembola species, specific for mofette sites (Russell et al., 2011).

Geological CO_2 in mofette areas induces changes in several abiotic soil factors, including acidification (Jamnik, 2005), higher concentrations of nutrients due to reduced mineralization rates (Maček et al., 2009), and hypoxia. The latter has been consistently shown as a major abiotic factor affecting soil microbes (Maček et al. 2009, Maček et al., 2011, 2013, Šibanc et al., 2012, Šibanc et al., under review). Hypoxia is not only limited to mofette sites, but is a wider phenomenon and a common transient property of soils that often appears in waterlogged and flooded areas or due to soil compaction. In a special issue of *New Phytologist* (*New Phytologist 190*, 2011) on 'Plant anaerobiosis' several mechanisms involved in plant response to flooding stress, the effects floods may have on patterns of plant distribution and biodiversity, and the devastating impact on crop growth are described (Perata et al., 2011). Interestingly, no reports on the response of plant symbiotic arbuscular mycorrhizal fungi or any other rhizosphere organisms to hypoxia were considered in this issue, though rhizosphere organisms represent an important ecosystem component affecting plant performance in practically all natural environments.

This however indicates a general rule, since reports on soil hypoxia impacts on rhizosphere and soil biota are scarce, inconsistent and often neglected. Thus, since the first rhizosphere study conducted within the Stavešinci mofette field, focusing on the research of high CO_2 concentrations and hypoxia on root respiration (Maček et al., 2005), hypoxia was chosen as the our stress of choice for further investigations: it is present in many natural ecosystems (Perata et al., 2011) and in addition, mofettes provide an unique example of plant and soil communities subject to well characterized (Vodnik et al. 2006, 2009), localized, long-term selection pressure (Maček et al., 2011). This represents a relatively rare opportunity for research of the different aspects of soil ecology and the driving forces of soil diversity in natural ecosystems, and therefore sheds some light on an important research issue that needs immediate attention in order to better understand soil biodiversity and its ecological functions.

3 MOFETTE RESEARCH INTO SOIL MICROBIAL DIVERSITY

There is a limited understanding of the importance of abiotic factors in regulating biodiversity and structure of many functionally important microbial communities in soil. Understanding the significance of the soil biota and the feedback between above- and belowground communities may be critical for designing sustainable production systems in the future, and for using the ecosystem services they can provide effectively (Gianinazzi et al., 2010, Mace et al., 2012). Soils represent a dynamic and complex system that requires intense, complex, and logistically difficult sampling strategies in order to get sufficient information that lead to solid conclusions on the biodiversity and the ecological drivers of this diversity. In the last few decades the development of the DNA- and RNA-based methods has increased our knowledge on soil microbial diversity and function with a big boost because of recent development in the high-throughput

sequencing methods (e.g. massively parallel pyrosequencing) (e.g. Schloss, 2009, Lemos et al., 2011). Thus, the fast development of the new molecular methods, especially in the fields of metagenetics, metagenomicas and metatranscriptomics, now give us a much better tool to study microbial diversity and its functions in practically all environments, including soils and extreme ecosystems like mofettes (Table 1).

Table 1: A list of studies on the different aspects of microbial biology and diversity in mofette soils.
Preglednica 1: Seznam študij s področja raziskav mikrobiologije in biodiverzitete talnih mikroorganizmov na območjih mofet.

Microbial group	**Gene region and/or methodology used**	**Mofette location**	**Study**
AM fungi	16S rRNA gene, T-RFLP, pyrosequencing (Roche 454 FLX), clone libraries	Stavešinci, SI, Bossoleto, IT, Cheb basin, CZ	Maček et al. (2013), Šibanc et al. (2013)
AM fungi	Plant root colonization, soil glomalin concentration	Stavešinci, SI	Maček et al. (2012)
AM fungi	16S rRNA gene, RFLP, clone libraries, plant root colonization	Stavešinci, SI	Maček et al. (2011)
Soil yeasts	26S rRNA D1/D2 domain, sequencing, isolation and culture techniques	Stavešinci, SI	Šibanc et al. (2012)
AM fungi	Plant root colonization, soil glomalin concentration	Hakanoa, New Zealand	Rillig et al. (2000)
Soil microbes	Cell number (qPCR) and activity measurements, *nirK* genes DGGE fingerprinting	Larcher See, DE	Krüger et al. (2009, 2011)
Soil archaea and bacteria	16S rRNA gene, DGGE, activity measurements	Larcher See, DE	Frerichs et al. (2013)
Soil archaea and bacteria	16S rRNA gene, T-RLFP, clone libraries	Stavešinci, SI	Šibanc et al. (under review)
CO_2-fixing bacteria	*cBBl* genes, RFLP	Stavešinci, SI	Videmšek et al. (2009)
Soil microbes	Substrate induced respiration (SIR)	Stavešinci, SI	Maček et al. (2009)
Soil microbes	Lipid biomarkers and ^{13}C analyses, cell numbers (qPCR), biomass, and activity measurements	Latera Caldera, IT	Beaubien et al. (2008), Oppermann et al. (2010)
Soil microbes	16S-23S spacer region, ITS region, Automated Ribosomal Intergenic Spacer Analysis (ARISA), qPCR, PLFA, enzyme analyses	Mammoth Mountain, U.S.A.	McFarland et al. (2013)

3.1 CASE STUDY 1 – ARBUSCULAR MYCORRHIZAL FUNGI

In terrestrial ecosystems, symbiotic associations between plant roots and mycorrhizal fungi are near ubiquitous, with 90 % of all plant species forming mycorrhizas (Smith and Read, 2008). The vast majority of all terrestrial plants receive inorganic nutrients indirectly from symbiotic associations with arbuscular mycorrhizal (AM) fungi (ph. Glomeromycota) (Fig. 2), via efficient exploration of the soil by fungal hyphae, and not by a direct uptake from the soil by plant roots (Smith and Read, 2008, Hodge et al., 2010). In exchange, the plants supply up to 20 % of photosynthates to the fungi as the only energy source of the fungus (ca five billion tonnes carbon per year) (Bago et al., 2000). The nutrient exchange within plant root cells mainly takes place at the fungus-plant symbiotic interface formed around the finely branched fungal arbuscules (Parniske, 2008). Yet, despite its ecological importance, astonishingly little is known about their ecological and physiological responses to hypoxia (Maček et al., 2011).

AM fungi are a functionally important microbial group with poorly understood community ecology (Helgason and Fitter, 2009). Different studies suggest that where an extreme environmental stress occurs in soils, there are a small number of AM fungal lineages that are better able to tolerate those conditions, which results in unique, adapted populations (Helgason and Fitter, 2009, Dumbrell et al., 2010, Maček et al., 2011). AM fungi form an extensive mycelial network in soil and therefore will be subject to strong selection pressures from the abiotic soil environment (e.g. Dumbrell et al., 2010, Maček et al., 2011). However, reports on molecular community analyses and diversity studies of AM fungi in extreme ecosystems are still very scarce (e.g. Appoloni et al., 2008, Maček et al., 2011). In the last 15 years several molecular techniques have been developed, typically targeting different regions of ribosomal rRNA genes that allow identification of the fungal endophytes within roots and soil (e.g. Helgason et al., 1998, Dumbrell et al., 2011). Only recently, some reports on using high-throughput sequencing techniques on the characterization of natural AM fungal communities were published (Öpik et al., 2009, Dumbrell et al., 2011). The newly developed methodology now allows us sufficient sampling intensity within different habitats to answer numerous ecological questions about this important group of soil fungi. However, to the best of our knowledge – apart from our research on mofettes (e.g. Maček et al. 2011, 2013) – there are no other studies on the direct effect of soil hypoxia on AM fungal communities (Table 1). Within the Stavešinci mofette area, studies on AM fungal root colonization (Maček et al., 2011, 2012), the concentration of glomalin-related soil protein, produced by AM fungi (Maček et al., 2012) and the structure of AM fungal communities (Maček et al., 2011, Maček et al., 2013, Šibanc et al., 2013) were conducted, investigating CO_2/hypoxia related responses of this fungal group. Maček et al. (2011) report on significant levels of AM fungal community turnover (beta diversity) between soil types and the numerical dominance of specific AM fungal taxa in hypoxic soils. This work strongly suggests that direct environmental selection acting on AM fungi is a major factor regulating AM fungal communities and their phylogeographic patterns. Consequently, some AM fungi are more strongly associated with local variations in the soil environment than with their host plant's distribution (Maček et al., 2011). There are more reports to follow this initial study of AM fungi in mofette areas, including the ones involving high-throughput sequencing techniques (Roche 454 FLX) (Maček et al., 2013, Šibanc et al., 2013), thus allowing more intensive sampling and more detailed analyses of the mofette AM fungal communities.

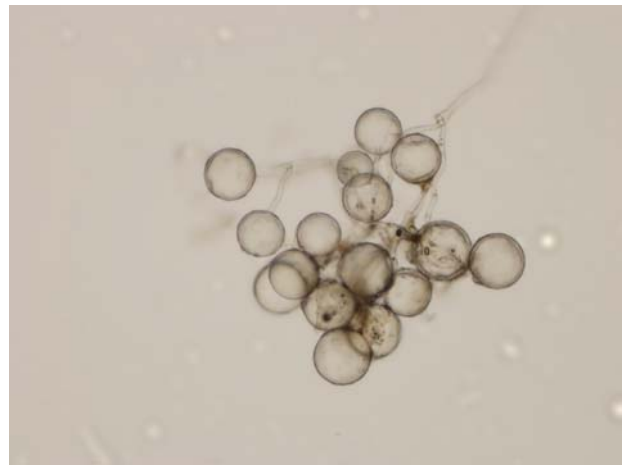

Figure 2: A cluster of AM fungal spores in a sporocarp, isolated from a glasshouse pot culture initiated with AM fungal inoculum from a location exposed to high geological CO_2 concentration (>60 % CO_2) within a Stavešinci mofette area. The isolate represents a potentially new AM fungal species. Photos taken by Olympus Provis AX70 microscope and digital camera.

Slika 2: Grozd spor AM gliv znotraj glivnega sporokarpa. Spore so bile izolirane iz lončne kulture iz rastlinjaka, inokulirane z vzorcem tal z AM glivami, vzorčenim iz območja velike koncentracije geološkega CO_2 znotraj območja mofet v Stavešincih. Izolat predstavlja potencialno še neopisano vrsto AM gliv. Posneto z mikroskopom Olympus Provis AX70 in digitalno kamero.

3.2 CASE STUDY 2 – SOIL ARCHAEA, BACTERIA and FUNGI

Soil is the most biologically diverse environment on Earth, with a biodiversity which can often be several orders of magnitude greater than that present aboveground (Heywood, 1995). A large portion of this diversity involves the greatly unknown diversity of different prokaryotic organisms, bacteria, and archaea. Up to now only a few studies of soil microorganisms from mofette areas were conducted (Table 1). In the Slovenian Stavešinci mofette soils Videmšek et al. (2009) examined the abundance and diversity of *cbbL* genes, encoding for the large subunit of RubisCO in CO_2-fixing bacteria. In this same area Maček et al. (2009) reported on reduced levels of substrate induced respiration (SIR), indicating reduced microbial biomass and activity in high geological CO_2 exposed soil. However, apart from the Slovenian Stavešinci mofette, at least two other mofette areas in Europe and one in U.S.A. have been involved in studies of microbial responses to geological CO_2 exhalations.

First, a terrestrial CO_2 vent located at the Laacher See, Germany was used by the group of Krüger et al. (2009, 2011) as a model ecosystem for investigating the impact of potential leakage from carbon capture and storage systems (CCS) on the surrounding environment. They reported on lower bacterial cell numbers, higher levels of bacterial non-isoprenoidical tetraethers lipids (most likely derived from anaerobic bacteria), and higher archaeal cell numbers at the vent compared to the control site. The investigation of archaeal and bacterial communities, based on potential sulphate reduction rates, methane production, and a lipid biomarkers study, showed a shift towards anaerobic and acidophilic species in high CO_2 sites. Moreover, recently a study employing molecular markers (community fingerprinting technique – denaturing gradient gel electrophoresis – DGGE) was used to identify the shifts in the communities of archaea and bacteria among geological CO_2 impacted and control soil samples in the mofette field near Laacher See (Frerichs et al., 2013). The study of the abundance of several functional and group-specific gene markers revealed differences in the composition of the mofette soil microbial communities, for example a decrease of Geobacteraceae and an increase in sulphate-reducing taxa in the vent core, reaching moderately elevated (up to 30%) soil CO_2 concentrations.

Second, within the Latera Caldera mofette in the volcanic district in Central Italy, Beaubien et al. (2008) reported on decreasing trends in adenosine triphosphate (ATP) biomass, bacterial cell counts, and the higher activity of strictly anaerobic, sulphate-reducing bacteria and methanogenic archaea in the centre of the CO_2 vent compared to the transit zone and background, while H_2 dependant methanogenesis was absent and aerobic methane oxidation was negatively correlated with increased CO_2. In addition to this study in the same mofette area, Oppermann et al. (2010) found CO_2-utilising methanogenic archaea, Geobacteraceae, and sulphate-reducing bacteria mainly at the CO_2 vent, only minor quantities were found at the reference site. Also, their results suggest a shift in the microbial community towards anaerobic and acidophilic microorganisms as a consequence of the long-term exposure of the soil environment to high geological CO_2 concentrations.

A very recent report comes from the Mammoth Mountain, a dormant volcano from eastern California (U.S.A.), and an area known for geological CO_2 induced tree mortality (McFarland et al., 2013). The authors of the study assessed the soil microbial community response to CO_2 disturbance that resulted in localised tree kill. As a result to reduced soil carbon availability soil microbial biomass decreased, which was linked to the loss of soil fungi. In contrast, archaeal populations responded positively to the CO_2 disturbance, presumably due to reduced competition of bacteria and fungi.

To our knowledge, however, there is no published data on the overall community structure or diversity of bacteria, and archaea in mofette areas based on clone libraries, and especially so in the most extreme locations (with the soil CO_2 concentrations well above 60 %). In these sites, however, CO_2 induced hypoxia could strongly affect microbial communities (Šibanc et al., under review).

4 CONCLUSIONS

All these studies are important not only for their use in the research of impacts of elevated atmospheric CO_2 concentrations on plants and possible leakage of CO_2 in CCS systems and related impacts on biota, but also from a biotechnological and ecological perspective. Extreme environments have previously served as a rich source of potentially useful organisms in different fields of applied biotechnology and agronomy (e.g. new antibiotics discovery, isolates in commercial inoculums of AM fungi). Little is known about what kind of organisms actually live in these habitats and even less about their ecological function. Moreover, as major shifts in microbial community composition have significant implications for ecosystem functioning (e.g. changes in carbon cycling driven by changes in methanogenic archaea populations), understanding their response to long-term environmental changes is of crucial ecological importance. Thus, a full phylogenetic characterisation of fungal, archaeal, and bacterial communities, their taxonomy, and an investigation into the processes regulating their diversity and community structure has yet to be reported (Maček et al., 2013, Šibanc et al., 2012, 2013, Šibanc et al., under review).

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6 REFERENCES

Appoloni S., Lekberg Y., Tercek M.T., Zabinski C.A., Redecker D. 2008. Molecular community analysis of arbuscular mycorrhizal fungi in roots of geothermal soils in Yellowstone national park (U.S.A.). Microbial Ecology 56: 649–659

Bago B., Pfeffer P.E., Shachar-Hill Y. 2000. Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiology 124, 3: 949–957

Beaubien S.E., Ciotoli G., Coombs P., Dictor M., Krüger M., Lombardi S., Pearce J., West J. 2008. The impact of a naturally occurring CO_2 gas vent on the shallow ecosystem and soil chemistry of a Mediterranean pasture (Latera, Italy). International Journal of Greenhouse Gas Control 2: 373–387

Dumbrell A.J., Ashton P.D., Aziz N., Feng G., Nelson M., Dytham C., Fitter A.H., Helgason T. 2011. Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. New Phytologist 190: 794–804

Dumbrell A.J., Nelson M., Helgason T., Dytham C., Fitter A.H. 2010. Relative roles of niche and neutral processes in structuring a soil microbial community. The ISME Journal 4: 337–345

Frerichs J., Oppermann B.I., Gwosdz S., Möller I., Herrmann M., Krüger M. 2013. Microbial community changes at a terrestrial volcanic CO_2 vent induced by soil acidification and anaerobic microhabitats within the soil column. FEMS Microbiology Ecology 84: 60–74

Gianinazzi S., Gollotte A., Binet M.N., van Tuinen D., Redecker D., Wipf D. 2010. Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20, 8: 519–530

Helgason T., Daniell T.J, Husband R., Fitter A.H., Young J.P.W. 1998. Ploughing up the wood-wide web? Nature 394, 6692: 431–431

Helgason T. and Fitter A. H. 2009. Natural selection and the evolutionary ecology of the arbuscular mycorrhizal fungi (phylum Glomeromycota). Journal of Experimental Botany 60: 2465–2480

Heywood V.H. 1995. Global Biodiversity Assessment. Cambridge University Press: 1152 pg.

Hodge A., Helgason T., Fitter A.H. 2010. Nutritional ecology of arbuscular mycorrhizal fungi. Fungal Ecology 3, 4: 267–273

Jamnik M. 2005. Temporal and spatial variability of soil CO_2 concentration on the natural CO_2 spring Stavešinci. Graduation thesis. University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Ljubljana: 45 pg.

Kaligarič M. 2001. Vegetation patterns and responses to elevated CO_2 from natural CO_2 springs at Strmec (Radenci, Slovenia). Acta Biologica Slovenica 44, 1/2: 31–38

Krüger M., Jones D., Frerichs J., Oppermann B.I., West J., Coombs P., Green K., Barlow T., Lister R., Shaw R., Strutt M., Möller I. 2011. Effects of elevated CO_2 concentrations on the vegetation and microbial populations at a terrestrial CO_2 vent at Laacher See, Germany. International Journal of Greenhouse Gas Control 5: 1093–1098

Krüger M., West J., Frerichs J., Oppermann B., Dictord M., Jouliand C., Jones D., Coombs P., Green K., Pearceb J., Maya F., Möllera I. 2009. Ecosystem effects of elevated CO_2 concentrations on microbial populations at a terrestrial CO_2 vent at Laacher See, Germany. Energy Procedia 1: 1933–1939

Lal R., 2008. Carbon sequestration. Philosophical Transactions of The Royal Society Biological Sciences 363: 815–830

Lemos L.N., Fulthorpe R.R., Triplett E.W., Roesch L.F.W. 2011. Rethinking microbial diversity analysis in the high throughput sequencing era. Journal of Microbiological Methods 86: 42–51

Mace G.M., Norris K., Fitter A.H. 2012. Biodiversity and ecosystem services: a multilayered relationship. Trends in Ecology and Evolution 27: 19–26

Maček I., Dumbrell A.J., Nelson M., Fitter A.H., Vodnik D., Helgason T. 2011. Local adaptation to soil hypoxia determines the structure of an arbuscular mycorrhizal fungal community in roots from natural CO_2 springs. Applied and Environmental Microbiology 77: 4770–4777

Maček I., Kastelec D., Vodnik D. 2012. Root colonization with arbuscular mycorrhizal fungi and glomalin-related soil protein (GRSP) concentration in hypoxic soils from natural CO_2 springs. Agricultural and Food Science 21: 62–71

Maček I., Pfanz H., Francetič V., Batič F., Vodnik D. 2005. Root respiration response to high CO_2 concentrations in plants from natural CO_2 springs. Environmental and Experimental Botany 54: 90–99

Maček I., Šibanc N., Dumbrell A.J., Helgason T. 2013. Impact of long-term soil hypoxia on arbuscular mycorrhizal fungal communities in mofette areas (natural CO_2 springs). In: 7th International conference on mycorrhiza "Mycorrhiza for all: an under-earth revolution", 6–11 January 2013, New Delhi, India. Abstracts. Adholeya A. (ed.). New Delhi, Department of Biotechnology, Ministry of Science and Technology, Government of India: 94

Maček I., Videmšek U., Kastelec D., Stopar D., Vodnik D. 2009. Geological CO_2 affects microbial respiration rates in Stavešinci mofette soil. Acta Biologica Slovenica 52: 41–48

McFarland J.W., Waldrop M.P., Haw M. 2013. Extreme CO_2 disturbance and the resilience of soil microbial communities. Soil Biology & Biochemistry 65: 274–286

Miglietta F., Raschi A., Bettarini I., Resti R., Selvi F. 1993. Natural CO_2 springs in Italy – A resource for examining long-term response of vegetation to rising atmospheric CO_2 concentrations. Plant, Cell and Environment, 16, 7: 873–878

New Phytologist, Special Issue: Plant anaerobiosis, April 2011, 190, 2, Wiley: 269-508

Noble R.R.P., Stalker L., Wakelin S.A., Pejcic B., Leybourne M.I., Hortle A.L., Michael K. 2012. Biological monitoring for carbon capture and storage – A review and potential future developments. International Journal of Greenhouse Gas Control 10: 520–535

Oppermann B.I., Michaelis W., Blumenberg M., Frerichs J., Schulz H.M., Schippers A., Beaubien S.E., Krüger M. 2010. Soil microbial community changes as a result of long-term exposure to a natural CO_2 vent. Geochimica et Cosmochimica Acta 74: 2697–2716

Öpik M., Metsis M., Daniell T.J., Zobel M., Moora M. 2009. Large-scale parallel 454 sequencing reveals host ecological group specificity of arbuscular mycorrhizal fungi in a boreonemoral forest. New Phytologist 184: 434–437

Parniske, M. 2008. Arbuscular mycorrhiza: the mother of plant root endosymbiosis. Nature Reviews Microbiology 6, 10: 763–775

Perata P., Armstrong W., Voesenek L.A.C.J. 2011. Plants and flooding stress. New Phytologist 19, 2: 269–273

Pfanz H., Vodnik D., Wittmann C., Aschan G., Raschi A. 2004. Plants and geothermal CO_2 exhalations – survival in and adaptation to a high CO_2 environment. Progress in Botany 65: 499–538

Pfanz H., Vodnik D., Wittmann C., Aschan G., Batič F., Turk B., Maček I., 2007. Photosynthetic performance (CO_2-compensation point, carboxylation efficiency, and net photosynthesis) of timothy grass (*Phleum pratense* L.) is affected by elevated carbon dioxide in post-volcanic mofette areas. Environmental and Experimental Botany 61: 41–48

Raschi A., Miglietta F., Tognetti R., Van Gardingen P.R. 1997. Plant responses to elevated CO_2. Cambridge University Press, Cambridge: 286 p.

Rillig M.C., Hernandez G.Y., Newton C.D. 2000. Arbuscular mycorrhizae respond to elevated atmospheric CO_2 after long-term exposure: evidence from a CO_2 spring in New Zealand supports the resource balance model. Ecology Letters 3: 475–478

Russell D.J., Schulz H., Hohberg H., Pfanz H. 2011. Occurrence of collembolan fauna in mofette fields (natural carbondioxide springs) of the Czech Republic. Soil Organisms 83, 3: 489–505

Schloss P.D. 2009. A high-throughput DNA sequence aligner for microbial ecology studies. PLoS ONE 4, 12: e8230

Smith S.E. and Read D.J. 2008. Mycorrhizal symbiosis, Third Edition, London, Academic Press: 787 p.

Šibanc N., Dumbrell A.J., Mandić-Mulec I., Maček I. (under review). Impacts of naturally elevated soil CO_2 concentrations on communities of soil archaea and bacteria. Soil Biology & Biochemistry

Šibanc N., Helgason T., Dumbrell A.J, Vodnik D., Pfanz H., Raschi A., Maček I. 2013. Biogeography of arbuscular mycorrhizal fungal communities in hypoxic soil – evidence from the Slovenian, Italian, and Czech mofette fields (natural CO_2 springs). In: 7th International conference on mycorrhiza "Mycorrhiza for all: an under-earth revolution", 6–11 January 2013, New Delhi, India. Abstracts. Adholeya A. (ed.). New Delhi, Department of Biotechnology, Ministry of Science and Technology, Government of India: 189

Šibanc N., Helgason T., Dumbrell A.J, Mandić-Mulec I., Zalar P., Schroers H, Maček I. 2012. Elevated CO_2 is changing soil microbial communities at natural CO_2 springs (mofettes). In: 14th International symposium on microbial ecology, 19–24 August 2012 Copenhagen, Denmark. Abstract book: ISME14: 71

Videmšek U., Hagn A., Suhadolc M., Radl V., Knicker H., Schloter M., Vodnik D. 2009. Abundance and diversity of CO_2-fixing bacteria in grassland soils close to natural carbon dioxide springs. Microbial Ecology 58: 1–9

Vodnik D., Kastelec D., Pfanz H., Maček I., Turk B. 2006. Small-scale spatial variation in soil CO_2 concentration in a natural carbon dioxide spring and some related plant responses. Geoderma 133: 309–319

Vodnik D., Pfanz H., Maček I., Lojen S., Batič F. 2002a. Photosynthesis of cockspur [*Echinochloa crus-galli* (L.) Beauv.] at sites of natural elevated CO_2 concentrations. Photosynthetica 40: 575–579

Vodnik D., Pfanz H., Wittmann C., Maček I., Kastelec D., Turk B., Batič F. 2002b. Photosynthetic acclimation in plants growing near a carbon dioxide spring. Phyton 42: 239–244

Vodnik D., Videmšek U., Pintar M., Maček I., Pfanz H. 2009. The characteristics of soil CO_2 fluxes at a site with natural CO_2 enrichment. Geoderma 150: 32–37

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Influence of arbuscular mycorrhiza on osmotic adjustment compounds and antioxidant enzyme activity in nodules of salt-stressed soybean (*Glycine max*)

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ABSTRACT

The influence of the colonization with arbuscular mycorrhizal (AM) fungus, *Glomus mosseae* (Nicolson and Gerdemann), on characteristics of growth, osmotic adjustment compounds and activity of antioxidant enzymes in nodules of salt-stressed soybean (*Glycine max* (L.) Merr.) was studied in this experiment. The pot experiment was arranged as a factorial in randomized complete block design with four replications at greenhouse of College of Agriculture, Tehran University, Iran. Results indicated that the contents of glycine betaine and proline in nodules were higher in inoculated than in non-inoculated plants. AM fungal colonization increased the activities of superoxide dismutase, catalase, and peroxidase in the nodules. The results indicate that the AM fungus is capable of alleviating the damage caused by salt stress on symbiotic nitrogen fixation of soybean plants by increasing the accumulation of compatible osmolytes and by increased antioxidant enzyme activity. Consequently, arbuscular mycorrhiza formation highly enhanced the salinity tolerance of soybean plant, which increased symbiotic nitrogen fixation and promoted plant growth.

Key words: antioxidants, nodules, osmolytes, salinity, soybean

IZVLEČEK

VPLIV ARBUSKULARNE MIKORIZE NA SPOJINE OSMOTSKE PRILAGODITVE IN ANTIOKSIDACIJSKO ENCIMSKO AKTIVNOST V NODULIH SOJE (*Glycine max* (L.) Merr.) V SLANOSTNEM STRESU

V poskusu je bil preučevan vpliv kolonizacije z arbuskularno mikorizno glivo (AM), *Glomus mosseae* (Nicolson and Gerdemann), na značilnosti rasti, snovi osmotskega prilagajanja in aktivnost antioksidacijskih encimov v nodulih soje (*Glycine max* (L.) Merr.) v slanostnem stresu. Lončni poskus je bil izveden kot naključni faktorski bločni poskus v štirih ponovitvah v rastlinjaku College of Agriculture, Tehran University, Iran. Izsledki so pokazali, da sta bili vsebnosti glicin betaina in prolina večji v inokoliranih kot v neinokuliranih rastlinah. Kolonizacija z AM glivo je povečala aktivnost superoksid dizmutaze, katalaze in peroksidaze v nodulih. Rezultati kažejo, da je AM gliva sposobna omiliti poškodbe, ki nastanejo ob slanostnem stresu v simbiontski vezavi dušika pri soji s povečano akumulacijo primernih osmotikov in povečano antioksidacijsko encimsko aktivnostjo. Posledično tvorba arbuskularne mikorize pri soji močno poveča toleranco na slanostni stres s povečano simbiontsko vezavo dušika, kar pospeši rast.

Ključne besede: antioksidanti, noduli, osmotiki, slanost, soja

1 INTRODUCTION

Symbiotic nitrogen fixation (SFN) in legumes such as soybean (*Glycine max*) is frequently limited, especially in semi-arid conditions by poor quality of soil and irrigation water. Soybean is classified as a salt-sensitive crop (Läuchli, 1984).The limitation in its productivity is associated with a decreased growth, poor symbiotic development of root-nodule bacteria (Georgiev and Atkins, 1993) and a consequent a reduction in the nitrogen-fixation capacity (Delgado et al., 1994).

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The establishment of the *Rhizobium*-legume symbiosis has been shown to be salt sensitive (Rao et al., 2002). Nodule initiation appears to be more sensitive to salt stress than nodule development (Zahran and Sprent, 1986). Cordovilla et al. (1994) and Soussi et al. (1999) found that the tolerance of the host plant to salt stress could be a determinant factor of symbiosis development. The effect of salt stress on symbiotic nitrogen fixation (SNF) and ion distribution in nodules has been studied in many crops such as soybean, common bean and alfalfa. It seems, the sensitivity of the symbiotic nitrogen fixation is not always associated with a high Na^+ accumulation in nodules. Salinity causes oxidative damage which affects nitrogen fixation and assimilation in nodules. Some studies have implicated reactive oxygen species (ROS) in nodule senescence (Becana et al., 2000; Garg and Manchanda, 2008). But plants are not defenseless; under salt stress some defense mechanisms are initiated which protect plants from harmful effects of oxidative stress. Reactive oxygen species (ROS) scavenging is one such common defense response against abiotic stress (Vranova et al., 2002). The major ROS scavenging system includes a complex enzymatic group such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and non-enzymatic molecules such as proline, glycine betain, sorbitol and manitol (Prochazkova et al., 2001).

Arbuscular mycorrhizal fungi (AMF) widely occur in saline soils (Aliasgharzadeh et al., 2001). These fungi exploit water and mineral salts from soils more effectively than plant roots (Kaya et al., 2003). Many studies have demonstrated that arbuscular mycorrhizal fungi (AMF) protected the host plants to improve the growth of plants under salt stress condition (Trimble and Knowles, 1995). Moreover, additive and sometimes synergistic effects on legume performance are frequently seen when both rhizobia and AMF are present (Goss and de Varennes, 2002; Sanginga et al., 1999; Fitter and Garbaye, 1995). Reports on the response of antioxidant defense system to stress factors in inoculated plants are contradictory; increase, no change, or even decrease in the activity of SOD, CAT, POD and APX were reported in mycorrhizal soybean (Porcel et al., 2003) subjected to drought and tomato subjected to salinity (He et al., 2007; Hajiboland et al., 2010).

The aim of this study was to evaluate the effect of root colonization with *Glomus mosseae* (Nicolson and Gerdemann) on growth parameters, nodulation, mineral uptake, osmotic adjustment compounds and antioxidant enzyme activity of soybean plants under salinity stress, in order to further understand salt tolerance mechanisms in inoculated plants.

2 MATERIALS AND METHODS

Experimental design

The experiment was conducted from 22th of April to 22th of November, 2011 in a greenhouse of the College of Agriculture, University of Tehran, Iran. Plants were grown in the greenhouse under natural sunlight with temperatures of 25 – 30°C (day) and 20 – 23°C (night). There were four replications for each treatment. The experiment was arranged as a factorial in completely randomized design.

Rhizobial and AM fungal inoculum

Mycorrhizal fungal and rhizobal inoculum were provided by the Institute of Soil and Water Research,Karaj, Iran. The AM fungal species used was *Glomus mosseae* (Nicolson and Gerdemann).The soybean seeds were rinsed with water and surface sterilized by dipping in 0.1% sodium hypochlorite for 2 min and then washed three times with distilled water. Seeds were pretreated with a standard rhizobial inoculum of *Bradyrhizobium japonicum*. The AM fungal spores were applied at 10 spores per seed (approximately 1500 spores/100 g of media). Seeds were inoculated by placing the fresh AM inoculum (30 g) in the hole under the seeds and covering with the soil.

The soil used for pots was collected from the uncultivated site located in Qom province, Iran. The soil used in this experiment was not sterilized (autoclaved). The basic soil properties were as follows: organic matter content 1.08%, total N 0.062%, total K 740.80 mg kg^{-1}, total P 10.90 mg kg^{-1}, available P ($NaHCO_3$-extractable) 2.78 mg kg^{-1}, water-soluble K 13.43 mg kg^{-1} and electrical conductivity 8.1 dSm^{-1}.

Five seeds were sown in each pot containing 2 kg of soil mixture. After 21 days, thinning was carried out to leave three uniform seedlings in each pot. When the seedlings were established (30 days after sowing), the plants were treated with saline solution with electrical conductivities 6 (S1 treatment) and 12 dSm (S2 treatment). The control plants (C) were treated with distilled water only. Pots were irrigated according to their weight at 80% field capacity moisture. Regular fortifications of saline solutions were made to maintain the desired soil salinity levels after monitoring the conductivity levels of the soils at weekly intervals, with the help of EC meter, till the end of the experiments. Parameters such as mycorrhizal colonization, nodule weight, leghemoglobin content, nitrogenase activity, osmolyte accumulation and antioxidant enzymes activities nodules were studied after 180 days of sowing. The plants and the adhering soil were transferred to the sieve and roots and nodules were collected from the sieve. For dry weight measurements, the samples were dried in an oven at 70°C for 72 h.

Mycorrhizal colonization

Mycorrhizal colonization was estimated by the method of Phillips and Hayman (1970). For AMF colonization analysis, 2.5 -cm root segments from three plants per treatment were sampled at harvest and pooled to assess colonization percentage. The roots were cut and dipped in 8 % KOH solution for 24 h and then kept in 2% HCl solution for 15 to 30 min. Staining solution containing 0.05% (v/v) cotton blue dye was added. The samples were kept for 24 to 36 h at room temperature condition. Twenty 2.5 -cm stained root pieces were placed on each slide and three observations (the top, the middle, and the bottom) per 2.5-cm root piece were made with microscope. There were four slides per treatment. Root pieces that contained even a single vesicle or arbuscules were considered as colonized. The percentage of AM colonization was calculated from the following equation: Percentage of AM colonization = (Root length colonized/Root length observed) ×100.

Leghemoglobin

Leghemoglobin content was determined in fresh uniform sized root nodules measuring 0.5 cm or more diameter. Nodules were carefully removed from the roots with sharp edged blade. These were washed with prechilled double distilled water. After washing, the nodules were blotted on filter paper, weighed and then finally crushed in prechilled sterilized pestle mortar containing 50.0 mM HCl, 5 mM MgCl2, 20 mM KCl and 5 mM mercapto ethanol. The slurry was centrifuged at 40°C at 8,000 rpm for 15 minutes. The pellets were discarded and supernatant (SN) was made to known volume i.e. 4 ml/ gm fresh weight of nodules. In this supernatant, lb content was estimated by using haemochromogen method (Hartree, 1955).

The 0.5 and 1 ml aliquot of clear extract was taken in the test tube. To each tube, 1.5 ml of 1 N NaOH was added and kept for half an hour at room temperature. After 30 minutes, 3 ml of pyridine solution and 1.5 ml of 10% (W/V) sodium bisulphide were added to each tube. Then distilled water was added to make the volume to 15 ml. The tubes were incubated for 30 minutes and the optical density was recorded at 535 and 556 nm. Calibration curve was prepared by using a standard solution of haemin 100 (µg/ml) by dissolving in 1N NaOH. Leghaemoglobin content is expressed as mg haemin / gm fresh weight of nodules. All observations were recorded in triplicates and data were subjected to statistical analysis of variance using three factorial randomized design methods (Bruning and Kintz, 1977).

Enzymeatic activity

Nitrogenase activity

Nitrogenase was determined by the acetylene-reduction-activity test (ARA) on the nodulated root portion of three plants, following the method of Herdina and Silsbury (1990). Nitrogen fixing complex (nitrogenase) of legumes is able to reduce C_2H_2 to C_2H_4. The nodulated root sample (1 g of root plus nodules) was immediately incubated at room temperature in vials containing acetylene (C_2H_2) (10%, V/V) and sealed with serum caps. The sample of 1 ml of gas from the incubation mixture was analyzed for ethylene in a Perkin Elmer 8600 gas chromatograph equipped with a Porapak R column (Ligero et al., 2007). From the standard values, n. moles of ethylene produced in each case was calculated, the nodules were dried in an oven at 70°C for 24 h, and their dry weights were taken. The rate of enzyme activity was calculated as n. moles of ethylene produced per mg dry weight of nodules per hour.

Superoxide dismutase (SOD) activity

For Superoxide dismutase (SOD) activity analysis, the plant samples were brought to the laboratory and the roots were thoroughly washed under running tap water without damaging roots and nodules. The nodulated root sample from three plants per treatment were sampled at harvest and pooled to assess (SOD) activity. The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to Stewart and Bewley (1980). The reaction mixture (3 ml) contained 13 mM methionine, 75 mM NBT, 100 mM EDTA, 50 ml of enzyme extract within 50 mM phosphate buffer (pH 7.8). The reaction was started with 2 mM riboflavin by exposing the cuvette to a 15-W fluorescent tube for 10 min. The absorbance of each reaction mixture was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme which causes 50% inhibition of the photochemical reduction of NBT.

Catalase activity

The activity of catalase (CAT) was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 (Chance and Meahly, 1955). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2.

Peroxidase activity

Peroxidase (POD) activity was measured by following the change of absorption at 470 nm due to guaiacol oxidation.The activity was assayed for 1 min in a reaction solution (3 ml final volume) composed of 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 m MH_2O_2 and 0.15 ml enzyme extract (Polle et al., 1994).

Calcium, sodium and potassium content

Ground samples were ashed at 580°C for 6 h. The white ash was taken up in 2 M hot HCl, filtered into a 50 ml volumetric flask, and made up to 50 ml with distilled water. Na, K, and Ca were determined in these sample solutions. Na and K in the sample solution were analyzed using a flame photometer and Ca with an atomic absorption spectrophotometry (Chapman and Pratt, 1961).

Phosphorus content

Phosphorus was estimated by the method given by Chapman and Pratt (1961). Vanadate solution was added to the molybdate solution and cooled to room temperature. Added 250 ml of concentrated HNO_3 and diluted to 1 L. A total of 0.5 g of material was taken in 50 ml volumetric flask and 10 ml of vandomolybdate reagent was added to each flask and made the volume by deionized water. The solution was kept for 30 min and took the absorbance at 420 nm with spectrophotometer. Appropriate standards were run simultaneously.

Proline content

Free proline content was determined following the method of Bates et al. (1973). Proline estimation is based on the formation of brick red colored proline- ninhydrin complex in acidic medium. Nodule sample (0.5 g) was homogenized in 5 ml of sulfosalicylic acid (3%) using mortar and pestle, the homogenate was filtered and filtrate was used for the estimation of proline content. Two milliliter of extract was taken in test tube and to it 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent were added and heated for 30 min. Six milliliter of toluene was added and then transferred to a separating funnel. The chromophore containing toluene was separated and its absorbance read was at 520 nm in spectrophotometer against toluene blank. Concentration of proline was estimated by referring to a standard curve made from known concentrations of proline.

Glycine betaine content

Glycine betaine estimation was done as per the method of Grieve and Grattan (1983). Betaine makes a betaine-periodite complex with iodide in acidic medium, which absorbs at 360 nm in UV range. Finely ground dry plant material (0.5 g) was mechanically shaken with 20 ml of deionized water for 48 h at 258°C. The samples were filtered. Thawed extracts were diluted 1:1 with 2 N sulphuric acid. Aliquot (0.5 ml) was cooled in ice water for 1 h and to it; cold potassium iodide-iodine reagent (0.2 ml) was added. The samples were stored at 0–48°C for 16 h and were centrifuged at 10,000 g for 15 min at 08°C. The supernatant was carefully aspirated. The periodite crystals were dissolved in 9 ml of 1, 2- dichloro ethane (reagent grade). After 2.0–2.5 h, the absorbance was measured at 365 nm with UV-visible spectrophotometer. Reference standards of

glycine-betaine (50–200 mg/ml) were prepared in 2 N sulphuric acid and the procedure for sample estimation was followed.

Statistical analysis

All data were subjected to analysis of variance using two-way ANOVA and means were compared by Duncan's multiple range test (Duncan, 1955).

3 RESULTS

The results pointed out that different level of salt stress had inhibitory effects on mycorrhizal colonization, although high mycorrhizal colonization was observed at the moderate level of salinity stress.

Salinity stress significantly reduced the root and shoot dry matter compared with the control treatment (Table 1). However, AM fungal colonization mostly improved dry matter in the salt-stressed plants. This effect of AM on dry matter was more pronounced in shoot biomass than root biomass.

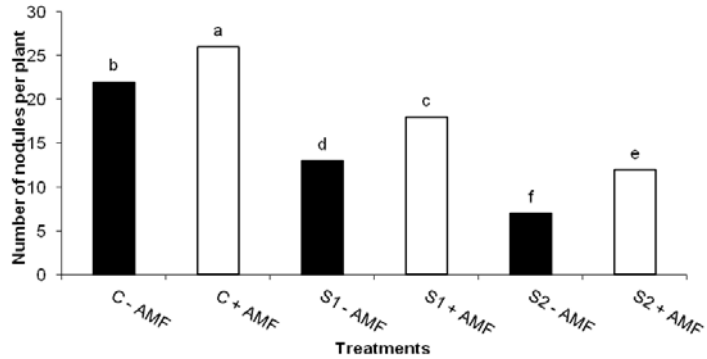
Table 1: Effect of salt stress on shoot length, root length, shoot DM, root DM and colonization in AM and non-AM soybean plants under salt stress.

Treatments	Shoot length (cm plant $^{-1}$± SD)	Root length (cm plant $^{-1}$± SD)	Shoot DM (g plant $^{-1}$± SD)	Root DM (g plant $^{-1}$± SD)	AMF colonization (%)
C - AMF	54.6±1.43^{b}	34.8±1.64^{a}	11.3±0.64^{b}	4.6±0.7^{b}	-
C + AMF	61.8±1.16^{a}	36.6±1.65^{a}	13.6±0.6^{a}	5.8±0.51^{a}	28.8± 1.05^{a}
S1 - AMF	44.3±1.82^{c}	31.2±0.48^{b}	7.9±1.1^{c}	3.8±0.67^{c}	-
S1 + AMF	58.8±0.68^{ab}	35.3±0.73^{a}	10.6±0.75^{b}	5.3±1.37^{a}	26.3±1.63^{a}
S2 - AMF	32.5±1.46^{d}	30.7±1.33^{b}	6.3±o.65^{c}	2.1±0.28^{d}	-
S2 + AMF	42.3±1.07^{c}	34.5±1.02^{a}	9.4±1.13^{bc}	3.6±0.7^{c}	18.8±0.23^{b}

Results represent the average of three experiments ± SD. Different letters represent significant differences ($p < 0.05$) between treatments at each column.

nodulation of vicia faba L. plants by Rhizobium leguminosarum. Planta, 167: 303-309.

Nodule number and dry mass of the nodules decreased under all saline treatments (Figure 1). AM fungal inoculation further boosted the nodulation under saline stress and the nodule number showed a significant increase in unstressed as well as stressed conditions.



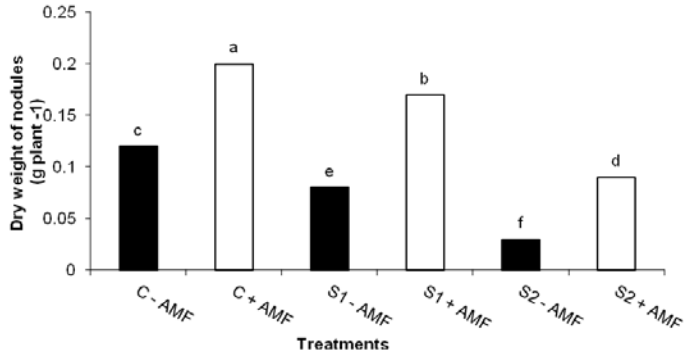


Figure 1: Effect of AM inoculation on number of nodules per plant (a) and dry weights of nodules per plant (b) of soybean under salt stress. Treatments are designed as uninoculated controls, saline stress (S1 = 6 and S2 = 12 dSm^{-1}) and arbuscular mycorrhiza (AM). Means followed by the same letter are not significantly different (p<0.05) as determined by Duncan's Multiple Range test.

Marked decline in the leghemoglobin content was observed in plants exposed to salt stress (Figure 2). The decrease in leghemoglobin content was smaller in inoculated plants, when compared to corresponding uninoculated-stressed plants. AM fungi conferred an advantage on the plants under saline stress and at 12 dSm salinity, inoculated plants had higher leghemoglobin content than the corresponding non-inoculated plants.

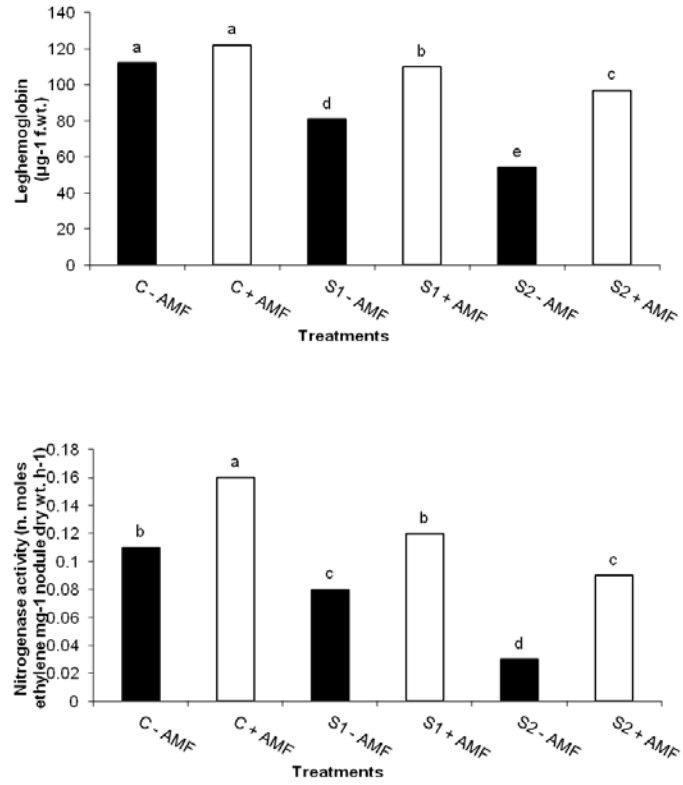


Figure 2: Effect of AM inoculation on leghemoglobin content (a) and nitrogenase activity (b) in the nodules of soybean under salt stress. Treatments are designed as uninoculated controls, saline stress (6 and 12 dSm^{-1}) and arbuscular mycorrhiza (AM). Means followed by the same letter are not significantly different (p<0.05) as determined by Duncan's Multiple Range test.

Nodule activity in terms of acetylene-dependent ethylene production was severely damaged by the presence of salt stress. The presence of fungi proved to be favorable and nitrogenase activity was significantly higher in AM inoculated plants, than in non-inoculated plants, regardless of the saline treatments.

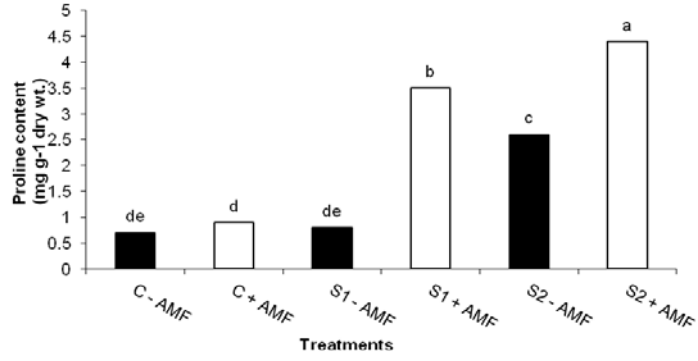
Potassium and phosphorus contents in the nodules declined with increase in the salt concentrations in the soil in all the stressed plants, whereas an increase in the sodium and calcium contents was observed in all the stressed plants (Table 2). Nodules of AM inoculated plants maintained significantly higher ion (potassium, phosphorus, and calcium) contents than the corresponding non-inoculated plants under all saline treatments.

Table 2: Effect of salt stress on potassium, sodium, calcium, and phosphorus content in nodules of AM and non-AM soybean plants under salt stress.

Treatments	Potassium (mg g^{-1} d.wt.± SD)	Sodium (mg g^{-1} d.wt.± SD)	Calcium (mg g^{-1} d.wt.± SD)	Phosphorus (mg g^{-1} d.wt.± SD)
C - AMF	22.22±1.08^{b}	1.52±0.08^{a}	2.61±0.65^{a}	8.32±1.27^{b}
C + AMF	27.11±1.64^{a}	1.34±0.09^{a}	3.41±0.76^{bc}	12.6±1.2^{a}
S1 - AMF	8.11±0.48^{d}	5.77±0.0.39^{c}	2.56±0.21^{a}	5.1±1.04^{d}
S1 + AMF	12.18±0.9^{c}	4.05±0.93^{b}	3.78±0.92^{c}	8.05±0.88^{b}
S2 - AMF	5.26±1.02^{e}	10.3±1.16^{e}	3.18±1.12^{bc}	4.21±0.64^{e}
S2 + AMF	8.92±0.47^{d}	9.1±0.77^{d}	4. 2±0.83^{d}	6.73±0.78^{c}

Results represent the average of three experiments ± SD. Different letters represent significant differences ($p < 0.05$) between treatments at each column.

Proline concentration increased in the nodules with salinity, however, in non-inoculated stressed plants, the increase was not significant at 6 dSm (Figure 3). Nodular proline levels in inoculated salt stressed plants were higher than in non-inoculated salt-stressed plants.



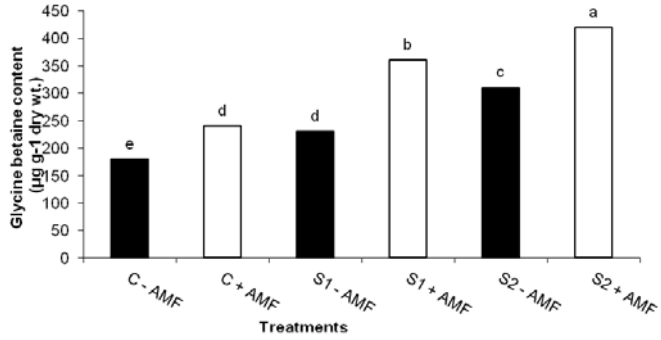


Figure 3: Effect of AM inoculation on the proline (a) and glycine betaine (b) content in the nodules of soybean under salt stress. reatments are designed as uninoculated controls, saline stress (6 and 12 dSm^{-1}) and arbuscular mycorrhiza (AM). Means followed by the same letter are not significantly different (p<0.05) as determined by Duncan's Multiple Range test.

Glycine betaine content increased significantly in nodules of all the stressed plants, however, stress induced increase in glycine betaine content was higher in nodules of AM inoculated plants than those of non- AM inoculated plants.

Exposure of the plants to salt stress resulted in general increment in the antioxidant enzyme activities of the nodules. Mycorrhizal inoculation further increased the antioxidant enzyme activities. Saline stress led to enhanced SOD activity in nodules of all the plants. SOD activity was higher in inoculated plants than in non- inoculated-stressed plants at 6 and 12 dSm, respectively. CAT activity increased in the nodules at 12 dSm, while the salt levels of 6 dSm did not bring a significant increase in CAT activity in comparison with the control. Symbiosis with the mycorrhizal fungi significantly increased the CAT activity at 6 and 12 dSm and it was higher than corresponding nodules of stressed non-AM inoculated plants. POX activity increased with application of saline doses of 6 and 12 dSm. Salinity induced increase in POX activity of non- inoculated nodules. A higher increase was observed in nodules of inoculated plants at 6 and 12 dSm, respectively.

Table 3: Effect of salt stress on SOD, CAT, POX activities in nodules of AM and non-AM soybean plants under salt stress.

Treatments	SOD (units mg^{-1}prot. min^{-1}± SD)	CAT (s mol H_2O_2 red. mg^{-1}prot. min^{-1}± SD)	POX (n mol tetra- guaiacol formed min^{-1} g^{-1} f.wt.± SD)
C - AMF	16.05± 0.48^{e}	7.86±0.23^{e}	204.23±3.22^{f}
C + AMF	20.4± 1.11^{d}	9.84±0.85^{d}	217.54±1.78^{e}
S1 - AMF	27.87±2.3^{c}	8.03±0.45^{e}	257.44±2.1^{d}
S1 + AMF	47.04±o.78^{b}	15.11±1.33^{c}	321.02±0.76^{b}
S2 - AMF	29.85±1.3^{c}	22.12±0.65^{b}	279.6±1.28^{c}
S2 + AMF	53.87±1.42^{a}	36.7±1.37^{a}	349.77±1.48^{a}

Results represent the average of three experiments ± SD. Different letters represent significant differences (p < 0.05) between treatments at each column

4 DISCUSSION

The colonization rate declined with increasing NaCl level, indicating that salinity suppressed the growth of AM fungi. It has been reported that addition of various salts to soil inhibits hyphal growth with a subsequent decrease in the spread of mycorrhizal colonization (Ruiz-Lozano and Azcon, 1996; Hajiboland et al., 2010).

Salinity stress significantly reduced the root and stem dry matter compared with the control treatment due to direct effects of ion toxicity or indirect effects of saline ions that cause soil /plant osmotic imbalance (Abdel Latef, 2010). Colonization with AMF significantly improved dry matter in the salt-stressed plants. This effect of AM fungi on dry matter was more pronounced in aerial biomass than root biomass which may be because of arbuscular mycorrhizal colonization can cause a proportionally greater allocation of carbohydrates to the shoot than root tissues (Shokri and Maadi, 2009). Enhanced growth of mycorrhizal tomato plants grown in saline environments has been related partly to mycorrhiza-mediated enhancement of host plant nutrition (Kaya et al., 2009). Cantrell and Linderman (2001) reported that AM fungi improved growth under salt stress condition. These findings indicated the benefits of AM fungi and the important role they play in increasing salinity tolerance.

Size and dry weights of root nodules decreased in soybean plants grown in saline environment. Our results indicated that the reductions in dry weights under salt-stressed conditions were more closely linked to the reductions in the size of nodules, rather than to the initiation of the nodules.

The process of nitrogen fixation was affected negatively by salt stress, as revealed by declined leghemoglobin content and reduced nitrogenase activity. Similar decline in nodulation and nodule activity has also been reported earlier by Serraj et al. (2001); Tejera et al. (2005); Bolanos et al. (2006); Garg and Manchanda (2008). Despite a decline in the functional efficiency of nodules, AM inoculated plants had considerably higher leghemoglobin content and nitrogenase activity than corresponding non-AM inoculated plants under salt stress. AM inoculation markedly increased nodulation at low saline concentration. Evidences from the previous studies (Johansson et al. 2004; Rabie and Almadini, 2005; Garg and Manchanda, 2008) indicate that the presence of AM fungi enhances nodulation and nitrogen fixation by legumes.

In this study, the contents of potassium and phosphorus declined under saline conditions. Phosphorus concentration of nodules was significantly lowered in salt-affected compared with control plants. Reduction of P uptake in saline soils was attributed to precipitation of $H_2PO_4^-$ with Ca^{2+} ions in soil and of K^+ and Ca^{2+} to a competition with Na^+ (Marschner, 1994). A marked effect of AMF on the uptake of P was observed even in the control plants. The enhancement of plant P (Giri et al., 2007) uptake by AMF has been reported and was considered one of the main reasons for amelioration of growth in salt-affected plants colonized by AMF (Ruiz-Lozano and Azcon, 1996).

The nodules of AM inoculated plants accumulated lesser Na^+ than the corresponding non-inoculated-stressed plants. Nodular potassium and calcium contents were higher in inoculated-stressed plants than in stressed non-inoculated plants, which could have been an important factor in maintaining higher nodulation and nitrogen fixation in these plants. Higher K^+ accumulation by inoculated plants in saline soil could be beneficial by maintaining a high K^+/Na^+ ratio and by influencing the ionic balance of the cytoplasm or Na^+ efflux from plants (Giri et al., 2007; Aleman et al., 2009). Improved ionic ratios in the aerial parts of inoculated-stressed plants have been reported earlier by Giri et al. (2003); Rabie (2005); Rabie and Almadini (2005).

Many plants accumulate proline as a nontoxic and protective osmolyte under saline conditions Parida et al., 2003). Marked increase in free proline occurs in many plants during moderate or severe water or salt stress; this accumulation, mainly as a result of increased proline biosynthesis, is usually the most outstanding change among the free amino acids (Hurkman et al., 1989). Data reported here revealed that proline and glycine betaine contents increased under salt stress. Synthesis and accumulation of both the osmolytes were significantly higher in the nodules of AM inoculated- stressed plants than the corresponding non-inoculated ones. The results suggested that higher accumulation of proline and glycine betaine contents in the nodules of inoculated-stressed plants was correlated with enhanced nitrogen fixing ability of these plants. High proline concentration was suggested to protect nodule metabolism by avoiding protein denaturalization and maintaining cell pH levels (Irigoyen et al., 1992).

A constitutively high antioxidant capacity under stress conditions can prevent damages due to ROS formation (Harinasut et al., 2003). There are reports showed that a greater SOD activity in salt tolerant plants (Benavides et al., 2000). Our results showed that moderate and high salinity caused a significant increase in SOD activity in nodules of both inoculated and non-inoculated soybean plants. These results are similar in part to results obtained by Garratt et al. (2002) who found enhanced SOD activity under salinity condition in cotton. Based on the induced SOD activity in the nodules of soybean plants grown under salinity, it could be concluded that SOD is important for soybean to tolerate salinity. Furthermore, enhanced SOD activity in inoculated plants as compared to non-inoculated plants supports the view that increased antioxidative enzyme activities could be involved in the beneficial effects of mycorrhizal colonization on the performance of plants grown under semi-arid conditions (Alguacil et al., 2003). Gradual exposure of the AM fungus to salinity enhanced its ability to increase SOD activity in the host plants. The great SOD activity in inoculated plants could increase the capacity of nodules to scavenge superoxide radicals.

Plant possesses hydrogen peroxide scavenging enzymes POD and CAT. Detoxifications of the reactive oxygen protect cells against harmful concentration of hydroperoxides (Castillo, 1992). The increased POD in response to salinity has been reported (Harinasut et al., 2003). In tolerant plants, POD activity was found to be higher to protect plants against the oxidative stresses (Sreenivasulu et al., 1999). Pacovsky et al. (1991) studied POX activity in *Phaseolus vulgaris* colonized by *Glomus etunicatum* and found that peroxidase activity increased in the mycorrhizal plants. Alguacil et al. (2003) reported that mycorrhizal inoculation increased CAT activity in *Olea europaea* grown under semi-arid conditions. On the other hand, since CAT is involved in decomposition of H_2O_2 in peroxisomes, similar increases in CAT activity of non-inoculated and inoculated plant at moderate and high NaCl indicate that under these conditions H_2O_2 is probably produced in higher concentrations in the peroxisome.

On the basis of the results presented here, our results support the view that AMF can contribute to protect plants against salinity by alleviating the salt induced oxidative stress. This ameliorative effect of mycorrhizal colonization shows significant interactions with salt exposure. Enhanced antioxidant enzymes activity in AM inoculated plants may contribute to better maintenance of the ion balance the reactions in nodules under salinity.

5 REFERENCES

Alguacil M.M., Hernandez J.A., Caravaca F, Portillo B., Roldan A. 2003. Antioxidant enzyme activities in shoots from three mycorrhizal shrub species afforested in a degraded semi-arid soil. Physiol. Plant, 118: 562–570.

Aliasgharzadeh N., Rastin N.S., Towfighi H., Alizadeh A. 2001. Occurrence of arbuscular mycorrhizal fungi in saline of the Tabriz Plain of Iran in relation to some physical and chemical properties of soil. Mycorrhiza, 11: 119-122.

Bates L.S., Waldran R.P., Teare I.D. 1973. Rapid determination of free proline for water studies. Plant Soil, 39: 205–208.

Becana M., Dalton D.A., Moran J.F., Iturbe-Ormaetxe I., Matamoros M.A., Rubio M.C. 2000. Reactive oxygen species and antioxidants in legume nodules. Physiol. Plant, 109: 372–81.

Bolanos L., Martín M., El-Hamdaoui A., Rivilla R., Bonilla I. 2006. Nitrogenase inhibition in nodules from pea plants grown under salt stress occurs at the physiological level and can be alleviated by B and Ca. Plant Soil, 280: 135–142.

Cantrell I.C., Linderman R.G. 2001. Preinoculation of lettuce and onion with VA mycorrhizal fungi reduces deleterious effects of soil salinity. Plant and Soil, 233:269-281.

Castillo F.J. 1992. Peroxidases and stress- In: Penel, C, Gasper T, Greppin H (Eds.), Plant Peroxidases. Topics and Detailed Literature on Molecular, Biochemical, and Physiological Aspect. University of Geneva, Geneva, pp., 187-203.

Chance B., Maehly A.C. 1955. Assay of catalase and peroxidase. Methods Enzymol, 2: 764-775.

Chapman H.D., Pratt P.F. 1961. Methods of analysis for soils, plants and water, 150–210. University of

California, Berkley, CA: Division of Agricultural Sciences.

Cordovilla M.P., Ligero F., Lluch C.1994. The effect of salinity on N fixation and assimilation in Vicia faba J. Exp. Bot., 45: 1483-1488.

Delgado M.J., Ligero F., Lluch C.1993. Effects of salt stress on growth and N2 fixation by pea, faba bean, common bean, and soybean plants. Soil Biol. Biochem., 26: 371-376.

Dhindsa R.S., Plumb-Dhindsa P., Thorpe T.A.1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. Journal of Experimental Botany, 32: 93–101.

Duncan D. B. 1955. Multiple range and multiple F-tests. Biometrics, 11: 1–42.

Fitter A.H. 1988. Water relations of red clover Trifoliumpratanse L. as affected by VA mycorrhizal infection and phosphorus supply before and during drought. J. Exp. Bot., 39: 599–608.

Garg N., Manchanda G. 2008. Effect of arbuscular mycorrhizal inoculation on salt-induced nodule senescence in C. cajan (Pigeonpea). J. Plant Growth Regul., 27: 115–124.

Garg N., Manchanda G. 2008. Effect of arbuscular mycorrhizal inoculation on salt-induced nodule senescence in Cajanus cajan (pigeonpea). Journal of plant growth, 27(2):115-124.

Georgiev G.I., Atkins C.A. 1993. Effects of salinity on N2 fixation, nitrogen metabolism and export and diffusive conductance of cowpea root nodules. Symbiosis, 15: 239-55.

Giri B., Kapoor R., Mukerji K.G. 2003. Influence of arbuscular mycorrhizal fungi and salinity on growth, biomass, and mineral nutrition of Acacia auriculiformis. Biol. Fertil. Soils, 38: 170–175.

Giri B., Kapoor R., Mukerji K.G. 2007. Improved tolerance of Acacia nilotica to salt stress by arbuscular mycorrhiza, Glomus fasciculatum may be partly related to elevated K:Na ratios in root and shoot tissues. Microb. Ecol., 54: 753–760.

Goss M.J., de Varennes A. 2002. Soil disturbance reduces the efficacy of mycorrhizal associations for early soybean growth and N2 fixation. Soil Biology & Biochemistry, 34: 1167–1173.

Goss M.J., de Varennes A., Smith P.S., Ferguson J.A. 2002. N2 fixation by soybeans grown with different levels of mineral nitrogen, and the fertilizer replacement value for a following crop. Canadian Journal of Soil Science, 82:139–145.

Grieve C. M, Grattan S.R. 1983. Rapid assay for determination of water soluble quaternary – amino compounds. Plant Soil, 70: 303–307.

Hajiboland R., Aliasgharzadeh A., Laiegh S.F., Poschenrieder C. 2010. Colonization with arbuscular mycorrhizal fungi improves salinity tolerance of tomato (Solanum lycopersicum L.) plants. Plant Soil, 331: 313-327.

Harinasut P., Poonsopa D., Roengmongkol K., Charoensataporn R. 2003. Salinity effects on antioxidant enzymes in mulberry cultivar. Sci. Asia, 29: 109-113.

Hartree E.F. 1955. "Haematin compounds". In Modern methods of plant analysis Edited by: Paech, K and Tracey, M V. 197–245. Berlin: Springer-Verlag.

He K., Gou X., Yuan T., Lin H., Asami T., Yoshida S., Russell S.D., Li J. 2007. BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. Curr. Biol., 17: 1109–1115.

Herdina J.A., Silsbury J.H. 1990. Estimating nitrogenase activity of faba bean (Vicia faba L.) by acetylene reduction (ARA) assay. Aust. J. Plant Physiol., 17: 489–502.

Hurkman W.J., Fornari C.S., Tanaka C.K. 1989. A comparison of the effect of salt on polypeptides and translatable mRNAs in roots of a salt-tolerant and a salt-sensitive cultivar of barley. Plant Physiol, 90: 1444–1456.

Irigoyen J.J., Emerich D.W., Sanchez-Diaz M. 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (Medicago sativa) plants. Physiol. Plant, 84: 55–60.

Johansson J. F., Paul L. R., Finlay R. D. 2004. Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol. Ecol., 48: 1–13.

Kaya C., Higgs Kirnak D.H., Tas I. 2003. Mycorrhizal colonisation improves fruit yield and water use efficiency in watermelon (Citrullus lanatus Thunb.) grown under well-watered and water-stressed conditions. Plant Soil, 253: 287–292.

Lauchli A. 1984. Salt exclusion: An adaptation of legumes for crops and pastures under saline conditions. In Salinity tolerance in plants – Strategies for crop improvement, Edited by: Staples, R C and Toenniessen, G H. 171–188. New York: Wiley and Sons.

Ligero F., Lluch C., Olivares J. 2007. Evolution of ethylene from roots of Medicago sativa plants inoculated with Rhizobium meliloti. J. Plant Physiol., 125: 361–365.

Marschner H., Dell B. 1994. Nutrient uptake in mycorrhizai symbiosis. Plant and Soil, 159:89-102.

Olivera M., Tejera N., Iribarne C., Ocana A., Lluch C. 2004. Growth, nitrogen fixation and ammonium assimilation in common bean (Phaseolus vulgaris): Effect of phosphorus. Physiol. Plant, 121: 498–505.

Paradi I., Bratek Z., Lang F. 2003. Influence of arbuscular mycorrhiza and phosphorus supply on polyamine content, growth and photosynthesis of Plantago lanceolata. Biol. Plant, 46: 563–569.

Phillips J.M., Hayman D.S. 1970. Improved procedures for clearing and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Tran. Br. Mycol. Soc., 55: 158–161.

Polle A., Otter T., Seifert F.1994. Apoplastic peroxidases and lignification in needles of Norway spruce (Picea abies L.). Plant Physiology, 106: 53–60.

Porcel R., Barea J.M., Ruiz-LozanoJ.M. 2003. Antioxidant activities in mycorrhizal soybean plants under drought stress and their possible relationship to the process of nodule senescence. New Phytol., 157: 135–143

Prochazkova D., Sairam R.K., Srivastava G.C., Singh D.V. 2001. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. Plant Sci., 161(4):765–771.

Rabie G.H., Almadini A.M. 2005. Role of bioinoculants in development of salt-tolerance of Vicia faba plants under salinity stress. Afr. J. Biotechnol., 4: 210–222.

Rabie G.H. 2005. Influence of arbuscular mycorrhizal fungi and kinetin on the response of mungbean plants to irrigation with seawater. Mycorrhiza, 15: 225–230.

Rao D.L.N., Giller K.E., Yeo A.R., Flowers T.J.2002. The Effects of Salinity and Sodicity upon Nodulation and Nitrogen Fixation in Chickpea (Cicer arietinum). Ann. Bot., 89: 563-570.

Ruiz-Lozano J.M., Azcon R., Gomez M. 1996. Alleviation of salt stress by arbuscular mycorrhizal Glomus species in Lactuca sativa plants. Physiol. Plant, 98: 767–772.

Shokri S., Maadi B. 2009. Effects of Arbuscular Mycorrhizal Fungus on the Mineral Nutrition and Yield of *Trifolium alexandrinum.* Plants under Salinity Stress., 79-83.

Sanginga N, Thottappilly G and Dashiell K. 1999. Effectiveness of rhizobia nodulating recent promiscuous soybean selections in the moist savanna of Nigeria. Soil Biol. Biochem., 32: 127–33.

Serraj R., Vasquez Diaz H., Hernandez G., Drevon J.J. 2001. Genotypic difference in the short-term response of nitrogenase activity (C_2H_2 reduction) to salinity and oxygen in the common bean. Agronomie, 21: 645–651.

Soussi M., Lluch C., Ocana A.1999. Comparative study of nitrogen fixation and carbon metabolism in two chick-pea (Cicer arietinum L.) cultivars under salt stress. J. Exp. Bot., 50: 1701- 1708.

Sprent J.I., Zahran H.H. 1988. Infection, development and functioning of nodules under drought and salinity. In: Beck DP, Materon LA, eds. Nitrogen fixation by legumes in Mediterranean agriculture. Dordrecht: Martinus Nijhoff, 145-151.

Sreenivasulu N., Ramanjulu S., Ramchandra-Kini K., Prakash H.S., Shekar-shetty H., Savithri H.S., Sudhakar C. 1999. Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet with differential salt tolerance. Plant Science, 141: 1-9.

Stewart R.R.C., Bewley J.D.1980. Lipid peroxidation associated aging of soybean axes. Plant Physiol., 65: 245-248.

Tejera N.A., Campos R., Sanjuan J., Lluch C. 2005. Effect of sodium chloride on growth, nutrient accumulation, and nitrogen fixation of common bean plants in symbiosis with isogenic strains. J. Plant Nutr., 28: 1907–1921.

Trimble M.R., Knowles N.R.1995. Influence of phosphorus nutrition and vesicular-arbuscular mycorrhizal fungi on growth and yield of greenhouse cucumber (Cucumis sativus L.). Can. J., 79-83.

Udvardi M.K., Day D.A. 1997. Metabolite transport across symbiotic membranes of legume nodules. Annu. Rev. Plant Physiol. Plant Mol. Biol., 48: 493–523.

Vranova E., Inze D., Van Brensegem F. 2002. Signal transduction during oxidative stress. J. Exp. Bot., 53: 1227-1236.

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Indirect plant regeneration in aromatic rice (*Oryza sativa* L.) var. 'Kalijira' and 'Chinigura'

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ABSTRACT

Mature seeds of two traditional rice genotypes (Kalijira and Chinigura) were used for callus induction and plant regeneration on different concentrations and combinations of plant growth regulators cultured on MS (Murashige and Skoog) basal medium. Callus induction frequency was different between the cultivars, as well as among the 2,4-dichlorophenoxyacetic acid (2,4-D) levels tested. Both tested cultivars exhibited highest callus frequency at 2 mgl^{-1} 2,4-D. The incorporation of benzylaminopurine (BAP) and kinetin (KIN) in the callus induction medium supplemented with 2 mgl^{-1} 2,4-D did not significantly improve the callus induction frequency but required days of callus initiation were decreased compared to single use of 2,4-D. After two subcultures, at 21 days interval, embryogenic callus was placed on medium containing different concentration and combination of auxin and cytokinin. Treatment T_4 (0.5 mg l^{-1} BAP and 0.1 mg l^{-1} IBA) showed the highest shoot induction: 91.67% in Kalijira and 83.33% in Chinigura. Similarly, the highest range of shoot number was also observed in both genotypes when treated with 0.5 mgl^{-1} BAP and 0.1 mgl^{-1} IBA. Plant regeneration efficiency was further observed best when treated with 1 mgl^{-1} 2,4-D along with 1 mgl^{-1} 2,4-D along with 1 mgl^{-1} BAP and 1 mgl^{-1} IBA. Furthermore, the highest number of callus derived shoot per culture was achieved in 2 mgl^{-1} 2,4-D along with 1 mgl^{-1} BAP and 1 mgl^{-1} IBA. Both rice genotypes are promising in terms of callus induction frequency and morphology, and regeneration ability of the embryogenic callus.

Key words: callus induction, plant regeneration, aromatic rice, shoots

IZVLEČEK

POSREDNA REGENERACIJA AROMATIČNEGA RIŽA (*Oryza sativa* L.), SORT 'KALIJIRA' IN 'CHINIGURA'

Zrela semena dveh tradicionalnih genotipov riža ('Kalijira' and 'Chinigura') so bila uporabljena za indukcijo kalusa in regeneracijo rastlin pri različnih koncentracijah in kombinacijah rastlinskih rastnih regulatorjev pri gojenju na osnovnem MS (Murashige and Skoog) mediju. Frekvenca indukcije kalusa je bila različna med sortama kot tudi glede na koncentracije 2,4-diklorfenoksi ocetne kisline (2,4-D). Obe preiskušeni sorti sta imeli največjo frekvenco kalusa pri 2 mgl^{-1} 2,4-D. Dodatek benzilaminopurina (BAP) in kinetina (KIN) v medij za indukcijo kalusa z dodatkom 2 mgl^{-1} 2,4-D ni značilno izboljšal indukcije kalusa, vendar so se potrebni dnevi za začetek tvorbe kalusa zmanjšali v primerjavi s postopkom, ko smo uporabili samo 2,4-D. Po dveh predkulturah, v interval 21 dni, je bil embriogeni kalus prenešen na medij, ki je vseboval različno koncentracijo in kombinacijo auksina in citokinina. Tretma T_4 (0.5 mg l^{-1} BAP in 0.1 mg l^{-1} IBA) je dal največjo indukcijo poganjkov: 91.67 % pri 'Kalijira' in 83.33 % pri 'Chinigura'. Podobno je nastalo največ poganjkov pri obeh sortah, kadar so jih tretirali z 0.5 mgl^{-1} BAP in 0.1 mgl^{-1} IBA. Nadalje je bila sposobnost regeneracije rastlin najboljša, če so jih tretirali z 1 mgl^{-1} 2,4-D z dodatkom 1 mgl^{-1} BAP in 1 mgl^{-1} IBA. Največje število iz kalusa nastalih poganjkov na kulturo je bilo doseženo pri 2 mgl^{-1} 2,4-D z dodatkom 1 mgl^{-1} BAP in 1 mgl^{-1} IBA. Oba genotipa riža sta obetavna v smislu morfologije in pogostosti indukcije kalusa kot tudi v regeneracijski sposobnosti embriogenega kalusa.

Ključne besede: indukcija kalusa, regeneracija rastlin, aromatični riž, poganjki

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1 INTRODUCTION

Global population is increasing very rapidly. Loss in crop production could lead to hunger and famine, especially in the developing countries. So it is time to tackle the challenges of a rapidly increasing population and stiffer global competition in the next millennium. Moreover these two challenges require better research to produce more and better quality food efficiently. The improvement can possibly be achieved by creating genetic variability. Rice (*Oryza sativa* L.) is the world most important food supplier cereal crop after wheat and maize (Ray, 1985). It provides half of total dietary carbohydrate, especially in Asian countries and it is suitable diet for more than three billion people, supplying 50-80% of their daily calorie intake (Khush, 2005). Thus a considerable improvement has been done through traditional rice breeding. Rice breeding has made significant progress towards higher yield, improved quality, greater disease resistance and other important characters of agricultural importance in the past and even in future, it will still play an important role. Due to its increasing importance in nutrition and economy, it is now felt that new varieties of rice, having good agronomic characters, should be evolved.

Kalijira and Chinigura are the most important aromatic rice varieties of Bangladesh and the rest of the world due to its attractive flavor, fine grain and good taste. Aroma and taste are caused by the chemical compound 2-acetyl-1-pyrroline (Ghareyazie *et al*., 1997). This rice is generally used to prepare dishes such as *polau, biriani* and different types of cake which are served on special occasions. Aromatic rice receives premium price and is profitable for the growers as well as the traders. Country can benefit by earning exchange by production and export of aromatic rice.

Most of the aromatic rice cultivars are traditional rice varieties which have tall stature, low yield, photoperiod-sensitivity, are susceptible to disease and pest and unresponsive to fertilizer. But due to the favorite flavor and some other dominant grain quality characteristics, they are the important resource for breeding and improving the aromatic rice cultivars for diverse demands of consumers in the world. Several laboratories have described regeneration of plants from various rice explants such as immature embryos, immature panicles (Ling *et al*., 1983), young inflorescence (Chen *et al*., 1985) and root (Abe and Futsuhara, 1985). Rashid *et al*. (2000) studied that rice seeds have more potential for callogenesis as compared to node or tip. Successful callus induction from rice seed has been reported by several researchers (Gonalz, 2000; Alam *et al*., 2003; Shahsavari *et al*., 2010). The use of mature seeds has the advantage, because they can be obtained at anytime throughout the year regardless of growing season (Alam, 1994). Despite the enormous importance of aromatics rice, knowledge on the *in vitro* propagation of these rice lines is still elusive.

Therefore, this study was aimed at evaluating two Bangladeshi aromatic rice genotypes (Kalijira and Chinigura) for callus induction and regeneration efficiency under different concentrations and combinations of growth regulators.

2 MATERIALS AND METHODS

2.1 Explant sterilization and culture establishment

Mature seeds of two genotypes of aromatic rice namely; Kalijira and Chinigura were dehusked and immersed in 70% ethanol for 3 min, after washing the explants were dipped in 0.1% $HgCl_2$ solution for 3 minutes. The seeds were then rinsed 5-6 times with sterile distilled water to remove $HgCl_2$ with vigorous agitation in the laminar air flow cabinet. After surface sterilization of seeds, they were kept on autoclaved filter paper on the petridish. When the water removed from the seeds surface it was inoculated into the culture tubes with sterilized forceps. The seeds were then placed on callus induction media and kept in the dark at 26 ± 2°C. MS (Murashige and Skoog, 1962) basal medium was used for callus induction and plant regeneration. In this study, 30 mgl^{-1} sugars was used and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8.

2.2 Callus induction

Different concentrations of 2,4-D (1, 2, 3 and 4 mgl^{-1}) were added into the MS medium for callus induction. Subculture was performed twice at 21-day interval using the same medium. Combinations of auxin and cytokinin (T_1=2.0 mgl^{-1} 2,4-D+0.25 mgl^{-1} KIN, T_2=2.0 mgl^{-1} 2,4-D+0.5 mgl^{-1} KIN, T_3=2.0 mgl^{-1} 2,4-D+1.0 mgl^{-1} KIN, T_4=2.0 mgl^{-1} 2,4-D+1.5 mgl^{-1} KIN, T_5=2.0 mgl^{-1} 2,4-D+0.25 mgl^{-1} BAP, T_6=2.0 mgl^{-1} 2,4-D+0.5 mgl^{-1} BAP, T_7=2.0 mgl^{-1} 2,4-D+1.0 mgl^{-1} BAP, T_8=2.0 mgl^{-1} 2,4-D+1.5 mgl^{-1} BAP mgl^{-1}) were also used in MS media for callus induction.

2.3 Plant regeneration

Embryogenic calli produced on MS medium containing 2 mgl^{-1} 2,4-D were cultured on different regeneration media for plantlet formation. MS basal media supplemented with different concentrations and combinations of cytokinin and auxins (T_1=0, T_2=0.1 mgl^{-1} IBA, T_3=0.1 mgl^{-1} BAP+0.1 mgl^{-1} IBA, T_4=0.5 mgl^{-1} BAP+0.1 mgl^{-1} IBA, T_5=1 mgl^{-1} BAP+0.5 mgl^{-1} IBA, T_6=0.5 mgl^{-1} BAP+0.1 mgl^{-1} IAA, T_7=0.5 mgl^{-1} BAP+0.5 mgl^{-1} IBA, T_8=3 mgl^{-1} KIN+0.5 mgl^{-1} NAA, T_9=3 mgl^{-1} KIN+0.5 mgl^{-1} IAA mgl^{-1}) were prepared for plantlet regeneration. Regenerated shoots were then transferred to half MS media immediately under light (2000 lux) provided by 40W white cool fluorescence tubes. The cultures were maintained in a growth chamber at 24 + 18^0C for a 16 h photoperiod under cool white fluorescent lamps (Phillips Bangladesh Ltd.) and the light intensity was maintained at 28–34 mol/m/s.Visual observation of culture was made every week.

2.4 Data recording

The frequency of callus induction and plant regeneration (%) were measured using the following formulas (Zaidi *et al*., 2006):

$$\text{Frequency of callus induction } (\%) = \frac{\text{no. of explants induced callus}}{\text{no. of cultured explants}} \times 100$$

$$\text{Frequency of shoot induction } (\%) = \frac{\text{no. of culture induced shoot}}{\text{no. of culture}} \times 100$$

$$\text{Frequency of root induction } (\%) = \frac{\text{no. of shoot induced root}}{\text{no. of culture inducted shoot}} \times 100$$

2.5 Statistical analysis

The experiments were arranged in a split plot design with three replications. Each replication per treatment contained 12 seeds for callus induction and 4-6 embryogenic calli for plant regeneration. Data were analyzed using the two way-factorial analysis of variance (factorial ANOVA), with plant growth regulator concentration as one treatment and genotype as the other treatment. Data were analyzed as means ± SE. IRRIState 7.2 software was also used to do ANOVA and DMRT.

3 RESULTS

3.1 Effect of 2,4-D on callus induction

Different concentrations (1.0, 2.0, 3.0 and 4.0 mgl^{-1}) of 2,4-D were used for producing sufficient amount of embryonic callus from mature seeds in MS medium. The results are presented in Table 1. Results indicate that growth regulators played a major role in callus induction. The callus induction was occurred at 7-12^{th} days after inoculation.

Result showed that MS medium supplemented with 2 mgl^{-1} of 2,4-D was most effective in callus induction in both Kalijira (97.22%) and Chinigura (94.44%). This indicates that the use of 2,4-D with 2 mgl^{-1} was enough for production of high amount of callus in rice. Lowest range of days for callus induction was observed in both Kalijira and Chinigura in higher (4.0 mgl^{-1}) concentration of 2,4-D. The color of all Kalijira callus was creamy yellowish and Chinigura was creamy white but both of those textures were friable.

Table 1: Effect of 2,4-D in MS media on quality and quantity of callus induction.

	Kalijira			Chinigura			
Concentration of 2,4-D mgl^{-1}	**Range**	**%**	**Degree with callus morphology**	**Range**	**%**	**Degree with callus morphology**	**Mean (varieties)**
1	10-12	91.66±1.3 a	+++Py,C	13-15	86.11±2.1 a	+++CrW,C	88.89±2.7
2	7-10	97.22±0.8 a	+++Py,C	11-13	94.44±0.8 a	+++CrW,C	95.83±1.4
3	7-10	94.44±0.8 a	+++Py,C	11-13	91.66±1.4 a	+++CrW,C	93.21±1.4
4	8-11	88.79±0.8 a	+++Py,C	12-15	88.78±0.8 a	++CrW,C	88.78±0.0
Mean (treatments)		93.0±1.7			90.2±1.7		

+ Slight callus, ++ Moderate callus, +++ Massive callus, Py= Pale yellow, C=Creamy and CrW= Creamy white; concentrations with the same letter were not significantly different at 0.05 probability level using LSD.

3.2 Effect of 2,4-D in combination with KIN and BAP on induce callus

Although 2,4-D (auxin) gave the highest result of callus induction in rice, some worker have showed a good result in other cereal crops (e.g. wheat) using 2,4-D in combination with low concentration of cytokinine.

The effect of cytokinin (BAP and KIN) along with (2,4-D, 2.0 mgl^{-1}) on callus induction was also tested in MS medium (result shown in Table 2). KIN was found more effective (95.8±1.4) than BAP (94.4±0.0) for high amount of callus formation. In addition, numerous callus (95.8±1.4) was observed when explants were treated with 2.0 mgl^{-1} of 2,4-D was supplemented with 0.5 mgl^{-1} of KIN . Similar result was also found when treated with 2.0 mgl-1 of 2,4-D (95.83 %). But required days of callus initiation were decreased (5-7 days) by using cytokinins along with 2,4-D than single use of 2,4-D in all cases.

Table 2: Effect of different combinations of growth regulator on callus initiation and callus growth.

	Kalijira			Chinigura			
Concentrations and combinations	**Range**	**%**	**Degree**	**Range**	**%**	**Degree**	**mean**
T_1	6-7	94.44±0.8 a	+++	7-8	88.89±0.8 a	+++	91.6±2.7
T_2	5-6	97.22±0.8 a	+++	5-7	94.44±0.8 a	+++	95.8±1.4
T_3	5-6	88.89±2.1 a	+++	5-6	86.11±0.8 a	+++	87.5±1.4
T_4	4-6	69.44±2.1 b	++	4-6	69.44±0.8 b	++	69.4±0.0
T_5	7-8	91.67±1.4 a	+++	7-8	88.89±2.1 a	+++	90.2±1.4
T_6	6-7	91.67±1.4 a	+++	7-8	91.67±1.4 a	+++	91.6±0.0
T_7	5-7	94.44±0.8 a	+++	6-7	94.44±0.8 a	+++	94.4±0.0
T_8	5-7	86.11±2.1 a	+++	5-7	83.33±2.4 a	+++	84.7±1.4
mean		88.23±3.08			86.15±2.8		

+ Slight callus, ++ Moderate callus, +++ Massive callus and same letter were not significantly different at 0.05 probability level using LSD. In each treatment, 36 explants were used.

3.3 Plantlet regeneration

The results indicate that, among different concentrations and combinations, treatment T_4 (0.5 mgl^{-1} BAP +0.1 mgl^{-1} IBA) showed better performance (Kalijira 91.67±0.18 and Chinigura 83.33±0.22) to produce plantlet while treatment T_2 (0.1 mgl^{-1} IBA) shows the lowest results (Kalijira 41.67% and Chinigura 41.33%). The range of shoot number (Kalijira 4-8 and Chinigura 3-8) and mean performance (Kalijira 6.63±0.18 and Chinigura 6.30±0.05) was also better for T_4 treatment and the lowest for T_2 (0.1 mgl^{-1} IBA). Overall, Kalijira was found to be more efficient in producing plantlets than that of Chinigura.

Another experiment has been performed to find out the effect of 2,4-D on plant regeneration. Calli obtain from different concentration of 2,4-D (i.e. 1.0, 2.0, 3.0 and 4.0 mgl^{-1}) were used for regeneration.

The result showed that the highest percentages of callus producing shoot (40%) were observed from the callus obtained from low concentration (Table 4). In addition, highest number of shoots was observed on the callus derived from 2.0 mgl^{-1} of 2,4-D treatment. However, callus induced from high concentration of 2, 4-D (3.0 mgl^{-1} or more) was found to be inefficient for plantlet regeneration.

Table 3: Regeneration efficiency of from callus derived from mature seeds (calli were obtained from 2.0 mgl^{-1} of 2,4-D)

	Kalijira				Chinigura			
Treatments	Shoot induction (%)	Number of shoot		(%) of shoots inducted root	Shoot induction (%)	Number of shoots		(%) of shoots inducted root
		Range	$\bar{X}$ ±SE			Range	$\bar{X}$ ±SE	
T_1	0	-	-	-	0	-	-	-
T_2	41.67±0.26 e	1-4	2.80±0.20 c	100	41.33±0.62 e	2-3	2.60±0.23 f	100
T_3	50.00±0.58 d	3-5	3.83±0.17 d	100	50.00±0.44 d	3-5	3.67±0.09 d	100
T_4	91.67±0.63 a	4-8	6.63±0.18 a	100	83.33±0.22 a	3-8	6.30±0.05 a	100
T_5	66.67±0.66 b	3-7	4.12±0.24 bcd	100	58.33±0.33 c	3-6	4.00±0.29 c	100
T_6	50.00±0.88 d	2-6	3.83±0.00 d	100	50.00±0.58 d	2-5	3.00±0.29 e	100
T_7	50.00±0.58 d	2-5	4.00±0.5 cd	100	41.67±0.55 e	2-5	3.20±0.11 e	100
T_8	66.67±0.41 b	3-8	4.37±0.25 b	100	66.67±1.20 b	3-8	4.75±0.07 b	100
T_9	58.33±1.01 c	3-7	4.28±0.42 bc	100	58.33±0.68 c	2-7	4.14±0.25 c	100
Mean (%)	59.37				56.25			

Treatments with the same letter were not significantly different at 0.05 probability level using LSD. In each treatment 12 explants were used.

Table 4: Plant regeneration efficiency of callus induced from different combinations of 2,4-D.

Calluses of different concentration of 2,4-D (mgl^{-1})	Concentration of BAP+IBA (mgl^{-1})	% of callus producing shoots	Average number of shoots per culture $\bar{X}$ ±SE	% of callus producing shoots	Average number of shoots per culture $\bar{X}$ ±SE
		Kalijira		Chinigura	
1.0	0.5+0.1	40	4.5±0.29	40	4.0±0.40
2.0	0.5+0.1	20	6.5±1.50	30	5.6±0.33
3.0	0.5+0.1	20	3.5±0.50	20	3.5±0.50
4.0	0.5+0.1	10	1.0±0.00	10	1.0±0.0

4 DISCUSSION

Despite the great importance of aromatic rice, little information is available on the callus induction and plant regeneration method through *in vitro* culture. The present study investigated the effect of various growth regulators on callus induction and plant regeneration efficiency in two Bangladeshi Traditional Aromatic Rice var. Kalijira and Chinigura.

4.1 Callus induction

The results showed that MS medium supplemented with 2 mgl^{-1} of 2,4-D was the most effective in callus induction for both cultivars Kalijira and Chinigura. This indicate that the use of 2,4-D with 2 mgl^{-1} was adequate for production of high amount of callus in rice. This finding is in

agreement with previous report by Rashid *et al.* (2003), where they showed that Basmati 370, Basmati 385 and KS 282 produced high amount of callus cultured on MS medium supplemented with 2.0 mgl^{-1} 2,4-D. Sikder *et al.* (2006) also reported that 2.0 mgl^{-1} 2,4-D is better for Chinigura callus induction. Similar results were also found in Thai aromatic rice KDML105 (Summart *et al*., 2008), ASD 16, ADT 43, Basmati 370, Pusa Basmati and Pokkali (Revathi and Pillai, 2011; Libin *et al*., 2012; Islam *et al*., 2005). Our results further revealed that the use of 2,4-D with cytokinin could be helpful for high and early production of callus. Similar observations were also reported in rice (Alam, 1994; Khondokar, 1999). Taken together, the findings from this study will be very useful for producing high frequency callus induction that is the prime step for crop improvement or rapid propagation through biotechnological approaches.

4.2 Plant regeneration

Among different concentrations and combinations, treatment T_4 (0.5 mgl^{-1} BAP +0.1 mgl^{-1} IBA) showed better performance to produce plantlets. The range of shoot number and mean performance was also found to be better for T_4 treatment. Similar results were reported on indica (Khanna and Raina, 1998) and Japonica rice cultivars (Lee et al., 2002). However, Sripichitt and Cheewasestatham (1994) reported that MS agar medium supplemented with 1 mgl^{-1} indol-3-acetic acid (IAA) and 4 mgl^{-1} benzyladenine (BA) induced highest percentage of calli forming shoots. Thadavong *et al.* (2002), Rashid *et al.* (2003), Sikder *et al.* (2006), Jubair *et al.* (2008) and Libin *et al.* (2012) also showed similar results. In our study, high concentrations of 2,4-D (2.0 mgl^{-1} or more) were found to be suitable for callus induction but these calli were not efficient for plant regeneration. In this study, results also showed that Kalijira is more efficient than Chinigura for producing plantlet from callus.

Our findings provide a simple *in vitro* protocol for generating high frequency callus formation and its subsequent regeneration for aromatic rice. These findings can also be manipulated for disease and pest resistant variety, stress and salt tolerance variety through tissue culture and gene transfer techniques.

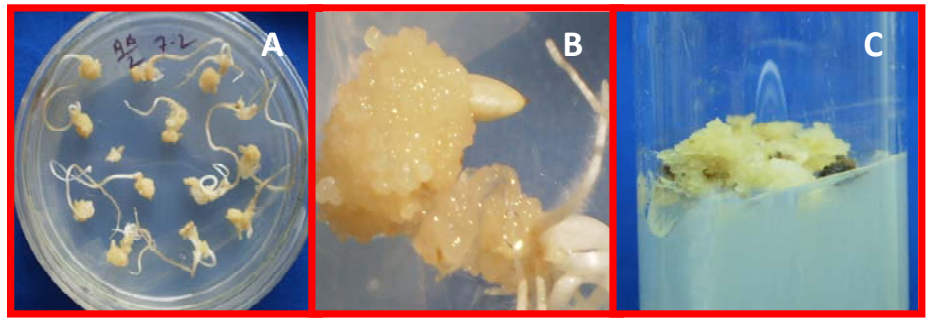

Figure 1: (A) Induction of callus from mature seeds in MS+2.0 mgl^{-1} of 2,4-D. (B) Highlight a single seed derived callus. (C) Proliferation of callus.

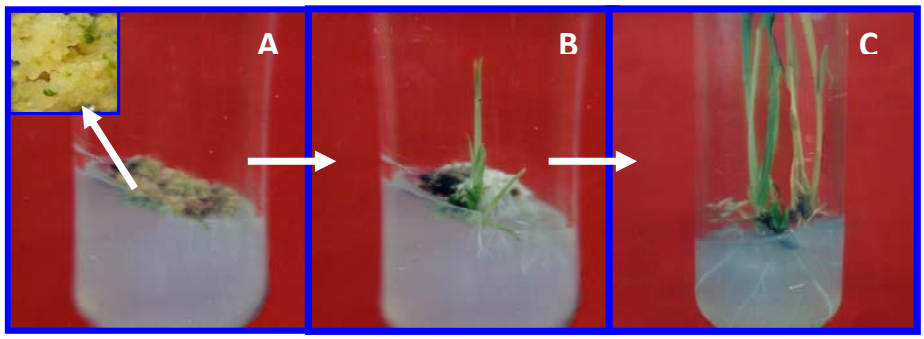

Figure 2: (A) Callus showing green spot on regeneration medium (MS +1.5 mgl^{-1} BAP +0.1 mgl^{-1} IBA). (B) Shoot formation from embryonic callus (MS+1.5 mgl^{-1} BAP+ 0.1 mgl^{-1} IBA). (C) Root proliferation of shoots in 1/2 MS without any growth regulators.

5 REFERENCES

Abe, T.; Futsuhara, Y. (1985). Genotypic variability for callus formation and plant regeneration in rice *(Oryza sativa L.).* Theoretical and Applied Genetics, 72: 3-10.

Alam M.F. (1994): Protoplast culture and transformation in rice *(Oryza sativa L.).* Ph.D. (Genetics) Thesis. Faculty of the Graduate School University of the Philippines, Lose Banos, Philippines.

Alam, M. F.; Khatun, S. M.; Khandakar, I. A.; Khalekuzzaman, M.; Shohael, A. M. and Parvez, S. (2003). Genetic parameter on callus induction and plant regeneration using three explants in four rice cultivars of Bangladesh. Bangladesh Journal of Genetics and Biotechnology, 4(1&2): 59-62.

Chen, T. H.; Lam, L. and Chen, S. C. (1985). Somatic embryogenesis and plant regeneration from cultured young inflorescence of *Oryza sativa* L. (rice). Plant Cell, Tissue and Organ Culture, 4: 51-54.

Ghareyazie, B.; Alinia, F.; Menguito, C. A.; Rubia, L. G.; Palma, J. M. D.; Liwanag E. A.; Cohen, M. B.; Khush, G. S. and Bennett, J. (1997): Enhanced resistance to two stem borers in an aromatic rice containing a synthetic *cryIA(b)* gene. Molecular Breeding, 3: 401–414.

Gonalz, M. C. (2000). Effects of different growth regulators on *in vitro* culture of rice cultivors. Tropicales, 21(1): 27-28.

Islam, M. M.; Ahmed, M. and Mahaldar D. (2005). *In vitro* callus induction and plant regeneration in seed explants of rice (*Oryza sativa* L.). Research Journal of Agriculture and Biological Sciences, 1(1): 72-75.

Jubair, T. A.; Salam, U.; Haque, N.; Akter, F.; Mukti, I. J.; Haque, A. K. M. F. and Ali M. R. (2008). Callus induction and regeneration of local rice (*Oryza sativa* L.) variety Topa. Asian Journal of Plant Sciences, 7(5):514-517.

Khanna, H. K. and Raina S.K. (1998). Genotype x media culture interaction effects on regeneration response of three indica rice cultivars. Plant Cell, Tissue and Organ Culture, 52: 145-153.

Khondokar, I. (1999). Callus induction and organogenesis of rice (*Oryza sativa* L.). M.Sc. Thesis, Department of Botany,University of Rajshahi, Bangladesh.

Khush, G.S. (2005). What it will take to feed 5.0 billion rice consumers in 2030. Plant Molecular Biology, 59: 1-6.

Lee, K., Jeon, H. and Kim, M. (2002., Optimization of mature embryo-based *in vitro* culture system for high frequency somatic embryogenic callus induction and plant regeneration from Japonica rice cultivars. Plant Cell, Tissue and Organ Culture, 71: 237-244.

Libin, A.; King, P. J. H.; Ong, K. H.; Chubo, J.K. and Sipen P. (2012). Callus induction and plant regeneration of Sarawak rice (*Oryza sativa* L.) variety Biris. African Journal of Agricultural Research, 7(30): 4260-4265.

Ling, D. H.; Chen, W. F.; Chen, M. F. and Ma, Z. R. (1983). Direct development of plantlets from immature panicles of rice *in vitro*. Plant Cell Reports, 2: 172-174.

Murashige, T. and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology, 15: 473-497.

Rashid, H.; Abbasi, F.M. and Quraishi, A. (2003). Plant regeneration from seed derived callus of three

varieties of basmati rice. Plant Tissue Culture, 13(1): 75-79.

Rashid, H.; Toriyama, A.; Qurashi, K. And Malik, K. A. (2000). An improved method for shoot regeneration from calli of Indica rice. Pakistan Journal of Biological Sciences, 3(12): 2229-2231.

Ray, J. K. (1985). Rice Research Institute in India. Indian Council of Agricultural Research, New Delhi, India. Introduction to Botany of the Rice Plant. 2nd Ed, p. 5.

Revathi, S. and Pillai, M.A. (2011). *In vitro* callus induction in rice (*Oryza sativa* L.). Research in Plant Biology, 1(5): 13-15.

Shahsavari, E.; Maheran, A. A.; Siti N. A. A. and Hanafi M. M. (2010). The effect of plant growth regulators on optimization of tissue culture system in Malaysian upland rice. African Journal of Biotechnology, 9(14): 2089-2094.

Sikder, M. B. H.; Sen P. K.; Mamun, M. A.; Ali, M. R. and Rahman S. M. (2006). *In Vitro* Regeneration of Aromatic Rice (*Oryza sativa* L.) International Journal of Agriculture & Biology, 6: 759–762.

Sripichitt, P. and Cheewasestatham P. (1994). Plant regeneration from embryo derived callus of aromatic rice (*Oryza sativa* L.) variety Khao Dawk Mali 105. Kasetsart Journal Natural Science, 28: 27-37.

Summart, J.; Panichajakul, S.; Prathepha P. and Thanonkeo P. (2008). Callus induction and influence of culture condition and culture medium on growth of thai aromatic rice, Khao Dawk Mali 105, Cell Culture. World Applied Sciences Journal, 5(2): 246-251.

Thadavong, S.; Sripichitt, P.; Wongyai, W. and Jompuk P. (2002). Callus induction and plant regeneration from mature embryos of glutinous rice (*Oryza sativa* L.) cultivar TDK1. Kasetsart Journal: Natural Science, 36: 334 – 344.

Zaidi, M.A.; Narayanan, M.; Sardana, R.; Taga, I.; Postel, S.; Johns, R.; McNulty, M.; Mottiar, Y.; Mao, J.; Loit, E. and Altosaar, I. (2006). Optimizing tissue culture media for efficient transformation of different indica rice genotypes. Agronomy Research, 4: 563–575.

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Phenotypic evaluation of scutellum-derived calluses in 'Indica' rice cultivars

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ABSTRACT

By using amenable MS based medium containing 4 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.4 mg l^{-1} benzyl-aminopurine (BAP), 30 g l^{-1} sucrose, 8 g l^{-1} Agar-agar, qualitative and quantitative traits of calluses initiated from four genetically and commercially valuable Northern Iranian rice cultivars including Hashemi, Hasani, Gerdeh, and Gharib were studied. Five seeds were placed in each Petri dish and three replicates of eight Petri dishes per replicate were incubated in a growth chamber at 25 ±2 °C in the dark and the averages for every replicate were employed in the analyses. Several important parameters related to callogenesis of the cultivars including rate of non-viable seeds, necrotic, scutellar, slow growing, and non-scutellar calluses, simultaneous callus induction from scutellar and non-scutellar tissues, seeds with appropriate callus, and root emergence were compared. Accordingly, calli of Gharib and Hashemi were highly responsive in callogenesis, while Gerdeh and Hasani produced dissatisfying calluses. Necrotic calluses, scutellar calli, and non-viable seeds were positively correlated with each other; although they were negatively correlated with non-scutellar calli, simultaneous scutellar and non-scutellar calli induction, and root emergence. The results of the present study are expected to be the first promising step to generate genetically manipulated Iranian indigenous rice cultivars.

Key words: tissue culture, callogenesis, necrosis, *Oryza sativa*, 'Gharib', 'Hashemi', 'Gerdeh', 'Hasani'

IZVLEČEK

FENOTIPSKO VREDNOTENJE IZ SKUTELUMA PRIDOBLJENIH KALUSOV IZBRANIH SORT 'INDICA' RIŽEV

Z uporabo MS medija, ki je vseboval 4 mg l^{-1} 2,4-diklorfenoksi ocetne kisline (2,4-D), 0.4 mg l^{-1} benzil-aminopurina (BAP), 30 g l^{-1} saharoze in 8 g l^{-1} agarja so bili preučevani kvalitativni in kvantitativni znaki kalusov, pridobljeni iz štirih genetsko in komercialno priznanih sort riža ('Hashemi', 'Hasani', 'Gerdeh', and 'Gharib') iz severnega Irana. Po pet semen je bilo položeno v vsako od osem petrijevk v treh ponovitvah, ki so jih inkubirali v rastni komori pri 25 ±2 °C v temi. Povprečje vsake ponovitve je bilo uporabljeno v analizah. Primerjani so bili pomembni parametri kalogeneze kot so: število nekalečih semen, nekrotični, skutelarni, počasi rastoči in neskutelarni kalusi, simultana indukacija kalusov iz skutelarnih in neskutelarnih tkiv, semena s primernim kalusom in izraščanje korenin. V kalogenezi sta bili zelo odzivni sorti 'Gharib' and 'Hashemi', medtem ko sta sorti 'Gerdeh' and 'Hasani'dali neustrezne kaluse. Nekrotični kalusi, skutelarni kalusi in nekaleča semena so bili med seboj v veliki pozitivni korelaciji in v negativni korelaciji z neskutelarnimi kalusi, simultano indukcijo skutelarnih in neskutelarnih kalusom in nastankom korenin. Iz rezultatov te raziskave pričakujemo prvi obetajoči korak v pridobivanju genetsko spremenjenih domačih sort iranskega riža.

Ključne besede: tkivne kulture, kalogeneza, nekroza, *Oryza sativa*, 'Gharib', 'Hashemi', 'Gerdeh', 'Hasani'

1 INTRODUCTION

Rice is the second most widely cultivated cereal in the world, after wheat, and is a staple food for over half the world's population. In recent years, considerable efforts have been directed towards the improvement of important agronomic traits of rice through tissue culture based *Agrobacterium*-mediated transformation techniques. However, Indica subspecies is the most recalcitrant one to

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Agrobacterium-mediated transformation and tissue culture techniques. Furthermore, differences in callus growth were even reported within Indica cultivars (Ge et al., 2006). These between-cultivar differences restrict the application of tissue culture techniques to a few rice cultivars (Lin and Zhang, 2005; Ge et al., 2006).

Healthy and actively growing calli are prerequisite for transformation by *Agrobacterium* (Hiei et al., 1994) and biolistic methods (Cao et al., 1992). Short period of tissue culture minimizes the possibility of somaclonal variation and thus improves the fertility of transgenic plants (Toki et al., 2006). Indeed, it has frequently been the plant tissue culture technology, rather than the transformation process itself, that has been the limiting step in achieving efficient transformation (Herrera-Estrella et al., 2005)

Several highly efficient tissue culture systems for japonica and Indica rices have recently been established (Hiei et al., 1994; Lin and Zhang, 2005; Toki et al., 2006; Hiei and Komari, 2008). MS (Murashige and Skoog, 1962) is a widely used medium in Indica rice tissue culture (Lin and Zhang, 2005; Ge et al., 2006; Yan et al., 2010). In Indica rices originated in Iran, robust and highly applicable techniques for tissue culture have never been established, regarding the lack of extensive researches in the *in vitro* culture of Iranian indigenous rice, particularly Northern Iranian cultivars. The mentioned issues impede rice tissue culture; so transformation in Indica subspecies, especially Iranian rice cultivars, faces difficulties.

Nowadays, Hashemi is the most popular cultivar in Northern Iran, which is cultivated in most rice fields of Guilan province in Iran. To the best of our knowledge, these cultivars have never been used to investigate their culturability. In the unique study reported here, mature embryos of four Northern Iranian indigenous rice cultivars (*Oryza sativa* L.) were used to assess the effect of an amenable MS based medium on the initiated calli quality and quantity.

2 MATERIALS AND METHODS

This experiment was conducted in the Biotechnology Laboratory of the faculty of Agricultural Sciences, The University of Guilan, Rasht, Iran.

2.1 Plant materials and sterilization

Mature seeds of four indigenous rice (*Oryza sativa* L.) cultivars, including Hashemi (Hm), Hasani (Hn), Gerdeh (Gr), and Gharib (Gb), obtained from the Rice Research Institute of Iran (http://berenj.areo.ir), were used. Rice caryopses were manually husked, sterilized for 30 min in sodium hypochlorite 10%, and then soaked in hydrogen peroxide 1% (W/W) for 3 h. They were sterilized again in sodium hypochlorite 10% for 20 min (Ozawa, 2009). After every stage of sterilization, seeds were rinsed in sterile distilled water three times. Seeds were carefully inspected for any malformation, immaturity, and stains, before and after hulling.

2.2 Callus induction and culture

After rinsing, embryos were placed on an induction medium with the scutellum facing upwards. The induction medium was MS basal medium (Murashige and Skoog, 1962) containing 4 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.4 mg l^{-1} N^6-Benzyladenine (BA), 30 g l^{-1} sucrose, 8 g l^{-1} Agar-agar. Medium was adjusted to pH 5.8 before the addition of Agar-agar prior to autoclaving. Five seeds were placed on each Petri dish and sealed with Parafilm. For each cultivar three replicates of eight Petri dishes per replicate were incubated in a growth chamber at 25 ±2 °C in the dark and the averages for every replicate were employed in the analyses.

2.3 Observation and statistical analysis

Appearance and proliferation of calli were surveyed and documented after 3 weeks. By using Microsoft Office Excel package the percentages of non-viable seeds (NVS), necrotic calli (NC), scutellar calli (SC), slow growing calli (SGC), non-scutellar calli (NSC), simultaneous callus induction (SCI) from scutellar and non-scutellar tissue, seeds with appropriate callus (AC) (at least 3mm in diameter is defined as appropriate callus,

here), and root emergence (RE) were evaluated for the four Northern Iranian indigenous rice cultivars.

3 RESULTS AND DISCUSSION

Before conducting this experiment, seeds of the four cultivars had been being kept in refrigerator (4 °C) for one year. Thus, for each of the cultivars, some of the cultured seeds were non-viable. However, Hn had the highest, while Gb had the lowest rate of NVS (Fig. 1). Long-term storage augments NVS, which is different for each cultivar. Loss of vigour and viability through dry storage comprises a wide range of degenerative events that accumulate over time and trigger loss of viability (Smith and Berjak, 1995). Several findings have shown that reactive oxygen species (O^{-}_{2} and H_2O_2), play an important role in seed deterioration during aging (Sung and 1996). However, there are compelling evidences that hydrogen peroxide may act as a signaling molecule in plants mediating some hormone-regulated processes (Kwak et al., 2006; Vranová et al., 2002). Contrary to earlier views, it is suggested that exogenous application of hydrogen peroxide promotes germination, that indicates a positive role of active oxygen species in germination (Ogawa and Iwabuchi, 2001; Sarath et al., 2007). Likely, seeds viability has been deteriorated during dry storage, albeit loss of viability differently intensified for each cultivar. Five months after starting the experiment, in a separate germination test, non-viable seeds were accounted for Hm 5.73%, Gr 10.3%, Hn 39.06%, and Gb 5.73%.

Hn produced 77% of NC, which was the highest one among Hm (22%), Gr (21%), and Gb (18%) (Fig. 1). In rice, callus necrosis was most likely to occur in cultivars that produced ethylene at a high rate. Callus growth of these plants was more strongly inhibited by a controlled gas mixture, which had higher ethylene as compared to necrosis-tolerant cultivars (Adkins et al., 1990). It has been shown that $AgNO_3$, an ethylene action inhibitor, increased type II callus production from immature embryos of maize (Songstad et al., 1991). In some cases, ethylene could increase putrescine levels (Lee and Chu, 1992). Similarly, addition of an inhibitor of putrescine synthesis to the culture also restored the ability to regenerate whereby the problem of loss of regeneration ability seen in rice callus cultures may be overcome (Bajaj and Rajam, 1996). Thus, it seems that callus necrosis of rice is due to ethylene which increases cellular putrescine levels. Several studies have been carried out on rice varieties tissue culture and it has been reported that those varieties could be divided into two groups: browning/poor-growth type and non-browning/good-growth type (Abe, 1992; Ogawa et al., 1999). It seems clear that rice cultivars are different in tissue culture necrosis. It is likely that the four cultivars have been examined here have had dissimilar rates of necrosis in callus culture.

In this experiment, NSCs were mostly originated from roots. Gr showed the highest rate (75%) for NSC, while Hn showed the lowest rate (4.54%) (Fig. 1). NSCs are not suitable materials for breeding purposes, for instance *Agrobacterium*-mediated transformation of rice (Hiei et al., 1994; Hiei et al., 1997). It has been reported that calli initiated from scutella had embryogenic potential, while calli that arose from the radicle tended to be non-embryogenic. It has also been suggested that the calli arose from the swollen radicle had a translucent, soft, moist, mucilaginous, and unorganized appearance. These calli did not have regeneration ability (Ge et al., 2006). Emergence of calli derived from non-scutellar tissue of the explants shows the medium has not been defined accurately (George and Sherrington, 1984). In the case of NSC, Gr showed the highest rate of NSC, two-thirds of the whole seeds, whereas Hn produced the lowest amount of NSC, nearly 5% of the whole seeds. SCs are the most suitable tissues for modern breeding methods, particularly in *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). Gr had the lowest rate of SC (25%), while three other cultivars had 65 ±10% of that for cultured seeds (Fig. 1). Results of several studies clearly Indicate that calli initiated from scutella are excellent materials for rice transformation by *Agrobacterium* (Hiei et al., 1994; Hiei et al., 1997). It was observed that calli induced from scutellum were mainly embryogenic. These calli were dry, compact, light yellowish, and nodular in appearance (Ge et al., 2006). In contrast to Gr, three other cultivars produced reasonable

amounts of scutellum-derived calli, especially Hn and Gb. Although Hn had the highest rate of calli initiation from scutella, at the same time it had the highest rate of SGC too. Callus induction from scutellar tissue sometimes coincides with calli from non-scutellar tissue of the same seed, hence termed SCI. While some specimens from each four cultivars had just calli either from scutellar or non-scutellar sources, others had both types of calli. For both types of calli, which mentioned earlier, Hn had about 5% coincident, whereas the percentage of coincident for Hm, Gr, and Gb were 13, 20.8, and 15.3, respectively (Fig. 1).

Rice genotypes are different regarding callogenesis or callus initiation as well as SGC. In this experiment, an appropriate size for a callus has been defined as 3 mm or higher. SGC is not ideal for *in vitro* tissue manipulation or transformation. In the present experiment, calli induced by Hn showed the highest rate of SGC (54.5%), whereas in Hm, Gr, and Gb the rate was 15.4%, 16.7%, and 3.5%, respectively (Fig. 1). The use of actively growing embryogenic calli is one of the most important factors in efficient transformation of rice (Hiei et al., 1997). A SGC needs more time to succeed in breeding aims, furthermore long-term culture increases risks of somaclonal variations. There are two options for cultivars with SGC: (i) optimizing callus initiation medium in favor of cultivar with SGC, or (ii) choosing highly responsive cultivars to callus initiation medium. Regarding the second option, apparently all of the examined cultivars were quite prolific in callogenesis, except for Hn with more than 50% SGC.

While all specimens had almost stunted shoots, they brought out different patterns of roots. In contrast with many cultured seeds that did not have any visible roots, some seeds had however roots. The highest percentage of roots were produced through Gr (62.5%), while in the case of Hn, it was 4.5 times fewer than that of Gr (Fig. 1). Based on the evidences from the present study, root formation is mostly accompanied by root-derived calli, an unintended outgrowth. Rice cultivars have, however, different sensitivity to exogenous hormones application (Khanna and Raina, 1998), hence diversely display altered growth, such as a stunted shoot or root and enhanced formation of adventitious roots. It has been seen that, germination process was inhibited, especially RE, as 2,4-D concentration increased to 2 mg l^{-1}, meanwhile callus proliferation commenced at scutellum region scutellum region. A similar findings were reported by Al-Khayri et al., 1996. It seems that excessive rhizogenesis may result in root-derived calluses, or be a prerequisite for such calluses. This is supported by present data showing Hn and Gr on the opposite sides, showed a positive correlation between RE and NSC formation.

Gb produced the most appropriate calli, whereas Gr showed the most inappropriate calluses (Fig. 1). As mentioned previously, calli derived from scutellar tissue should have satisfactory growth rate and healthy appearance to be promising. During the present study, seemingly, calli, which grew smaller than 3 mm in diameter (the slow growing ones) were not fruitful, and needed to be avoided. NC, also, are not preferable for breeding purposes, as discussed earlier. For instance, unlike the highest rate of scutellum-derived calli, the lowest amount of AC produced by Hn resulted from the highest rate of NC, and SGC, which spoiled its yield.

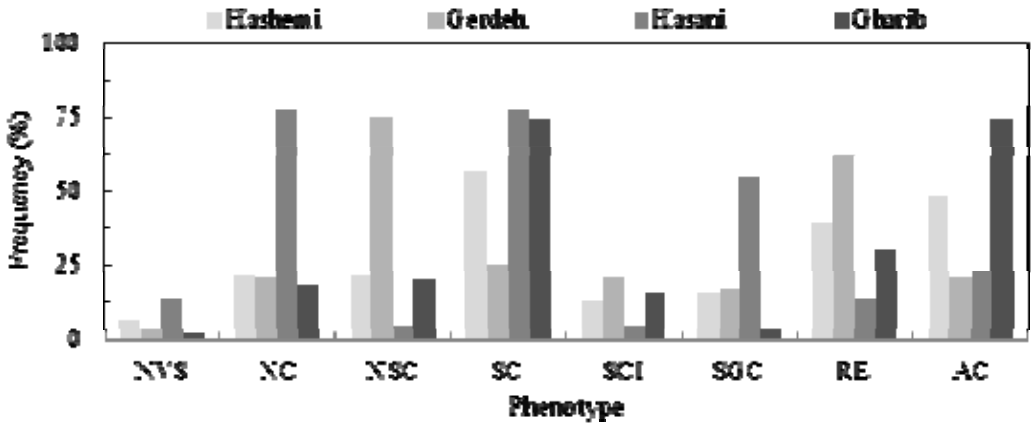

Figure 1: Evaluated rates of: non-viable seeds (NVS), necrotic calli (NC), non-scutellar callus (NSC), scutellar callus (SC), simultaneous callus induction (SCI) from scutellar and non-scutellar tissue, slow growing callus (SGC), root emergence (RE), and seeds with appropriate callus (AC), for the four Northern Iranian indigenous rice cultivars, Hashemi, Gerdeh, Hasani, and Gharib.

Interestingly, it is thought that origin of all the four cultivars were Guilan province, Iran. Gb and Hm have originated in a common region, whereas Hn and Gr originated in two distinct areas from each other and from two previously mentioned. It is assumed that the origin of Hm was Fuman and Soma'e-sara, Gb was Soma'e-sara and Fuman, Hn was Hashtpar, Talesh, Masal and Astarara, and lastly Gr was originated in Tarom. However, there is to our knowledge no support for this claim in the published scientific literature. The Alborz Mountain range forms a barrier between the supposed origins of Gr and the three other cultivars, in particular for Hn (Fig. 2). Geographical barriers can contribute to speciation (Darwin, 1985; Doebeli and Dieckmann, 2003). Interestingly, our findings support the presumed origins of the cultivars and geographical isolation effect on them.

Comparing recorded parameters of Gr and Hn, as two on the opposite sides, revealed that most parameters had a reasonable correlation. The parameters of NC, SC, and NVS showed a positive correlation; meanwhile they were negatively correlated with NSC, SCI, and RE. As discussed previously, there are some evidences that callus necrosis of rice resulted from ethylene through increase in cellular putrescine levels (Bajaj and Rajam, 1996). It was suggested that application of exogenous spermidine, which increases cellular spermidine levels and decreases cellular putrescine levels, adjusts putrescine/spermidine ratio (Bajaj and Rajam, 1996). Interestingly, it was indicated that indole-3-butyric acid (IBA) considerably enhances putrescine biosynthesis result in an increase of the putrescine/spermidine ratio; furthermore, auxin-induced root formation is thought either require or induce the promotion of polyamine (putrescine) synthesis (Friedman et al., 1985). There is increasing evidence that callus-browning trait is genetically controlled. However, either the root formation or callus necrosis obtained in the present investigation may be explained by considering all evidences, including the positive effect of ethylene on callus necrosis (Adkins et al., 1990; Songstad et al., 1991), ethylene-regulated putrescine/spermidine ratio, callus necrosis affected by putrescine/spermidine ratio (Bajaj and Rajam, 1996), casual relationship of ethylene and roots (Lorbiecke and Sauter, 1999), root formation through polyamine (putrescine) synthesis (Friedman et al., 1985), auxin-induced ethylene synthesis (Lorbiecke and Sauter, 1999).

However, the results of the present study by negative correlation between root formation and necrotic calli invitingly need more intense investigations.

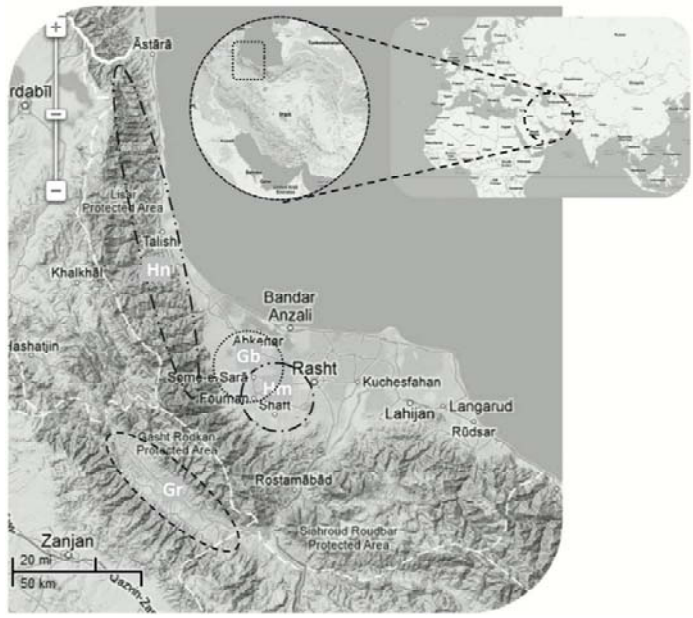

Figure 2: Origin of all the four cultivars was the province of Guilan, Iran (Google Maps 2012). Hasani (Hn), Gharib (Gb), Hashemi (Hm), and Gerdeh (Gr).

4 CONCLUSION

The results of this experiment proved that the most responsive and appropriate cultivar in callus initiation is in decreasing order: Gb > Hm >> Hn ≥ Gr. While Gb appeared very similar in the callogenesis and observed parameters to Hm, Hn and Gr represented dissimilarity among each other and the every other cultivar. The parameters of NC, SC, and NVS indicated a positive correlation; meanwhile they were negatively correlated with NSC, SCI, and at last RE. Gb and Hm produced actively, healthy and scutellar calli, therefore can be employed in tissue culture mediated breeding programs of Iranian Indica rice cultivars (Figure 3).

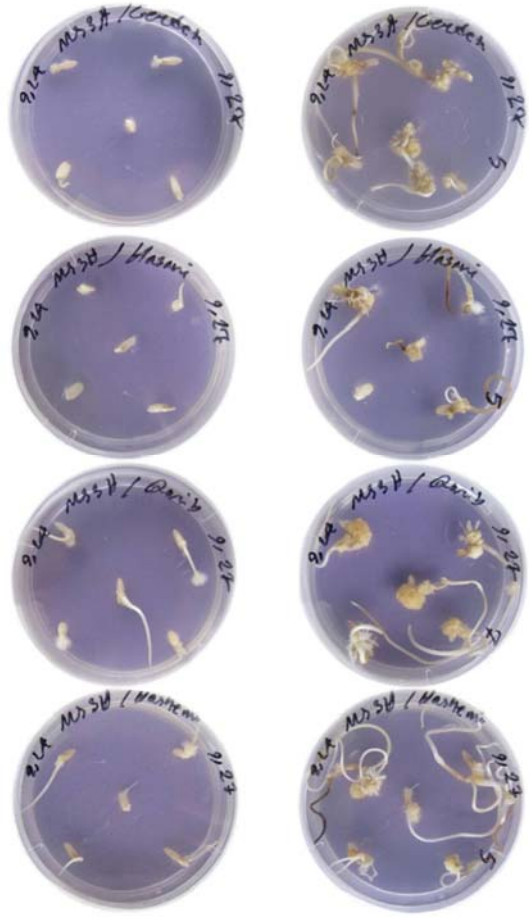

Figure 3: Callus growth from the four rice cultivars 4 days (left) and 24 days (right) after culturing on the callogenesis medium. Rice cultivars from up to down: Gerdeh (Gr), Hasani (Hn), Gharib (Gb), and Hashemi (Hm). Gr produced the lowest rate of scutellar calli (SC), while it had the highest rate of non-scutellar callus (NSC) and root emergence (RE). Although Hn produced the highest SC and the lowest NSC and RE, it showed the highest non-viable seeds (NVS), necrotic calli (NC), and slow growing callus (SGC). Gb produced the highest rate of appropriate callus (AC) because it had the highest SC and the second lowest NSC after Hn, furthermore with the lowest rate of NC and SGC in contrast to Hn. Hm produced high amount of AC but stood after Gb, since it had higher rate of NC, NSC, and above all grew four times slower than Gb.

5 ACKNOWLEDGMENT

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6 REFERENCES

Abe K. 1992. Genealogical study on callus formation ability in anther culture of rice variety Koshihikari. Japanese Journal of Breeding, 42: 403-413. NII Article ID (NAID): 10006567791

Adkins S. W., Shiraishi T., Mccomb J. A. 1990. Rice callus physiology: identification of volatile emissions and their effects on culture growth. Physiologia Plantarum, 78: 526-531. doi: 10.1111/j.1399-3054.1990.tb05237.x

Al-Khayri J. M., Shambun C. E., Ronald W., McNew R. W., Anderson E. J. 1996. Callus induction and plant regeneration of U.S. rice genotypes as affected by

medium constituents. In Vitro Cellular and Developmental Biology - Plant, 32: 227-232. doi: 10.1007/BF02822692

Bajaj S., Rajam M. V. 1996. Polyamine accumulation and near loss of morphogenesis in long-term callus cultures of rice. Plant Physiology, 122: 1343-1348. PMCID: PMC158062

Cao J., Duan X., McElroy D., Wu R. 1992. Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Reports, 11: 586-591. doi: 10.1007/BF00233098

Darwin C. R. 1859. On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. London, John Murray.

Doebeli M., Dieckmann U. 2003. Speciation along environmental gradient. Nature, 42: 259-264. doi: 10.1038/nature01274

Friedman R., Altman A., Bachrach U. 1985. Polyamines and root formation in mung bean hypocotyl cuttings. II. Incorporation of precursors into polyamines. Plant Physiology, 79: 80-83. doi: 10.1104/pp.79.1.80

Ge X., Chu Z., Lin Y., Wang S. 2006. A tissue culture system for different germplasms of *Indica* rice. Plant Cell Reports, 25: 392-402. doi: 10.1007/s00299-005-0100-7

George E. F., Sherrington P. D. 1984. Plant Propagation by Tissue Culture: Handbook and Directory of Commercial Laboratories. Hants, Exegetics Limited: ISBN: 0950932507

Google Maps. 2012. [Guilan Province, Iran, Asia] [Terrain map] Retrieved June 6, 2012, from http://maps.google.com/maps?q=Gilan&hl=en&ie=UTF8&view=map&ftid=0x401fd79dd371a61f:0x4a78900f0f0b7b0b&ftt=545&geocode=FepcNgIdwLzzAg&split=0&sll=37.530671,49.561731&sspn=1.906436,2.072472&hq=&hnear=Gilan,+Iran&t=p&z=9&vpsrc=0

Herrera-Estrella L., Simpson J., Martinez-Trujillo M. 2004. Transgenic plants: an historical perspective. Methods in Molecular Biology, 286: 3-32. doi: 10.1385/1-59259-827-7:003

Hiei Y., Komari T. 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. Nature Protocols, 3: 824-834. doi: 10.1038/nprot.2008.46

Hiei Y., Komari T., Kubo T. 1997. Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Molecular Biology, 35: 205-218. doi: 10.1023/A:1005847615493

Hiei Y., Ohta S., Komari T., Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant Journal, 6: 271-282. doi: 10.1046/j.1365-313X.1994.6020271.x

Khanna H. K., Raina S. K. 1998. Genotype x culture media interaction effects on regeneration response of three Indica rice cultivars. Plant Cell, Tissue and Organ Culture, 52: 145-153. doi: 10.1023/A:1006032303195

Kwak J. M., Nguyen V., Schroeder J. I. 2006. The role of reactive oxygen species in hormonal responses. Plant Physiology, 141: 323-329. doi: 10.1104/pp.106.079004

Lee T. M., Chu C. 1992. Ethylene-induced polyamine accumulation in rice (*Oryza sativa* L.) coleoptiles. Plant Physiology, 100: 238-245. doi: 10.1104/pp.100.1.238

Lin Y. J., Zhang Q. 2005. Optimising the tissue culture conditions for high efficiency transformation of Indica rice. Plant Cell Reports, 23: 540-547. doi: 10.1007/s00299-004-0843-6

Lorbiecke R., Sauter M. 1999. Adventitious root growth and cell-cycle induction in deepwater rice. Plant Physiology, 119: 21-29. PMCID: PMC32222

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

Ogawa K., Iwabuchi M. 2001. A mechanism for promoting the germination of *Zinnia elegans* seeds by hydrogen peroxide. Plant Cell Physiology, 42: 286-291. doi: 10.1093/pcp/pce032

Ogawa T., Fukuoka H., Yano H., Ohkawa Y. 1999. Relationships between nitrite reductase activity and genotype-dependent callus growth in rice cell cultures. Plant Cell Reports, 18: 576-581. doi: 10.1007/s002990050625

Ozawa K. 2009. Establishment of a high efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.). Plant Science, 176: 522-527. doi: 10.1007/978-1-61779-558-9_5

Sarath G., Hou G., Baird L. M., Mitchell R. B. 2007. Reactive oxygen species, ABA and nitric oxide interactions on the germination of warm-season C_4-grasses. Planta, 226: 697-708. doi: 10.1007/s00425-007-0517-z

Smith M. T., Berjak P. 1995. Deteriorative changes associated with the loss of viability of stored desiccation-tolerant and desiccation-sensitive seeds. In: Seed Development and Germination, Kigel J., Galili G. (eds.). New York, Marcel Dekker: 701-746 ISBN-13: 978-0824792299.

Songstad D. D., Armstrong C. L., Petersen W. L. 1991. $AgNO_3$ increases type II callus production from immature embryos of maize inbred B73 and its derivatives. Plant Cell Reports, 9: 699-702. doi: 10.1007/BF00235361

Sung J. M. 1996. Lipid peroxidation and peroxide-scavenging in soybean seeds during aging. Physiologia Plantarum, 97: 85-89. doi: 10.1111/j.1399-3054.1996.tb00482.x

Toki S., Hara N., Ono K., Onodera H., Tagiri A., Oka S., Tanaka H. 2006. Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. The Plant Journal, 47: 969-976. doi: 10.1111/j.1365-313X.2006.02836.x

Vranovà E., Inzé D., Van Breusegem F. 2002. Signal transduction during oxidative stress. Journal of Experimental Botany, 53: 1227-1236. doi: 10.1093/jexbot/53.372.1227

Yan L., Li X., Wu D. 2010. The comparison in tissue culture ability of mature embryo in different cultivars of rice. Agricultural Sciences in China, 9: 840-846. doi: 10.1016/S1671-2927(09)60162-0

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Biogenic amines in red wine: The impact of technological processing of grape and wine

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ABSTRACT

The knowledge of the biogenic amines present in wine is important to consumers in terms of their potential threats of toxicity to human and to wine producers as a result of market impact. In the scientific field, biogenic amines have the potential to be applied as indicators of food spoilage. Biogenic amines are essential at low concentrations for metabolic and physiological functions in animals, plants, and microorganisms, but at high concentrations can induce adverse reactions in susceptible individuals. Despite the intensive research aimed at determining and reduction of biogenic amines, our current knowledge remains far from complete. However, a number of factors that influence the biogenic amines concentration in red wine have been already described. Most of them are related to the winemaking conditions in the cellars and some of them are environmental factors. During winemaking it is important to consider all factors beginning from viticulture practices, alcoholic and malolactic fermentation and physiochemical composition of wine, as well as, aging and storage of wine. This paper reviews changes of the concentration of biogenic amines depending on technological processing of grape and wine.

Key words: biogenic amines, red wine, winemaking conditions, fermentation, microbiological decarboxylation

IZVLEČEK

BIOGENI AMINI V RDEČEM VINU: VPLIV TEHNOLOŠKE PREDELAVE GROZDJA IN VINA

Poznavanje prisotnih biogenih aminov v vinu je pomembno za potrošnike in pridelovalce zaradi potencialne nevarnosti toksičnosti za človeka in posledično tržnih vplivih. Na znanstvenem področju imajo biogeni amini potencial, ki se uporablja kot pokazatelji kvarjenja hrane. Biogeni amini so v majhnih koncentracijah bistvenega pomena za normalne metabolne in fiziološke funkcije pri živalih, rastlinah in mikroorganizmih, lahko pa imajo škodljive učinke pri velikih koncentracijah ter predstavljajo tveganje za zdravje občutljivih posameznikov. Kljub intenzivnim raziskavam, usmerjenim v določanje in zmanjšanje vsebnosti biogenih aminov, naše sedanje znanje še zdaleč ni dokončno. Opisanih je več dejavnikov, ki vplivajo na vsebnost biogenih aminov v rdečih vinih. Večina od njih je povezanih z vinarskimi razmerami v kleti, od katerih so nekateri tudi okoljski dejavniki. V vinarstvu je pomembno upoštevati vse dejavnike, ki se začnejo z vinogradniških vplivi, alkoholno in jabolčno-mlečnokislinsko fermentacijo, fizikalno-kemijsko sestavo vina, kakor tudi staranjem in skladiščenjem vina. V tem članku so pregledno podane spremembe vsebnosti biogenih aminov glede na tehnološke postopke predelave grozdja in pridelave vina.

Ključne besede: biogeni amini, rdeče vino, pogoji med pridelavo vina, fermentacija, mikrobiološka dekarboksilacija

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1 INTRODUCTION

Biogenic amines (BA) are low molecular weight compounds, derived from aromatic or cationic amino acids and all of them have one or more positive charges and a hydrophobic skeleton. The chemical structure of BA can be aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine). The most frequently found BA in wine are histamine, cadaverine, putrescine, phenylethylamine and tyramine (Figure 1) (Smit et al., 2008; Čuš et al., 2013). Amines are mainly formed in foods in fermentative processes and during aging and storage by microbiological decarboxylation of the corresponding amino acid precursors, which is why they are referred to as biogenic. The non-volatile BA (histamine, putrescine, cadaverine, spermine, spermidine, agmatine, tyramine, tryptamine and volatile amine phenylethylamine are formed mainly by microbial decarboxylation of corresponding amino acids (Halász et al., 1994): histidine – histamine; tyrosine – tyramine; phenylalanine – phenylethylamine; arginine and/or ornithine – putrescine; arginine – agmatine; lysine – cadaverine (Buňka et al., 2012) . Volatile amines, except phenylethylamine, are believed to be formed by the reductive amination or transamination of the corresponding aldehyde or ketone (Smith, 1980; Ough et al., 1981). In spite of toxicological implications no legal limit has been defined for BA in wine. Because of these reasons, some countries have established regulations regarding either their content in various kinds of food or their maximum limit requirements (Lehtonen, 1996). In the wine industry, the occurrence of BA has been receiving increasingly attention. There are trade implications due to the recommended or suggested existing limits for histamine in wine in some European countries. Switzerland and Austria reject wines which contain more than 10 mg l^{-1}, and lower limits have been recommended in Germany (2 mg l^{-1}), Holland (3 mg l^{-1}), Finland (5 mg l^{-1})), Belgium (5-6 mg l^{-1}) and France (8 mg l^{-1}) (Lehtonen, 1996; Smit et al., 2008). Generally the toxic dose in alcoholic beverages is considered to be between 8 and 20 mg l^{-1} for histamine, 25 and 40 mg l^{-1} for tyramine, while as little as 3 mg l^{-1} of phenylethylamine can cause negative physiological effect (Soufleros et al., 1998).

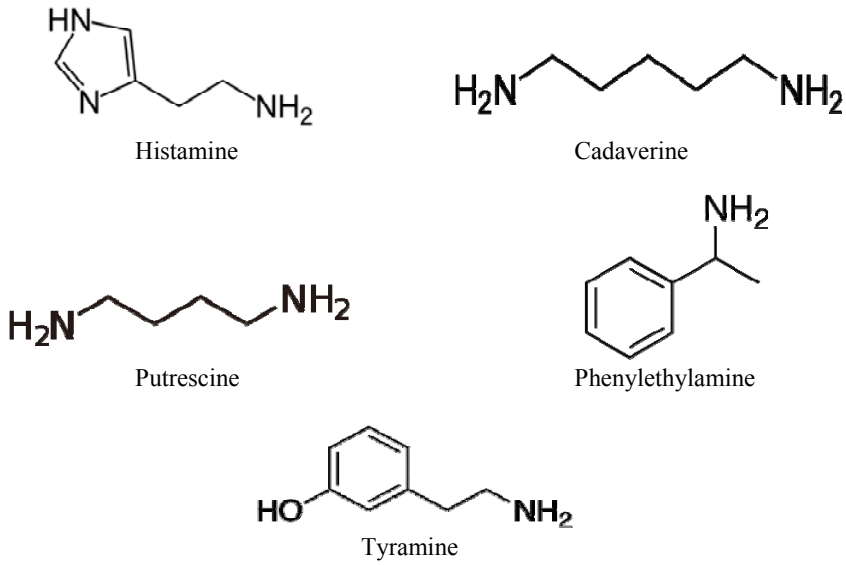

Figure 1.: Chemical structures of the biogenic amines most frequently found in wine.

Some studies have found that BA are formed by yeasts and their concentration is increased during alcoholic fermentation. BA formation in winemaking takes place predominantly during malolactic fermentation (MLF) by lactic acid bacteria (LAB) (García-Ruiz et al., 2011). Contamination may occur from poor sanitary conditions of both grape berries and processing cellar equipment (Moreno-Arribas and Polo,

2009). The results of some studies indicate that vintage can clearly influence the BA contents in wine (Martín-Álvarez et al., 2006). Many actions for increasing the complexity of wine, such as skin maceration and aging on lees, strongly influenced the final content of BA in wines. Some studies have shown significant correlation between some BA and the physico-chemical parameters of wine (pH, total acidity, alcohol and total SO_2 concentration) (Martín-Álvarez et al., 2006).

2 BIOGENIC AMINES (BA)

Bioactive or biologically active amines are low molecular weight organic bases, formed by biochemical processes and are involved in metabolic and physiologic functions in every living organism, playing several important roles (Halász et al., 1994). In humans, the BA involved in brain function, regulation of body temperature and the pH of the stomach, gastric acid secretion, and immune response, the cellular growth and differentiation, etc. The main BA associated with wine are putrescine, histamine, tyramine and cadaverine (Čuš et al., 2011; 2013), followed by phenylethylamine, spermidine, spermine, agmatine and tryptamine (Smit, 2008). Histamine, tyramine and especially putrescine were found in some wines by Buňka et al. (2012) and by Čuš et al. (2011; 2013), while the white wines showed lower content of BA in comparison to the red wines (Table 1) (Bodmer et al., 1999).

Table 1.: Comparison of biogenic amines concentration (mg l^{-1}) in red and white wine (Bodmer et al., 1999).

wine	tyramine	histamine	putrescine	cadaverine	phenylethylamine	spermidine
red	18.2	19.6	99.9	1.0	1.4	2.6
white	2.3	1.1	9.7	0.6	1.7	1.5

There are also recent studies which confirm the fact that histamine and tyramine are the most abundant BA produced by bacterial isolates from experimental wines (Sebastian et al., 2011), in contrast to the lower amounts found by Kaschak et al. (2009) in commercial wines of average quality. Literature data on the levels of biogenic amines in the Montenegrin red wine is not available, but we find more recent data for Slovenian wines (Baša Česnik et al., 2012; Čuš et al., 2011; 2013). The authors found out that the microbiological stability of the wines was poor and should be improved, but however, the levels of BA in the traded Cviček and Blaufränkisch wines were low (Čuš et al., 2013).

Spermine, spermidine and putrescine are involved in DNA, RNA and protein synthesis, growth, membrane stabilization and senescence prevention of organism (Souza et al., 2005). Histamine and serotonine are vaso- and neuroactive and can also protect plants from insects and predators (Smith, 1985). Some amines are frequent constituents of grapes with amounts varying with variety, soil type and composition, fertilization and climatic conditions during the grape growth and stage of maturation (Souza et al., 2005). Putrescine and spermidine are usually abundant in grapes, whereas agmatine, cadaverine, spermine, histamine, tyramine and phenylethylamine have been found in small amounts (Ough, 1971; Zee et al., 1983; Vidal-Carou et al., 1990; Glória et al., 1998; Hajos et al., 2000; Sass-Kiss et al., 2000).

BA can be produced during fermentation processes, aging or storage, when wine is exposed to the undesirable activity of decarboxylase-positive microorganisms. However, reports on development of BA are contradictory. There are reports indicating the possibility that amines are formed in wine by the action of contaminant microorganisms or by those not directly implicated in the fermentation process, for example enteric bacteria (Buteau et al., 1984). In this case, formation of amines was related to the lack of hygiene during winemaking. Based on this assumption, histamine alone or together with other amines could be an indicator of the quality of raw materials employed or poor sanitary conditions prevailing during wine production (Buteau et al., 1984; Vidal-Carou et al., 1990; Soufleros et al., 1998).

A number of studies have reported no remarkable rise in the content of BA during alcoholic fermentation, concluding that yeasts do not appear to be responsible for the production of most amines found in industrial commercial red wines (Herbert et al., 2005; Marcobal et al., 2005). Most researchers attribute the formation of amines, especially tyramine and histamine, to the action of bacteria involved in MLF (Buteau et al., 1984; Vidal-Carou et al., 1990; Soufleros et al., 1998; Sebastian et al., 2011; Buňka et al., 2012). According to Soufleros et al. (1998), during MLF carried out by indigenous LAB, amino acid contents decreased significantly, while content of bioactive amines increased. Lactic acid bacteria are present in low populations in healthy grapes and are transferred to the cellar equipment where they reproduce rapidly. These indigenous bacteria are responsible for spontaneous MLF. However, the metabolic characteristics of the microbiological flora are not well known and in some strains enzymatic decarboxylase activities could be involved in BA production (Soufleros et al., 1998; Arena and Manca de Nadra, 2001).

2.1 Toxicological effect of biogenic amines (BA) in wines

In alcoholic drinks, especially wine, BA received more attention, because ethanol can increase the toxic effects by directly or indirectly inhibiting the enzymes responsible for detoxification of these compounds (Maynard and Schenker, 1996; Smit et al., 2008). The human organism easily tolerates low contents of BA since these are efficiently broken down by mono- and diaminoxidase enzymes in the intestinal tract (Moreno-Arribas and Polo, 2009). Although there are differences in individual susceptibility to intoxication by BA, several pharmacological reactions can take place after excess intake of these compounds. The best known reactions are those caused by histamine. Histamine is known to cause rash, edema, headaches, hypotension, vomiting, palpitation, diarrhea, and heart problems (Ladero et al., 2010). Tyramine and phenylethylamine can produce hypertension through the release of noradrenalin and norepinephrine, respectively, which are vasoconstrictors. Putrescine and cadaverine although non-toxic themselves, aggravate the adverse effects of histamine, tyramine and phenylethylamine, as they interfere with the enzymes that metabolize them (Shalaby, 1996; Silla Santos, 1996). Moreover, putrescine and cadaverine can have negative effects on wine aroma, giving them flavors of putrefaction or rotting flesh, respectively (Moreno-Arribas and Polo, 2009).

Beside the toxic effect (Ladero et al., 2010), some BA also have other negative consequences, particularly regarding sensory characteristics of wine and thus economic implications. A study carried out by Rohn et al. (2005) showed that high contents of histamine in wines identify well-trained wine assessors. In that study to describe the feeling in the mouth (mouthfeel descriptors) used two, namely: "deep throat irritation" and "creep language." No special taste, it can not be attributed to histamine. Putrescine, which is the most common BA in wine, may reduce the sensory quality of wine at concentration of 15-20 mg l^{-1} in white and 20-30 mg l^{-1} in red wines, respectively (Arena and Manca de Nadra, 2001).

2.2 Microorganisms related to production of biogenic amines in the winemaking

In the winemaking process, all groups of wine microorganisms may participate in production of BA. There is general agreement that yeasts make a less significant contribution than LAB to the final content of BA in wine. There is also a fact that yeasts form different BA than LAB. On the other hand, there is much more data about the biochemistry, genetics and regulations of amine production by LAB, compared with the data available for yeasts. Beside yeasts and LAB, fungus *Botrytis cinerea* can cause biotic stress of grapevine and therefore can lead to a rise in the amine content of the grape berries (Hayos et al., 2000).

2.2.1 YEAST

A large species of indigenous yeasts can grow and perform alcoholic fermentation in wine, along with commercial *Saccharomyces cerevisiae* strains. Few studies have been conducted on the formation of BA by yeasts, and most of these only compared different yeast species and only quantified histamine (Torrea and Ancín, 2002). Somavilla et al. (1986), using six yeast strains, demonstrated that small amounts of histamine are produced during alcoholic fermentation and that the

association of yeasts and LAB can reduce the histamine content (Moreno-Arribas and Polo, 2009). The highest histamine concentrations (from 3.7 to 8.3 mg l^{-1}) were produced when histidine was added to the must (34 mg l^{-1}), in other experiments histamine concentrations were lower than 1.2 mg l^{-1}. Vidal-Carou et al. (1990) did not detect formation of histamine during alcoholic fermentation, although they detected tyramine formation, but at very low concentrations (0.60 mg l^{-1}). In contrast, other authors disagree with the hypothesis that BA are formed by LAB during MLF. Torrea-Goñi and Ancín-Azpilicueta (2001) found a slight BA production by *Saccharomyces cerevisiae* depending on the strain. Landete et al. (2007) screened 36 strains of different yeast genera isolated from must and wines for production of BA (*Aureobasidum, Candida, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia,* strains of the species *Saccharomyces cerevisiae*), and no BA were produced by any of these strains. These results are consistent with previous studies in which neither histamine, tyramine, nor putrescine production were detected in 50 yeasts strains isolated from grape and/or wine (Moreno-Arribas and Polo, 2009). These results, therefore, indicate that yeast does not appear to be directly involved in the direct origin of most amines found in wine.

2.2.2 LACTIC ACID BACTERIA (LAB) AND THE CONDITIONS FOR THEIR GROWTH IN MUST AND WINE

Usually BA production results from the presence of bacteria that are capable of decarboxylating amino acids (Gale, 1946). The LAB are a group of Gram positive bacteria, non-respiring, non-spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. Beside the positive aspects of LAB, they are also able to form redundant metabolites in wine (Bartowsky, 2009). Only few species of LAB can grow in media such as must and wine, which are very selective type of media. Bacteria from the genera *Lactobacillus, Pediococcus* and *Oenococcus* are the main strains involved in BA production. Different strains of *Lactobacillus hilgardii, L. brevis, L. buchneri* and *L. mali* have been found to be able to produce a variety of BA in wine (Moreno-Arribas and Lonvaud-Funel, 1999; Moreno-Arribas et al., 2000; Moreno-Arribas et al., 2003; Constantini et al., 2006; Landete et al., 2007).

Among LAB, *O. oeni* is the main species present in wine and the best adapted to carry out the MLF at low pH of wine (Wibowo et al., 1985). If BA formation is associated with MLF, it would be expected that *O. oeni* has the enzymes for breakdown of peptides and decarboxylation of amino acids present in wine at this stage (Leitão et al., 2000). Some authors found that *O. oeni* significantly contribute to the overall content of histamine in wines and that the ability of the species to produce this amine varies among strains (Coton et al., 1998; Guerrini et al., 2002). Marcobal et al. (2004) isolated and identified a strain of the *O. oeni* species, a producer of putrescine, and also studied the ability of another 42 strains of this species to produce putrescine at a molecular level. The gene that encodes biosynthesis of this amine was not present in any of them.

Other authors found that inoculation with commercial starter culture of LAB could reduce the incidence of BA in comparison with spontaneous MLF in wines (Martín-Álvarez et al., 2006; Schneider et al., 2011). Actually, starter cultures could inhibit indigenous bacteria, or possibly could decrease the production of BA by undesirable strains.

Amine build-up usually results from decarboxylation of free amino acids by enzymes of bacterial origin. Hystidine decarboxylase catalyzes decarboxylation of histidine to histamine. Tyramine decarboxylase is responsible for the production of tyramine from tyrosine. Number of tyramine-producing LAB in wine that had undergone MLF were identified and isolated by Moreno-Arribas et al. (2000) and all of them belong to *Lactobacilli*. As the literature suggest, no tyramine-producing *O. oeni* strain has yet been reported except of one strain (*O. oeni* DSM 2025) that was shown to be able to produce tyramine in a defined growth medium (Choudhury et al., 1990). This is confirmed by Sebastian et al. (2011) who observed formation of BA for all 57 strains of *Lactobacillus brevis.* The dominant BA found in this study was tyramine, which was formed by 96% of the strains, while histamine was produced by 19% of *Lactobacillus brevis* strains.

Lactobacillus paracasei formed histamine and ethylamine, while species such as *Lactobacillus delbrueckii* and the most important *Oenococcus oeni* did not show any production of BA (Sebastian et al., 2011).

There are a lot of factors affecting the activity of LAB in wine.

Sulphur dioxide: The antimicrobial activity of SO_2 is based on its ability to pass across cell membrane. Free forms of sulphur dioxide are inhibitorier than bound forms. Of the free forms, molecular SO_2 has the greatest antimicrobial activity. The pH has a marked influence on the toxicity of sulphur dioxide (Jackson, 2008). Therefore, maintaining low pH is helpful in making SO_2 the most effective tool to control LAB. In wine, SO_2 is bound to certain carbonyl compounds such as acetaldehyde. When LAB metabolize the carbonyl compound, the bound SO_2 is released. It is this liberated free form of SO_2 that prevents further growth of the bacteria. Different species and strains vary in their sensitivity to sulphur dioxide. In general, it appears that *O. oeni* is the most sensitive (Jackson, 2008). After MLF the wine is sulphited with the objective of eliminating the yeasts and residual bacteria, but due to the rise in pH and also to the fact that it is found in part combined with the polyphenols, the activity of the SO_2 decreases. Thus can give rise to some LAB remaining viable months after the winemaking and conserving certain biological activity, fundamentally that which helps their survival (García-Marino et al., 2010).

pH: Wine pH is one of the most important factors influencing the growth of LAB. It affects the initiation and duration of MLF, it influences the type of species of bacteria that may develop in wine and it also affects the metabolic behavior of the organism and thereby determines the kind of byproducts formed as the result of bacterial activity (Dharmadhikari, 1992). In the wine pH range mostly from 3.0 to 4.0, the time needed for the completion of MLF decreases with an increase of pH. Bousbouras and Kunkee (1971) reported that at pH 3.15 it took 23.4 weeks to complete MLF; whereas at pH 3.83, it was completed in just two weeks. Many researchers have noted the effect of pH on the species of bacteria that can grow in wine. Generally at pH below 3.5, the MLF is often dominated by *Oenococcus*; whereas, above pH 3.5, species of *Pediococcus* and *Lactobacillus* seem to flourish. It should be noted here that many strains of *Lactobacillus* are involved in wine spoilage. Another important pH effect not commonly realized is the effect of pH on the metabolic behavior of the organisms. For example at pH 3.5 and above, LAB are more likely to decompose sugars, tartaric acid and citric acid. As mentioned earlier, fermentation of sugars leads to higher content of volatile acids in wine (Dharmadhikari, 1992). Lopez et al. (2012) established that the elaboration of 'Tempranillo' grapes at lower pH did not prevent BA formation.

Ethanol: LAB are sensitive to ethanol at 8-10 %vol. Cocci are more sensitive than bacilli. There is some variation between various species regarding alcohol tolerance. The alcohol tolerance is influenced by pH and storage temperature (Bousbouras and Kunkee, 1971; Kelly et al., 1989).

Temperature of fermentation: LAB can normally grow in the range of 10-30 °C, out of this range their metabolism is reduced or stopped. The optimal temperature is 20-25 °C for *Oenococcus* and 25-30 °C (Kelly et al., 1989) for *Lactobacillus*. A growth of LAB can be stopped at 35 °C (Schieri, 1991). Temperature is influenced by ethanol; if the alcohol content is 13-14 % vol., the optimum temperature decreases (Ribéreau-Gayon et al., 1998). If MLF starts, LAB can complete it also at decreased temperature (Ribéreau-Gayon et al., 1998). The influence of temperature on LAB growth is also related to wine pH and SO_2 content.

Nutrition: The LAB need organic compounds for its growth: sugars, amino acids and organic acids. Sugars are the best nutrient for LAB because they provide energy and further stored in ATP molecules. Also citric acid and arginine provide energy to LAB. MLF and histidine decarboxylation are useful to conserve energy (Ribéreau-Gayon et al., 1998). LAB are not able to synthesize amino acids, to the contrary of yeasts (Schieri, 1991). Amino acids must be present in wine to induce LAB growth (Coton et al., 1999). The different strains have different needs: cocci are more exigent than bacilli. Normally, alanine, arginine, cysteine, glutamine, histidine, leucine, phenylalanine, serine, tryptamine, tyramine and valine are necessary all together or partly. Amino

acids are usually used to synthesize new proteins or to provide energy (arginine and histidine) (Ribéreau-Gayon et al., 1998). After alcoholic fermentation yeast lees undergo proteolysis and release amino acids and peptides in the medium.

Oxygen: LAB benefit from the increase of the oxidoreductive potential of wine in order to multiply or at least to improve their existence temporarily (Millet et al., 1995).

3 VITICULTURE AND WINEMAKING FACTORS AFFECTING PRODUCTION OF BIOGENIC AMINES (BA)

The contents of BA produced in wine largely depend on the abundance of amino acid precursors in the grape must, since on the whole, BA increase with an increase in amino acids contents. Amino acid content may be influenced by vinification methods, grapevine variety, geographical origin and vintage (Soufleros et al., 1998; Moreno-Arribas et al., 2000). While some factors increase the content of amino acid precursors, other factors influence the growth and the enzyme activity of microorganisms that can form BA.

3.1 Geographical origin, variety, viticultural practices and vintage year

Some amines, such as putrescine and spermidine, may already be present in grape berries (Solange et al., 2005). According to Broquedis et al. (1989) these amines are found in the pericarp of 'Cabernet sauvignon' berries. Del Prete et al. (2009) found some amines in grapes, such as ethanolamine, ethylamine and putrescine. Therefore, putrescine content in wine may be influenced more by geographical origin and grapevine variety than by winemaking practices (Landete et al., 2005). Potassium deficiency in the soil has been linked to an increase in putrescine content in plants (Adams, 1991); while water deficiency does not seem to influence the content of BA in grape berries and wines (Bover-Cid and Holzapfel, 1999). The stage of grape maturation and the soil type can also influence BA contents in the produced wine (Glória et al., 1998).

Glória et al. (1998) observed that in Cabernet Sauvignon wines from Oregon, USA, putrescine was the prevalent amine (63.5%), followed by histamine (16.8%) and spermidine (9.8%). The prevalence of these amines was also observed in Rioja wines (Vazquez-Lasa et al., 1998). Prevalence of other types of amines has also been reported in the literature, for example, 2-phenylethylamine in wines from Hungary (Hajos et al., 2000; Sass-Kiss et al., 2000). Histamine, tyramine and putrescine contents in Brazilian wines were lower compared to the red wines from other countries (Solange et al., 2005).

The mean contents of all BA, except for cadaverine, can vary significantly over vintages (Martín-Álvarez et al., 2006). In this study, results can be explained partially by the fact that the contents of most of the precursor amino acids varied between years. Moreover, differences in BA contents between vintages could also be due to the diversity of yeast and bacteria strains that are present on the grapes each year.

3.2 Alcoholic fermentation

During alcoholic fermentation, the duration of skin contact is the first factor that affects the extraction of some compounds present in grape skin, especially phenolic compounds and also of other components such as proteins, polysaccharides and amino acids which are precursors of BA. In most red wines alcoholic fermentation takes place in contact with the grape skin. During cold maceration, grape must is left in contact with the grape skins at a cold temperature prior to alcoholic fermentation. Extended maceration after alcoholic fermentation can also be applied at cool temperature to extent the extraction period. Pectolytic enzymes are added to grape musts to increase the yield of juice, to clarify the must or wine, to extract more grape derived compounds such as phenols and to facilitate pressing and filtration (Smit et al., 2008).

Soufleros et al. (1998), determined low content of BA (histamine, tyramine and putrescine) after alcoholic fermentation. Kovačević Ganić et al. (2009) found that cryomacerated wines have higher content of BA, then press wines or free-run wine. Soleas et al. (1999) found no correlation between duration of skin contact and content of

BA. On the other hand, Martín-Álvarez et al. (2006) and Bauza et al. (1995) found that duration of skin maceration is a very important variable which affects the content of BA in wine, and that longer maceration time could favor increased production of BA. These authors noted that the mean content of phenylethylamine and cadaverine were affected by the use of pectolytic enzymes, i.e. the mean contents of these amines were lower in the wines with supplements of pectinases compared with the wines produced without enzymes. They also compared wines aged and not aged with yeast lees and they found that the mean content of methylamine and putrescine were higher in wines aged on yeast lees. This was probably because through the contact of wine with lees, the proteins are initially hydrolyzed to peptides of different molecular weight and these peptides are later degraded further to amino acids and amines as the consequence of yeast and bacteria lysis (Lonvaud-Funel, 2001). These results agree in part with those of Bauza et al. (1995), who also found a higher production of tyramine and putrescine in matured wines in contact with yeast lees, where lactic acid bacteria find more peptides and free amino acids to hydrolyze and decarboxylate. Intense and prolonged maceration produce wines with higher contents of histamine, tyramine, putrescine and cadaverine (Lonvaud-Funel and Joyeux, 1994). In this respect, pH is the most important factor determining not only the biological activity of bacteria in wine but also their variety. At higher pH is more complex the bacterial microflora, because pH acts as a selective factor of microorganisms in wine. At high pH, BA are always produced in high amounts (Lonvaud-Funel, 1991; Lonvaud-Funel and Joyeux, 1994).

Also during alcoholic fermentation, yeast can play indirectly an important role in the subsequent production of BA by LAB, altering the composition of amino acids that might also be released during autolysis (Villamiel-Guerra et al., 2008; Moreno-Arribas and Polo, 2009). The first gene of ornithine decarboxylase was identified, in LAB of oenological origin, isolated from wine lees (Marcobal et al., 2004). In 2011, the OIV adopted a guide, which established and accurately described the various actions to be implemented in vineyards and cellars to minimize the presence of BA in wines. Nitrogenous fertilization of the soil, the poor state of health of the grapes combined with mould, a high must pH and the development of certain yeasts during alcoholic fermentation can all favor a moderate content of BA; thereafter, certain bacteria can, during MLF, significantly increase the presence of BA in wines. Post-fermentative maceration can also favor the formation of BA. The mentioned actions in the document are particularly recommended when a wine has high pH and is aged with few prior oenological treatments (OIV code …, 2011).

3.3 Malolactic fermentation (MLF)

MLF is an important biological process in winemaking because it reduces wine acidity and, if carried out by proper strains of LAB, it improves the flavor and the microbial stability during the wine aging (Davis et al., 1985). MLF is therefore considered essential for most red and some white wines. *Oenococcus oeni*, due to its acid tolerance, is the most frequent bacterial species occurring in wine performing spontaneous MLF and thus it is also the preferred bacterium used as a starter culture in the induced MLF. However, *O. oeni* has been found capable of producing a wide range of BA (Lonvaud-Funel, 2001; Guerrini et al., 2002).

It is considered that the main increase in content of BA in wine is related to MLF. According to *in vitro* studies conducted by Moreno-Arribas et al. (2000), none of the four commercial malolactic starter cultures examined could produce histamine, tyramine or putrescine. Inoculation with *O. oeni* starter cultures that are unable to produce BA is a feasible option for the control of these compounds in wine (Martín-Álvarez et al., 2006). It seems that co-inoculation of *O. oeni* starter cultures during the alcoholic fermentation has the potential to curb BA production even more than conventional inoculation for MLF after the completion of alcoholic fermentation (Moreno-Arribas and Polo, 2009). Recent studies by Schneider et al. (2011) have shown that the wine inoculation of starter cultures after alcoholic fermentation results in lower histamine contents than in wines with spontaneous MLF. Regarding to Lopez et al. (1971) inoculation with a commercial bacterial starter culture resulted in lower BA content after MLF has already be finished, but this advantage was lost after seven months due to the development of indigenous LAB during this period. According to their studies, in order to

reduce BA formation during conservation, it is necessary to remove LAB or inhibit their activity suddenly after the completion of MLF.

3.4 Physiochemical composition of wine

Wine physiochemical factors such as pH, temperature, SO_2 and the variety of substrates and products of fermentation can influence the content and diversity of microorganisms in the wine but can also affect decarboxylase enzyme activity and gene expression.

The product of MLF, lactic acid, was found to inhibit histidine decarboxylase activity (Rollan et al., 1995; Lonvaud-Funel, 2001), while on the contrary, lactic acids does not appear to inhibit ornithine decarboxylase activity (Mangani et al., 2005). Citric acid, as well as succinic acid, D-sorbitol, and malic acid, may also inhibit histidine decarboxylase activity and tyramine decarboxylase activity to a small extent at contents usually present in wines after MLF (Rollan et al., 1995; Moreno-Arribas and Lonvaud-Funel, 1999; Smit et al., 2008; Naila et al., 2010). Other compounds found to inhibit tyramine decarboxylase activity to different extents include glycerol, β-mercaptoethanol, lactic acid and ethanol. However, Moreno-Arribas and Lonvaud-Funel (1999) concluded that even the highest contents of these compounds likely to be present in wine will not be sufficient to prevent the formation of tyramine.

Wine pH and ethanol content at values found in wine could inhibit decarboxylase enzyme activity (Leitão et al., 2000). Histidine decarboxylase activity and consequent histamine production is enhanced at pH 3.5 and by ethanol concentrations up to 10 %vol., where the conditions for histidine transport inside the cells are more favorable due to the fluidification of the cell membrane by ethanol (Lonvaud-Funel and Joyeux, 1994). A high ethanol concentration (12 %vol. or more), as is most often found in wine, reduces the histidine decarboxylase activity by altering the physiochemical properties of the membrane and slowing down histidine transport (Rollan et al., 1995).

According to some authors, the addition of SO_2 in grape must does not affect the formation of BA during alcoholic fermentation (Gárde-Cerdan et al., 2007). Studies carried out throughout the process of industrial wine production indicate that adding of SO_2 to red wines prevents the formation of BA during wine aging and maturation (Marcobal et al., 2006). Use of SO_2 is less effective due to the high pH values of many wines, and often the content of BA can rise in sulfited wines during aging. In fact several studies have shown that red wines with high histamine concentration (>10 mg l^{-1}) are characterized by pH values above 3.7 (Landete et al., 2005; Marcobal et al., 2006).

3.5 Conditions during aging and storage of wine

After MLF Landete et al. (2005) noticed a further increase of histamine content during the first six months of storage in bottles. Other studies showed an increase of histamine contents between four and eight months after MLF in Pinot noir and Chardonnay, while some studies showed an increase of histamine after eighteen months after MLF, while putrescine and tyramine contents seemed to increase immediately following MLF in red wines (Gerbaux and Monamy, 2000; Herbert et al., 2005). A reason for increased contents of BA can be aging wine in contact with yeast lees. Martín-Álvarez et al. (2006) left the wines in contact with the lees for two months after alcoholic fermentation, before aging in barrels. The average contents of methylamine and putrescine were higher in the wines aged on lees.

Other factors of wine aging could also play an important role in the accumulation of BA. These include wine filtration using diatoms that can adsorb amino acids and cationic proteins at their surface, affecting changes in BA content during aging.

It has also been shown that the type of oak used to make barrel (American, French, etc.) used for wine aging does not affect the accumulation of BA in the final product (Jiménez-Moreno et al., 2003). On the other hand, the type of container used for MLF seems to affect the final content of BA. Significantly higher contents of BA were detected in wines undergoing MLF in stainless steel tanks compared to those in which MLF was carried in oak barrels (Alcaide-Hidalgo et al., 2007).

3.6 Prevention of biogenic amine (BA) formation and decrease of their content in wine

The most practical way to control the problem of BA production is based on inhibiting the growth of indigenous decarboxylase-positive bacteria and other microorganisms responsible for this alteration. As mentioned above, SO_2 can prevent growth of these bacteria. There is also possibility to use together lysozyme with SO_2 to delay or inhibit the growth of LAB. Lysozyme is an enzyme that can cause lysis of the cell wall of Gram-positive bacteria, and pH value of grape must or wine can be high for maintaining the activity of lysozyme.

Clarification is the best oenological treatment to decrease the BA content of wine. Clarification can be carried out by physical methods (sedimentation, flotation, centrifugation and filtration) or by fining agents addition (gelatin, albumin, casein) or by pectolytic enzymes addition (Ribéreau-Gayon et al., 1998). Other authors showed that of these oenological coadjuvants, the most effective in dropping BA content is bentonite; a decrease in BA contents was namely directly related to the amount of bentonite used (Mannino et al., 2006). Kally and Body-Szalkai (1996) observed that in red wines, 80 g hl^{-1} of bentonite reduced histamine content by 60%. According to the research by Grossmann et al. (2007), the bentonite is more effective for removal of BA, when used in the must in comparison with the wine fining, where can be removed only minor amount of BA and especially aliphatic histamine, which is adsorbed on the surface of the bentonite.

4 CONCLUSION

The occurrence of BA in wines has been extensively studied in last few years, because these substances are potentially toxic to human health in high contents. In the available literature, a lot of different factors were shown to be involved in the production of BA in wines. Most of them are related to the winemaking conditions in the cellars and some of them are viticultural factors. During winemaking it is important to consider all factors beginning from viticultural practices, alcoholic and malolactic fermentation and physiochemical composition of wine, as well as aging and storage of wine. In the majority of studies MLF appears to be the stage causing the greatest increase of BA contents. Therefore, it can be concluded that the presence of LAB, which are capable of decarboxylation of amino acids, is the main reason of BA incidence in wine. It is very important to take care of cellar sanitation, because contamination with BA can be due to the poor sanitation status. The best way to decrease BA content is wine clarification, actually the use of good clarifiers (bentonite etc).

5 REFERENCES

Adams D.O. 1991. Accumulation of putrescine in grapevine leaves showing symptoms of potassium deficiency or spring fever. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wine. Rantz J. (ed.). American Society for Enology and Viticulture, Davis, California: 126-131

Alcaide-Hidalgo J.M., Moreno-Arribas M.V., Martín-Álvarez P.J., Polo, M.C. 2007. Influence of malolactic fermentation, postfermentative treatments and ageing with lees on nitrogen compounds of red wines. Food Chem., 103: 572-581

Arena M.E., Manca de Nadra M.C. 2001. Biogenic amine production by *Lactobacillus*. J. Appl. Microbiol., 90: 158-162

Bartowsky E.J. 2009. Bacterial spoilage of wine and approaches to minimize it. Lett. Appl. Microbiol., 48: 149-159

Baša Česnik H., Žnidaršič Pongrac V., Velikonja Bolta Š., Čuš F., Butinar L., Rakar A., Žabar R., Trebše P., Franko M., Lisjak K. 2012. Spojine, ki jih v vinu ne želimo. V: Bioaktivne spojine terana: zbornik prispevkov simpozija. Lisjak K. (ur.). Ljubljana, Kmetijski inštitut Slovenije: 63-81

Bauza T., Blaise A., Daumas F., Cabanis J.C. 1995. Determination of biogenic amines and their precursor amino acids in wines of the Vallée du Rhône by high-performance liquid chromatography with precolumn derivatization and fluorimetric detection. J. Chromatogr. A, 707: 373-379

Bodmer S., Imark C., Kneubühl M. 1999. Biogenic amines in foods: Histamine and food processing. Inflammation Research, 48: 296-300

Bousbouras G.E., Kunkee R.E. 1971. Effect of pH on Malo-Lactic Fermentation in Wine. Am. J. Enol. Vitic., 22: 121-126

Bover-Cid S., Holzapfel W.H. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int. J. Food Microbiol., 53: 33-41

Broquedis M., Dumery B., Boucard J. 1989. Ise en evidence de polyamines (putrescine, cadaverine, nor-spermidine et spermine) dans les feuilles et les grappes de *Vitis vinifera* L. Connaiss. Vigne Vin, 23: 1-6

Buňka F., Ivičičová B., Buňková L., Flasarová R., Kráčmar S. 2012. Biogenic amines content in selected wines during winemaking. JMBFS, 4: 785-793

Buteau C., Duitschaever C.L., Ashton G.C. 1984. A study of the biogenesis of amines in a Villard Noir wine. Am. J. Enol. Vitic., 35: 228-236

Choudhury N., Hansen W., Engesser D., Hammes W.P., Holzapfel, W.H. 1990. Formation of histamine and tyramine by lactic acid bacteria in decarboxylase assay medium. Lett. Appl. Micriobiol., 11: 278-281

Constantini A., Cersosimo M., Del Prete V., Garcia-Moruno E. 2006. Production of biogenic amines by lactic acid bacteria: Screening by PCR, thin-layer chromatography, and HPLC of strains isolated from wine and must. J. Food Protect., 69: 391-396

Coton E., Torlois S., Bertrand A. and Lonvaud-Funel A. 1999. Biogenic amines and wine lactic acid bacteria. Bull. OIV., 72, 815-816: 22-34

Coton E., Rollan G., Bertrand A., Lonvaud-Funel A. 1998. Histamine-producing lactic acid bacteria in wines: Early detection, frequency, and distribution. Am. J. Enol. Vitic., 49: 199-204

Čuš F., Bach B., Barnavon L., Žnidaršič Pongrac V. 2013. Analytical determination of Dolenjska region wines quality. Food control, 33: 274-280

Čuš F., Gerič Stare B., Bach B., Barnavon L. 2011. Vsebnost biogenih aminov in hlapnih fenolov ter prisotnost kvasovke Brettanomyces bruxellensis v slovenskih vinih. V: Vinarski dan 2011, Ljubljana, 30. november 2011, (Prikazi in informacije, 275). Čuš F. (ur.). Ljubljana, Kmetijski inštitut Slovenije: 5-24

Davis C.R., Wibowo D., Eschenbruch R., Lee R. 1985. Practical implication of malolactic fermentation: a review. Am. J. Enol. Vitic., 36: 175-177

Del Prete V., Constatini A., Cecchini F., Morassut M., Garcia-Moruno E. 2009. Occurrence of biogenic amines in wine: The role of grapes. Food Chem., 112: 474-481

Dharmadhikari M. 1992. Lactic acid bacteria and wine spoilage. Vineyard and vintage view, 7: 4-7

Gale E.F. 1946. The bacterial amino acid decarboxylases. Adv. Enzymol., 6: 1-32

García-Marino M., lvaro Trigueros A., Escribano-Bailon T. 2010. Influence of oenological practices on the formation of biogenic amines in quality red wines. Journal of Food Compostion and Analysis, 23: 455-462

García-Ruiz A., González-Rompinelli E.M., Bartolomé B., Moreno-Arribas M.V. 2011. Potential of wine-associated lactic acid bacteria to degrade biogenic amines. Int**.** J**.** Food Microbiol**.,** 148: 115-120

Gárde-Cerdan T., Arias-Gil M., Romano P. 2007. Formation of biogenic amines through spontaneous and inoculated wine alcoholic fermentations: effect of SO_2. Food Control, doi: 10.1016/j.foodcont.2006.07.003

Gerbaux V., Monamy C. 2000. Biogenic amines in Burgundy wines. Contents and origin in wines. Rev. Fr. Oenol., 183: 25-28

Glória M.B.A., Watson B.T., Simon-Sarkadi L., Daeschel, M.A. 1998. A survey of biogenic amines in Oregon Pinot noir and Cabernet Sauvignon wines. Am. J. Enol. Vitic., 49: 279-282

Guerrini S., Mangani S., Granchi L., Vincenzini M. 2002. Biogenic amine production by *Oenococcus oeni*. Curr. Microbiol., 44: 374-378

Grossmann M., Smit I., Loehnertz O., Ansorge A. 2007. Biogenic amines and grapes: Effect of microbes and fining agents. In: Proceeding of international symposium of microbiology and food safety of wine. Vilafranca, Spain: 20-21, November 2007.

Hajos G., Sass-Kiss A., Szerdahelye E., Bardocz S. 2000. Changes in biogenic amine content of Tokaj grapes, wines, and Aszu-wines. J. Food Sci., 65: 1142-1144

Halász A., Baráth A., Simon-Sarkadi L., Holzapfel W. 1994. Biogenic amines and their production by microorganisms in food. Trends Food Sci. Tech., 5: 42-49

Herbert P., Cabrita M.J., Ratola N., Laureano O., Alves A. 2005. Free amino acids and biogenic amines in wines and musts from the Alentejo region. Evolution of amines during alcoholic fermentation and relationship with variety, sub-region and vintage. J. Food Eng., 66: 315-322

Jackson R.S. 2008. Wine Science: Principles and Applications. 3rd ed. London, Elsevier Inc.: 751 str.

Jiménez-Moreno N., Goñi D.T., Anzín Azpilicueta C. 2003. Changes in amine concentrations during aging of red wine in oak battels. J. Agric. Food. Chem., 51: 5732-5737

Kallay M., Body-Szalkai M. 1996. Ammine biogene nei vini ungheresi. Riv. Vitic. Enol., 3: 29-38

Kelly W.J., Asmundson R.V., Hopcraft D.H.. 1989. Growth of Leuconostoc oenus under anaerobic conditions. Am. J. Enol. Vitic., 40: 277-282.

Kovačević Ganić K., Gracin L. Komes D., Ćurko N., Lovrić, T. 2009. Changes of the content of biogenic amines during winemaking of Sauvignon wines.Croat. J. Food. Sci., 2: 21-27

Kaschak E., Göhring N., König H., Pfeiffer P. 2009. Biogenic amines in German wines: analysis and assessment according to the application of different HPLC-process. DLR, 105: 375-384

Ladero V., Calles-Enriquez M., Fernandez M., Alvarez M.A. 2010. Toxicological effects of dietary biogenic amines. Curr. Nutr. Food Sci., 6: 145-156. doi: 10.2174/157340110791233256.

Landete J.M., Ferrer S., Pardo I. 2007. Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. Food Control, 18: 1569-1574

Landete J.M., Ferrer S., Polo L., Pardo I. 2005. Biogenic amines in wines from three Spanish regions. J. Agr. Food Chem., 53: 1119-1124

Lehtonen P. 1996. Determination of amines and amino acids in wine – a review. Am. J. Enol.Vitic., 47: 127-133

Leitão M.C., Teixeira H.C., Barreto Crespo M.T., San Romão M.V. 2000. Biogenic amines occurrence in wine: Amino acid decarboxylase and proteolytic activities expression by *Oenococcus oeni*. J. Agr. Food Chem., 48: 2780-2784

Lonvaud-Funel A., Joyeux A. 1994. Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of *Leuconostoc oenos.* J. Appl. Bacteriol., 4: 401-407

Lonvaud-Funel A. 2001. Biogenic amines in wine: role of lactic acid bacteria. FEMS Microbiology Letters, 1: 9-13

López R., Tenorio C., Rosa Gutiérrez A., Garde-Cerdán T., Garijo P., González-Arenzana L., López-Alfaro I., Santamaría P. 2012. Ellaboration of Tempranillo wines at two different pHs. Influence on biogenic amine contents. Food Control, 25: 583-590

Mangani S., Geurrini S., Granchi L., Vincenzini, M. 2005. Putrescine accumulation in wine: role of *Oenococcus oeni*. Curr. Microbiol., 51: 6-10

Mannino M., Vassanelli G., Triulzi G. 2006. Trattamenti al vino per ridurre il contenuto in ammine biogene e loro quantificazione. Vigne Vini, 1-2: 72-75

Marcobal Á., De Las Rivas B., Moreno-Arribas M.V., Muñoz R. 2004. Identification of the ornithine decarboxylase gene in the putrescine-producer *Oenoccocus oeni* BIFI-83. FEMS. Microbiol. Lett., 239: 213-220

Marcobal A., De Las Rivas B., Moreno-Arribas M.V., Muñoz R. 2005. Multiplex PCR method for the simultaneous detection of histamine-, tyramine-, and putrescine producing lactic acid bacteria in foods. J. Food Protect, 68: 874-878

Marcobal Á., Martin-Álvarez P.J., Moreno-Arribas M.V., Muñoz R. 2006. A multifactorial design for studying factors influencing growth and tyramine production of the lactic acid bacteria *Lactobacillus brevis* CECT4669 and *Enterococcus faecium* BIFI-58. Res. Microbiol., 157: 417-424

Martín-Álvarez P.J., Marcobal Á., Polo M.C., Moreno-Arribas M.V. 2006. Technological factors influencing biogenic amine production during red wine manifacture. Eur. Food. Res. Technol., 222: 420-424

Maynard L.S., Schenker V.J. 1996. Monoamine-oxidase inhibition by ethanol in vitro. Nature, 196: 575-576

Millet V., Vivas N., Lonvaud-Funel A. 1995. The development of the bacterial microflora in red wine during aging in barrels. Sci. Techn. Tonnellerie, 1: 123-150

Moreno-Arribas V., Lonvaud-Funel A. 1999. Tyrosine decarboxylase activity of *Lactobacillus brevis* IOEB 9809 isolated from wine and *L. brevis* ATCC 367. FEMS Microbiol. Lett., 180: 55-60

Moreno-Arribas V., Polo C.M. 2009. Amino acids and biogenic amines. In: Wine chemistry and biochemistry. Moreno-Arribas M.V. (ed.), Polo M.C. (ed.). Chapter 6A. New York, Springer: 163-189

Moreno-Arribas V., Polo C.M., Jorganes F., Muñoz R. 2003. Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. Int. J. Food Microbiol., 84: 117-123

Moreno-Arribas V., Torlois S., Joyex A., Bertrand A., Lonvaud-Funel A. 2000. Isolation, properties and behaviour of tyramine-producing lactic acid bacteria from wine. J. Appl. Microbiol., 88: 584-593

Naila A., Flint S., Fletcher G., Bremer P., Meerdink G. 2010. Control of Biogenic Amines in Food - Existing and Emerging Approaches. J. Food Sci., 75: R139-R150

OIV code of good vitivinicultural practices in order to minimise the presence of biogenic amines in vine-based products. 2011. OIV, Resolution OIV-CST 369-2011: 1-5

Ough C.S., Daudt C.E., Crowel E.A. 1981. Identification of new volatile amines in grapes and wines. J. Agric Food Chem., 29: 938-94

Ough C.S. 1971. Measurement of histamine in California wines. J. Agr. Food Chem., 19: 241-244

Ribéreau-Gayon P., Dubourdieu D., Doneche B., Lonvaud A. 1998. Trattato di Enologia I Ed. Bologna, Edagricole: 329-402

Rohn L., Page L., Borck H., Horr B., Diel F. 2005.Can histamine be tasted in wine? Inflammation Research, 54: S66-S67. doi: 10.1007/s00011-004-0430-x

Rollan G.C., Coton E., Lonvaud-Funel A. 1995. Histidine decarboxylase activity of *Leuconostoc oenos* 9204. Food Microbiol., 12: 455-461

Sass-Kiss A., Szerdahelyi E., Hajos G. 2000. Study of biologically active amines in grapes and wines by HPLC. Chromatography, 52: S316-S320

Schieri G. 1991. Industrie agrarie. U. Hoepli, Milano, ISBN 88-203-1885-5:123-133

Schneider I., Ansorge A., Herr P. 2011. The biogenic amine histamine: Physiological effect and concentrations in wine. Journal of Plant Pathology, 93: 39-42

Sebastian P., Herr P., Fischer U., König H. 2011. Molecular identification of lactic acid bacteria occurring in must and wine. S. Afr. J. Enol. Vitic., 32: 300-309

Shalaby A.R. 1996. Significance of biogenic amines to food safety and human health. Food Res. Int., 29: 675-690

Silla Santos M.H. 1996. Biogenic amines: Their importance in foods. Int. J. Food Microbiol., 29: 213-231

Smit A.Y., du Toit W.J., du Toit M. 2008. Biogenic amines in wine: Understanding the headache. S. Afr. J. Enol. Vitic., 29: 109-127

Smith T.A. 1980. Amines in food, Food Chem., 6: 169-200

Smith T.A. 1985. Polyamines, Ann. Rev. Plant Physiol., 36: 117-143

Souza S.C., Theodoro K.H., Souza E.R., da Motta S., Glória M.B.A. 2005. Bioactive amines in Brazilian wines: types, levels and correlation with physico-chemical parameters. Food science and technology, 48: 53-62

Soleas G.J., Carey M., Goldberg D.M. 1999. Method development and cultivar-related differences of nine biogenic amines in Ontario wines. Food Chem., 64: 49-58

Somavilla C., Bravo F., Iñigo B., Burdaspal P. 1986. Acumulacion de histamineen medios naturales y semisinteticos. Alimetari, 86: 37-42

Soufleros E., Barrios M., Bertrand A. 1998. Correlation between the content of biogenic amines and other wine compounds. Am. J. Enol. Vitic., 49: 266-278

Torrea D., Ancín C. 2002. Content of biogenic amines in a Chardonnay wine obtained through spontaneous and inoculated fermentation. J. Agr. Food Chem., 50: 4895-4899

Torrea-Goñi D.T., Anzín-Azpilicueta C. 2001. Influence of yeast strain on biogenic amine content in wines: Relationship with utilization of amino acids during fermentation. Am. J. Enol. Vitic., 52: 185-190

Vazquez-Lasa M.B., Iñiguez-Crespo M., González-Larraina M., González-Guerrero A. 1998. Biogenic amines in Rioja wines. Am. J. Enol. Vitic., 49: 229-229

Vidal-Carou M.C., Codony-Salcedo R., Mariné-Font A. 1990. Histamine and tyramine in Spanish wines: Relationships with total sulfur dioxide level, volatile acidity and malo-lactic fermentation intensity. Food Chem., 35: 217-227

Villamiel-Guerra M., Polo M.C., Moreno-Arribas M.V. 2008. Nitrogen compounds and polysaccharides changes during the biological ageing of sherry wines. LWT-Food Sci. Technol., 41: 1842-1846

Wibowo D., Eschenbruch R., Davis C.R., Fleet G.H., Lee T.H. 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic., 36: 302-313

Zee J.A., Simard R.E., Heureux L.L., Tremblay J. 1983. Biogenic amines in wines. Am. J. Enol. Vitic., 34: 6-9

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Biological Control of Root-Knot Nematodes (*Meloidogyne* spp.): Microbes against the Pests

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ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) are important pests of many cultivated plants. Recently, the most efficient chemical control products (e.g. methyl bromide) have now been restricted due to their toxic characteristics. Research on agents that work against root-knot nematodes and do not have a detrimental impact on the environment is becoming increasingly important. Advances in the last decades produced quite a number of biocontrol products that are already marketed. Some of the well-accepted commercial products contain bacteria *Bacillus firmus* and *Pasteuria penetrans*, and fungus *Purpureocillium lilacinus*. In this review we summarize the antagonistic activity of bacteria and fungi, with their advantages and limitations in biocontrol of root-knot nematodes.

Key words: biological control, *Meloidogyne* spp., antagonisms, bacteria, fungi, commercial products

IZVLEČEK

BIOTIČNO ZATIRANJE OGORČIC KORENINSKIH ŠIŠK (*Meloidogyne* spp.): MIKROORGANIZMI PROTI ŠKODLJIVCEM

Ogorčice koreninskih šišk (*Meloidogyne* spp.) uvrščamo med pomembne škodljivce številnih kmetijskih rastlin. Najbolj učinkovita kemična sredstva za njihovo zatiranje so močno strupena, zato je njihova uporaba močno omejena ali celo prepovedana (npr. metil bromid). Razvoj na področju pripravkov za zatiranje ogorčic koreninskih šišk z okoljsko sprejemljivimi lastnostmi se povečuje. Napredek v zadnjih desetletjih je viden v večjem številu biotičnih pripravkov, mnogi med njimi se danes že tržijo. Aktivne snovi v uveljavljenih biotičnih sredstvih sta bakteriji *Bacillus firmus* in *Pasteuria penetrans* ter gliva *Purpureocillium lilacinus*. V članku je predstavljen pregled zaviralnih mehanizmov delovanja bakterij in gliv, prav tako omenjamo največje prednosti in slabosti njihove uporabe v biotičnem zatiranju ogorčic koreninskih šišk.

Ključne besede: biotično varstvo rastlin, *Meloidogyne* spp., antagonizem, bakterije, glive, tržni pripravki

1 INTRODUCTION: OLD VS. MODERN PLANT PEST CONTROL STRATEGIES

The success of pesticides in the middle of the 20th century enabled control of many harmful organisms. Unfortunately, the adaptation of plant-damaging organisms was not accounted for. The pesticides introduced new environmental conditions to which plant pathogens had to adapt, frequently by becomming resistant. Recently, the importance of healthly food and identification of environmental hazards inclined the research field toward alternative control disease strategies by focusing on biological control agents.

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Plant parasitic nematodes are important pests of many cultivated plants. The *Meloidogyne* genus belongs to a group of root-knot nematodes (RKN) and is represented by over 90 species that have been described so far (Moens *et al*., 2009). These are ubiquitous soil organisms with a wide host range. From financial standpoint the most damaging species are *M. incognita*, *M. javanica*, and *M. arenaria* (Sasser et al., 1982). The RKN produce galls on roots that eventually lead to reduced water uptake to shoots. The severeness of yield loss can range from minimal to total depending on the infesting RKN species and crop variety, season, soil type and use of crop rotation (Sikora and Fernández, 2005; reviewed in Wesemael *et al*., 2011). The tropic group of *Meloidogyne* spp. thrive in hot climates but can survive in temperate climate conditions also (Strajnar *et al*., 2011). Importing plants and seedlings infested with RKN from tropic to temperate climates promotes their spread, which is especially important in greenhouses where temperatures are suitable for RKN reproduction (reviewed in Wesemael *et al*., 2011).

The concerns at this point are methods of controlling *Meloidogyne* spp. in soil because no effective nematicides are available. The public concern over the chemical nematicides is not only their toxicity but also their loss of efficiency after a prolonged use. In 2005, the EU banned the use of methyl bromide which was the most effective nematicidal agent. The use of other nematicides has been restricted or withdrawn recently (reviewed in Wesemael *et al*., 2011). Still useful but not entirely effective are management strategies focusing on prevention rather than curation. These practices are an improvement of old practices. Among them are agrotechnical measures to restore and maintain healthy soils (removal of plant debris, solarisation of soil, crop rotation with plant species immune to pathogens that harm other rotation crops, soil fallow, and addition of organic amendments), use of pathogen-free seeds and resistant varieties, and biological control, which emerged as an alternative to chemical control (reviewed in Collange *et al*., 2011).

2 BIOLOGICAL CONTROL – NATURAL INTERACTIONS IN FOCUSED ACTIONS

Soil is a complex ecosystem, one that harbours many different organisms with a complex network of interactions. In rhizosphere where nutrients are abundant the soil organisms have to compete for food sources. Biological control exploits these interactions to either protect the host plant from infections or to reduce the severity of the disease. In short, biological control uses microbes to control plant pathogens. The pioneer of nematode biocontrol was Duddington in 1951. Since then the research has led to a production of various commercial biological control products containing live microorganisms or their metabolites that target specific nematode hosts, though their low efficacy on the fields remains an issue. We will focus on live microbe action towards the RKN; products based on microbial metabolites are classified as biopesticides and their registration resembles that of chemical pesticides.

2.1 The action: specific vs. non-specific

The microorganisms with the ability to control plant parasitic nematodes belong to bacteria, fungi, and actinomycetes. They exert antagonistic action through various mechanisms. Non-pathogenic bacteria antagonize the nematodes by (1) inducing plant resistance (induced or systemic resistance), by (2) degrading signalling compounds to which the nematodes are attracted to, or (3) simply by colonizing the roots thus blocking the penetration of infective juveniles. Some microbes produce toxic compounds that kill the nematodes, others (e.g. fungi) parasitize on them. All these mechanisms can be affected by multiple factors, biotic or abiotic, which limit their use in biological control (Sikora, 1992).

3 ACTIVE INDIGREDIENTS IN BIOLOGICAL CONTROL PRODUCTS

Each soil has the capacity to limit the *Meloidogyne* spp. reproduction to a certain degree, the rest depends on the activity of native microbial community in soil (Sikora, 1992). Research on *Meloidogyne*-suppresive soils revealed a high microbial diversity (Bent et al., 2008). Microbial groups with highest suppressive potential are (1) pathogenic fungi infecting nematode eggs; (2) rhizobacteria; (3) fungi with a general antagonising effect; (4) endophytic fungi, and (5) obligate parasitic bacteria (Whipps and Davies, 2000).

Most promise for RKN (*Meloidogyne* spp.) biological control show fungi from *Trichoderma* and *Purpureocillium* genera (Dababat *et al*., 2006; Affokpon *et al*., 2011; Wilson and Jackson, 2013), endospores of *Pasteuria penetrans*, and rhizobacteria (e.g. *Bacillus firmus*) that are already marketed (Wilson and Jackson, 2013).

3.1 Bacteria and antagonists

Plant-parasitic nematodes co-exist in rhizosphere with biologically diverse bacterial communities. These bacteria impact the nematode life cycle as endoparasites or antagonists (Table 1). Most of the antagonistic bacteria are saprophytes living in the rhizosphere.

3.1.1 Endoparasites: *Pasteuria penetrans*

Well-studied endoparasites of nematodes are bacteria from the *Wolbachia* genus. These are bacteria with a virus-like lifestyle; they are obligate intracellular parasites of invertebrates. Isolation of bacteria from *Meloidogyne* sp. revealed the presence of *Pasteuria* sp., an endoparasite of many economically important plant parasitic nematodes and water fleas (*Daphni*a spp.) (Starr and Sayre, 1988). The genus *Pasteuria* belongs to a *Bacillus-Clostridium* group that produces very resilient endospores (Charles et al., 2005). The most common endoparasite of *Meloidogyne* spp. is *P. penetrans* (Stirling, 1985) and *P. hartismeri* in *Meloidogyne ardenensis* (Bishop *et al*., 2007).

Pasteuria-infected female nematodes produce low numbers of eggs. The endospores are resistant to drying and have good shelf-life; they also reduce infectivity of the juveniles and fecundity of the females (Mankau and Prasad, 1977; Davies *et al*., 1988; Chen *et al*., 1996). Unfortunatelly, their narrow host range limits their wide use, and mass endospore production is currently hard to achieve. The *Pasteuria Biosciences* LLC (recently aquisited by Syngenta) is the only company able to produce enough endospores in a bioreactor to accomodate small field trials (Hewlett *at al*., 2004; 2006). They overcame the obstacle of obligate living conditions by regulating the activity of the sporulating protein Spo0F (Kojetin *et al*., 2005).

Endospores have different binding affinities to infective juveniles J2. The attachment of endospores to cuticle varies between and within populations of *P. penetrans* (Davies *et al*., 2001). Further, the nematode cuticle which determines the success of the endospore attachment shows equal variability in composition (Wishart *et al*., 2004). The level of soil suppression depends on the density of the *P. penetrans* endospores with the lowest limit of 10^4 endospores per gram of soil (Stirling , 1991). It is extremely difficult to assess adequate endospore concentration in soil. Endospore detection limit is currently around 100 endospores per gram of soil as achieved with immunological and molecular techniques. Currently, no mathematical equation correctly describes the relationship between the number of soil endospores and the level of soil suppression (reviewed in Hallmann *et al*., 2009).

Table 1: Bacterial pathogens and antagonists affect different developmental stages of *Meloidogyne* spp. (adapted from Hallmann *et al*., 2009).

Developmental stage	Nematode behaviour intercepted	Mode of action	Place of action	Examples of Bacteria	References
Egg or egg mass	Development, hatching	Toxins, lytic enzymes, parasitism	soil	*Telluria chitinolytica, Bacillus firmus*	Spiegel *et al*., 1991; Wilson and Jackson, 2013
Infective juveniles	Vitality, host attraction, host recognition, penetration	Toxins, lectins, degradation of root exudates, induced resistance, parasitism	Soil, rhizosphere	*Pasteuria penetrans, Pseudomonas fluorescens, Pseudomonas aeroginosa, Rhizobium etli*	Kretchel *et al*., 2002; Siddiqui and Shaukat (2004); Siddiqui *et al*., 2006; Sikora *et al*., 2007; Oliveira *et al*., 2007
Sedentary juvenile	Formation of feeding site, development	Toxins, induced resistance, parasitism	endorhiza	*P. penetrans, R. etli*	Davies *et al*., 1991; Reitz *et al*., 2002
Female	Fecundity		Rhizosphere, endorhiza	*P. penetrans*	Davies *et al*., 2008

3.1.2 Endosymbionts of entomopathogenic nematodes

Lewis *et al*. (2001) found that entomopathogenic nematodes exibit biocontrol activity toward *Meloidogyne* spp. These nematodes (*Steinernema* and *Heterorhabditis*) carry endosymbiotic bacteria that produce exo- and endometabolites with a suppressive effect on *Meloidogyne* spp. (Grewal *et al*., 1999; Vyas *et al*., 2006). The symbiotic bacteria from genera *Xenorhabdus* and *Photorhabdus* produce metabolites that reduce egg hatch and juvenile's penetration, exibit repellent effect and can also paralyse juveniles (Hu *et al*., 1999). The metabolites are only effective in soil and do not affect nematode development inside the roots. Both genera of entomopathogenic nematodes were classified among exotic organisms in Slovenia until 2008, and consecutively their usage in biocontrol was prohibited according to the Rules on Biological Protection of Plants (the Official Gazette of the Republic of Slovenia, No. 45/06). Between 2007 and 2009 the presence of *Steinernema affine* (Laznik and Trdan, 2007), *S. carpocapsae* (Laznik *et al.*, 2008), *S. feltiae* (Laznik *et al*., 2009a), *S. kraussei* (Laznik *et al*., 2009b), and *Heterorhabditis bacteriophora* (Laznik *et al.,* 2009c) was confirmed in Slovenia, and the last four species and now allowed to use in biological control programs.

3.1.3 Rhizobacteria

Soil microbiota is attracted to roots. Root exudates are excellent food source for soil organisms that accumulate around the roots. Diversity of microbes in this area called the rhizosphere transcends the diversity in bulk soil. The bacteria that colonize the rhizosphere of the host plant are called rhizobacteria. These are mostly non-pathogenic bacteria that provide the first line of defence much like microbiota in human intestines (Weller, 1988). By colonizing the host roots the bacteria can also benefit the plant. Many rhizobacteria can stimulate the plant growth and are termed as plant-growth promoting rhizobacteria or PGPR (Kloepper *et al*., 1980; reviewed in Ahemad and Kibret, 2013). Most frequently studied antagonistic rhizobacteria to affect the RKN are *Bacillus subtilis*, *B. sphaericus* and *Pseudomonas fluorescens* (Becker *et al*., 1988; Sikora, 1992; Tian *et al*., 2007). Among other representatives are genera of *Agrobacterium*, *Alcaligenes*, *Aureobacterium*, *Chryseobacterium*, *Corynebacterium*, *Enterobacter*, *Klebsiella*, *Paenibacillus*, *Phyllobacillus*, *Rhizobium*, *Telluria,* and *Xanthomonas* (Spiegel *et al*., 1991; Kloepper *et al*.,

1992; Hallmann *et al*., 1995; Krechel *et al*., 2002; Oliveira *et al*., 2007; Son *et al*., 2009).

Plant parasitic nematodes are also attracted to roots. Moreover, they use the exudate concentration and CO_2 gradient in the rhizosphere to sense the root's proximity (reviewed in Curtis, 2008). Rhizobacteria consume the exudates thereby truncating the nematode's recognition of root penetration points. They are also able to provoke a plant defence response that controls *Meloidogyne* spp. on tomato (Siddiqui and Shaukat, 2004) and other plant pathogens (Ramamoorthy *et al*., 2001). Root-nodulating bacterium *Rhizobium etli* G12 can induce systemic resistance by cell surface lipopolysaccharides (LPS) (Reitz *et al*., 2002). The resistance response decreases the nematode penetration but has no effect on nematode attraction and only slight effect on development inside the roots. Actually, the application of plant defence response elicitors could potentially provide a broad-spectrum and a long-term protection against different plant pathogens (reviewed in Hallmann *et al*., 2009). In support, it has been established that some pesticides act by primming plant defence to enable a rapid response to pathogen attack (Beckers and Conrath, 2007).

The rhizobacteria are easily grown *in vitro* and in bioreactors. Besides having a beneficial effect on host plant they also reduce plant damage. To maximise the biocontrol efficiency many of the marketed products are sold as seed treatments (Oostendrop and Sikora, 1989). It is vital that bacteria colonize the root surface before the nematodes can compete for entry points. Due to many positive effects, the rhizobacteria are considered ideal for nematode biocontrol, but are limited by a number of factors. The seed treatment provides a short-term control even though it induces systemic resistance and reduces the root invasion of the juveniles. The protection is only effective against nematodes having a single generation in a growing season. Also, the activity of the rhizobacteria is affected by the crop cultivar and nematode species (Kerry, 1990; 1992). The antagonistic activity of rhizobacteria is affected by factors that are difficult to control. The key factors are field conditions, environmental or edaphic factors, nematode species, and developmental stage of the nematode (Table 1), or physiological and genetic characteristics of the host plant (Sayre and Walter, 1991; reviewed in Hallmann *et al*., 2009).

3.1.3.1 *Bacillus firmus*

Bacillus firmus is a Gram-positive, endospore-producing soil bacterium sparsely represented in nature. Not all strains exhibit nematicidal activity. Those that do, destroy the eggs of *Meloidogyne* spp. by colonising egg sacs (Keren-Zur *et al*., 2000), some have also suggested the involvement of toxins (Mendoza *et al*., 2008). Recently, Wilson and Jackson (2013) examined the interest of growers for bionematicides, and *B. firmus* preparations received the most attention. Bayer CropScience markets a seed-treatment product (VOTiVO™) and a drench product (Nortica™) (see Table 2) that are currently being sold in the USA.

3.1.4 Actinomycetes

Another group of soil bacteria with potent antagonistic activity toward *Meloidogyne* spp. are actinomycetes. These bacteria are known producers of secondary metabolites with antibiotic activity towards many fungi and bacteria. Most studied are *Streptomyces* species that act against various fungal species and *Meloidogyne* spp. (Krechel *et al*., 2002). *S. avermitilis* produces antibiotic compounds avermectins that are the most effective nematicides. This antibiotic kills infective juveniles, reduces egg hatching, and it has been suggested recently that avermectins inhibit RNA synthesis (Takatsu *et al*., 2003). A commercial product available on the market is Avicta (Syngenta, Switzerland) used as a seed treatment for vegetables and cotton.

3.2 Fungal biocontrol agents

Well-known anatagonists of *Meloidogyne* spp. are ubiquitous soil fungi from genera *Trichoderma* and *Fusarium*. They live in the rhizosphere and colonize the root surface. Their antagonistic activity is focused at fungal pathogens, but they affect the RKN life cycle also (reviewed in Sikora *et al*., 2008). *Trichoderma* spp. prevents nematode penetration and improves plant growth. The conidia of *Trichoderma* attach to nematode cuticle or to egg shell and parasitize on them (Sharon *et al*., 2007). The attachment affinities to *Meloidogyne* spp. eggs, cuticle or gelanious matrix of egg masses are species-specific (Sharon *et al*.,

2001). Like rhizobacteria the *Trichoderma* species should be present in soil before the crop planting to completely colonize the root (Dababat *et al*., 2006). Adding organic amendments to the soil (e.g. chicken litter) can maximize the *Trichoderma* control activity (Islam *et al*., 2005).

Production of fungi for wide use is fairly simple, and some even produce resistant resting spores (*Pochnia* sp.). Most soil fungi are rhizosphere competent with a wide host range. Endophytic fungi may improve plant growth and reduce damage caused by the nematodes. Like bacteria, fungi have specific temperature, moisture, and density requirements; therefore it is difficult to predict their control activity in soil. The biocontrol efficiency depends on the nematode species, plant host and their root exudates, and other crops in rotation (reviewed in Hallman *et al*., 2009).

3.2.1 Nematode-trapping fungi

Some fungi are predators and feed on nematodes, either by attacking eggs or juveniles and/or by forming special hyphal structures to prey on moving nematodes. Nematophagous fungi are classified into Hyphomycetes species, Zygomycetes (*Stylopage* and *Cystopage*) and Ascomycetes (*Monacrosporium cionopagum*) (Stirling, 1991). Hyphae of nematophygous fungi form trapping structures with an adhesive to catch the nematodes. Most commonly found structures are adhesive nets of *Arthrobotrytis* spp. with a three-dimensional network. The fungal hyphae form rings which constrict upon nematode passage then the hyphae penetrate through the cuticle and feed on nematode (review in Hallmann *et al*., 2009). Adding *A. dactyloides* to soil at an early developmental plant stage provides protection against *M. incognita* penetration for 10 weeks (Kumar and Singh, 2006); long enough to prevent major plant damage.

3.2.2 Parasites of eggs and females

Fungi that parasitize on eggs and/or females are facultative parasites. The most important and well studied pathogen of *Meloidogyne* spp. is *Pochonia chlamydosporia* (= *Verticillium chlamydosporium*). The fungus wraps abound the egg, penetrates the shell and destroys the insides of the egg with a cocktail of proteases (reviewed in Hallmann *et al*., 2009; Esteves *et al*., 2009). *Pochonia chlamydosporia* densities in soil can maintain high levels for up to five months in controlled conditions, which makes this fungus suitable for biological control (Atkins *et al*., 2003). There are a few limitations, though. Siddiqui *et al*. (2009) found biotypes of the fungus with a preference to RKN nematodes but with high differences in virulence. The RKN-biotypes with highest virulence had lowest soil densities indicating a fitness cost.

Widely used in marketed control products is *Purpureocillium lilacinus* (former *Paecilomyces lilacinus)* (Table 2) that parasitizes on eggs and other developmental stages of several nematode species. Its antagonistic activity resembles that of *P. chlamydosporia* (Jatala *et al*., 1986). Strain PL251 reduces infestation with *M. incognita* by 66 %, but does not provide a long-term protection. Establishment of *P. lilacinus* in soil varies with soil type and one single application of condia might not suffice, even if the inoculum was high (10^6 conidia/g soil), as proposed by Kiewnick and Sikora (2006). Anastasiadis *et al*. (2008) suggested repeated applications to soil and addition of fungicides to prevent secondary infections by soil fungi. Commercial products with *P. lilacinus* are marketed in Europe (in Italy), North Africa and Central America (Wilson and Jackson, 2013).

3.2.3 Endoparasitic fungi

Other biocontrol fungi are endoparasitic soil fungi of *Hirsutella* spp. Similar to *Pasteuria penetrans* the fungi produce adhesive conidia that attach to nematode cuticle in a manner much like *P. penetrans*, and also have special requirements to grow *in vitro* (Stirling, 1991). The *H. rhossiliensis* and *H. minnesotensis* have the potential to be used in biological control though they are limited by their low density in soil and short-term protection (Tedford *et al*., 1993; Mennan *et al*., 2007).

3.2.4 Mycorrhizal fungi

Symbiotic association between plant roots and fungi is termed mycorrhiza. Mycorhizas form on the root surface (ectomycorrhiza) or grow inside the roots (endomycorrhiza). Endomycorrhizae with hyphae extending inside were found to effectively control the RKN which spend majority of their life-time settled inside the gall. The fungal appresorium penetrates the root cortex, grows inter- and intracellulary forming vesicles and arbuscules. The fungal-plant symbiosis provides

the plant with nutrients and protects the plant against the RKN attack. The mechanisms underlying biocontrol activity of mycorrizae are (1) alteration or reduction of plant exudates upon endomycorrhizae symbiosis which affects egg hatch or nematode attraction, (2) competition for nutrients and impediment of nematode reproduction, and (3) parasitism on female nematodes and their eggs (reviewed in Hallmann *et al*., 2009).

The arbuscular mycorrhizal fungus *Glomus mosseae* gave the most successful results in controlling *Meloidogyne* spp. (Stirling, 1991; Robab *et al*., 2012). Recently, Vos *et al*. (2012) demonstrated an induction of systemic resistance response in tomato roots colonized by *G. mosseae* against *M. incognita*. The combination of *G. intraradices* with mycorhiza-helper bacteria (e.g. *Rhizobium etli* G12) could further enhance the protection of crops plants and extend the time-frame of the biocontrol activity to whole growing season (Reimann *et al*., 2008).

3.2.5 *Myrothecium verrucaria*

Myrothecium verurrucaria is an ascomycete that produces nematicidic compounds. These compounds are the result of *in vitro* fermentation in bioreactors. The biocontrol activity of the fermented broth is not clear at the moment. It is known, however, that the product reduces egg hatching, inhibits development or even kills the nematodes, hinders nematode perception of the host, and enhances microbial antagonism in the rhizosphere (reviewed in Wilson and Jackson, 2013).

Table 2: Commercially available biological control products to control RKN (adapted from Hallman *et al*., 2009).

Product	Antagonist	Product Form	Application	Crop	Company/ country
Bioact WG PL Gold	*Purpureocillium lilacinus*	Water-dispersible granulate; Wettable powder	Drench, drip irrigation	Vegetables, banana Tobacco, citrus	Bayer CropScience, USA ; BASF Worldwide
BioNem-WP Nortica VOTiVO	*Bacillus firmus*	Wettable powder; Solution	Drench, drip irrigation, Seed treatment	Vegetables; Turfgrass; Corn, soybean, cotton	AgroGreen, Israel; Bayer CropScience, USA
KlamiC	*Pochonia chlamydosporia*	Granulate	Soil incorporation	Vegetables	Cuba
Econem	*Pasteuria penetrans*	Solution or powder	Irrigation, kapljično namakanje	Vegetables, turf, soybean	Syngenta; Nematech, Japan
Deny Blue Circle	*Burkholderia cepacia*	Powder or Solution	Seed treatment, Irrigation	Alfalfa, barley, beans, clover, cotton, peas, grain sorghum, vegetable crops and wheat	CCT Corp, USA; Stine Microbial Products, USA;
Biostart	*Bacillus* spp. mixture	Liquid	Soil drench, irrigation	General use	Microbial Solutions, S Africa
Nemix	*Bacillus* spp.	Powder	Drench/drip	Vegetables, Fruit trees	AgriLife/Chr Hansen, Brazil
DiTera	*Myrothecium verrucaria*	Powder	Ground or chemigation	Almonds	Valent Biosciences Corporation, Canada

4 BIOCONTROL PRODUCTS ON THE MARKET

In the last few decades the number of marketed biological control products has increased substancially. Some are summarized in Table 2. In recent years the multinational companies acquisited small biotechnology companies. In 2012-2013, the BASF acquisited Becker Underwood, Bayer CropScience merged with Agraquest and Prophyta, and Syngenta aquisited Pasteuria Bioscience. According to a study of Wilson and Jackson (2013), the key products at the moment are VOTiVO (*B. firmus*), DiTera (*Myrothecium verrucaria*), and BioAct (*P. lilacinus*). The factors affecting selection of an appropriate biocontrol agent are summarized in Hallmann et al. (2009).

5 CONCLUSION: MANY CHALLENGES AHEAD

Many of the biocontrol agents are effective at a specific nematode developmental stage. Attacking the infective juveniles of the RKN may decrease the infection but will not decrease the nematode population, especially of those RKN that have more than one generation in a growing season. On the other hand, the control of females and eggs does not prevent the root invasion and plant damage, but the multiplication of the nematodes is reduced. Another issue is a sedentary stage of RKN that cannot be parasitized by all rhizosphere fungi. The life cycle of the *Meloidogyne* completes when a sedentary female inside the gall produces eggs that extrude from the root surface. The female, however, stays hidden inside the gall. At high temperatures the eggs hatch early and the egg-parasitizing fungi are unable to destroy the eggs in time. Introducing the chitin-degrading bacteria that degrade soil amendments into ammonium can kill most of the nematodes in soil (Kerry, 1992; reviewed in Hallmann *et al.*, 2009).

To maximise the antagonistic control activity many of the commercial products contain one or a few biocontrol organisms. The combinations of biocontrol agents in a product have to be carefully selected as they might not compatibly interact (Roberts *et al*., 2005). Recently, it has been demonstrated that addition of one microbial species to soil has low impact on indigenious microbial community structure (reviewed in Shade *et al*., 2012). This finding will hopefully facilitate biocontrol product registration. One thing to keep in mind though, is the possible facultative pathogenesis to human as many rhizobacteria and soil fungi (e.g. *Trichoderma*) can be excellent biocontrol agents and simultaneously opportunistic human pathogens (Berg *et al*., 2005; Druzhinina *et al*., 2011).

The EU now faces a challenge. We have reduced or banned many of the toxic chemical nematicides even though the yield losses due to RKN are increasing. Moreover, the climatic changes have presented favourable conditions for RKN that are already spreading or are expected to spread throughout the Medditerranean countries (Strajnar *et al.*, 2011; Castagnone-Sereno, 2012). In Slovenia, four species of RKN have been found since 2003: *M. incognita*, *M. hapla*, *M. arenaria*, and *M. ethiopica* (reviewed in Strajnar, 2012). The infestation is found mainly in greenhouses on tomato and pepper. Controlling RKN in greenhouses is challenging and expensive as frequently the whole greenhouse is contaminated. According to data from Agricultural Institute of Slovenia infestations with RKN are increasing. Like in many EU countries we try to restrain the spread with agrotechnical management techniques and recommend the planting of resistant varieties (Širca S., personal communication).

In conclusion, biological control will never be a substitute for chemical control because of its inherent limitations: inconsistency and lower effectiveness. But, its added value on a long-term scale is much higher: clean environment, safe food and water, and most importantly healthy people. Based on current knowledge we have a long road ahead. Fortunately, the use of biocontrol agents is widely accepted among the growers, which is a strong stimulus for a continued research. On the other hand, the most important impediment that we have to deal with is the bureaucracy of product registration.

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7 REFERENCES AND RECOMMENDED READING

Affokpon A., Coyne D.L., Htay C.C., Agbede R.D., Lawouin L., Coosemans J. 2011. Biocontrol potential of native *Trichoderma* isolates against root-knot nematodes in West African vegetable production systems. Soil Biology and Biochemistry, 13: 600-608

Ahemad M., Kibret M. 2013. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. Journal of King Saud University – Science. http: // dx.doi.org /10.1016 /j.jksus.2013.05.001

Anastasiadis I.A., Giannakou I.O., Prophetou-Athanasiadou D.A, Gowen S.R. 2008. The combined effect of the application of a biocontrol agent *Paecilomyces lilacinus*, with various practices for the control of root-knot nematodes. Crop Protection, 27: 352-361

Atkins S.D., Hidalgo-Diaz L., Kalisz H., Mauchline T.H., Hirsch P.R., Kerry B.R. 2003. Development of a new management strategy for the control of root-knot nematodes (*Meloidogyne* spp.) in organic vegetable production. Pest Management Science, 59, 2: 183-189

Becker J.O., Zavaleta.Mejia E., Colbert S.F., Schroth M.N., Weinhold A.R., Hancock J.G., Van Gurdy S.D. 1988. Effects of rhizobacteria on root-knot nematodes and gall formation. Phytopathology, 78, 11: 1466-1469

Beckers G.J.M., Conrath U. 2007. Primming for stress resistance: from lab to the field. Current Opinion in Plant Biology, 10: 425-431

Bent E., Loffredo A., McKenry M.V., Becker J.O., Borneman J. 2008. Detection and investigation of soil biological activity against *Meloidogyne incognita*. Journal of Nematology, 40, 2:109-118

Berg G., Eberl L., Hartmann A. 2005. The rhizosphere as a reservoir for opportunistic pathogenic bacteria. Environmental Microbiology, 7, 11: 1673-1685

Bishop A.H., Gowen S.R., Pembroke B., Trotter J.R. 2007. Morphological and molecular characteristics of a new species of *Pasteuria* parasitic on *Meloidogyne ardenensis*. Journal of Invertebrate Pathology, 96: 28-33

Castagnone-Sereno P. 2012. *Meloidogyne enterlobii* (= *M. mayaguensis*): profile of an emerging, highly pathogenic, root-knot nematode species. Nematology, 12, 2: 133-138

Charles L., Carbonne I., Davies K.G., Bird D., Burke M., Kerry B.R., Opperman C.H. 2005. Phylogenetic analysis of *Pasteuria penetrans* using multiple loci. Journal of Bacteriology, 187: 5700-5708

Chen Z.X., Dickson D.W., McSorley R., Mitchell D.J., Hewlett T.E. 1996. Suppression of *Meloidogyne arenaria* race 1 by soil application of endospores of *Pasteuria penetrans*. Journal of Nematology, 28: 159-168

Collange B., Navarrete M., Peyre G., Mateille T., Tchamitchian M. 2011. Root-knot nematode (*Meloidogyne*) management in vegetable crop production: The challenge of an agronomic system analysis. Crop Protection, 30: 1251-1262.

Curtis R.H.C. 2008. Plant-nematode interactions: Environmental signals detected by the nematode's chemosensory organs control changes in the surface cuticle and behavour. Parasite, 15: 310-316

Dababat A.A., Sikora R.A., Hauschild R. 2006. Use of *Trichoderma harzianum* and *Trichoderma viridae* for biological control *of Meloidogyne incognita* on tomato. Communications in agricultural and applied biological sciences, 71: 953-961

Davies K.G., de Leij F.A.A.M., Kerry B.R. 1991. Microbial agents for the biological control of plant-parasitic nematodes in tropical agriculture. Tropical Pest Management, 37: 303-320

Davies K.G., Fargette M., Balla G., Daudi A., Duponnois R., Gowen S.R., Mateille T., Phillips M.S., Sawadogo A., Trivino C., Vouyoukalou E., Trudgill D.L. 2001. Cuticle heterogeneity as exhibited by *Pasteuria* spore attachment is not linked to the phylogeny of the parthenogenetic root-knot nematodes (*Meloidogyne* spp.). Parasitology, 122: 111-120

Davies K.G., Kerry B.R., Flynn C.A. 1988. Observations on the pathogenicity of *Pasteuria penetrans*, a parasite of root-knot nematodes. Annals of Applied Biology, 112: 1491-1501

Davies K.G., Rowe J., Williamson V.M. 2008. Cuticle variation amongst amphimictic and parthenogenetic populations of nematode (*Meloidogyne* spp.) as exhibited by a bacterial parasite (*Pasteuria penetrans*). Journal of Parasitology, 38: 851-860.

de Leij F.A.A.M., Kerry B.R. 1991. The nematophagous fungus, *Verticillium chlamydosporium,* as a potential biological control agent for *Meloidogyne arenaria.* Revue de Nematologie,14: 157-164

Druzhinina I.S., Seidl-Seiboth V., Herrera-Estrella A., Horwitz B.A., Kenerley C.M., Monte E., Mukherjee P.K., Zeilinger S., Grigoriev I.V., Kubicek C.P. 2011. *Trichoderma*: the genomics of opportunistic success. Nature Reviews Microbiology, 9: 749-759

Duddington C.L. 1951. *Dactylella lobata*, predacious on nematodes. Transactions of the British Mycological Society, 34, 4: 489-491

Esteves I., Peteira B., Atkins S.D., Magan N., Kerry B. 2009. Production of extracellular enzymes by different isolates of *Pochonia chlamydosporia*. Mycological Research, 113, 8: 867-876

Grewal P.S., Lewis E.E., Venkatachari S. 1999. Alleopathy: a possible mechanism of suppresion of plant-parasitic nematodes by entomopathogenic nematodes. Nematology, 1: 735-743

Hallman J., Davies K.G., Sikora R. 2009. Biological control using microbial pathogens, endophytes and antagonists. In: Root-knot Nematodes. Perry R.N., Moens M., Starr J.L (eds.). Wallingford, UK, CAB International: 380-411

Hallmann J., Kloepper J.W., Rodriguez-Kabana R., Sikora R.A. 1995. Endophytic rhizobacteria as antagonists of *Meloidogyne incognita* on cucumber. Phytopathology, 85: 1136

Hewlett T.E., Gerber J.F., Smith K.S. 2004. In vitro culture of *Pasteuria penetrans*. In: Nematology monographs and perspectives. Cook R., Hunt D.J. (eds.). Vol. 2. Koninklijke Brill, Leiden and Boston: 175-185.

Hewlett T.E., Griswold S.T., Smith K.S. 2006. Biological control of *Meloidogyne incognita* using *in vitro* produced *Pasteuria penetrans* in a microplot study. Journal of Nematology, 38: 274

Hu K., Li J., Webster J.M. 1999. Nematicidal metabolites produced by *Photorhabdus luminescens* (Enterobacteriaceae) bacterial symbiont of entomopathogenic nematodes. Nematology, 1: 457-469.

Islam M.N., Ali M.B., Froz M.J., Mondol A.T.M.A.I., Jahan M.A.H.S. 2005. Integrated management of root-knot (*Meloidogyne* spp.) disease on tomato using antagonistic isolates of *Trichoderma harzianum* and its combination with organic amendments. Journal of Subtropical Agricultural Research and Development, 3: 78-81

Jatala P. 1986. Biological control of plant parasitic nematodes. Annual Review of Phytopathology, 24: 453-489

Keren-Zur M., Antonov J., Bercovitz A., Feldman K., Husid A., Kenan G., Markov N., Rebhun M. 2000. *Bacillus firmus* formulations for the safe control of root-knot nematodes. In: Proceedings of the brighton crop protection conference on pests and diseases. Vol. 2A, UK: 47-52

Kerry B. 1992. Biological control of nematodes: prospects and opportunities. In: Plant Nematode Problems and their Control in the Near East Region (FAO Plant Production and Protection Paper - 144). Maqbool M.A., Kerry B. (eds.). Proceedings of the Expert Consultation on Plant Nematode Problems and their Control in the Near East Region Karachi, Pakistan 22-26 November 1992

Kerry B.R. 1990. An assessment of progress towards microbial control of plant-parasitic nematodes. Journal of Nematology, 22: 621-631

Kiewnick S., Sikora R.A. 2006. Biological control of the root-knot nematode *Meloidogyne incognita* by *Paecilomyces lilacinus* strain 251. Biological Control, 38, 2: 179-187

Kloepper J.W., Leong J., Teintze M., Schroth M.N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature, 286: 885-886

Kloepper J.W., Rodriguez-Kabana R., McInroy J.A., Young R.W. 1992. Rhizosphere bacteria antagonistic to soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne incognita*) nematodes: identification of fatty acid analysis and frequency of biological control activity. Plant and Soil, 139: 75-84

Kojetin D.J., Thompson R.J., Benson L.M., Naylor S., Waterman J., Davies K.G., Opperman C.H., Stephenson K., Hoch J.A., Cavanah J. 2005. The structural analysis of divalent metals to the *Bacillus subtillis* response regulator Spo0F: the possibility for *in vitro* metalloregulation in the initiation of sporulation. Biometals, 18: 449-466

Krechel A., Faupel A., Hallmann J., Ulrich A., Berg G. 2002. Potato-associated bacteria and their antagonistic potential toward plant-pathogenic

fungi and the plant-parasitic nematode *Meloidogyne incognita* (Kofoid & White) Chitwood. Canadian Journal of Microbiology, 48: 772-786

Kumar D., Singh K.P. 2006. Assessment of predacity and efficacy of *Arthrobotrys dactyloides* for biological control of root knot disease of tomato. Journal of Phytopathology, 154: 1-5

Laznik Ž., Tóth T., Lakatos T., Trdan S. 2008. Entomopathogenic nematode *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae), a new member of Slovenian fauna. Acta agriculturae Slovenica, 91: 351-359

Laznik Ž., Tóth T., Lakatos T., Trdan S. 2009a. First record of *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) in Slovenia. Helminthologia, 46: 135-138.

Laznik Ž., Tóth T., Lakatos T., Trdan S. 2009b. First record of a cold active entomopathogenic nematode *Steinernema kraussei* (Steiner) (Rhabditida: Steinernematidae) in Slovenia. Acta Agriculturae Slovenica, 93: 37-42

Laznik Ž., Tóth T., Lakatos T., Trdan S. 2009c. *Heterorhabditis bacteriophora* (Poinar) – the first member from Heterorhabditidae family in Slovenia. Acta agriculturae Slovenica, 93: 181-187

Laznik Ž., Trdan, S. 2007. Po prvi najdbi entomopatogenih ogorčic v Sloveniji. Lectures and papers presented at the 8th Slovenian Conference on Plant Protection (ed. Maček, J.), Radenci, March 6-7 2007. Ljubljana, Plant Protection Society of Slovenia: 99-106.

Lewis E.E., Grewal P.S., Sardanelli S. 2001. Interactions between the *Steinernema feltiae* – *Xenorhabdus bovienii* insect pathogen complex and the root-knot nematode *Meloidogyne incognita*. Biological Control, 21: 55-62

Mankau R., Prasad N. 1977. Infectivity of *Bacillus penetrans* in plant-parasitic nematodes. Journal of Nematology, 9: 40-45

Mendoza A.R:, Kiewnick S., Sikora R.A. 2008. *In vitro* activity of *Bacillus firmus* against the burrowing nematode *Radopholus similis* the root-knot nematode *Meloidogyne incognita* and the stem nematode *Ditylenchus dipsaci*. Biocontrol Science and Technology, 18: 377-389

Mennan S., Chen S.Y., Melakeberhan H. 2007. Effects of *Hirsutella minnesotensis* and N-Viro Soil (R) on populations of *Meloidogyne hapla*. Biocontrol Science andTechnology, 17: 233-246

Moens M., Perry R.N., Starr J.L. 2009. *Meloidogyne* species - a diverse group of novel and important plant parasites. In: Root-knot Nematodes. Perry R.N., Moens M., Starr J.L. (eds.). CABI International, Cambrige, MA, USA: 1-17

Oliveira D.F., Campos V.P., Amaral D.F., Nunes A.S., Pantaleão R.A., Costa D.A. 2007. Selection of rhizobacteria able to produce metabolites active against *Meloidogyne exigua*. European Journal of Plant Pathology, 119: 477-479

Oostendorp M., Sikora R.A. 1989. Seed treatment with antagonistic rhizobacteria for the suppression of *Heterodera schachtii* early root infection of sugar beet. Revue de Nematologie, 12: 77-83

Padgham J.L., Sikora R.A. 2007. Biological control potential and modes of action of *Bacillus megaterium* against *Meloidogyne graminicola* on rice. Crop Protection, 26: 971-977

Ramamoorthy V., Viswanathan R., Raghuchander T., Prakasam V., Samiyappan R. 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Protection, 20: 1-11

Reimann S., Hauschild R., Hildebrandt U., Sikora R. A. 2008. Interrelationships between *Rhizobium etli* G12 and *Glomus intraradices* and multitrophic effects in the biological control of the root-knot nematode *Meloidogyne incognita* on tomato. Journal of Plant Diseases and Protection, 115, 3: 108–113

Reitz M., Oger P., Meyer A., Niehaus K., Farrand S.K., Hallmann J., Sikora R.A. 2002. Importance of the O-antigen, core-region and lipid A of rhizobial lipopolysaccharides for the induction of systemic resistance in potato to *Globodera pallida*. Nematology, 4: 73–79

Robab M.I., Shaikh H., Azam T. 2012. Antagonistic effect of *Glomus mosseae* on the pathogenicity of root-knot nematode infected *Solanum nigrum*. Crop Protection, 42: 351-355

Roberts P., Lohrke S., Meyerb L.F., Buyer S., Bowers H., Backed C., Jorge T., Lewis C.S. 2005. Biocontrol agents applied individually and in combination for suppression for of soilborne disease of cucumber. Crop Protection 24: 141–155

Sasser J.N., Carter C.C., Taylor A.L. 1982. A guide to the development of a plant nematology program. A Cooperative Publication of The Department of Plant Pathology, North Carolina State University and The United States Agency for International Development. Raleigh, North Carolina, USA: 21

Sayre R.M., Walter D.E. 1991. Factors affecting the efficacy of natural enemies of nematodes. Annual Review of Phytopathology, 29: 149-166

Shade A., Peter H., Allison S.D., Baho D.L., Berga M., Bürgmann H., Huber D.H., Langenheder S., Lennon J.T., Martiny J.B., Matulich K.L., Schmidt T.M., Handelsman J. 2012. Fundamentals of microbial community resistance and resilience. Frontiers in Microbiology, 3: 1-19

Sharon E., Bar-Eyal M., Chet I., Herrera-Estrella A., Kleifeld O., Spiegel Y. 2001. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology, 91: 687-693

Sharon E., Chet I., Viterbo A., Bar-Eyal M., Nagan H., Samuels G.J., Spiegel Y. 2007. Parasitism of *Trichoderma* on *Meloidogyne javanica* and role of the gelatinous matrix. European Journal of Plant Pathology, 118: 247-258

Siddiqui I. A., Shaukat S. S. 2004. Systemic resistance in tomato induced by biocontrol bacteria against the root-knot nematode, *Meloidogyne javanica* is independent of salicylic acid production. Journal of Phytopathology, 152: 48–54

Siddiqui I. A., Shaukat S. S., Sheikh I.H., Khan A. 2006. Role of cyonide production by *Pseudomonas fluorescens* CHA0 in the suppression of root-knot nematode, *Meloidogyne javanica* in tomato. World Journal of Microbiology and Biotechnology, 22: 641-650

Siddiqui I.A., Atkins S.D., Kerry B.R. 2009. Relationship between saprotrophic growth in soil of different biotypes of *Pochonia chlamydosporia* and the infection of nematode eggs. Annals of Applied Biology, 155: 131-141

Sikora R.A. 1992. Management of the antagonistic potential in agricultural ecosystems for the biological control of plant-parasitic nematodes. Annual Review of Phytopathology, 30: 245-270

Sikora R.A., Fernández E. 2005. Nematodes parasites of vegetables. In: Plant parasitic nematodes in subtropical and tropical agriculture. Liuc M., Sikora R.A., Bridge J. (eds.). CAB International, Wallingford, UK. 319-392

Sikora R.A., Pocasangre L., zum Felde A., Niere B., Vu T.T., Dababat A.A. 2008. Mutualistic endophytic fungi and in-planta suppressiveness to plant parasitic nematodes. Biological Control, 46: 15–23

Sikora R.A., Schäfer K., Dababat A.A. 2007. Mode of action associated with microbially induced in planta suppression of plant-parasitic nematodes. Australasian Plant Pathology, 36: 124-134

Son S.H., Khan Z., Kim S.G., Kim Y.H.J. 2009. Plant growth-promoting rhizobacteria, *Paenibacillus polymyxa* and *Paenibacillus lentimorbus* suppress disease complex caused by root-knot nematode and fusarium wilt fungus. Applied Microbiology, 107, 2: 524-532

Spiegel Y., Cohn E., Galper S., Sharon E., Chet I. 1991. Graduation of a newly isolated bacterium, *Pseudomonas chitinolytica* sp.nov., for controlling the root-knot nematode *Meloidogyne javanica*. Biocontrol Science Technology, 1: 115-125

Starr M.P., Sayre R.M. 1988**.** *Pasteuria thornei* sp. nov. and *Pasteuria penetrans* sensu stricto emend., mycelial and endospore-forming bacteria parasitic, respectively, on plant-parasitic nematodes of the genera *Pratylenchus* and *Meloidogyne*. Annales de l'Institut Pasteur Microbiology, 139: 11–31

Stirling G.R. 1985. Host specificity of *Pasteuria penetrans* within the genus *Meloidogyne*. Nematologica, 31: 203-209

Stirling G.R. 1991. Biological control of plant-parasitic nematodes. Wallingford, UK, CAB International: 282

Strajnar P. 2012. Bionomija, virulenca in genetska karakterizacija ogorčice *Meloidogyne ethiopica* Whitehead (Tylenchida: Meloidogynidae) ter njen vpliv na fiziološke procese v rastlini. Doktorska disertacija, Biotehniška fakulteta, Univerza v Ljubljani, Ljubljana: 61 pp

Strajnar P., Širca S., Knapič M., Urek G. 2011. Effect of Slovenian climatic conditions on the development and survival of the root-knot nematode *Meloidogyne ethiopica***.** European Journal of Plant Pathology, 129, 1: 81-88

Takatsu T., Horiuchi N., Ishikawa M., Wanibuchi K., Moriguchi T., Takahashi S. 2003. 1100-50, a novel nematicide from *Streptomyces lavendulae* SANK 64297. Journal of Antibiotics, 56: 306-309

Tedford E.C., Jaffee B.A., Muldoon A.E., Anderson C.E., Westerdahl B.B. 1993. Parasitism of *Heterodera schachtii* and *Meloidogyne javanica* by *Hirsutella rhossiliensis* in microplots over two growing seasons. Journal of Nematology, 25, 3: 427-433

Tian B., Yang J., Zhang K.Q. 2007. Bacteria used in biological control of plant-parasitic nematodes: populations, mechanisms of action, and future aspects. FEMS Microbial Ecology, 61: 197-213

Vos C.M., Tesfahun A.N., Panis B., De Waele D., Elsen A. 2012. Arbuscular mycorrhizal fungi induce systemic resistance in tomato against the sedentary nematode *Meloidogyne incognita* and the migratory nematode *Pratylenchus penetrans*. Applied Soil Ecology, 61: 1-6

Vyas R.V., Maghodia A.B., Patel B.A., Patel B.J. 2006. Isolation of native *Xenorhabdus* bacteria from *Steirnernema* spp. and role of their exo and endo metabolites for suppresion of root-knot nematodes (*Meloidogyne* spp.) on tomato. Indian Journal of Nematology, 36: 241-246

Weller D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology, 26: 379-407

Wesemael W.M.L.; Viaene N.; Moens M. 2011. Root-knot nematodes (*Meloidogyn* spp.) in Europe. Nematology, 13, 1: 3-16

Whipps J.M., Davies K.G. 2000. Success in biological control of plant pathogens and nematodes by microorganisms. In: Measures of success in biological control. Gurr G., Wratten S.D. (eds.). Dordrecht, The Netherlands, Kluwer Academic Publishers: 231–269

Wilson M.J., Jackson T.A. 2013. Progress in the commercialization of bionematicides. BioControl. doi 10.10077s10526-013-9511-5

Wishart J., Blok V.C:, Phillips M.S., Davies K.G. 2004. *Pasteuria penetrans* and *P. nischizawae* attachment to *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla*. Nematology, 6: 507-510

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Ohranjanje semena vrste *Brassica napus* L. v talni semenski banki

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IZVLEČEK

Najbolj razširjena oblika vrste *Brassica napus* L. je oljna ogrščica, njeno seme pa je zaradi svojih fizikalnih lastnosti zelo mobilno in zato nagnjeno k raztrosu. Nenadzorovane izgube semena predstavljajo potencial za pojavljanje samosevnih in podivjanih populacij te vrste znotraj in zunaj pridelovalnih površin, saj se njeno seme ohranja in ostaja viabilno v tleh tudi več let. Dinamika pojavnosti teh rastlin je odvisna od potenciala talne semenske banke in kompleksih interaktivnih lastnosti genotipa semena ter pedoloških in agro-klimatskih dejavnikov. Prisotnost nedefiniranih opraševalnih virov, ki izvirajo iz talne semenske banke, pa v naravi predstavlja potencial za spontane intra- in inter-speciesne oprašitve vrste *B. napus*, kar vpliva na spremembe v njeni genetski strukturi.

Ključne besede: *Brassica napus*, samosevci, podivjane populacije, talna semenska banka, tla, izgube semena, ohranjanje semena

ABSTRACT

PRESERVATION OF *Brassica napus* L. SEED IN SOIL SEED BANK

The most common form of the *Brassica napus* L. is oilseed rape. Because of its physical characteristics the seed is very mobile and therefore disposed to spillage. Uncontrolled seed loss represents the potential for the appearance of volunteer and feral populations of *B. napus* inside and outside production areas; *B. napus* seed remains viable in the soil for several years. The appearance dynamics of these plants is dependent on the soil seed bank potential and complex interactive characteristics of the genotype seeds and soil and agro-climatic factors. The presence of undefined pollinating resources originated from soil seed bank in the nature presents the potential for spontaneous intra- and inter-species pollination of *B. napus* reflected also in its genetic structure.

Key words: *Brassica napus*, volunteers, feral populations, soil seed bank, soil, seed losses, seed preservation

1 UVOD

Vrsta *Brassica napus* L. je vsesplošno uporabna rastlina, ki spada v raznoliko družino križnic (Brassicaceae). Vrsta vključuje dve po namenu pridelave precej različni podvrsti. Prva podvrsta obsega zelenjadne predstavnike, med njimi pomembno podzemno (rumeno) kolerabo (*B. napus* L. subsp. *napobrassica* (L.) Hanelt), druga podvrsta (*B. napus* L. subsp. *napus* L.) pa združuje ozimno in jaro oljno ogrščico ter krmno obliko navadne ogrščice (Snowdon in sod., 2007). Vrsta *B. napus* se prideluje kot krmna rastlina za krmo govedu v vegetativni fazi razvoja ali se seje za podor z namenom obogatitve tal z organsko snovjo. V največji meri pa se prideluje kot oljnica z veliko vsebnostjo olja v semenu (Kocjan Ačko, 1999).

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Vrsta *B. napus* je ena izmed najbolj perspektivnih oljnic tudi v Sloveniji, in to predvsem zaradi možnosti večnamenske uporabe (prehrana, živalska krma, biogorivo, farmakologija, ekološka funkcija). Kot pomemben člen v kolobarju se pojavlja v vseh sistemih kmetijske pridelave, s svojo prisotnostjo pa omogoča opraševanje s samosevnimi in podivjanimi populacijami znotraj in zunaj pridelovalnih površin (Pipan in sod., 2011). V osnovi je vrsta *B. napus* samoprašna rastlinska vrsta, vendar pa se lahko v odvisnosti od posameznega genotipa in specifičnih vplivov okolja delež tujeprašnosti spreminja (Friedt in Snowdon, 2009). Oprašujejo jo predvsem čebele, lahko pa tudi veter. Zaradi variabilne stopnje tujeprašnosti v naravi prihaja do oprašitev znotraj vrste (intraspeciesna križanja), in sicer med posevki, samosevnimi rastlinami (znotraj pridelovalnih površin) ter podivjanimi populacijami (zunaj pridelovalnih površin). Prisotnost teh rastlin v pridelovalnem prostoru je posledica neustreznih agrotehničnih ukrepov, izgub semena med žetvijo (spravilom), transportom in skladiščenjem, saj je seme zelo drobno, mobilno in zato nagnjeno k raztrosu. Poleg tega so možna tudi nenadzorovana medvrstna opraševanja (interspeciesna križanja) vrste *B. napus* z njenimi spolno kompatibilnimi sorodniki (SKS) iz družine križnic. Te rastline se v naravi pojavljajo predvsem kot plevelne rastline ob obrobjih njiv, lahko pa tudi kot divje rastoče na neobdelanih območjih. Križanja znotraj vrste *B. napus* in med vrstami znotraj družine križnic kratkoročno vplivajo na kakovost posevkov in sortno čistost, dolgoročno pa ohranjanje takega semena v tleh omogoča nenadzorovano spreminjanje genetskega potenciala rastlin vrste *B. napus*, saj se v naslednjih letih lahko pojavljajo kot neopredeljen opraševalni vir (z ohranjenimi geni spolno kompatibilnih rastlin, ki so lahko kultivirane, podivjane ali celo gensko spremenjene) (Treu in Emberlin, 2000).

Seme vrste *B. napus* je drobno, gladko in okroglo, zato se ob spravilu, pri dodelavi, predelavi in transportu zelo lahko nenadzorovano izgublja. Zaradi svoje majhnosti se lepi oziroma obdrži na kmetijskih strojih in strojih za dodelavo zrnja. S stališča ohranjanja izgubljenega semena oljne ogrščice v tleh so zelo pomembne kompleksne interakcije bioloških lastnosti semena, talnih in klimatskih razmer ter agrotehničnih ukrepov, ki se na določenem območju izvajajo. Izraba potenciala talne semenske banke pa je v različnih letih različna in se vedno znova obnavlja. Izgubljeno seme ostane v tleh kalivo več let in v času vegetacije v naslednjih letih s svojo prisotnostjo predstavlja opraševalni vir. V naravnih habitatih lahko tako pride do prenosa genov med podivjanimi populacijami ter kultiviranimi oblikami vrste *B. napus* in njenimi spolno kompatibilnimi sorodniki (Pipan in sod., 2011).

Namen prispevka je podati pomen talne semenske banke pri ohranjanju semena vrste *B. napus* v tleh. Vrsta *B. napus* perspektivna rastlinska vrsta z vsestransko uporabno vrednostjo za hrano, krmo, industrijo ter naravo zato je pomembno poznati tudi sposobnost viabilnosti semena, ki je shranjeno v talni semenski banki in predstavlja potencial za nenadzorovano pojavljanje rastlin te vrste v različnih habitatih pridelovalnega prostora. V prispevku je podan pregled dejavnikov, ki lahko vplivajo na kratkoročno in dolgoročno ohranjanje in samoohranjanje semena v tleh ter pregled raziskav o simulacijskih modelih s pomočjo katerih je mogoče, na podlagi kompleksne sheme vplivov iz okolja, predvideti pojavnost rastlin vrste *B. napus*, ki izvirajo iz talne semenske banke tudi z vidika soobstoja različnih sistemov kmetijske pridelave.

2 TALNA SEMENSKA BANKA

Talna semenska banka je skupno ime za shranjevanje semena, ki je pogosto dormantno in se v tleh nahaja v različnih kopenskih ekosistemih (Csontos, 2007). Talne semenske banke so naravno skladišče semen v tleh za številne rastlinske vrste. Imajo pomembno vlogo pri dinamiki vegetacije, še posebej ob različnih oblikah motenj v naravi ali ob destrukcijah habitatov (požari, goloseki, (pre)intenzivna paša, erozija, oranje), saj lahko rastlinske vrste, katerih seme je že v tleh, hitro kolonizirajo nastali prostor. Vrstna sestava in dolgoživost semen v tleh vplivata na hitrost obnove habitata, njegovo vrstno sestavo, proizvodnost in ekološko vrednost (Batič, 2009). Talne semenske banke za posamezne vrste se razlikujejo glede na dolžino obstojnosti in ohranjanja kalivosti semen v tleh, kar pomeni, da je prisotnost nekaterih semen v tleh le začasna in

se potencial talne semenske banke izčrpa v naslednjem vegetacijskem obdobju. V tleh se nahajajo tudi semena rastlin, ki so vitalna in sposobna reprodukcije tudi po nekaj letih shranjevanja v tleh (v glavnem so to predstavniki plevelnih vrst); mednje spada na primer bela metlika (*Chenopodium album* L.) v primerih samosevnega in podivjanega pojavljanja pa tudi vrsta *B. napus* L. Obstajajo pa tudi semena rastlin, ki se v tleh sploh ne ohranjajo (razen v sušnem obdobju med zrelostjo in med prvimi jesenskimi padavinami); tak primer je navadni kokalj (*Agrostemma githago* L.) (Strokovni predlog …, 2009), ki je v Sloveniji uvrščen na rdeči seznam rastlinskih vrst.

Talna semenska banka ima pomembno vlogo v različnih ekosistemih, saj pomeni izvor semena za hitro obnavljanje degradiranih površin, ki so lahko posledica naravnih nesreč ali človeške aktivnosti v določenem ekosistemu. Vsako seme, ki ni odstranjeno iz polja v času žetve ali ostane na polju, postane del talne semenske banke, kjer je nato izpostavljeno različnim vplivom v tleh, ki pogojujejo naslednja stanja semena: neposredna kalitev, doba dormance (mirovanja), ki ji sledi kalitev, datiranje, napad mikrobov in propad semena.

2.1 Dormantnost semena vrste *B. napus* L.

Dormantnost semena je opredeljena kot nesposobnost kalitve semena, ki je sicer sposobno za življenje, vendar pod določenimi optimalnimi pogoji. Ločimo primarno in sekundarno dormanco. Slednja je bila dokazana na evropskih genotipih vrste *B. napus* (Schlink, 1994). Primarna dormanca opredeljuje stanje semena, kjer je kalitev potomcev onemogočena, vse dokler seme ne dozori na starševski rastlini in še nekaj časa po tem, ko je ločeno od matične rastline. Obdobje do popolne razvitosti semena (fiziološki zrelosti) je pogosto skrajšano s primarno dormanco. Sekundarna dormanca pa je opredeljena kot zmanjšanje kalivosti semena, ki se razvije po raztrositvi in lahko v določenih primerih prednostno vpliva na hitrejše skrajšanje primarne dormance (Baskin in Baskin, 1998).

Dormantnost pri vrsti *B. napus* je v glavnem inducirana prek določenih temperaturnih in vodnih razmer. Inducirana ali sekundarna dormanca po podatkih raziskav ni pogojena z izpostavljenostjo semena normalnim temperaturam od 15 °C do 20 °C (Schlink, 1994). Na podlagi študij, ki so jih izvedli Perkun in sod. (1997 in 1998), Marshall in sod. (2000) ter Squire (1997, 1999), je razvidno, da so za večino vrst razmere, ki inducirajo dormanco, predvsem nizke temperature, sušne razmere ali tema. Iz navedenega izhaja trditev, da je pojavnost podivjanih populacij odvisna tudi od genetskih preddispozicij semena in ne samo od razmer *in vivo*.

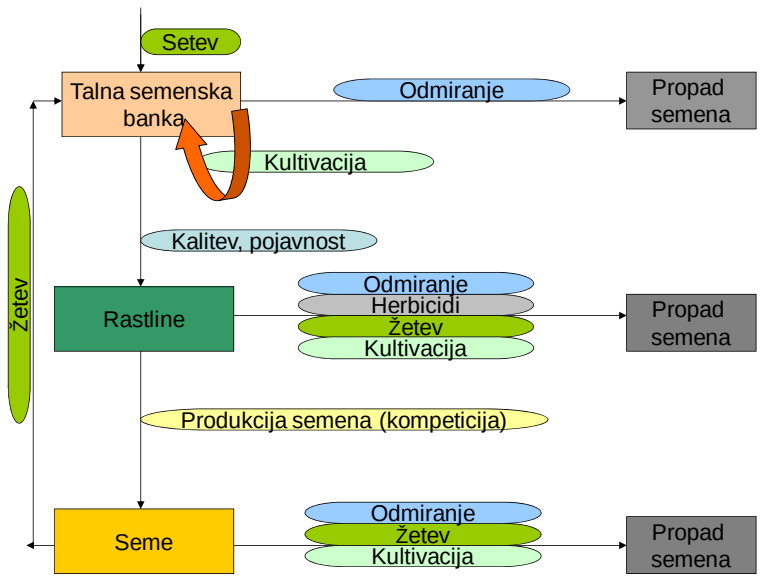
Podrazdelitev sistema dormantnosti semena je opredeljena s stopnjo dormance, pri čemer sta tako primarna kot sekundarna dormanca lahko pogojni (inducirani zaradi specifičnih okoljskih dejavnikov) ali prirojeni (genetsko določeni) (Baskin in Baskin, 1985). Za razliko od nedormantnega semena pogojno dormantno seme kali pod vplivom omejenega števila specifičnih optimalnih dejavnikov, medtem ko seme s prirojeno dormanco ne kali v nobenih okoljskih razmerah, četudi so še tako optimalne. Baskin in Baskin (1985) navajata, da lahko seme v tleh prehaja iz stanja nedormance v stanje pogojne sekundarne dormance, iz nje v naravno (prirojeno) sekundarno dormanco in nato nazaj v nedormantno stanje za kratko obdobje znotraj enega leta. Ta krog se lahko ponavlja iz leta v leto v katerem koli semenu v talni semenski banki in služi kot blažilni mehanizem proti hitri genetski adaptaciji (genetski zastanek), s čimer se prepreči prilagoditev na specifične razmere v kratkih obdobjih vegetacije enoletnih rastlin. Sinhronizacija tega cikla je pod velikim vplivom temperature (Probert, 2000), življenjski krog pa je odvisen tudi od sorte (jara, ozimna).

Poročil o primarni dormanci pri vrsti *B. napus* je kar nekaj, vendar so zaključki pri vseh zelo dvomljivi in nedorečeni. Majhna kalivost pri ozimni in jari obliki obstaja izjemoma med dozorevanjem semena ter pada z njegovo naraščajočo zrelostjo (Schlink, 1994). Primarna dormanca pri ozimni obliki je med 10 in 20 % (Perkun in Lutman, 1998), pri nekaterih genotipih pa je med osmimi in dvanajstimi tedni po cvetenju lahko zastopana tudi v 60 % semena. Po žetvi primarna dormanca ni več prisotna (Schlink, 1994). Četudi ima primarna dormanca malenkostno vlogo pri celotno dozorelem semenu, seme vrste *B. napus* lahko razvije tudi sekundarno dormanco.

2.2 Samoohranjanje semen vrste *B. napus* v tleh

Seme, ki je prisotno v tleh, lahko kali, lahko postane hrana talnim organizmom ali pa ga začnejo razgrajevati saprofitni organizmi, lahko pa tudi samo od sebe propade. Veliko poskusov so na temo sposobnosti ohranjanja v tleh in kalitve teh semen izvedli pri rodu *Brassica* ter tudi pri ostalih sorodnikih vrste *B. napus* iz družine Brassicaceae, kjer so seme vrste *B. napus* posejali po različnih setvenih metodah, vendar se kljub temu, da so semena kalila, rastlinice niso pojavile na površini (semena niso prodrla skozi površinsko plast šote). Seme vrste *B. napus* ostane v tleh kalivo tudi do 16 let, v nekaterih primerih pa lahko le eno leto. Ko so primerjali kalivost gensko spremenjenega in gensko nespremenjenega semena, so ugotovili le majhno razliko v njegovi dolgoživosti. Spolno kompatibilni sorodniki, natančneje vrsti navadna repa (*B. rapa* L.) in vrsta *Hirschfelda incana* (L.) Lagr.-Foss., pa sta v glavnem zastopani v večjih odstotkih kot ostali. Na podlagi zgornjih ugotovitev Squire in sod., (2003) navajajo, da večina semena rastlin iz rodu *Brassica,* ki se nahaja v talni semenski banki, hitro propade. Le manjši del semen (manj kot 1 %) v tleh postane dormanten in živ ter sposoben kalitve več let, še posebej, če se nahaja na globini 15–20 cm. Dolgoživost je odvisna od sorte in razmer v tleh.

Ob primernem času, ko rastline začnejo semeniti in se življenjski krog zaključuje, se zaradi stresanja semen iz luskov samosevnih rastlin začne tudi talna semenska banka dopolnjevati in obnavljati s semeni samosevne vrste *B. napus* v tekočem letu. V vzhodni Kanadi, kjer posamezne samosevne rastline preživijo zimo, lahko obogatijo talno semensko banko tudi z do 3000 semeni (Simard in sod., 2002). Tako se življenjski krog dokončno zaključi s sproščanjem semen v tla, s tem pa se razširi dolžina življenjskega kroga, ki vključuje tudi izčrpavanje in dopolnitev semena v tleh iz leta v leto. Če je življenjski krog končan, preden je v celoti izčrpan potencial talne semenske banke, obseg podivjanih (samoohranjenih) populacij naraste (Stump in Westra, 2000) (slika 1). Prisotnost podivjane ozimne oblike (rastline izven pridelovalnih površin) je bila proučevana tudi v več državah Evrope. Po navedbah Crawleyja in Browna (1995) je prisotnost ozimne oblike v Veliki Britaniji majhna in te populacije v zadnjih nekaj letih izginjajo. Avtorja med drugim navajata, da so transgene linije manj trdovratne od netransgenih. V Franciji podivjane ozimne oblike ostanejo v tleh ob transportnih poteh kalive tudi do osem let (Pessel in sod., 2001). Shema na sliki 1 prikazuje povezave med fazami v življenjskem krogu (zgornji del sheme) ter med življenjsko zgodovino in pridelovalnim procesom (nižji del sheme).



Slika 1: Strukturni model ohranjanja semena v talni semenski banki (Squire in sod., 2003)

Figure 1: Structural model of self-recruited seed in soil seed bank (Squire et al., 2003)

Prvotne poti vstopa semen v talno semensko banko so bile prek izgub semena ob žetvi, o čemer je znanega veliko, vendar zelo specifično usmerjenega znanja, ki le redko zajema izgube ob žetvi sami (naravno izgubljeno seme in strojne izgube). V Veliki Britaniji neposredne izgube ob žetvi ozimne oblike v optimalnih razmerah spravila znašajo 2–5 % pridelka, v primeru neugodnih razmer pa lahko ob spravilu pride tudi do 50-odstotnih izgub (Price in sod., 1996). Nekatere študije navajajo, da je časovno usklajevanje žetvenih dejavnosti s stališča minimaliziranja izgub pomembnejše kot žetvena tehnika (Price in sod., 1996). Zhu in sod. (2012) so ugotovili, da se kar tri četrtine vseh izgub semena vrste *B. napus* zgodi v času žetve in da te izgube znašajo 0,7–1,1 % mase celotnega pridelka. To seme nato preide v talno semensko banko znotraj pridelovalne površine. Dokazali so, da če je nato ta pridelovalna površina kakor koli obdelana, da se niti v treh mesecih po vnosu v tla samosevne rastline ne pojavijo. Prav tako so ugotovili, da globina nahajanja semena v tleh ne vpliva na kalitev (Zhu in sod., 2012).

2.3 Dejavniki, ki vplivajo na kakovost semen vrste *B. napus* v tleh

Splošno znano dejstvo je, da različne kombinacije kompleksnih dejavnikov vplivajo na kalitev semena v tleh. Prepletajo se vplivi lastnosti tal, specifične talne razmere in klimatski dejavniki, prisotnost rastlinskih hormonov v semenu ter agrotehnični ukrepi. Tako veliko število vplivov pa potencira možnosti napovedi pojavnosti rastlin, ki izvirajo iz talne semenske banke. Sinergistični in anatgonistični vplivi kombinacij dejavnikov iz različnih okolij biosfere vključujejo nepredvidljivo dinamiko pojavljanja rastlin iz talne semenske banke. V osnovi pa so zgoraj našteti dejavniki v naravnem okolju spremenljivi, njihov vpliv pa je odvisen tudi od genotipa semena v talni semenski banki in od njegovih bioloških lastnosti.

Na podlagi navedb López-Granadosa in Lutmana (1998) je znano, da tekstura tal pomembno vpliva na prisotnost in dolgoživost semen v tleh. V muljasti ilovici je namreč tendenca semen ozimnih genotipov za obstoj večja kot na peščenih tleh (podatki so povzeti iz študije, opravljene v Veliki Britaniji). Na indukcijo sekundarne dormance in s tem na skrajšanje prisotnosti semen v tleh (hitrejša kalitev) vplivajo različni talni dejavniki. Pri poskusih so kombinacije teme in majhnih koncentracij kisika (3 % kisika in 97 % dušika) inducirale sekundarno dormanco, vendar v manjši meri kot vpliv kombinacije osmotskega stresa in teme (Momoh, 2002).

Raziskave potekajo tudi na področju vpliva temperature na kalitev semen v tleh. Na podlagi temperaturne razlike med začetno temperaturo za sekundarno dormanco in testno temperaturo, ki je bila potrebna za kalitev, so proučevali razvoj sekundarne dormance. Ugotovili so, da čim večja je bila absolutna temperaturna razlika, manj je vplivala na sekundarno dormanco semena v tleh (Momoh, 2002). Pri ozimni obliki je sicer potrebno obdobje nizkih temperatur za nemoten potek kasnejših fenofaz (cvetenja), vendar v času kalitve semena nizke temperature sicer inducirajo kalitev, a imajo take rastline zelo šibek rastni vigor (Larsen in sod., 1998). Ugotavljali so tudi vplive konstantnega osvetljevanja z bliskavico fotoaparata. S pomočjo konstantne svetlobe dormantnega semena so dosegli skupno 98,1-odstotno kalivost teh semen (Schlink, 1994). Izpostavljenost beli svetlobi pri nizkih temperaturah in v kombinaciji z vodnim stresom pa naj bi po podatkih Schlinka (1995) inhibirala kalitev semen v tleh.

Za biosintezo posameznih rastlinskih hormonov, ki so vključeni v regulacijo procesov v dormantnih stanjih semena, stojijo kompleksne encimske reakcije. Perkun in sod. (1998) so uporabili eksogeno giberelinsko kislino (0,2 mg/l) in dokazali, da je njena prisotnost nasprotujoča sekundarni dormanci semena. Tudi abscizinska kislina (ABA) je bila pri rastlinah dokazana kot pomemben element različnih odzivov rastline v različnih stanjih, tudi v dormanci. Poskusi so vključevali aplikacijo eksogenih hormonov ABA in giberelinov na seme, kar je posledično privedlo do antagonističnega učinka med tema dvema rastnima rastlinskima hormonoma. Velika razmerja ABA–giberelini pomenijo pospeševanje dormance, majhna razmerja teh dveh rastnih hormonov pa vodijo v končno kalitev semena (Wareing in Saunders, 1971). Vloga ABA-hormona pri dormanci semena je bila dokazana pri različnih rastlinskih vrstah, fluridon, znan kot biosintezni

inhibitor ABA, pa tako kot eksogena giberelinska kislina omogoča učinkovito prekinitev dormantnosti semena (Grappin in sod., 2000). Za ABA je znano, da je največja koncentracija tega hormona v semenu prav v času njegovega dozorevanja, zmanjševati pa se začne z izsuševanjem semena (Juricic in sod., 1995).

Zasip semena v tla prav tako bogati talno semensko banko vrste *B. napus*, enako pa velja tudi za plevelne rastline. Rezultati raziskave so pokazali, da je bilo na zemljiščih, ki so jih takoj po žetvi preorali, naslednje leto vzkaljenih kar 30 % rastlin vrste *B. napus* iz talne semenske banke, kjer je seme prezimilo. Samo 0,1 % rastlin pa je naslednje leto vzklilo na zemljiščih, kjer po žetvi niso obdelali tal, temveč so izgubljeno seme pustili na površini tal (Perkun in Lutman, 1998). Gruber in sod. (2010) so ugotovili, da je bolj kot globina obdelave tal pomemben termin obdelave.

Na podlagi teh ugotovitev lahko trdimo, da je s pomočjo različnih agrotehničnih ukrepov mogoče regulirati obseg talne semenske banke na pridelovalnih površinah. V Sloveniji se v praksi izvaja podoben sistem, kot je opisan. Zlasti v intenzivnem načinu pridelave kmetje takoj po žetvi tal ne obdelajo, temveč pustijo, da izgubljeno seme na površini tal vzkali, s čimer se izrabi potencial izgubljenega semena, in potem te mlade rastline v vegetativni fazi bodisi zaorjejo ali pokosijo za krmo, lahko pa jih uničijo tudi s kemičnimi pripravki (totalni herbicid). S takim načinom preprečijo pojav samosevcev v posevku, ki se bo v naslednjem letu prideloval na isti površini. Z oranjem spreminjamo mikroklimatske dejavnike v rizosferi in s tem vplivamo tudi na semena v talni semenski banki, ki izvirajo predvsem iz plevelnih rastlin. Sprememba trenutnih mikroklimatskih dejavnikov v tleh pa posredno vpliva na kalitev semen. Ob oranju se zaradi obračanja plasti in prezračevanja tal spremeni plinska sestava v rizosferi (CO_2, O_2), prihaja pa tudi do povečane vsebnosti organske snovi, zaradi česar se spremenijo tudi procesi v tleh. Ob vsem tem se spreminja tudi temperatura (Probert, 2000).

3 SIMULACIJSKI MODELI DINAMIKE POJAVLJANJA RASTLIN VRSTE *B. NAPUS* IZ TALNE SEMENSKE BANKE

Napovedovanje dinamike pojavljanja tako samosevnih kot podivjanih rastlin vrste *B. napus* predstavlja nadgradnjo in povezovanje vsega dosedanjega znanja o genetskih, bioloških, agrotehničnih, klimatoloških, pedoloških in geografskih področjih, ki neposredno in posredno vplivajo na ohranjanje semena v naravnih in polnaravnih habitatih. Zaradi možnosti uvajanja gensko spremenjenih rastlin so se začele oblikovati povezane skupine strokovnjakov iz vseh omenjenih področij, saj želijo vse te dejavnike vključiti v model, ki bo s pomočjo dejanskih podatkov s terena poskušal napovedati kratkoročno in dolgoročno pojavnost rastlin vrste *B. napus* v prostoru. Ti modeli pa bodo z dopolnjenimi podatki karakteristik transgenih rastlin uporabni tudi v primeru soobstoja gensko spremenjene in gensko nespremenjen pridelave v določenem prostoru.

Garnier in Lecomte (2006) sta razvila invazivni model, ki združuje stopnjo strukturne dinamike pojavnosti rastlin (prek tranzitnih poti) z izgubo semena (raztrošeno zrnje), kar omogoča osnovo za strukturno integrativno stopnjo različnih modelov, ki sta jih razvila Neubert in Caswell (2000). Vsekakor se ob tem pojavi potreba po simulaciji pojavnosti rastlin vrste *B. napus* v naravi ter po vrednotenju njene invazivnosti na prostorski in časovni ravni. Middelhoff in Breckling (2003) sta razvila model na individualni ravni (angl. Generic Transgene Movement and Persistence – GeneTraMP), ki omogoča natančno spremljanje pojavnosti rastlin v relaciji s pridelovalnimi površinami. Model vključuje obstoječe znanje, ki temelji na bioloških temeljih, ter prenos genov iz transgenih rastlin v prostoru in času. Begg in sod. (2007) so razvili model za pojavnost obstoječih genskih dogodkov pri vrsti *B. napus*. Ta model je vključeval vplive demografskih in agronomskih dejavnikov na prostorski ravni, temeljil pa je na predhodnem modelu, ki so ga razvili Begg in sod. (2006), ter predstavlja pomembno odkritje med dosedanjimi modeli, ki vključujejo poenostavljeno obravnavanje genetskih karakteristik transgenih rastlin in ne vključujejo prostorske heterogenije (Begg in sod., 2006; Colbach in sod., 2001a, b; Pekrun in Lutman, 2005).

Colbach in sod. (2001a in 2001b) so razvili model GeneSys za ugotavljanje vpliva kmetijske prakse na pretok genov iz posevkov gensko spremenjenih rastlin, odpornih na herbicid, na samosevce v naslednjih letih pridelave. Podrobno so opisali prenos peloda na majhnih parcelah in časovno ovrednotili pojavnost samosevnih populacij na polju. Ta model so aplicirali tudi na razmere pridelave na Danskem v primeru soobstoja gensko spremenjene in gensko nespremenjene pridelave vrste *B. napus* (Østergard in Colbach, 2006). V model so vključili spremenljivke, kot so kolobar, tehnologija pridelave, sortne značilnosti, klimatski podatki, oblika polja, razdalje prenosa cvetnega prahu, ter izračunali število in genotipsko zgradbo v talni semenski banki za posamezno fenofazo razvoja vrste *B. napus* iz genske banke ter napovedali pojavnost samosevnih rastlin v prihodnjih letih za različne sisteme pridelave (ekološko, konvencionalno). Tudi Debeljak in sod. (2011) so v svoji študiji proučevali časovno pojavnost samosevnih rastlin vrste *B. napus* v posevkih glede na različne agrotehnične ukrepe znotraj agroekosistema. Glede na rezultate spremljanja so izdelali sheme, ki vključujejo tudi okoljske dejavnike in prek katerih je mogoče predvideti pojavnost plevelnih (samosevnih) rastlin znotraj pridelovalnih površin.

Zaenkrat še ni povsem jasno, ali so modeli, ki so jih uporabili za prikaz podatkov, neustrezni ali podatki niso pravilni ali pa še vseeno niso zajeli tistih dejavnikov, ki zares vplivajo na kalitev semena vrste *B. napus* iz talne semenske banke. Ugotavljajo namreč, da naj bi prisotnost čisto naključnih sprememb na dani mikrolokaciji vplivala na kalitev v talni semenski banki (npr. zaradi prometne nesreče ob cesti je prišlo do degradacije rastlinskega pokrova in razgolitve tal). Predvidevajo, da se rastline vrste *B. napus* pojavijo predvsem na tistih rastiščih, katerih površina je v času pričetka kalitve gola in na njej ni rastlinskega pokrova, saj naj bi bila vrsta *B. napus* v kalitvenem obdobju zelo slabo kompatibilna z ostalimi semeni v talni semenski banki. Na območjih, na primer ob cesti, kjer je rasla podivjana oblika in semenila, se je seme otreslo, padlo na tla in ostalo v talni semenski banki, ni za pričakovati, da bo to seme zopet kalilo v prihodnjem letu. Raziskave kažejo, da to seme sicer ostane v talni semenski banki, vendar pa načeloma v prihodnjem letu ne kali zaradi neznanih vzrokov (Debeljak in sod., 2008).

Zaradi potrebe po proučevanju in upravljanju okoljskih procesov poskušajo podatke, ki te procese opisujejo, formalizirati v obliki modelov, s katerimi proučujejo povezave med elementi modela kot tudi njegovo obnašanje v daljšem časovnem obdobju v (Colbach in sod., 2012).

4 ZAKLJUČEK

Poglavitna vloga talne semenske banke se kaže predvsem v njenem potencialu za ohranjanje semena vrste *B. napus* v različnih habitatih pridelovalnega prostora. Vznik semena vrste *B. napus* iz tal je težko predvideti, saj je dinamika pojavljanja rastlin te vrste tako časovno kot tudi prostorsko nepredvidljiva in posledično vpliva na opraševalne odnose znotraj pridelovalnega prostora ob sobivanju različnih sistemov pridelave, samoohranjanje semena v tleh skozi generacije pa na njeno genetsko strukturo. Zato je nadvse pomembno omejiti nenadzorovane izgube semena predvsem ob žetvi in transportu (tranzitu), saj je tako mogoče preprečiti dodaten vnos in kontinuirano obnavljanje prisotnosti semena vrste *B. napus* v tleh znotraj in zunaj pridelovalnih površin.

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6 VIRI

Baskin C. C., Baskin J. M. 1998. Seeds, ecology, biogeography, and evolution of dormancy and germination. San Diego, Academic Press: 395 str.

Baskin J. M., Baskin C. C. 1985. The annual dormancy cycle in buried weed seeds: A continuum. BioScience, 35: 492–498

Batič F. 2009. Pomen in trajnost talnih semenskih bank v različnih tipih habitatov. Katedra za aplikativno botaniko, ekologijo in fiziologijo rastlin, Oddelek za agronomijo, Biotehniška fakulteta: 4 str.
http://web.bf.uni-lj.si/ag/botanika/Diplome.html (junij 2013)

Begg G. S., Elliott M. J., Copeland J., Squire G. R. 2007.Sources of uncertainty in the quantification of genetically modified oilseed rape contamination in seed lots. Transgenic Research, 16: 51–63

Begg G. S., Hockaday S., McNicol J. W., Askew M., Squire G. R. 2006. Modelling the persistence of volunteer oilseed rape (*Brassica napus*). Ecological Modelling, 198: 195–207

Colbach N., Clermont-Dauphin C., Meynard J. M. 2001a. GeneSys: a model of the influence of cropping system on gene escape from herbicide tolerant rapeseed crops to rape volunteers. II. Genetic exchanges among volunteer and cropped populations in a small region. Agriculture, Ecosystems and Enironment, 83: 255–270

Colbach N., Clermont-Dauphin C., Meynard J. M. 2001b. GeneSys: a model of the influence of cropping system on gene escape from herbicide tolerant rapeseed crops to rape volunteers. I.Temporal evolution of a population of rapeseed volunteers in a field. Agriculture, Ecosystems and Enironment, 83: 235–253

Colbach N., Granger S., Mézière D. 2012. Using a sensitivity analysis of a weed dynamics model to develop sustainable cropping systems. II. Long-term effect of past crops and management techniques on weed infestation. The Journal of Agricultural Science, CJO doi:10.1017/S0021859612000160

Crawley M. J., Brown S. L. 1995. Seed limitation and the dynamics of feral oilseed rape on the M25 motorway. Proceedings of Royal Society of London, 259: 49–54

Csontos P. 2007. Seed banks: ecological definitions and sampling considerations. Community Ecology, 8, 1: 75–85

Debeljak M., Squire G., Demšar D., Young M. W., Džeroski S. 2008. Relations between the oilseed rape volunteer seedbank, and soil factors, weed functional groups and geographical location in the UK. Ecological Modelling, 212: 138–146

Debeljak M., Squire G., Kocev D., Hawes C., Young M. W., Džeroski S. 2011. Analysis of time series data on agroecosystem vegetation using predictive clustering trees. Ecological Modelling, 222, 14: 2524–2529

Friedt W., Snowdon R. 2009. Oil crops, Handbook of Plant Breeding 4. V: Oilseed rape. Vollman J., Rajcan I. (eds.). Giessen, Springer Science+Business Media: 91–126

Garnier A., Lecomte J. 2006. Using a spatial and stage-structured invasion model to assess the spread of feral populations of transgenic oilseed rape. Ecological Modelling, 194: 141–149

Grappin P., Bouinot D., Sotta B., Miginiac E., Jullien M. 2000. Control of seed dormancy in *Nicotiana plumbaginifolia*: Post-imbibition abscisic acid synthesis imposes dormancy maintenance. Planta, 210: 279–285

Gruber S., Bühler A., Möhring J., Claupein W. 2010. Sleepers in the soil-Vertical distribution by tillage and long-term survival of oilseed rape seeds compared with plastic pellets. European Journal of Agronomy, 33: 81–88
http://www.infogm.org/spip.php?page=imprimer&id_article=1310 (september 2010)

Juricic S., Orlando S., Lepage-Degivry M. T. 1995. Genetic and ontogenic changes in sensitivity to abscisic acid in *Brassica napus* seeds. Plant Physiology and Biochemistry, 33: 593–598

Kocjan Ačko D. 1999. Pozabljene poljščine. Ljubljana, Založba Kmečki glas: 187 str.

Larsen S. U., Povlsen F. V., Eriksen E. N., Pedersen H. C. 1998. The influence of seed vigour on field performance and the evaluation of the applicability of the controlled deterioration vigour test in oilseed rape (*Brassica napus*) and pea (*Pisum sativum*). Seed Science Technology, 26: 627–641

López-Granados F., Lutman P. J. W. 1998 Effect of environmental conditions on the dormancy and germination of volunteer oilseed rape seed (*Brassica napus*). Weed Science, 46: 419–423

Marshall B., Dunlop G., Ramsay G., Squire G. R. 2000. Temperature-dependent germination traits in oilseed rape associated with 5.-anchored simple sequence repeat PCR polymorphisms. Journal of Experimental Botany, 51, 353: 2075–2084

Middelhoff U., Breckling B. 2003. Modelling population interactions and dispersal of oilseed rape (*Brassica napus* L., *Brassicceae*). Verhandlungen der Gesellschaft für Ökologie 33, 273

Momoh E. J. J., Zhou W. J., Kristiansson B. 2002. Variation in the development of secondary seed dormancy in oilseed rape genotypes under conditions of stress. Weed Research, 42: 446–455

Neubert M. G., Caswell H. 2000. Demography and dispersal: calculation and sensitivity analysis of invasion speed for structured populations. Ecology, 81: 1613–1628

Østergard H., Colbach N. 2006. Simulation of gene flow using GeneSys under Danish conditions for oilseed rape co-existence. Montpellier, Agropolis Productions: 2 str.

Perkun C., Hewitt J. D. J., Lutman P. J. W. 1998. Cultural control of volunteer oilseed rape (*Brassica napus*). Journal of Agricultural Science, 130: 155–163

Perkun C., Hewitt J. D. J., Lutman P. J. W. 1998. Cultural control of volunteer oilseed rape (*Brassica napus*). Journal of Agricultural Science, 130: 155–163

Perkun C., Lane P. W., Lutman P. J. W. 2005. Modelling seed bank dynamics of volunteer oilseed rape (*Brassica napus*). Agricultural Systems, 84: 1–20

Perkun C., Lutman P. J. W., Baeumer K. 1997. Induction of secondary dormancy in rape seeds by prolonged imbibition under conditions of water stress or oxygen deficiency in darkness. European Journal of Agronomy, 6: 245–255

Perkun C., Lutman P. J. W. 1998. The influence of post-harvest cultivation on the persistence of volunteer oilseed rape. Aspects of Applied Biology, 51: 113–118

Pessel F. D., Lecomte J., Emeriau V., Krouti M., Messean A., Gouyon P. H. 2001. Persistence of oilseed rape (*Brassica napus* L.) outside of cultivated fields. Theoretical Applied Genetics, 102: 841–846

Pipan B., Šuštar Vozlič J., Meglič V. 2011. Cultivation, varietal structure and possibilities for cross-pollination of *Brassica napus* L. in Slovenia. Acta agriculturae Slovenica, 97, 3: 247–258

Price J. S., Hobson R. N., Neale M. A., Bruce D. M. 1996. Seed loses in commercial harvesting of oilseed rape. Journal of Agricultural Engineering Research, 65: 183–191

Probert R. J. 2000. The role of temperature in the regulation of seed dormancy and germination. V: Seeds: The ecology of regeneration in plant communities, 2nd edition. Fenner M. (ed.). New York, CABI Publishers: 42 str.

Schlink S. 1994. Ökologie der Keimung und Dormanz von Körnerraps (*Brassica napus* L.) und ihre Bedeutung für eine Überdauerung der Samen im Boden. Dissertationes Botanicae, 222

Schlink S. 1995. Überdauerungsvermögen und Dormanz von Rapssamen (*Brassica napus* L.) im Boden.V: Challenges for Weed Science in a Changing Europe. Budapest, European Weed Research Society: 65–72

Simard M. J., Légère A., Pageau D., Lajeunesse J., Warwick S. I. 2002. The frequency and persistence of volunteer canola (*Brassica napus*) in Québec cropping systems. Weed Technology, 16: 433–439

Snowdon R., Lühs W., Friedt W. 2007. Genome Mapping and Molecular Breeding in Plants. V: Oilseeds, Volume 2. Kole C. (ed.). Berlin, Springer-Verlag: 55–11

Squire G. R. 1999. Temperature and heterogeneity of emergence time in oilseed rape. Annals of Applied Biology, 135: 439–447

Squire G. R., Begg G. S., Askew M. 2003. The potential for oilseed rape feral (volunteer) weeds to cause impurities in later oilseed rape crops. London, Department of Environment, Food and Rural Affairs: 2 str.

Squire G. R., Marshall B., Dunlop G., Wright G. 1997. Genetic basis of rate-temperature characteristics for germination in oilseed rape. Journal of Experimental Botany, 48: 869–875

Strokovni predlog za zavarovanje Krajinskega parka Dragonja. 2009. Piran, Zavod RS za varstvo narave: 60 str.

Stump W. L., Westra P. 2000. The seedbank dynamics of feral rye (*Secale cereale*). Weed Technology, 14: 7–14

Treu R., Emberlin J. 2000. Pollen dispersal of the crops Maize (*Zea mays*), Oilseed rape (*Brassica napus*), Potatoes (*Solanum tuberosum*), Sugar beet (*Beta vulgaris*) and Wheat (*Triticum aestivum*). Bristol, University College, Soil Association: 54 str.

Wareing P. F., Saunders P. F. 1971. Hormones and dormancy. Annual Reviews of Plant Physiology, 22: 261–288

Zhu Y. M., Li Y. D., Colbach N., Ma K. P., Wei W., Mi X. C. 2012. Seed losses at harvest and seed persistence of oilseed rape (*Brassica napus*) in different cultural cinditions in Chinese farming systems. Weed Research, 52: 317–326

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Možnosti varstva oreha (*Juglans* spp.) pred orehovo muho (*Rhagoletis completa* Cresson, 1929 Diptera,: Tephritidae) s poudarkom na biotičnem zatiranju škodljivca

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IZVLEČEK

Orehova muha (*Rhagoletis completa*) je gospodarsko pomembna sadna muha, ki napada različne vrste oreha (*Juglans* spp.). Žuželka izvira iz Severne Amerike, za najbolj učinkovito metodo lovljenja njenih odraslih osebkov pa velja trikotna rumena lepljiva plošča, skupaj z amonijevim karbonatom, ki deluje kot atraktant. V prispevku so predstavljeni bionomija, razširjenost, načini spremljanja in zatiranja orehove muhe, pri čemer je poseben poudarek namenjen biotičnemu zatiranju škodljivca. O slednjem je v strokovni literaturi relativno malo informacij, z njihovim upoštevanjem, upoštevanjem domače zakonodaje in našimi izkušnjami z razširjenostjo in učinkovitostjo različnih biotičnih agensov v Sloveniji, za biotično zatiranje orehove muhe predlagamo foliarni nanos entomopatogene glive *Beauveria bassiana* proti odraslim osebkom, jesensko talno aplikacijo entomopatogenih ogorčic proti ličinkam ter spomladansko talno aplikacijo entomopatogenih ogorčic proti odraslim osebkom v obdobju njihovega izleganja iz bub. Na območjih razširjenosti navadnega oreha pa bo potrebno v prihodnje načrtno spremljati zastopanost potencialnih domorodnih parazitoidov orehove muhe, saj na različnih koncih sveta prav nekatere predstavnike iz omenjene skupine naravnih sovražnikov (*Coptera occidentalis, Diachasmimorpha juglandis*) omenjajo kot dovolj ustrezne alternative sintetičnim insekticidom.

Ključne bedsede: orehova muha, *Rhagoletis completa*, parazitoidi, entomopatogene glive, entomopatogene ogorčice, biotično varstvo

ABSTRACT

POSSIBILITIES OF WALNUTS (*Juglans* spp.) PROTECTION AGAINST WALNUT HUSK FLY (*Rhagoletis completa* Cresson) WITH SPECIAL EMPHASIS ON BIOLOGICAL CONTROL

Walnut husk fly (*Rhagoletis completa*) is an economically important fruit fly, which attacks several species of walnuts (*Juglans* spp). The insect is indigenous to North America, the best method for trapping the walnut husk fly adults is a yellow sticky board with ammonium carbonate as an attractant. In the present paper the bionomics, geographical distribution, methods of monitoring and controlling the walnut husk fly with special emphasis on biological control of the pest are presented. In a scientific literature is a lack of information regarding biological control, however if we take into consideration the foreign researches, Slovenian legislation and our experiences we suggest for biological control of walnut husk fly the foliar application of entomopathogenic fungi *Beauveria bassiana* against adults, soil application of entomopathogenic nematodes against larvae in autumn and spring soil application of entomopathogenic nematodes against adults, when they emerge from pupas. Monitoring of domestic parasitoids of walnut husk fly will be in the future needed in areas where the walnuts are expanded. On different areas of the world several species of parasitoids (*Coptera occidentalis*, *Diachasmimorpha juglandis*) are mentioned as an alternative biological control agents to chemicals.

Key words: walnut husk fly, *Rhagoletis completa*, parasitoids, entomopathogenic fungi, entomopathogenic nematodes, biological control

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1 UVOD

Iz rodu *Rhagoletis* poznamo okoli 60 vrst žuželk, nekatere vrste predstavljajo gospodarsko pomembne rastlinske škodljive organizme. Orehova muha (*Rhagoletis completa* Cresson, 1929; Diptera: Tephritidae) je sadna muha, ki napada navadni ali evropski oreh (*Juglans regia* L.), pa tudi črni oreh (*J. nigra* L. in *J. californica* S. Wats.) (Solar s sod., 2007). Bush (1966) navaja, da se lahko orehova muha pojavlja tudi na breskvi (*Prunus persica* [L.] Stokes), medtem ko Yee in Goughnour (2008) poročata o poškodbah orehove muhe na navadnem glogu (*Crataegus laevigata* [Poir.] DC.). Navadni oreh je zaenkrat edina gostiteljska vrsta orehove muhe v Evropi (Duso in Dal Lago, 2006).

Ličinke (žerke) orehove muhe se hranijo z zeleno lupino orehov. Te vrtajo zavite rove v lupino in tkivo spremenijo v zdrizasto gmoto. Lupina se na napadenem mestu zmehča in počrni, zunanja povrhnjica pa ostane nepoškodovana. Lupina se prilepi na olesenelo luščino, ki počrni in se je ne da očistiti. Napadeni orehi odpadejo ali ostanejo prek zime na drevesu. Pri zgodnjem napadu so prizadeta tudi jedrca, ki potemnijo, se zgrbančijo in postanejo grenka, pogosto tudi plesniva (Solar s sod., 2007).

Žuželka izvira iz južnega in osrednjega dela ZDA ter skrajnega severa Mehike (Duso in Dal Lago, 2006). V Evropi je bila prvič ugotovljena leta 1991 v Švici (Merz, 1991), od koder se je razširila v sosednjo Italijo (Trematerra, 1995). V Sloveniji so jo prvič odkrili v Vipavski dolini leta 1997 (Seljak, 1999), do leta 2011 se je razširila po celotni Sloveniji (Miklavc s sod., 2013). Trenutno je ta škodljivec razširjen tudi v nekaterih ostalih državah območja EPPO (Avstrija, Hrvaška, Madžarska, Francija) (Duso in Dal Lago, 2006). Bionomija škodljivca je dobro preučena (Duso in Dal Lago, 2006). Vrsta je univoltilna (ima en rod na leto), let odraslih osebkov pa je mogoče spramljati med julijem in septembrom (Miklavc s sod., 2009). Škodljivec prezimi v razvojnem stadiju bube v tleh (Chen s sod., 2006), v omenjenem stadiju pa lahko preživi tudi do dve leti (Opp in Zermeno, 2000), kar lahko pripelje do sporadičnega pojavljanja škodljivca v posameznih nasadih.

2 SPREMLJANJE ŠTEVILČNOSTI IN KEMIČNO ZATIRANJE OREHOVE MUHE

Za najbolj učinkovito metodo lovljenja odraslih osebkov orehove muhe velja trikotna rumena lepljiva plošča, skupaj z amonijevim karbonatom, ki deluje kot atraktant (Riedl s sod., 1989; Yokoyama in Miller, 1996). Vzorčenje populacije škodljivca na orehih je priporočljivo po prvem ulovu odraslih osebkov, z namenom, da ugotovimo čas začetka odlaganja jajčec, ki predstavlja najpomembnejše obdobje za zatiranje omenjenega škodljivca (Riedl in Hoying, 1980). Solar in sod. (2007) poročajo, da kritično število za orehovo muho še ni določeno, velja pa, da je tretiranje orehov z insekticidi potrebno, če je bil napad močan v preteklem letu in če se je v tekočem letu na plošče ujelo nekaj muh. Od insekticidov so se v preteklosti za učinkovite izkazali organski fosforjevi estri in piretroidi (Barnes in Ortega, 1959; Madsen in Davis, 1964), v preizkušanju pa so tudi različne kombinacije okolju prijaznejših insekticidov in proteinskih vab (Van Steenwyk in sod., 2003).

Solar in sod. (2007) so preučevali učinkovitost različnih kemičnih pripravkov za zatiranje orehove muhe na prostem. Ugotovili so, da je najboljše delovanje (65 %) pokazal pripravek, katerega aktivno snov sta predstavljala tiakloprid in deltametrin. Omenjeni pripravek je bil nanesen dvakrat na spodnjo tretjino krošnje. Primerljiva učinkovitost (63 %) je bila v poskusu dosežena tudi ob uporabi aktivne snovi spinosad ob dodatku hidroliziranega proteina (atraktant), ki je bila dvakrat nanesena po celotnih krošnjah orehov. Avtorji še ugotavljajo, da se različne sorte orehov med seboj razlikujejo po občutljivosti za napad preučevanega škodljivca (Guillén s sod., 2011). Miklavc in sod. (2009) so preizkušali delovanje treh kemičnih insekticidov (aktivne snovi spinosad, dimetoat in tiakloprid) na dveh sortah oreha ('Novosadski kasni' in 'Franquette'), vendar pa so bili rezultati učinkovitosti zatiranja v primerjavi z raziskavo, ki so jo opravili Solar in sod. (2007), slabši. V isti raziskavi so ugotovili, da se je največ

muh ulovilo na rumeno lepljivo ploščo Rebell® amarillo (Miklavc s sod., 2009).

3 MOŽNOSTI BIOTIČNEGA ZATIRANJA OREHOVE MUHE

3.1 Parazitoidi

Parazitoidna osica *Coptera occidentalis* Muesebeck (Hymenoptera: Diapriidae) je solitarni parazitoid, ki izvira iz Kalifornije (ZDA) in parazitira bube predstavnikov iz rodu *Rhagoletis* (Granchietti in sod., 2012). Muesebeck (1980) poroča, da omenjeni parazitoid parazitira orehovo muho ter vrsto *Rhagoletis cingulata* (Loew). Razen v naravnih gostiteljih pa se lahko razvija tudi v drugih sadnih muhah, kot sta vrsti *R. indifferens* Curran in breskova muha (*Ceratitis capitata* Wiedemann) (Hagen in sod., 1995). Ob koncu 70' let se je masovno namnoževanje parazitoidne osice *C. occidentalis* začelo v Kaliforniji, z namenom zatiranja orehove muhe, v 80' letih je bil prvi masovni izpust tega naravnega sovražnika v okolje (Hagen in sod., 1995). Kljub 30-letnem vnašanju te vrste v naravno okolje, pa je za zdaj njen učinek pri zatiranju orehove muhe nezadovoljiv. Hagen in sod. (1995) poročajo, da je slabši učinek delovanja parazitoidne osice predvsem posledica posebne bionomije vrste, saj gre za solitarnega parazitoida, ki parazitira bube v tleh. Zaradi prenizke koncentracije atraktantov (sinomonov/kairomonov), ki se sproščajo v tleh, priporoča uporabo kemičnih stimulantov, ki bi dodatno aktivirali parazitoida v okolju. Vrsta *C. occidentalis* je bila na Slovaškem sicer vnesena kot biotični agens za zatiranje češnjeve muhe (*Rhagoletis cerasi* L.) (Vallo, 1996).

Parazitoidna osica *Diachasmimorpha juglandis* Muesebeck (Hymenoptera: Braconidae) je solitarni endoparazit, ki parazitira ličinke in bube predstavnikov iz rodu *Rhagoletis* (Henneman s sod., 2002). Henneman in sod. (2002) poročajo, da omenjeni parazitoid poišče svoj plen z zaznavanjem hlapnih komponent, ki izhajajo iz napadenih orehovih plodov, vendar zaenkrat omenjena vrsta še ni vključena v programe biotičnega zatiranja orehove muhe. Sorodna parazitoidna vrsta, *Diachasmimorpha longicaudata* (Ashmead), je bila na Havajih vključena v program biotičnega varstva breskove muhe ter vrste *Bactrocera dorsalis* (Hendell) (Henneman s sod., 2002).

3.2 Entomopatogene glive

Entomopatogeni glivi *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) ter *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales) predstavljata potencialno učinkovita kandidata za zatiranje odraslih osebkov orehove muhe. Številne raziskave so pokazale, da entomopatogeni glivi uspešno zatirata nekatere sorodne vrste iz rodu *Rhagoletis*; češnjevo muho (Daniel in Wyss, 2009) in druge predstavnike sadnih muh, na primer breskovo muho (Castillo s sod., 2000; Dimbi s sod., 2003). Obe vrsti gliv lahko ob talnem nanosu prideta v stik z ličinkami tretje larvalne stopnje (L3), bubami in odraslimi osebki. Yee in Lacey (2005) poročata, da je delovanje entomopatogenih gliv na ličinke in bube omejeno, medtem ko je delovanje na odrasle osebke precej boljše. Daniel in Wyss (2010) sta v svoji raziskavi prišla do podobnih zaključkov, kjer sta preučevala učinkovitost entomopatogene glive *B. bassiana* za zatiranje različnih razvojnih stadijev češnjeve muhe. Učinkovitost zatiranja ličink in bub je v njuni raziskavi znašala med 25 in 30 %, medtem ko je foliarni nanos glive v obdobju leta muhe zmanjšal napadenost plodov kar za 65 %.

3.3 Entomopatogene ogorčice

Entomopatogene ogorčice iz rodov *Steinernema* in *Heterorhabditis* veljajo za učinkovite biotične agense pri zatiranju različnih vrst škodljivih žuželk (Laznik in Trdan, 2011). Yee in Lacey (2003) poročata o uporabi različnih vrst entomopatogenih ogorčic iz rodu *Steinernema* za zatiranje vrste *Rhagoletis indifferens* Curran. Rezultati njune raziskave so pokazali, da so entomopatogene ogorčice iz rodu *Steinernema* učinkoviti biotični agensi za zatiranje ličink (do 80 % smrtnost) in odraslih osebkov (do 50 % smrtnost), medtem ko na bube entomopatogene ogorčice niso pokazale zadovoljive učinkovitosti. Entomopatogene ogorčice lahko s talnim nanosom pridejo v stik z ličinkami tretje larvalne stopnje (L3), bubami in odraslimi osebki. Prvi optimalni termin zatiranja orehove muhe bi tako bilo obdobje, ko se iz bub začnejo masovno izlegati odrasli osebki, njihovo

pojavljanje v sadovnjaku pa bi bilo mogoče opazovati z rumenimi lepljivimi ploščami. Drugo ustrezno obdobje za aplikacijo entomopatogenih ogorčic bi bilo obdobje, ko se ličinke premaknejo v tla, z namenom, da se tam zabubijo.

4 ZAKLJUČKI

Dobrih 15 let po vnosu v Slovenijo je orehova muha pri nas daleč najpomembnejši škodljivec navadnega oreha. Za njeno zatiranje imamo med insekticidi registriran le tiakloprid, ki ga lahko do dvakrat v rastni dobi nanašamo na krošnje, dovoljena pa je tudi njegova uporaba v integrirani pridelavi orehov. Za zmanjšanje populacije in posledično zmanjšanja škode, ki jo orehova muha povzroči na orehih, pa je potrebno izvajati predvsem naslednje agrotehnične ukrepe: redno rez dreves in skrb za dobro osvetlitev krošnje, odstranjevanje in sežiganje počrnelih odpadlih plodov (zlasti če so v lupini še žerke), jesensko ali spomladansko plitvo obdelavo tal pod krošnjami dreves in prekrivanje tal z vrtnarsko tkanino pod krošnjami dreves v obdobju izletanja odraslih osebkov (Olson in Buchner, 2002).

Med biotičnimi agensi, ki bi jih lahko v prihodnje v Sloveniji uporabljali za zatiranje tega vse pomembnejšega škodljivca so entomopatogena gliva *B. bassiana* ter entomopatogene ogorčice *Steinernema feltiae*, *S. carpocpasae*, *S. kraussei* ter *Heterorhabditis bacteriophora*, ki so na Seznamu domorodnih vrst organizmov za namen biotičnega varstva rastlin, s čimer je njihova uporaba pri nas tudi zakonsko dovoljena. Upoštevajoč rezultate dosedanjih tujih raziskav (Yee in Lacey, 2005; Daniel in Wyss, 2010) bi veljalo glivo *B. bassiana* uporabiti zlasti proti odraslim osebkom škodljivca (foliarni nanos), saj talna aplikacija proti ličinkam in bubam ni dala zadovoljivih rezultatov. Entomopatogene ogorčice bi bilo smotrno uporabiti zlasti proti ličinkam (jesenska talna aplikacija), ki z napadenimi orehi padejo na tla, veljalo pa bi poskusiti tudi s spomladansko talno aplikacijo v obdobju izleganja odraslih osebkov, ki so v dosedanjih raziskavah (Yee in Lacey, 2003) pokazali določeno dovzetnost za napad teh pri nas vse bolj pogosto uporabljenih biotičnih agensov. Vsekakor velja na območjih razširjenosti navadnega oreha v prihodnje načrtno spremljati zastopanost potencialnih domorodnih parazitoidov orehove muhe, saj na različnih koncih sveta prav nekatere predstavnike iz omenjene skupine naravnih sovražnikov (*Coptera occidentalis, Diachasmimorpha juglandis*) omenjajo kot dovolj ustrezne alternative uporabi sintetičnih insekticidov.

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6 VIRI

Barnes, M.M., Ortega, J.C. 1959. Experiments with protein hydrolysate bait sprays for control of the walnut husk fly. Journal of Economic Entomology 52: 279-285.

Bush, G.L. 1966. The taxonomy, cytology and evolution of the genus *Rhagoletis* in North America (Diptera: Tephritidae). Bulletin of the Museum of Comparative Zoology 134: 431-562.

Castillo, M.A., Moya, P., Hernándeza, E., Primo-Yúferab, E. 2000. Susceptibility of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) to entomopathogenic fungi and their extracts. Biological Control 19: 274-282.

Chen, Y. H., Opp, S.B., Berlocher, S.H., Roderick, G.K. 2006. Are bottlenecks associated with colonizationj? Genetic diversity and diapause

variation of native and introduced *Rhagoletis completa* population. Oecologia 149: 656-667.

Daniel, C., Wyss, E. 2009. Susceptibility of different life stages of the European cherry fruit fly, *Rhagoletis cerasi*, to entomopathogenic fungi. Journal of Applied Entomology 133: 473-483.

Daniel, C., Wyss, E. 2010. Field applications of *Beauveria bassiana* to control the European cherry fruit fly *Rhagoletis cerasi*. Journal of Applied Entomology 134: 675-681.

Dimbi, S., Maniania, N.K., Lux, S.A., Mueke, J.M. 2003. Host species, age and sex as factors affecting the susceptibility of the African Tephritid fruit fly species, *Ceratitis capitata*, *C. cosyra* and *C. fasciventris* to infection by *Metarhizium anisopliae*. Journal of Pest Science 76: 113-117.

Duso, C., Dal Lago, G. 2006. Life cycle, phenology and economic importance of the walnut husk fly *Rhagoletis completa* Cresson (Diptera: Tephritidae) in northern Italy. Annales de la Société Entomologique de France (n.s.) 42: 245-254.

Granchietti, A., Sacchetti, P., Rosi, M.C., Belcari, A. 2012. Fruit fly larval trail acts as a cue in the host location process of the pupal parasitoid *Coptera occidentalis*. Biological Control 61: 7-17.

Guillén, L., Aluja, M., Rull, J., Höhn, H., Schwizer, T., Samietz, J. 2011. Influence of walnut cultivar on infestation by *Rhagoletis completa*: behavioural and management implications. Entomologia Experimentalis et Applicata 140: 207-217.

Hagen, K.S., Tassan, R.L., Fong, M., Aliniazee, M.T. 1995. Walnut husk fly. In: Biological control in the Western United States (Nechols, J.R., Andres, L.A., Beardsley, J.W., Goeden, R.D., Jackson, C.G. (Eds.). University of California 61: 224-227.

Henneman, M.L., Dyreson, E.G., Takabayashi, J., Raguso, R.A. 2002. Response to walnut olfactory and visual cues by the parasitic wasp *Diachasmimorpha juglandis*. Journal of Chemical Ecology 28: 2221-2244.

Laznik, Ž., Trdan, S. 2011. Entomopathogenic nematodes (Nematoda: Rhabditida) in Slovenia: from tabula rasa to implemantation into crop production systems. In: Perveen, F. (Ed.). Insecticides - advances in integrated pest management. Rijeka, InTech: 627-656.

Madsen, H.F., Davis, C.S. 1964. War on the husk fly. Diamond Walnut News 46: 12-13.

Merz, B. 1994. Diptera: Tephritidae. Insecta. Helvetica Fauna 10: 198.

Miklavc, J., Mešl, M., Matko, B., Solar, A. 2009. Spremljanje sezonske dinamike orehove muhe (*Rhagoletis completa* Cresson) v letu 2008 z rumenimi lepljivimi ploščami in rezultati preizkušanja insekticidov. V: Maček, J. (ur.). Zbornik predavanj in referatov 9. slovenskega posvetovanja o varstvu rastlin z mednarodno udeležbo, Nova Gorica, 4.-5. marec 2009. Ljubljana, Društvo za varstvo rastlin Slovenije: 343-348.

Miklavc, J., Mešl, M., Matko, B., Solar, A., Trdan, S. 2013. Izkušnje z zatiranjem orehove muhe (*Rhagoletis completa* Cresson) v SV Sloveniji v letih 2011 in 2012. V: Maček, J. (ur.). Zbornik predavanj in referatov 11. slovenskega posvetovanja o varstvu rastlin z mednarodno udeležbo, Bled, 5.-6. marec 2011. Ljubljana, Društvo za varstvo rastlin Slovenije: 114-119.

Muesebeck, C.F.W. 1980. The nearctic parasitic wasp of the genera *Psilus* Panzer and *Coptera* Say (Hymenoptera, Proctotrupoidea, Diapriidae). Technical Bulletin, Science and Education Administration, USDA 1617, iv+71.

Olson, W.H., Buchner, R.P. 2002. Leading edge of plant protection for walnuts. Horttechnology 12: 615-618.

Opp, S., Zermeno, J. 2000. Timing and susceptibility of walnut cultivars to walnut husk fly attack-episode 2. In: Walnut research reports 2000. California. Walnut Mktg. Board, Sacramento: 293-295.

Riedl, H., Barnett, W.W., Coates, W.W., Coviello, R., Joos, J., Olson, W.H. 1989. Walnut husk fly (Diptera: Tephritidae): evaluation of traps for timing of control measures and for damage predictions. Journal of Economic Entomology 82: 1191-1196.

Riedl, H., Hoying, S.A. 1980. Seasonal patterns of emergence, flight activity and oviposition of the walnut husk fly in Northern California. Environmental Entomology 9: 567-571.

Seljak, G., 1999. Orehova muha (*Rhagoletis completa* Cresson) – nov nevaren škodljivec orehov v Sloveniji. Revija za sadjarstvo, Vinogradništvo in Vinarstvo 10: 12-15.

Solar, A., Miklavc, J., Seljak, G., Mešl, M., Matis, G., Matko, B., Pliberšek, T. 2007. Prve izkušnje z zatiranjem orehove muhe (*Rhagoletis completa* Cresson) v severovzhodni Sloveniji. V: Maček, J. (ur.). Zbornik predavanj in referatov 8. slovenskega posvetovanja o varstvu rastlin, Radenci, 6.-7. marec 2007. Ljubljana, Društvo za varstvo rastlin Slovenije: 220-224.

Trematerra, P., Paparatti, B., Girgenti, P. 1995. Attenzione alla presenza della mosca delle noci. Informatore agrario 47: 74-76.

Vallo, V. 1996. Comparison of laboratory and natural population of *Coptera occidentalis* (Mues.) (Hymenoptera, Proctrotrupoidea, Diapriidae). In: Proc. Of Symposium, Ecological problems of plant protection and contemporary agriculture. The High Tatras Stará Lesná, 71-72.

Van Steenwyk, R.A., Zolbrod, S.K., Nomoto, R.M., Fernandez, T.K. 2003. Control of walnut husk fly using reduced risk products. http.//ncce.ucdavis.edu/files/filelibrary/1214/25338.

Yee, W.L., Goughnour, R.B. 2008. Host plant use and new host records of apple maggot, western cherry fruit fly, and other *Rhagoletis species* (Diptera: Tephritidae) in western Washington state. The Pan-Pacific Entomologist 84: 179-193.

Yee, W.L., Lacey, L.A. 2003. Stage-specific mortality of *Rhagoletis indifferens* (Diptera: Tephritidae) exposed to three species of *Steinernema* nematodes. Biological Control 27: 349-356.

Yee, W.L., Lacey, L.A. 2005. Mortality of different life stages of *Rhagoletis indifferens* (Diptera: Tephritidae) exposed to the entomopathogenic fungus *Metarhizium anisopliae*. Journal of Entomological Science 40: 167-177.

Yokoyama, V.Y., Miller, G.T. 1996. Response of walnut husk fly (Diptera: Tephritidae) to low temperature, irrigation, and pest-free period for exported stone fruits. Journal of Economic Entomology 89: 1186-1191.

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Tehnike indukcije haploidov in podvojenih haploidov

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IZVLEČEK

Haploidi so samostojne rastline (sporofiti) z gametnim (haploidnim, n) številom kromosomov. Čeprav se spontano v naravi redko pojavijo, so dandanes poznane številne tehnike s katerimi lahko sprožimo njihov nastanek. Indukcija in regeneracija haploidnih (in podvojenih haploidnih) rastlin omogoča pridobivanje popolnoma homozigotnih linij v eni generaciji, kar lahko bistveno pospeši žlahtniteljski proces in genetske študije. Prav zato se haploidi intenzivno uporabljajo pri številnih vrstah za katere so poznani uspešni protokoli kot so pšenica, ječmen, koruza, tobak, čebula, kumara, oljna ogrščica in druge kmetijsko pomembne križnice. Prispevek povzema glavne lastnosti haploidov in podvojenih haploidov, načine njihove indukcije in regeneracije s poudarkom na njihovi uporabnosti v žlahtnjenju rastlin.

Ključne besede: Ginogeneza, androgeneza, mikrospora, homozigotnost, heterozigotnost, obsevan pelod, rastlinske tkivne kulture, ploidnost

ABSTRACT

TECHNIQUES FOR HAPLOID AND DOUBLED HAPLOID PRODUCTION

Haploids are plants (sporophytes) that contain a gametic chromomosome number (n). They rarely occur spontaneously in nature but several techniques are nowadays available for their production. Induction and regeneration of haploids (doubled haploids) enables the production of completely homozygous lines in one generation, thus shortening this process by many years. They are broadly used in breeding programs of plant species for which efficient protocols have been developed, such as barley, wheat, maize, tobacco, onion, cucumber, rapeseed and other *Brassica* species. This article presents the main characteristics of haploids, doubled haploids and inducing techniques, with an emphasis on their role in plant breeding.

Key words: Gynogenesis, androgenesis, microspore, homozygosity, heterozygosity, irradiated pollen, plant tissue culture, ploidy level

1 UVOD

Danes obsega žlahtnjenje rastlin poleg tradicionalnih tehnik kot so selekcija, načrtna medsortna in medvrstna križanja ter inducirane mutacije, tudi sodobnejše - biotehnološke - metode med katere spadajo *in vitro* vzgoja zdravih rastlin, reševanje nedozorelih embrijev težavnih križanj, *in vitro* opraševanje, fuzija protoplastov, *in vitro* mutageneza, poliploidizacija, genske transformacije in proizvodnja podvojenih haploidnih rastlin.

Haploidi in podvojeni haploidi (DH) so se v žlahtnjenju rastlin začeli uporabljati v drugi polovici prejšnjega stoletja pri koruzi (Forster in sod., 2007) in se sedaj uporabljajo za žlahtnjenje številnih kmetijskih rastlin. V zadnjih letih se je raziskovanje indukcije haploidov (in DH) razširilo iz žit in zelenjadnic na okrasne (Bal in Touraev, 2009; Ferrie in Caswell, 2011; Murovec in Bohanec, 2013), aromatične in zdravilne rastline (Ferrie, 2009) in zato je pričakovati, da se bo

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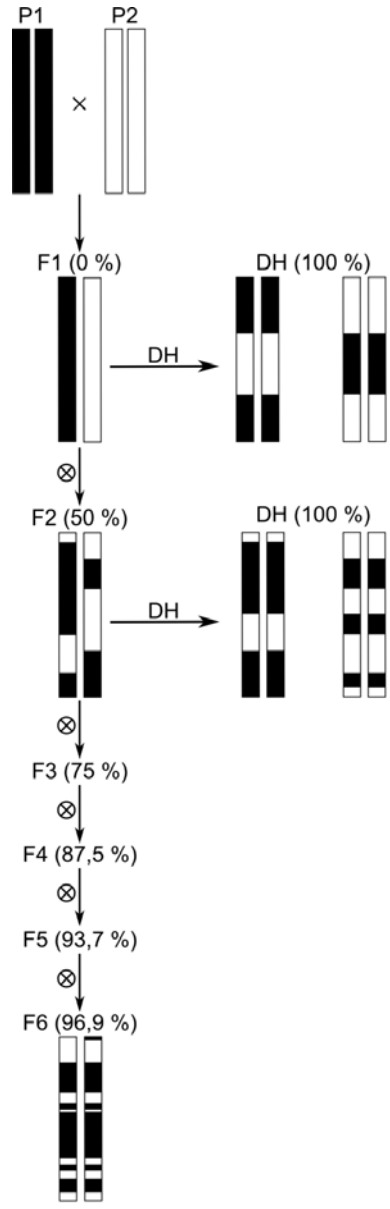
kmalu njihova uporaba razširila tudi na te ekonomsko manj pomembne vrste.

Prednost žlahtnjenja rastlin z uporabo tehnik indukcije haploidov je v hitrejši proizvodnji popolnoma homozigotnih rastlin, ki se pri tujeprašnicah uporabljajo kot starševske linije za pridobivanje hibridov, pri samoprašnicah pa že predstavljajo komercialno linijo. Zaradi enkratnega nastanka popolne homozigotnosti, se takoj izrazijo vsi škodljivi recesivni geni, ki bi pri samoopraševanju povzročali inbriding depresijo. Tako indukcija haploidov služi tudi kot selekcijski pritisk proti škodljivim recesivnim genom. Žlahtnjenje se kakor pri klasičnih metodah začne z izborom in križanjem starševskih rastlin in se nadaljuje z indukcijo haploidov iz različnih generacij, kakor je prikazano na sliki 1. Žlahtnitelji najpogosteje pridobivajo haploidne linije iz F1 generacije, katerih gamete predstavljajo F2 generacijo. Kljub temu mnogi priporočajo kasnejši pričetek pridobivanje haploidnih linij (iz F2 donorskih rastlin), saj je na ta način omogočena še ena mejotska preureditev kromosomov in s tem večja variabilnost. Nekateri žlahtnitelji se celo odločajo za haploidizacijo šele po nekaj letih selekcije in samoopraševanja na polju in s podvojenimi haploidi le stabilizirajo najbolj perspektivne linije. Pridobivanje haploidov iz poliploidnih rastlin (npr. iz tetraploidnega krompirja) omogoča njihovo križanje z diploidnimi divjimi sorodnimi vrstami, s tem prenos zanimivih genov med vrstami in žlahtnjenje na diploidnem nivoju. Pri žlahtnjenju s pomočjo induciranja mutacij na haploidem nivoju je hitrejše in lažje odkrivanje recesivnih sprememb, saj se le-te zaradi homozigotnosti izrazijo takoj in tudi vsakršna mutacija se takoj fiksira v genom. Haploidni protoplasti so idealno orodje za študij genetike somatskih celic, aplikacijo indukcije mutacij (veliko število individualnih genotipov, odsotnost himernosti, izraženost mutiranih genov), za fuzijo protoplastov (nastanek alodiploidnih organizmov namesto alotetraploidnih), prenos DNK (takojšnje izražanje vključenih genov in s tem povezana hitrejša selekcija). Zaradi popolne homozigotnosti in z njo povezane možnosti večkratnega spolnega razmnoževanja preko semen brez segregacije v naslednjih generacijah, so podvojeni haploidi zelo primerni tudi za mapiranje genov in genomske študije. Zaradi popolne homozigotnosti, se v zadnjih letih haploidi uporabljajo tudi za sekvenciranje celotnih genomov kot na primer pri *Citrus clementina* Hort. ex Tan. (Aleza in sod., 2009).



Slika 1: Primerjava pridobivanja homozigotnih linij s samooprševanjem (levo) in s postopki indukcije haploidov (desno). Številke prikazujejo pričakovane odstotke homozigotnosti posameznih generacij.

Figure 1: Comparison between self-pollination (left) and doubled haploid induction for production of homozygous lines. The numbers represent the expected homozygosity of each generation.

2 TEHNIKE INDUKCIJE IN REGENERACIJE HAPLOIDOV (in PODVOJENIH HAPLOIDOV)

Haploidi so samostojne rastline (sporofiti) z gametnim (haploidnim) številom kromosomov. Od odkritja prvega spontanega haploida vrste *Datura stramonium* L. leta 1922 pa do danes, so razvili postopke indukcije haploidov za več kot 250 rastlinskih vrst (Maluszynski in sod., 2003). Najbolj uspešni in široko uporabljeni so protokoli indukcije haploidov pri ječmenu (*Hordeum vulgare* L.), pšenici (*Triticum aestivum* L.), oljni ogrščici (*Brassica napus* L.), tobaku (*Nicotiana tabacum* L.) in koruzi (*Zea mays* L.). Pri teh rastlinskih vrstah intenzivno preučujejo tudi gene odgovorne za prehod iz gametofitnega v sporofitni

razvoj mikrospor (Hosp in sod., 2007; Segui-Simarro in Nuez, 2008).

Na uspeh indukcije haploidov vplivajo:

- genotip in starost matičnih rastlin,
- rastne razmere in pred-tretiranje matičnih rastlin (temperatura, osvetlitev),
- razvojna faza gamet,
- pred-tretiranje gamet (temperaturni in/ali osmotski šok, stradanje mikrospor, obsevanje z gama žarki)
- pH in sestava gojišča (predvsem dodani rastni regulatorji),
- temperatura, osvetlitev in fotoperioda v rastni komori.

Haploidi nastali iz diploidnih rastlin vsebujejo samo eno garnituro kromosomov, so monoploidi (2n=1x), medtem ko jih haploidi nastali iz poliploidnih vrst vsebujejo več. Tako na primer haploid iz tetraploidnega (2n=4x) krompirja vsebuje dve kromosomski garnituri (2n=2x) in ga imenujemo dihaploid. Po istem principu je haploid iz heksaploidnega (2n=6x) kivija triploid (2n=3x) in vsebuje tri garniture kromosomov. Dihaploidi, trihaploidi itd. zaradi večjega števila kromosomskih garnitur niso homozigotni. Pogosto se izraz dihaploid napačno uporablja tako v slovenski kakor v tuji literaturi za označevanje podvojenih haploidov. Le-ti so popolnoma homozigotne diploidne rastline, ki nastanejo iz haploidov po podvajanju števila kromosomov. So končni cilj in uporabni produkt vseh tehnik indukcij haploidov in se kot taki uporabljajo pri žlahtnjenju rastlin in genetskih študijah.

Med postopki androgeneze in ginogeneze lahko pride tudi do spontanega podvajanja kromosomov in se zato poleg haploidov regenerira tudi določen delež spontanih DH. Pojav je pogost pri androgenezi, kjer je delež spontanih DH do 90 % (Maluszynski in sod., 2003) in ga povzroča predvsem fuzija jeder (Sunderland, 1974; Kasha in sod., 2001; Testillano in Risueno, 2009). Med *in vitro* ginogenezo in po različnem opraševanju je spontano podvajanje kromosomov redek pojav in ne presega 10-15 % (Maluszynski in sod., 2003). Najvišji delež spontanih DH med *in vitro* ginogenezo so Alan in sodelavci (2004) odkrili pri čebuli (15 %).

Nizek odstotek induciranih in regeneriranih haploidov oz. DH, odvisnost uspeha od genotipa matičnih rastlin in s tem povezane omejitve pri prenosu tehnik iz modelnih genotipov v komercialno zanimive sorte, ter težavnost podvajanja kromosomov haploidov, še vedno predstavljajo določene ovire pri uporabi DH v žlahtnjenju rastlin, kjer je potrebna hitra proizvodnja fertilnih DH iz vseh zanimivih križancev.

Haploide lahko pridobivamo iz moških gamet (t.i. androgeneza) s pomočjo kulture prašnic ali kulture izoliranih mikrospor in iz ženskih gamet. Nastanek haploidov iz ženskih gamet lahko sprožimo *in vitro* (ginogeneza) ali *in situ* z različnimi vrstami opraševanja.

2.1 *In vitro* indukcija haploidov (ginogeneza)

Značilnost *in vitro* ginogeneze je kultura neoprašenih socvetij, cvetov, plodnic ali njihovih delov na primernem hranilnem gojišču, ki ob ugodnih fizikalnih pogojih sproži nastanek haploidnih rastlin. Za to je v večini primerov potrebna inokulacija nezrelih ženskih gametofitov (Musial in sod., 2001; Gémes-Juhász in sod., 2002), ki, za razliko od mikrospor, v tkivni kulturi dozorijo (Musial in sod., 2005). Večinoma se haploidi regenerirajo iz jajčnih celic (Ferrant in Bouharmont, 1994; Musial in sod., 2005; Thomas, 2004).

Pri večini rastlinskih vrst je metoda manj uspešna od androgeneze, predvsem zaradi majhnega števila semenskih zasnov (potencialnih haploidnih embrijev) na cvet in nizkih odstotkov uspeha. Poleg tega je tudi odstotek spontano podvojenih haploidov bistveno nižji kakor pri androgenezi. Zaradi naštetega se *in vitro* ginogeneza raziskuje predvsem pri vrstah, kjer androgeneza ni bila uspešna, kot so čebula (Bohanec in Jakše, 1999; Alan in sod., 2004), sladkorna pesa (Ferrant in Bouharmont, 1994) in drugih vrstah (Bohanec, 2009). Ginogeneza je edina možnost pridobivanja haploidov iz moško sterilnih linij in ženskih klonov dvodomnih ženskih rastlin.

V žlahtnjenju se metoda *in vitro* ginogeneze uporablja pri vrstah *Gerbera jamesonii* H. Bolus, *Allium* sp., *Beta* sp. (Wedzony in sod., 2009) in kumarah (ustni vir).

2.2 *In situ* indukcija haploidov

2.2.1 Medvrstno opraševanje

In situ indukcijo haploidov iz ženskih gamet lahko sproži opraševanje s pelodom sorodne ali nesorodne vrste (rodu) po katerem pride do oploditve jajčne celice in naknadno, v zgodnji embriogenezi, do izločanja kromosomov opraševalca. Kromosomi opraševalca se pogosto izločijo tudi iz endosperma, zaradi česar le-ta ne omogoča normalne rasti in razvoja embrija. V izogib propadu embrijev jih je v takih primerih potrebno nekaj dni po opraševanju rešiti z gojenjem *in vitro*.

Metodo so odkrili pri križanju ječmena *Hordeum vulgare* L. z divjo sorodno vrsto *H. bulbosum* L. (Kasha in Kao, 1970). Zaradi slabše razvitosti endosperma je 12-15 dni po opraševanju potrebno reševanje embrijev in njihovo gojenje *in vitro*. Tako imenovana bulbosum metoda je še vedno zelo učinkovita metoda pridobivanja haploidov pri ječmenu (Devaux in Kasha, 2009), s katero so požlahtnili že preko 60 sort (Thomas in sod., 2003). Je edina metoda indukcije haploidov pri ječmenu, katere uspeh ni odvisen od genotipa in je uspešna tudi pri kultivarjih, kjer androgeneza ni. Opraševanje s *H. bulbosum* je uspešno sprožilo nastanek haploidnih embrijev tudi pri posameznih genotipih pšenice in tritikale, vendar zaradi inkombatibilnosti metoda ni širše uporabna za druga žita.

Podoben način z oploditvijo in naknadnim izločanjem kromosomov opraševalca deluje po opraševanju ječmena s pelodom koruze. Uspešno indukcijo haploidov so tako dosegli še pri opraševanju pšenice (*Triticum aestivum* L.) (Laurie in Bennett, 1988), ječmena (Furusho, 1991, cit. po Wedzony in sod., 2009), tritikale (x *Triticosecale*) (Wedzony, 2003), ovsa (*Avena sativa* L.) (Rines, 2003) in rži (*Secale cereale* L.) (Deimling in Fleihinghaus-Roux, 1996).

Tudi pri krompirju (*Solanum tuberosum* L., 2n=4x) medvrstno opraševanje s *S. phurea* (2n=2x) povzroča nastanek embrijev z gametnim številom kromosomov. Ker je krompir v osnovi tetraploiden, so nastali embriji dihaploidni (2n=2x), saj vsebujejo dve garnituri kromosomov, in niso homozigotni. Princip delovanja temelji na lastnosti določenih genotipov *S. phurea* pri katerih obe spermalni jedri v pelodnem mešičku oplodita polarni jedri embrionalne vrečke. Tako nastane 6x endosperm, ki omogoča rast in razvoj 2x embrija. Čeprav je odstotek semen z dihaploidnimi embriji relativno nizek, jih je mogoče hitro in enostavno odbrati zaradi homozigotnega dominantnega markerja za vijolične pege semen, ki jih vsebujejo IVP genotipi *S. phurea* (Maine, 2003).

Indukcija haploidov z medvrstnim opraševanjem je bila uspešna tudi pri citrusih, saj je po *in vitro* opraševanju diploidne vrste *Citrus clementina* Hort. Ex Tan. cv. Nules s pelodom triploidne grenivke Oroblanco uspela regeneriracija 14 haploidov (Germanà in Chiancone, 2001).

2.2.2 Opraševanje znotraj vrste z opraševalnimi ('inducer') linijami ali obsevanim pelodom

Poznani sta dve vrsti *in situ* indukcije haploidov iz ženskih gamet, ki temeljita na opraševanju s pelodom iste vrste. Prva metoda je poznana pri koruzi, pri kateri so odkrili opraševalno ('inducer') linijo 'Stock 6', ki je po opraševanju sprožila nastanek do 2,3 % spontanih haploidov (Coe, 1959, cit. po Bohanec, 1994). Linijo so križali s številnimi drugimi linijami in križance uporabili za opraševanje z namenom pridobivanja haploidov. Poročajo, da s sodobnimi opraševalnimi linijami dosegajo že 8-10 % uspeh (Geiger in Gordillo, 2009; Melchinger in sod., 2013). Tako kot pri krompirju, tudi pri opraševanju s koruzo opraševalne linije vsebujejo homozigotni dominantni morfološki marker (za vijolično barvo embrija in alevrona) s pomočjo katerega lahko ločijo haploidne od hibridnih embrijev. Poleg tega ni potrebno *in vitro* reševanje embrijev, saj semena s haploidnimi embriji vsebujejo normalen endosperm. Oboje olajša indukcijo haploidov in povečuje uporabnost tehnike za žlahtnjenje rastlin, saj omogoča izločanje hibridov brez dodatnih laboratorijskih analiz (Zhang in sod., 2008). V raziskavi Barreta in sodelavcev (2008) so odkrili *ggi1* lokus na kromosomu 1, ki je odgovoren za izločanje kromosomov opraševalca.

Druga možnost *in situ* ginogeneze z opraševanjem znotraj vrste je opraševanje z obsevanim pelodom. Metoda je bila uspešna pri številnih rastlinskih vrstah, kar prikazuje preglednica 1.

Preglednica 1: Seznam rastlinskih vrst in virov pri katerih so z opraševanjem z obsevanim pelodom spodbudili nastanek haploidnih rastlin.

Table 1: List of plant species and available publications about haploid induction protocols by pollination with irradiated pollen.

Vrsta	Viri
buče	Kurtar in sod., 2002, 2009; Kurtar and Balkaya, 2010; Košmrlj in sod., 2013
čebula	Dore in Marie, 1993
črni ribez	Naess in sod., 1998
divja češnja	Höfer in Grafe, 2003
hruška	Bouvier in sod., 1993
jablana	Zhang in Lespinasse, 1991; De Witte in Keulemans, 1994; Hofer in Lespinasse, 1996
kivi	Pandey in sod., 1990; Chalak in Legave, 1997; Musial in Przywara, 1998, 1999
kumara	Przyborowski in Niemirowicz-Szczytt, 1994; Faris in sod., 1999; Faris in Niemirowicz-Szczytt, 1999; Claveria in sod., 2005
lubenica	Sari in sod., 1994
mandarina	Froelicher in sod., 2007; Aleza in sod., 2009
melona	Sauton in Dumas de Vaulx, 1987; Cuny in sod., 1993; Katoh in sod., 1993; Lotfi in sod., 2003; Gonzalo in sod., 2011; Godbole in Murthy, 2012
nagelj	Sato in sod., 2000
oreh	Grouh in sod., 2011
krinkar	Murovec in sod., 2013
petunija	Raquin, 1985
pomelo	Yahata in sod., 2010
sliva	Peixe in sod., 2000
sončnica	Todorova in sod., 1997
Nicotiana	Pandey, 1980; Pandey in Phung, 1982
vrtnica	Meynet in sod., 1994

Metoda temelji na lastnosti peloda obsevanega z UV, γ ali X žarki, ki po opraševanju na brazdi normalno kali in pelodni mešiček napreduje skozi vrat pestiča do embrionalne vrečke, kjer se tvori embrij. Ali jedri peloda oplodita jajčno celico in polarni jedri in se kromosomi opraševalca izločijo šele v kasnejši embriogenezi ali pa nastanek haploidnega embrija sproži že sama kalitev peloda, je za enkrat še nedorečeno. Novejše raziskave nakazujejo na možnost, da do oploditve dejansko pride, vendar se kromosomi iz močno obsevanega peloda čez čas izločijo iz jedra (Murovec in Bohanec, 2013). Uspeh metode je, poleg dejavnikov naštetih v drugem poglavju tega prispevka, odvisen od doze obsevanja in razvojne faze embrijev ob reševanju.

Vpliv doze sevanja na preživetje haploidnih embrijev in križancev je prvi preučil Hertwig leta 1911 v svojih poskusih oplojevanja jajčnih celic žab. Po njem so kasneje pojav poimenovali 'Hertwigov efekt'. V svojih poskusih je opazil, da po opraševanju s spermalnimi celicami predhodno obsevanimi z nizkimi dozami, velik odstotek embrijev propade zgodaj v razvoju. Tisti, ki preživijo, pa kažejo različne morfološke spremembe. Opraševanje s spermalnimi celicami obsevanimi z visokimi dozami, povzroči višji odstotek živih embrijev, ki so normalnega fenotipa (Hertwig, 1912 cit. po Pandey in Phung, 1982). Kasneje so pojav temeljito preučili in ugotovili, da so močno obsevane spermalne celice zmožne prodreti v jajčno celico, vendar je niso zmožne oploditi. Kljub temu pa stimulirajo delitev jajčne celice in ginogenetski razvoj embrija. Pri nižjih dozah obsevanja, spermalne celice obdržijo sposobnost oploditve jajčne celice, vendar zaradi obsevanja na hibrida prenesejo mutacije, ki povzročajo nenormalen fenotip ali prezgodnjo smrt (različni avtorji, cit. po Pandey in Phung, 1982). Pandey in Phung (1982) sta 'Hertwigov efekt' prva opazila pri rastlinah med opraševanjem štirih vrst rodu *Nicotiana*. Pri nižjih dozah obsevanja (100-200 Gy) je z višanjem doze padal odstotek pridobljenih sejancev, od katerih jih je veliko kmalu po kalitvi propadlo. Sejanci, ki so preživeli do cvetenja, so bili morfološko različni, niso bili podobni materinim rastlinam in so bili večinoma sterilni. Citološke analize so pokazale, da so bile rastline aneuploidni križanci, ki so vsebovali vse kromosome materine rastline in različno število kromosomov od opraševalne rastline. Z višanjem doze obsevanja peloda nad 500 Gy, se je število aneuploidov manjšalo, večalo pa se je število dihaploidov in podvojenih dihaploidov. Kasneje so vpliv doze sevanja na izid opraševanja preučevali še pri drugih kmetijsko pomembnih rastlinskih vrstah. Tako so tudi pri kumarah (Claveria in sod., 2005), kiviju (Chalak in Legave, 1997; Musial in Przywara, 1998) in orehu (Grouh in sod., 2011), z višanjem doze obsevanja, uspeli regenerirati večje število haploidov v primerjavi z nižjimi dozami. Po drugi strani pa višanje doze močno vpliva na manjšanje števila nastalih plodov (Przyborowski in Niemirowicz-Szczytt, 1994; Kurtar in sod., 2002; Froelicher in sod., 2007; Košmrlj in sod., 2013), manjšanje števila normalno razvitih semen (Cuny in sod., 1993; Przyborowski in Niemirowicz-Szczytt, 1994; Chalak in Legave, 1997; Musial in Przywara, 1998; Sugiyama in Morishita, 2000; Lotfi in sod., 2003; Froelicher in sod., 2007), manjšo kalivost semen (Chalak in Legave, 1997) in na manjše število nastalih embrijev (Grouh in sod., 2011; Košmrlj in sod., 2013).

Opraševanje z obsevanim pelodom je najbolj raziskano pri bučevkah (družina Cucurbitaceae) pri katerih se metoda uporablja že vrsto let v žlahtniteljskih programih (Sugiyama in Morishita, 2000; Sari in Yetisir, 2002; Kuzuya in sod., 2003; Claveria in sod., 2005). Od prvih poskusov leta 1987 (Sauton in Dumas de Valux, 1987) pa do danes, so s pomočjo opraševanja z obsevanim pelodom, pridobili haploide pri melonah, lubenicah, kumarah in različnih bučah. Metoda temelji na opraševanju z obsevanim pelodom in kasnejšim reševanjem embrijev na E20A gojišču, ki sta ga uporabila že Sauton in Dumas de Valux leta 1987 pri melonah. Reševanje embrijev poteka 3-5 tednov po opraševanju, ko se iz polnih semen izolira embrije. Ob izolaciji so embriji v različnih razvojnih fazah: pri bučah so od točkaste do kotiledonske faze (Kurtar in sod., 2002), pri kumarah so od zgodnje srčaste do kotiledonske faze (Faris in sod., 1999) in pri melonah so od globularne do kotiledonske faze (Cuny in sod., 1993). Kurtar in sodelovci (2002) so dokazali, da je uspešnost regeneracije rastlin odvisna od razvojne stopnje embrijev ob reševanju. Najvišji odstotek regeneracije so dosegli iz embrijev inokuliranih v kotiledonski fazi. Kasneje so opazili, da je z razvojno fazo ob reševanju povezana tudi ploidnost embrijev. Embriji v zgodnejših fazah razvoja so bili haploidni, medtem

ko so bili embriji v kotiledonski fazi izključno diploidni. Rezultati so skladni z rezultati Faris in Niemirowicz-Szczytt (1999), ki sta spremljala razvoj embrijev in endospermov kumare po opraševanju s svežim pelodom in pelodom obsevanim pri 100 ali 300 Gy. Ugotovila sta, da se v primerjavi s kontrolnim opraševanjem, embriji in endospermi nastali po opraševanju z obsevanim pelodom, razvijajo počasneje in kažejo odstopanja od normalne morfologije. Poleg tega so, po opraševanju z obsevanim pelodom, embriji v kasnejših razvojnih fazah (mutanti) začeli propadati že 9 (100 Gy) oz. 3 dan (300 Gy) po opraševanju, tako da sta 15 dni po opraševanju v semenskih zasnovah zasledila samo še embrije v globularni razvojni fazi.

Velika prednost indukcije haploidov s pomočjo opraševanja z obsevanim pelodom je, da pri nekaterih vrstah kot so kivi (Pandey in sod., 1990; Chalak in Legave, 1997), čebula (Dore in Marie, 1993), mandarina (Froelicher in sod., 2007) in vrste rodu *Nicotiana* (Pandey in Phung, 1982) ni potrebe po reševanju embrijev. Tako pri kiviju puščajo oprašene cvetove na trsih do fiziološke zrelosti plodov, jih nato vernalizirajo in izločijo semena. Pandey in sodelavci (1990) so slabšo *in vivo* kalivost zrelih partenogenetskih semen izbojšali z *in vitro* kalitvijo semen na štirih različnih hranilnih gojiščih, Chalak in Legave (1997) pa sta kasneje kalitev semen poenostavila in dobila zadovoljiv odstotek trihaploidnih regenerantov tudi po neposredni setvi v vermikulit.

Spontana diploidizacija haploidnega jedra po opraševanju z obsevanim pelodom je bila na podlagi morfološkega opazovanja regenerantov opažena pri vrstah rodu *Nicotiana* (Pandey in Phung, 1982), čebuli (Dore in Marie, 1993), sončnici (Todorova in sod., 1997), nageljnu (Sato in sod., 2000) in meloni (Lotfi in sod., 2003). Pri sončnicah so opazili zanimiv pojav spontane diploidizacije haploidov med gojenjem v rastlinjaku. Od 296 regenerantov, katerim so s pretočno citometrijo opravljeno v fazi 2-3 listov dokazali haploidnost, so po 20 dneh rasti v rastlinjaku dobili 239 (81 %) diploidov, 32 (11 %) miksoploidov in le preostalih 25 (8 %) je ostalo haploidnih (Todorova in sod., 1997). Spontano podvojene haploide so samooprašili, linije odbrali glede na lastnosti zanimive za žlahtnjenje in 17 odbranih DH linij analizirali s 4 izoencimskimi markerji. Rastline znotraj vseh linij so bile uniformne, linije pa so bile homozigotne in so vsebovale izoencime materinih ali očetovskih rastlin.

2.3 Androgeneza

S pojmom androgeneza (ali embriogeneza mikrospor) označujemo pridobivanje haploidnih embrijev iz moških gamet. Poznani sta dve metodi in sicer *in vitro* kultura prašnic, s katero so pred 50 leti pridobili prve haploide (vrsta *Datura innoxia*; Guha in Maheshwari, 1964, 1966) in kultura izoliranih mikrospor, ki je sledila 10 let kasneje (tobak; Nitsch, 1974). Zaradi velike uspešnosti in uporabnosti pri številnih rastlinskih vrstah, je androgeneza najbolj pogosto uporabljena metoda indukcije haploidov pri žlahtnjenju rastlin in genetskih raziskavah. V komercialne namene se redno uporablja pri ječmenu, pšenici, koruzi, rižu, tritikali, rži, tobaku, oljni ogrščici in drugih vrstah rodu *Brassica* (podrobni protokoli so zbrani v Maluszynski in sod., 2003). Glavne slabosti androgeneze so močna odvisnost uspeha od genotipa rastline, neodzivnost določenih kmetijsko pomembnih vrst (drevesne vrste, stročnice) in modelne rastline *Arabidopsis thaliana* ter relativno velik delež regeneriranih albino rastlin. Metoda temelji na sposobnosti nezrelega peloda – mikrospor, da ob primernih dražljajih, spremeni razvojno pot iz gametofitne (dozorevanja peloda) v sporofitno (nastanek embrija). Ob ugodnih pogojih v *in vitro* razmerah, nastane haploidni embrij neposredno ali pa posredno preko kulture haploidnega kalusa.

Androgeneza iz prašnic je najbolj preprosta metoda pri kateri se po površinski sterilizaciji predtretiranih cvetnih brstov, prašnice aseptično izločijo in gojijo na hranilnih gojiščih. Kultura izoliranih mikrospor se začne podobno, le da se izolirane prašnice potopi v tekoče gojišče, kjer se ob stalnem tresenju izločijo mikrospore. Druga možnost izolacije mikrospor je trenje steriliziranih cvetnih brstov v tekočem gojišču in kasnejše ločevanje mikrospor od somatskega tkiva s filtriranjem in centrifugiranjem. Čeprav je izolacija mikrospor bolj zahtevna metoda, je njena velika prednost ločevanje mikrospor od sporofitnega tkiva. Tako je onemogočen vpliv sporofitnega tkiva na embriogenezo mikrospor in hkrati se prepreči nevarnost regeneracije heterozigotov iz somatskih celic.

Za uspešno spremembo razvojne poti iz gametofitnega v sporofitni razvoj, je najpomembnejši dejavnik razvojna faza mikrospor. Za večino rastlinskih vrst je najbolj primeren čas prve haploidne mitoze, ko so mikrospore v pozni enojedrni in zgodnji dvojedrni fazi (Touraev in sod., 1997; Maraschin in sod., 2005). Stresni dejavniki, ki se uporabljajo v ta namen (temperaturno pred-tretiranje, stradanje mikrospor in osmotski stres), se razlikujejo glede na rastlinsko vrsto. Tako se pri ječmenu, pšenici, koruzi, rižu, tritikali in rži uporablja pred-tretiranje na nizkih temperatureh, pri vrstah rodu *Brassica* in tobaku pa na visokih temperaturah, oboje več ur ali dni. Pri oljni ogrščici in tobaku so dokazali, da lahko embriogenezo sprožijo različni stresni dejavniki, odvisno od razvojne faze mikrospor. Tako za enojedrne mikrospore oljne ogrščice zadostuje temperatura 32 ºC, za dvojedrne pa je potrebna že temperatura 41 ºC (Maraschin in sod., 2005). Pri tobaku, temperaturni šok uspešno vodi v embriogenezo enocelične mikrospore, medtem ko dvojedrnih mikrospor ne in jih je potrebno izpostaviti stradanju (Touraev in sod., 1997).

3 DOLOČANJE PLOIDNOSTI IN PREVERJANJE HOMOZIGOTNOSTI REGENERANTOV

Med *in vitro* kulturo cvetov in njihovih delov (prašnice, plodnice, itd.) se lahko na hranilnih gojiščih regenerirajo poleg haploidnih tudi diploidne rastline in celo rastline višjih stopenj ploidnosti (Sunderland, 1974; Germanà 2005, 2006, 2009; Košmrlj in sod., 2013; Murovec in Bohanec, 2013). V preteklosti so za določanje ploidnosti uporabljali posredne metode kot so spremljanje morfoloških lastnosti rastlin (velikost rastlin in listov, oblika cvetov), fertilnosti rastlin, bujnost rastlin, število kloroplastov v celicah zapiralkah in velikost listnih rež. Metode so bile zamudne, nenatančne in pod vplivom nepredvidljivih okoljskih dejavnikov. Dandanes se uporabljajo predvsem citološke tehnike štetja metafaznih kromosomov (primer protokola je predstavljen v Maluszynska, 2003) in merjenje količine DNA v jedrih s pomočjo pretočne citometrije (primer protokola je predstavljen v Bohanec, 2003). Slednja metoda predstavlja najhitrejšo in najbolj zanesljivo tehniko določanja ploidnosti regenerantov. Omogoča zelo zgodnje določanje, saj za analizo zadostujejo že majhne količine rastlinskega materiala, tako da se analiza lahko opravi še v fazi tkivne kulture. Poleg tega je s pomočjo pretočne citometrije mogoče odkrivanje miksoploidnih regenerantov, kar z ostalimi tehnikami ni mogoče.

Diploidni regeneranti so lahko posledica spontane diploidizacije gamet (so spontani podvojeni haploidi), somatski regeneranti iz diploidnih starševskih celic ali križanci po samoopraševanju oz. tujeopraševanju. Zato je za dokončno potrditev DH potrebna analiza homozigotnosti. V ta namen se uporabljajo številne metode, odvisno od rastlinske vrste in dostopnih markerskih sistemov. V preteklosti je analiza diploidnih regenerantov temeljila predvsem na fenotipskih markerjih (Raquin, 1985; Dore in Marie, 1993; De Witte in Keulemans, 1994; Maine, 2003) ter na samoopraševanju pridobljenih regenerantov in morfološkem testiranju potomstva (Sato in sod., 2000). Potomci DH naj bi bili izenačeni za vse lastnosti in pri njih naj ne bi bilo opaziti segregacije lastnosti. Kasneje so se uveljavili številni molekulski markerji, med njimi sprva izoencimi (Campion s sod., 1995; Bohanec in Jakše, 1999; Germanà in Chiancone, 2001; Höfer in Grafe, 2003), kasneje pa še DNA molekulski markerji kot npr. AFLP (Eeckhaut s sod., 2001) in RAPD (Bohanec in sod., 1995; Eimert in sod., 2003; Yahata in sod., 2005 a, b).

Zaradi kodominantnega načina dedovanja, relativne pogostosti v genomu evkariontov, visoke stopnje polimorfizma, preprostega in nedvoumnega vrednotenja (PCR namnoževanje, določanje dolžine z avtomatskimi sekvenčnimi aparati), mikrosateliti v zadnjih letih nadomeščajo ostale metode preverjanja homozigotnosti regenerantov. V primerih indukcije haploidov z inokulacijo prašnic ali drugih delov ne-oprašenih cvetov, je za potrditev DH dovolj analiza enega mikrosatelitnega lokusa, ki je pri izvorni rastlini heterozigoten. Pri indukciji haploidov s pomočjo opraševanja, kjer obstaja možnost nenamerne samooploditve ali oploditve s strani opraševalca, pa je potrebno analizirati več lokusov. Tako so Košmrlj in sodelavci (2013) odkrili, da je za nedvoumno potrditev izvora diploidnih

regenerantov buč v večini primerov zadostna analiza na treh lokusih.

Mikrosatelite so uporabili za določanje genetskega izvora diploidov pri številnih rastlinskih vrstah kot so *Citrus clementina* (Germanà in Chiancone, 2003; Germanà in sod., 2005), jablana (Höfer in sod., 2002; Vanwynsberghe in sod., 2005), hruška (Bouvier in sod., 2002), pšenica (Muranty in sod., 2002), koruza (Aulinger in sod., 2003; Tang in sod., 2006), mandarina (Froelicher in sod., 2007), oranžni krinkar (Murovec in Bohanec, 2013) in oljne buče (Košmrlj in sod., 2013).

Za hitro in poceni ločevanje med haploidi in nezaželjenimi heterozigotnimi diploidi po indukciji z opraševanjem, so najbolj primerni morfološki znaki, ki se izrazijo zgodaj v razvoju. Tako pri koruzi poznajo gen *R1-nj,* ki ob prisotnosti dominantnih genov *A1* ali *A2* in *C2* povzroči rdeče obarvanje alevronske plasti endosperma in v predelu skuteluma – ščitka (Geiger in Gordillo, 2009). Gen *R1-nj* mora biti v materini rastlini homozigotno recesiven, v opraševalni liniji pa homozigotno dominanten. Po opraševanju, se selekcija opravi že med zrnjem, saj se pri zrnju z nezaželenimi hibridnimi embriji opazi rdeče obarvano krono v predelu alevronske plasti endosperma in skuteluma, zrnje s haploidnim embrijem pa vsebuje rdečo krono samo v alevronski plasti zaradi normalne oploditve polarnih jeder. Nedavno so predstavili nov morfološki znak, ki naj bi v prihodnje služil za ločevanje haploidov od križancev koruze. S križanjem so ustvarili dve novi opraševalni liniji z izredno visoko vsebnostjo olja, hibridne potomce pa so določili s pomočjo jedrne magnetne resonance (nuclear magnetic resonance, NMR), saj so vsebovali večjo vsebnost olja od haploidnih (Melchinger in sod., 2013). Glavna prednost predstavljene novitete je v veliki zanesljivosti in možnosti avtomatizacije, hitrost pa je trenutno 20 semen na minuto.

Podoben princip z barvnim morfološkim znakom uporabljajo tudi pri krompirju, po opraševanju s *S. phurea*. Dominantni gen povzroča vijoličaste pike na semenih in vijoličen obroč v predelu nodija stebla, tako da lahko selekcija poteka v dveh razvojnih fazah. Tako pri koruzi kakor pri krompirju pa opisana barvna morfološka znaka ne moreta ločiti haploidnih embrijev od hibridov po nenamernem samoopraševanju, zato so potrebni še dodatni morfološki ali molekulski markerji.

4 PODVAJANJE ŠTEVILA KROMOSOMOV

Haploidi zaradi enojnega števila kromosomov ne tvorijo funkcionalnih gamet (so sterilni) in je za pridobivanje fertilnih linij potrebno število njihovih kromosomov podvojiti. Za pridobivanje t.i. podvojenih haploidov (doubled haploids, DH), homozigotnih na vseh lokusih, se uporabljajo različne tehnike, ki večinoma vključujejo tretiranje z antimitotičnimi sredstvi kot so kolhicin (v začetku izoliran iz jesenskega podleska *Colchicum autumnale* L.), orizalin, trifluralin, amiprofos-metil (AMP) in drugi. Ta sredstva se lahko uporabijo v različnih fazah indukcije haploidov od najzgodnejše faze mikrospor, ko jih dodajajo v indukcijsko gojišče, pa vse do faze aklimatiziranih haploidnih rastlin, pri katerih se sredstva nanašajo na meristeme. Novejše raziskave nakazujejo možnost podvajanja kromosomov preko adventivne regeneracije (Maine, 2003; Škof in sod., 2007) brez uporabe antimitotičnih sredstev. Tako so pri šalotki na gojišču za indukcijo ginogeneze opazili spontano podvajanje kromosomov somatskih regenerantov, v tem primeru iz diploidnega v tetraploidno število (Sulistyaningsih in sod., 2006). Metodo so uspešno uporabili za podvajanje kromosomov haploidnih rastlin čebule Alan in sodelavci (2007), ki so s somatsko regeneracijo na gojiščih za indukcijo ginogeneze pridobili 61 % diploidnih regenerantov. Odstotek regeneriranih diploidov se je ob dodajanju 12,5, 25 ali 50 μM kolhicina v gojišče celo zmanjšal, ob hkratnem povečanju odstotka regeneriranih tetraploidov in miksoploidov. S somatsko regeneracijo so iz miksoploidnega matičnega materiala uspeli regenerirati same diploide. Še boljše rezultate so dosegli Jakše in sodelavci (2010) s somatsko regeneracijo iz kulture cvetnih brstov, kjer so dobili do 83 % uspešnost podvajanja kromosomov haploidnih in do 100 % uspešnost podvajanja kromosomov miksoploidnih čebul.

5 ZAKLJUČEK

Petdeset let od prvega sproženega nastanka haploidnih rastlin so tehnike indukcije in regeneracije haploidov še vedno oz. vedno bolj aktualne. Od prvotne uporabe v žlahtnjenju najpomembnejših poljščin, se njihovo pridobivanje širi na druge vrste kot so zelenjadnice, okrasne, zdravilne, aromatične in krmne rastline. Poleg tega dobivajo (podvojeni) haploidi z novimi tehnologijami (npr. določanje zaporedij celotnih genomov) nov pomen in vlogo v sodobnih genetskih študijah. Zato menim, da bodo haploidi in podvojeni haploidi tudi v prihodnje igrali pomembno vlogo v kmetijstvu in bazičnih raziskavah.

6 VIRI

Alan A.R., Brants A., Cobb E., Goldschmied P.A., Mutschler M.A., Earle E.D. 2004. Fecund gynogenic lines from onion (*Allium cepa* L.) breeding materials. Plant Science, 167, 5: 1055-1066

Alan A.R., Lim W., Mutschler M.A., Earle E.D. 2007. Complementary strategies for ploidy manipulations in gynogenic onion (*Allium cepa* L.). Plant Science, 173: 25-31

Aleza P., Juarez J., Hernandez M., Pina J.A., Ollitrault P., Navarro L. 2009. Recovery and characterization of a *Citrus clementina* Hort. ex Tan. 'Clemenules' haploid plant selected to establish the reference whole Citrus genome sequence. BMC Plant Biology, 9, št. članka 110

Aulinger I.E., Peter S.O., Schmid J.E., Stamp P. 2003. Rapid attainment of a doubled haploid line from transgenic maize (*Zea mays* L.) plants by means of anther culture. In Vitro Cellular and Developmental Biology- Plant, 39: 165-170

Bal U., Touraev A. 2009. Microspore embryogenesis in selected medicinal and ornamental species of the *Asteraceae*. V: Advances in haploid production in higher plants. Touraev A., Forster B.P., Jain S.M. (ur.). Springer: 219-229

Barret P., Brinkmann M., Beckert M. 2008. A major locus expressed in the male gametophyte with incomplete penetrance is responsible for *in situ* gynogenesis in maize. Theoretical and Applied Genetics, 117: 581-594

Bohanec B. 1994. Induction of gynogenesis in agricultural crops: a review. V: Proceedings of the international colloquium on impact of plant biotechnology on agriculture, December 5th - 7th 1994, Rogla, Slovenia. Javornik B., Bohanec B., Kreft I. (ur.). Ljubljana, Biotechnical Faculty, Agronomy Department, Centre for Plant Biotechnology and Breeding: 43-55

Bohanec B. 2003. Ploidy determination using flow cytometry. V: Doubled haploid production in crop plants, a manual. Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. (ur.). Dordrecht, Kluwer Academic Publishers: 397-403

Bohanec B. 2009. Doubled haploids via gynogenesis. V: Advances in haploid production in higher plants. Touraev A., Forster B.P., Jain S.M. (ur.). Springer: 35-46

Bohanec B. Jakše M., Ihan A., Javornik B., 1995. Studies of gynogenesis in onion (*Allium cepa* L.): induction procedures and genetic analysis of regenerants. Plant Science, 104: 215-224

Bohanec B., Jakše M. 1999. Variations in gynogenic response among long-day onion (*Allium cepa* L.) accessions. Plant Cell Reports, 18: 737-742

Bouvier L., Guérif P., Djulbic M., Durel C.E., Chevreau E., Lespinasse Y. 2002. Chromosome doubling of pear haploid plants and homozygosity assessment using isozyme and microsatellite markers. Euphytica, 123: 255-262

Bouvier L., Zhang Y.X., Lespinasse Y. 1993. Two methods of haploidization in pear, *Pyrus communis* L.: greenhouse seedling selection and *in situ* parthenogenesis induced by irradiated pollen. Theoretical and Applied Genetics, 87: 229-232

Campion B., Bohanec B., Javornik B. 1995. Gynogenic lines of onion (*Allium cepa* L.): evidence of their homozygosity. Theoretical and Applied Genetics, 91: 598-602

Chalak L., Legave J.M. 1997. Effects of pollination by irradiated pollen in Hayward kiwifruit and spontaneous doubling of induced parthenogenetic trihaploids. Scientia Horticulturae, 68: 83-93

Claveria E., Garcia-Mas J., Dolcet-Sanjuan R. 2005. Optimization of cucumber doubled haploid line production using *in vitro* rescue of *in vivo* induced

parthenogenic embryos. Journal of the American Society for Horticultural Science, 130, 4: 555-560

Cuny F., Grotte M., Dumas de Vaulx R., Rieu A. 1993. Effects of gamma irradiation of pollen on parthenogenetic haploid production in muskmelon (*Cucumis melo* L.). Environmental and Experimental Botany, 33, 2: 301-312

De Witte K., Keulemans J. 1994. Restrictions of the efficiency of haploid plant production in apple cultivar Idared, through parthenogenesis *in situ*. Euphytica, 77: 141-146

Deimling S., Fleihinghaus-Roux T. 1996. Haploids in rye. V: In vitro haploid production in higher plants. Jain S.M., Spory S.K., Veilleux R.E. (ur.). Dordrecht, Kluwer Academic Publishers: 181-204

Devaux P., Kasha K.J. 2009. Overview of barley doubled haploid production. V: Advances in haploid production in higher plants. Touraev A., Forster B.P., Jain S.M. (ur.). Springer: 47-63

Dore C., Marie F. 1993. Production of gynogenetic plants of onion (*Allium cepa* L.) after crossing with irradiated pollen. Plant Breeding, 111: 142-147

Eeckhaut T., Werbrouck S., Dendauw J., Bockstaele E.V., Dobergh P. 2001. Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis. Plant Cell, Tissue and Organ Culture, 67:181-189

Eimert K., Reutter G., Strolka B. 2003. Fast and reliable detection of doubled-haploids in *Asparagus officinalis* by stringent RAPD-PCR. Journal of Agricultural Science, 141: 73-78

Faris N.M., Niemirowicz-Szczytt K. 1999. Cucumber (*Cucumis sativus* L.) embryo development *in situ* after pollination with irradiated pollen. Acta Biologica Cracoviensia Series Botanica, 41: 111-118

Faris N.M., Nikolova V., Niemirowicz-Szczytt K. 1999. The effect of gamma irradiation dose on cucumber (*Cucumis sativus* L.) haploid embryo production. Acta Physiologiae Plantarum, 21, 4: 391-396

Ferrant V., Bouharmont J. 1994. Origin of gynogenetic embryos of *Beta vulgaris* L. Sexual Plant Reproduction, 7: 12-16

Ferrie A.M.R. 2009. Current status of doubled haploids in medicinal plants. V: Advances in haploid production in higher plants. Touraev A., Forster B.P., Jain S.M. (ur.). Springer: 209-217

Ferrie A.M.R., Caswell K.L. 2011. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell, Tissue and Organ Culture, 104, 3: 301-309

Forster B.P., Heberle-Bors E., Kasha K.J., Touraev A. 2007. The resurgence of haploids in higher plants. Trends in Plant Science, 368-375

Froelicher Y., Bassene J.B., Jedidi-Neji E., Dambier D., Morillon R., Bernardini G., Costantino G., Ollitrault P. 2007. Induced parthenogenesis in mandarin for haploid production: induction procedures and genetic analysis of plantlets. Plant Cell Reports, 26:937-944

Geiger H.H., Gordillo G.A. 2009. Doubled haploids in hybrid maize breeding. Maydica, 54: 485-499

Gémes-Juhász A., Baloh P., Ferenczy A., Kristóf Z. 2002. Effect of optimal stage of female gametophyte and heat treatment on in vitro gynogenesis induction in cucumber (*Cucumis sativus* L.). Plant Cell Reports, 21: 105-111

Germanà M.A. 2006. Doubled haploid production in fruit crops. Plant Cell Tissue and Organ Culture, 86: 131–146

Germanà M.A. 2009. Haploid and doubled haploids in fruit trees. V: Advances in haploid production in higher plants. Touraev A., Forster B.P., Jain S.M. (ur.). Springer: 241–263

Germanà M.A., Chiancone B, Lain O., Testolin R. 2005. Anther culture in *Citrus clementina*: a way to regenerate tri-haploids. Australian Journal of Agricultural Research, 56: 839-845

Germanà M.A., Chiancone B. 2001. Gynogenetic haploids of Citrus after in vitro pollination with triploid pollen grains. Plant Cell, Tissue and Organ Culture, 66: 59-66

Germanà M.A., Chiancone B. 2003. Improvement of *Citrus clementina* Hort. Ex Tan. microspore-derived embryoid induction and regeneration. Plant Cell Reports, 22: 181-187

Godbole M., Murthy H.N. 2012. Parthenogenetic haploid plants using gamma irradiated pollen in snapmelon (*Cucumis melo* var. momordica). Plant Cell, Tissue and Organ Culture, 109: 167-170

Gonzalo M.J., Claveria E., Monforte A.J., Dolcet-Sanjuan R. 2011. Parthenogenic haploids in melon: Generation and molecular characterization of a doubled haploid line population. Journal of the American Society for Horticultural Science, 136: 145-154

Grouh M. S.H., Vahdati K., Lotfi M., Hassani D., Biranvand N. P. 2011. Production of haploids in persian walnut through parthenogenesis induced by gamma-irradiated pollen. Journal of the American Society for Horticultural Science, 136, 3: 198–204

Guha S., Maheshwari S.C. 1964. *In vitro* production of embryos from anthers of *Datura*. Nature, 204, 4957: 497

Guha S., Maheshwari, S.C. 1966. Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature, 212: 97-98

Höfer M., Gomez A., Aguiriano E., Manzanera J.A., Bueno M.A. 2002. Analysis of simple sequence repeat markers in homozygous lines of apple. Plant Breeding, 121: 159-16

Höfer M., Grafe Ch. 2003. Induction of doubled haploids in sweet cherry (*Prunus avium* L.). Euphytica, 130: 191-197

Höfer M., Lespinasse Y. 1996. Haploidy in apple. V: In vitro haploid production in higher plants, Vol. 3: Important selected plants. Jain S.M., Sopory S.K., Veilleux R.E. (ur). Dordrecht, Kluwer Academic Publishers: 261-276

Hosp J., Maraschin S.F., Touraev A., Boutilier K. 2007. Functional genomics of microspore embryogenesis. Euphytica, 158: 275-285

Jakše M., Hirschegger P., Bohanec B., Havey M.J. 2010. Evaluation of gynogenic responsiveness and pollen viability of selfed doubled haploid onion lines and chromosome doubling via somatic regeneration. Journal of the American Society for Horticultural Science, 135, 1: 67-73

Kasha K.J., Hu T.C., Oro R., Simion E., Shim Y.S. 2001. Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (*Hordeum vulgare* L.) microspores. Journal of Experimental Botany, 52, 359: 1227-1238

Kasha K.J., Kao K.N. 1970. High frequency haploid production in barley (*Hordeum vulgare* L.). Nature, 225: 874-876

Katoh N., Hagimori M., Iwai S. 1993. Production of haploid plants of melon by pseudofertilized ovule culture. Plant Tissue Culture Letters, 10: 60-66

Košmrlj K., Murovec, J., Bohanec B. 2013. Haploid induction in hull-less seed pumpkin through parthenogenesis induced by X-ray-irradiated pollen. Journal of the American Society for Horticultural Science, 138: 310-316

Kurtar E.S., Balkaya A. 2010. Production of in vitro haploid plants from *in situ* induced haploid embryos in winter squash (*Cucurbita maxima* Duchesne ex Lam.) via irradiated pollen. Plant, Cell, Tissue and Organ Culture, 102: 267–277

Kurtar E.S., Balkaya A., Ozbakir M., Ofluoglu T. 2009. Induction of haploid embryo and plant regeneration via irradiated pollen technique in pumpkin (*Cucurbita moschata* Duchesne ex. Poir). African Journal of Biotechnology, 8: 5944–5951

Kurtar E.S., Sari N., Abak K. 2002. Obtention of haploid embryos and plants through irradiated pollen technique in squash (*Cucurbita pepo* L.). Euphytica, 127: 335-344

Kuzuya M., Hosoya K., Yashiro K. Tomita K., Ezura H. 2003. Powdery mildew (*Sphaerotheca fuliginea*) resistance in melon is selectable at the haploid level. Journal of Experimental Botany, 54, 384: 1069-1074

Laurie D.A., Bennett M.D. 1988. The production of haploid wheat plants from wheat x maize crosses. Theoretical and Applied Genetics, 76: 393-397

Lotfi M., Alan A.R., Henning M.J., Jahn M.M., Earle E.D. 2003. Production of haploid and doubled haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. Plant Cell Reports, 21: 1121-1128

Maine M.J. 2003. Potato haploid technologies. V: Doubled haploid production in crop plants, a manual. Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. (ur.). Dordrecht, Kluwer Academic Publishers: 241-247

Maluszynska J. 2003. Cytogenetic tests for ploidy level analyses – chromosome counting. V: Doubled haploid production in crop plants: A manual. Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. (ur). Dordrecht, Kluwer Academic Publishers: 391-395

Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. 2003. Doubled haploid production in crop plants, a manual. Dordrecht, Kluwer Academic Publishers: 428 str.

Maraschin S.F., de Priester W., Spaink H.P., Wang M. 2005. Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. Journal of Experimental Botany, 56, 417: 1711-1726

Melchinger A.E., Schipprack W., Würschum T., Chen S., Technow F. 2013. Rapid and accurate identification of *in vivo*-induced haploid seeds based on oil content in maize. Scientific Reports, 3, št. članka 2129

Meynet J., Barrade R., Duclos A., Siadous R. 1994. Dihaploid plants of roses (*Rosa x hybrida*, cv 'Sonia') obtained by parthenogenesis induced using irradiated pollen and *in vitro* culture of immature seeds. Agronomie, 2: 169-175

Muranty H., Sourdille P., Bernard S., Bernard M. 2002. Genetic characterization of spontaneous diploid

androgenetic wheat and triticale plants. Plant Breeding, 121: 470-474

Murovec J., Bohanec B. 2013. Haploid induction in *Mimulus aurantiacus* Curtis obtained by pollination with gamma irradiated pollen. Scientia Horticulturae, 162: 218-225

Musial K., Bohanec B., Jakše M., Przywara L. 2005. The development of onion (*Allium cepa* L.) embryo sacs *in vitro* and gynogenesis induction in relation to flower size. In vitro Cellular and Developmental Biology- Plant, 41: 446-452

Musial K., Bohanec B., Przywara L. 2001. Embryological study on gynogenesis in onion (*Allium cepa* L.). Sexual Plant Reproduction, 13: 335-341

Musial K., Przywara L. 1998. Influence of irradiated pollen on embryo and endosperm development in kiwifruit. Annals of Botany, 82: 747-756

Musial K., Przywara L. 1999. Pollination with heavily irradiated pollen in *Nicotiana*: induced parthenogenesis and embryological study. Acta Biologica Cracoviensia Series Botanica, 41: 127-137

Naess S.K., Swartz H.J., Bauchan G.R. 1998. Ploidy reduction in blackberry. Euphytica, 99: 57-73

Nitsch C, 1974. Pollen culture - a new technique for mass production of haploid and homozygous plants. V: Haploids in higher plants: advances and potential : proceedings of the first international symposium, Guelph, Ontario, Canada. Kasha K.J. (ur). University of Guelph: 123–135

Pandey K.K. 1980. Parthenogenetic diploidy and egg transformation induced by irradiated pollen in *Nicotiana*. New Zeland Journal of Botany, 18, 2: 203-207

Pandey K.K., Phung M. 1982. 'Hertwig effect in plants: induced parthenogenesis through the use of irradiated pollen. Theoretical and Applied Genetics, 62: 295-300

Pandey K.K., Przywara L., Sanders P.M. 1990. Induced parthenogenesis in kiwifruit (*Actinidia deliciosa*) through the use of lethally irradiated pollen. Euphytica, 51: 1-9

Peixe A., Campos M.D., Cavaleiro C., Barroso J., Pais M.S. 2000. Gamma-irradiated pollen induces the formation of 2n endosperm and abnormal embryo development in Euroean plum (*Prunus domestica* L., cv. 'Rainha Claudia Verde'. Scientia Horticulturae, 86, 4: 267-278

Przyborowski J., Niemirowicz-Szczytt K. 1994. Main factors affecting cucumber (*Cucumis sativus* L.) haploid embryo development and haploid plant characteristics. Plant Breeding, 112: 70-75

Raquin C. 1985. Induction of haploid plants by *in vitro* culture of *Petunia* ovaries pollinated with irradiated pollen. Zeitschrift für Pflanzenzüchtung, 94: 166-169

Rines H.W. 2003. Oat haploid from wide hybridization. V: Doubled haploid production in crop plants, a manual. Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. (ur.). Dordrecht, Kluwer Academic Publishers: 155-159

Sari N., Abak K., Pitrat M., Rode J.C., Dumas de Vaulx R. 1994. Induction of parthenogenetic haploid embryos after pollination by irradiated pollen in watermelon. HortScience, 29, 10: 1189-1190

Sari N., Yetisir H. 2002. Some agronomical characteristics of doubled haploid lines produced by irradiated pollen technique and parental diploid genotypes in melons. Turkish Journal of Agriculture and Forestry, 26: 311-317

Sato S., Katoh N., Yoshida H., Iwai S., Hagimori M. 2000. Production of doubled haploid plants of carnation (Dianthus caryophyllus L.) by pseudofertilized ovule culture. Scientia Horticulturae, 83: 301-310

Sauton A., Dumas De Vaulx R. 1987. Obtention de plantes haploides chez le melon (*Cucumis melo* L.) par gynogenese induite par pollen irradie. Agronomie, 7, 2: 141–148

Segui-Simarro J., Nuez F. 2008. How microspores transform into haploid embryos: changes addociated with embryogenesis induction and microspore-derived embryogenesis. Physiologia Plantarum, 134: 1-12

Sugiyama K., Morishita M. 2000. Production of seedless watermelon using soft-X-irradiated pollen. Scientia Horticulturae, 84: 255-264

Sulistyaningsih E., Aoyagi Y., Tashiro Y. 2006. Flower bud culture of shallot (Allium cepa L. Aggregatum group) with cytogenetic analysis of resulting gynogenic plants and somaclones. Plant Cell, Tissue and Organ Culture, 86: 249-255

Sunderland N. 1974. Anther culture as a means of haploid production. V: Haploids in higher plants: advances and potential : proceedings of the first international symposium, Guelph, Ontario, Canada. Kasha K.J. (ur). University of Guelph: 91-122

Škof S., Bohanec B., Kastelec D., Luthar Z. 2007. Spontaneous induction of tetraploidy in hop using adventitious shoot regeneration method. Plant Breeding, 126, 4: 416-421

Tang F., Tao Y., Zhao T., Wang G. 2006. *In vitro* production of haploid and doubled haploid plants from pollinated ovaries of maize (*Zea mays* L.). Plant Cell Tissue Organ Culture, 84: 233-237

Testillano P.S., Risueno M.C. 2009. Tracking gene and protein expression during microspore embryogenesis by confocal laser scanning microscopy. V: Advances in haploid production in higher plants. Touraev A., Forster B.P., Jain S.M. (ur.). Springer: 339-347

Thomas T.D. 2004. Embryological observations on unpollinated ovary culture of mulberry (*Morus alba* L.). Acta Biologica Cracovensia Series Botanica, 46: 87-94

Thomas W.T.B., Forster B.P., Gertsson B. 2003. Doubled haploids in breeding. V: Doubled haploid production in crop plants, a manual. Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. (ur.). Dordrecht, Kluwer Academic Publishers: 337-349

Todorova M., Ivanov P., Shindrova P., Christov M., Ivanova I. 1997. Doubled haploid production of sunflower (*Helianthus annuus* L.) through irradiated pollen-induced parthenogenesis. Euphytica, 97: 249-254

Touraev A., Stoger E., Voronin V., Heberle-Bors E. 1997. Plant male germ line transformation. Plant Journal, 12, 4: 949-956

Vanwynsberghe L., De Witte K., Coart E., Keulemans J. 2005. Limited application of homozygous genotypes in apple breeding. Plant Breeding, 124: 399-403

Wedzony M. 2003. Protocol for doubled haploid production in hexaploid triticale (*xTriticosecale* Wittm.) by crosses with maize. V: Doubled haploid production in crop plants, a manual. Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. (ur.). Dordrecht, Kluwer Academic Publishers: 135-140

Wedzony M., Forster B.P., Zur I., Golemiec E., Szechynske-Hebda M., Dubas E., Gotebiowska G. 2009. Progress in doubled haploid technology in higher plants. V: Advances in haploid production in higher plants. Touraev A., Forster B.P., Jain S.M. (ur.). Springer: 1-33

Yahata M., Harusaki S., Komatsu H., Takami K., Kunitake H., Yabuya T., Yamashita K., Toolapong P. 2005a. Morphological characterization and molecular verification of a fertile haploid pummelo (Citrus grandis Osbeck). Journal of the American Society for Horticultural Science, 130: 34-40

Yahata M., Kunitake H., Yabuya T., Yamashita K., Kashihara Y., Komatsu H. 2005b. Production of a doubled haploid from a haploid pummelo using colchicine treatment of axillary shoot buds. Journal of the American Society for Horticultural Science. 130: 899-903

Yahata M., Yasuda K., Nagasawa K., Harusaki S., Komatsu H., Kunitake H. 2010. Production of haploid plant of 'Banpeiyu' pummelo (*Citrus maxima* (Burm.) Merr.) by pollination with soft x-ray-irradiated pollen. Journal of the Japanese Society for Horticultural Science, 79: 239-245

Zhang Y.X., Lespinasse Y. 1991. Pollination with gamma-irradiated pollen and development of fruits, seeds and parthenogenetic plants in apple. Euphytica, 54: 101-109

Zhang Z.L., Qiu F.Z., Liu Y.Z., Ma K.J., Li Z.Y., Xu S.Z. 2008. Chromosome elimination and *in vivo* haploid production induced by Stock 6-derived inducer line in maize (*Zea mays* L.). Plant Cell Reports, 27, 12: 1851-1860

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Novejši podatki o vsebnosti semen vrst iz rodu *Ambrosia* v krmi za prostoživeče ptice v Sloveniji

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IZVLEČEK

Vdihavanje peloda vrst iz rodu *Ambrosia* lahko povzroči preobčutljivostne reakcije. Krma za prostoživeče ptice je eden od dejavnikov, ki pripomorejo k širjenju omenjenih rastlin. Leta 2010 so zato k Direktivi o nezaželenih snoveh v živalski krmi (2002/32/ES) dodali aneks, da lahko krma za živali, ki vsebuje nezmleta žita, vsebuje do 50 mg semen vrst iz rodu *Ambrosia* v kilogramu krme (UL L 290/54). Podatkov o vsebnosti semen te rastline v krmi je zelo malo, zato smo z mikroskopsko metodo preiskali 40 vzorcev krme za prostoživeče ptice. Semena ambrozije je vsebovalo 20 vzorcev oziroma 50 %. Ugotovljeno število semen v kilogramu posameznega vzorca je bilo od 2 do 146 (10 mg do 774 mg). V skladu s predpisi smo vsebnosti preračunali relativno na vzorec z 12 % vlage in ugotovili, da je dovoljeno mejo presegalo 5 vzorcev (12,5 %).

Ključne besede: *Ambrosi*a, semena, krma – analize, mikroskopija, ptice

ABSTRACT

RECENT DATA ON *Ambrosia* spp. SEEDS CONTENT IN FEED FOR WILD BIRDS IN SLOVENIA

Inhalation of pollen belonging to the species of *Ambrosia* may cause hypersensitivity reactions. Feed for wild birds is one of the factors that contribute to the spread of these plants. For this reason an amendment to the Directive on undesirable substances in animal feed (2002/32/EC) was added, in 2010 stating that animal feed made of unground cereals can contain up to 50 mg of *Ambrosia* spp. seeds per kilogram (UL L 290/54). Due to the lack of data, 40 samples of feed for wild birds were examined with a microscopic method. *Ambrosia* spp. seeds were found in 20 samples (50%). The number of seeds was from 2 to 146 (10 mg to 774 mg). In accordance with the legislation, results were expressed relative to a feed with the moisture content of 12%. Five samples (12.5%) exceeded the permitted value.

Key words: *Ambrosia*, seeds, feed – analysis, microscopy, birds

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Prispevek je del Prešernove naloge z naslovom Ugotavljanje semen rastline *Ambrosia* spp. v krmi za prostoživeče ptice. Delo je bilo opravljeno na Veterinarski fakulteti v Ljubljani leta 2012 pod mentorstvom doc. dr. Brede Jakovac Strajn.

1 UVOD

Med vrstami iz rodu *Ambrosia* je v Sloveniji najbolj poznana in razširjena pelinolistna ambrozija ali pelinolistna žvrklja (*Ambrosia artemisiifolia* L.). Spada v družino nebinovk (*Asteraceae*). Značilnost pelinolistne ambrozije je, da ima moške in ženske cvetove v ločenih koških na isti rastlini (Buttenschon in sod., 2009). Količina cvetnega prahu ene rastline niha med sto milijoni in tremi milijardami pelodnih zrnc. Oprašena rastlina ima lahko več kot 6000 semen. Na tvorbo semen vplivata velikost rastlin in okolje, v katerem uspeva (Fumanal in sod., 2007a, b). Rastline, ki rastejo med poljščinami tvorijo večje količine cvetnega prahu in semen (Fumanal in sod., 2007a, b). Največ cvetnega prahu je v krogu s premerom enega kilometra okoli rastline (Simard in sod., 2011).

Velike količine cvetnega prahu ambrozije se z vetrom dvignejo v zrak in tako lahko pridejo v dihala ljudi in živali. Najpogostejši preobčutljivostni reakciji na vdihovanje cvetnega prahu sta vnetje nosne in očesne sluznice (rinokonjunktivitis) ter naduha (astma). Če pa pride cvetni prah na kožo, bodisi z zrakom bodisi z dotikom, lahko povzroči atopijski in kontaktni dermatitis (EFSA, 2010).

Vrste iz rodu *Ambrosia* so avtohtone v Severni Ameriki, od koder so se razširile po svetu najverjetneje z izvozom žit (EFSA, 2010). Najprej so jih opisovali kot plevel na podeželju, kjer so rastle v bližini kmetij, ob poteh, na pašnikih, njivah in travnikih, tam, kjer je bila uničena prvotna vegetacija (Kofol Seliger, 2001).

Ena od možnih poti širjenja je tudi širjenje semen ambrozije s krmo za prostoživeče ptice (Frick in sod., 2011). Že pred slabimi tremi desetletji sta Hanson in Mason (1985) uspešno vzgojila pelinolistno ambrozijo iz semen, zbranih iz krme za prostoživeče ptice. S tem sta opozorila, da je ptičja krma možni vektor pri širjenju rastline.

Danes je dobro znano, da se vrste iz rodu *Ambrosia* pojavljajo v vrtovih in območjih, kjer ljudje krmijo prostoživeče ptice (Dahl, 1999; Bohren, 2005), kar potrjuje prej omenjeno teorijo.

Po trenutnih ocenah kar 20 – 91 % krme za prostoživeče ptice vsebuje semena vrst iz rodu *Ambrosia,* od katerih jih do 25 % vzkali (EFSA, 2010). Ohranjanje kaljivosti semen je odvisno predvsem od temperature in globine tal, v katerih se seme nahaja. Na globini 35 do 45 cm so semena zelo obstojna in lahko v zemlji preživijo tudi 30 do 40 let (Baskin in sod., 1977). Zanje je značilno, da za klitje potrebujejo nizke temperature (Basset in Crompton, 1975). Po vzklitju se vrste iz rodu *Ambrosia* na ruderalnih rastiščih zelo uspešno širijo, kajti na njih ni sklenjene konkurenčne vegetacije (Šilc, 2006).

Do širjenja semen s krmo za prostoživeče ptice lahko pride z neposrednim prenosom, ko ljudje trosijo krmo za prostoživeče ptice po tleh ali ko odvržejo ptičje iztrebke in odpadke iz kletk v okolje (Essl in sod., 2009). Posredno se semena širijo s pticami in malimi sesalci, ki med hranjenjem prebirajo semena ali pa jih odnesejo v svoja gnezda in shrambe. Prav tako obstaja možnost, da semena nepoškodovano potujejo skozi prebavni trakt semenojedih živali in se tako širijo z njihovimi iztrebki (Alberternst in sod., 2008). Veliko rastlin zato najdemo v zasebnih vrtovih, kakor tudi na območjih reje perutnine, ki se giblje na prostem (EFSA, 2010).

Za ugotavljanje vsebnost semen v krmi moramo uporabljati ustrezne hitre in zanesljive metode. Ta čas priporočajo mikroskopsko preiskavo (IAG – Method 5). Na ta način smo v letih 2008 in 2009 v Sloveniji preiskali 20 vzorcev hrane za prostoživeče ptice in ugotovili, da je kar 13 od 20 vzorcev vsebovalo od 1 do 235 semen različnih vrst ambrozij (Ujčič-Vrhovnik in sod., 2008).

Ker od takrat za ptičjo krmo dostopno na slovenskem tržišču ni bilo veliko novih podatkov, smo želeli preveriti, kakšno je trenutno stanje glede vsebnosti semen vrst iz rodu *Ambrosia* v krmi za ptice.

2 MATERIAL IN METODE

2.1 Material

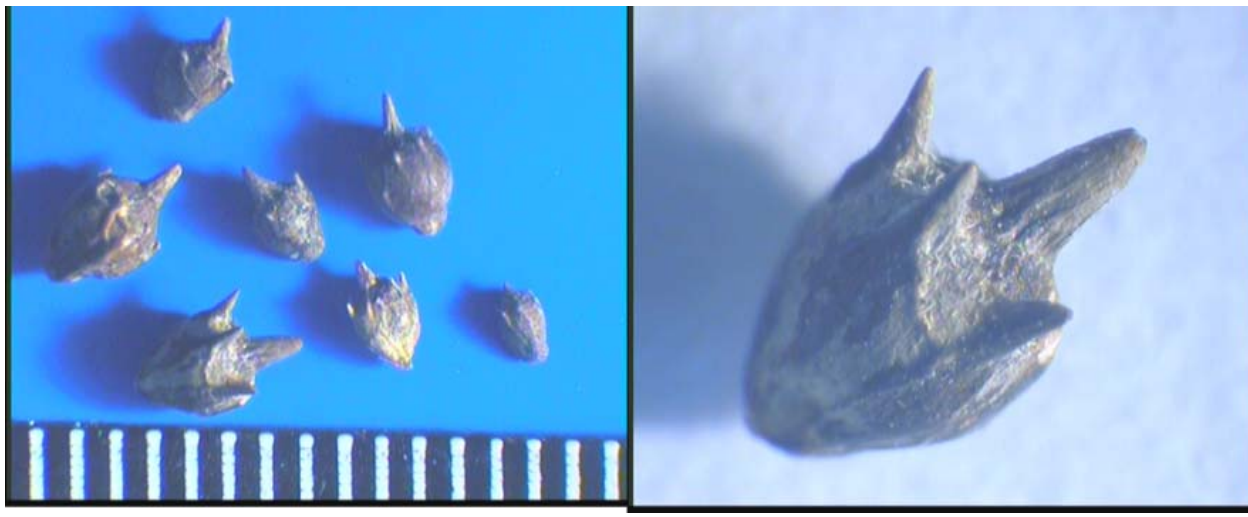
Raziskavo smo opravili na 40. vzorcih krme za prostoživeče ptice. Krmo smo kupili v različnih trgovskih centrih in specializiranih trgovinah za male živali po Sloveniji pozimi 2011/12. Izbrali smo 40 različnih vzorcev: 26 vzorcev semen sončnic (označili smo jih od S1 do S26) in 14 vzorcev mešanih semen (oznake M1 do M14). Krma je bila pakirana v vrečkah po 1 kg. Surovine za pripravo krme so izvirale iz območij izven ozemlja Slovenije.

2.2 Metode dela

Mikroskopska metoda

Za preiskavo vzorcev krme na vsebnost semen vrst iz rodu *Ambrosia* smo uporabili mikroskopsko metodo, pri čemer smo si pomagali z lupo in stereomikroskopom (IAG – Method 5).

V skladu s protokolom smo vsak vzorec razdelili z razdelilnikom na dva manjša vzorca po 500 g. S tehtanjem smo preverili maso, nato pa smo enega zavrgli, drugega pa presejali na sitih z različno velikostjo odprtin: < 1,6 mm, 1,6 - 4,0 mm in > 4,0 mm. Preiskali smo vse tri frakcije. Sumljiva semena smo odbrali v označene petrijevke in jih pregledali s primerno povečavo pod lupo in stereomikroskopom. Pri identifikaciji enosemenskih plodičev smo si pomagali z referenčnim materialom in morfološkimi opisi iz botanične literature (Bessett in Crompton, 1975; 1982). Preučevana ptičja krma je skoraj v popolnosti vsebovala nepoškodovane enosemenske plodiče s celotno ovojnico. Čistih semen brez ovojnice praktično naši vzorci niso vsebovali. V tem besedilu iz praktičnih razlogov namesto botanično ustreznega izraza enosemenski plodič uporabljamo izraz seme. Semena vrst iz rodu *Ambrosia* smo identificirali glede na njihovo značilno obliko in zgradbo (Slika 1). Nato smo jih prešteli in stehtali na analitski tehtnici. Botanične določitve do ravni vrste nismo opravili, ker zakonodaja (UL L 290/54) ne razmejuje mejnih vsebnosti med različnimi vrstami ambrozij. Semena vseh vrst obravnava enako.



Slika 1: Enosemenski plodiči, rožke (»semena«) vrst iz rodu *Ambrosia* pod stereomikroskopom.
Figure 1: One seed fruitlets, achenes («seeds«) of *Ambrosia* spp. under stereomicroscope.

Maso semen v vzorcih krme smo podali v mg kg^{-1}, pri čemer smo maso odbranih semen (v mg) množili s 1000 in zmnožek delili s celotno maso vzorca, uporabljenega za preiskavo (v kg).

Določanje vlage v krmi

Vlago v krmi smo določili s sušenjem vzorca pri temperaturi 103 °C 3 ure. Vzorce, v katerih smo ugotovili semena vrst iz rodu *Ambrosia*, smo zmleli ter natehtali približno 5 g v tehtič za določanje vlage. Tehtič smo pred tem označili in

stehtali. Po sušenju smo vzorec skupaj s tehtičem ponovno stehtali. Iz dobljenih rezultatov smo izračunali odstotek vlage v vzorcu.

3 REZULTATI IN DISKUSIJA

Semena vrst iz rodu *Ambrosia* je vsebovalo 8 vzorcev krmnih mešanic (57 %) (Preglednica 1) in 12 vzorcev semen sončnic (46 %) (Preglednica 2). V preostalih vzorcih semen ambrozije nismo našli.

Preglednica 1: Ugotovljena semena vrst iz rodu *Ambrosia* v mešanicah, ki se uporabljajo za krmljenje prostoživečih ptic (v 500 g).
Table 1: Identified seeds of *Ambrosia* spp. in seed mixtures, used for feeding wild birds (in 500 g).

Vzorec Sample	Sejane frakcije (mm) Fractions			Ugotovljeno število semen vrst iz rodu *Ambrosia* Number of identified seeds	Skupna masa semen vrst iz rodu *Ambrosia* (mg) Total mass of seeds (mg)	Preračunana masa semen v kg krme (mg/kg) Equivalent of seeds per kg (mg/kg)	Vlaga % Moisture %
	<1,6	*1,6 - 4,0*	*>4,0*				
M3	1	3	-	4	14,46	28,92	5,60
M4	-	6	-	6	28,07	56,14	4,51
M5	-	1	-	1	5,04	10,08	8,42
M6	1	6	-	7	17,55	35,10	10,90
M9	-	2	-	2	10,26	20,52	5,83
M10	-	8	-	8	58,34	116,68	5,84
M12	3	-	-	3	6,88	13,76	11,90
M14	-	1	-	1	4,94	9,80	7,28

Preglednica 2: Ugotovljena semena vrst iz rodu *Ambrosia* med semeni sončnic, ki se uporabljajo za krmljenje prostoživečih ptic (v 500 g).
Table 2: Identified seeds of *Ambrosia* spp. intermixed to sunflower seeds, used for feeding wild birds (in 500 g).

Vzorec Sample	Sejane frakcije (mm) Fractions			Ugotovljeno število semen vrst iz rodu *Ambrosia* Number of identified seeds	Skupna masa semen vrst iz rodu *Ambrosia* (mg) Total mass of seeds (mg)	Preračunana masa semen na kg krme (mg/kg) Equivalent of seeds per kg (mg/kg)	Vlaga % Moisture %
	<1,6	*1,6 - 4,0*	*>4,0*				
S1	-	4	-	4	21,28	42,56	5,84
S6	-	73	-	73	387,23	774,46	6,40
S7	-	2	-	2	7,13	14,26	5,56
S8	-	2	-	2	9,99	19,98	5,77
S11	9	27	-	36	121,89	243,78	5,94
S12	-	1	-	1	5,80	11,60	5,29
S13	13	27	1	41	126,14	252,28	5,86
S14	1	2	-	3	12,52	25,04	3,42
S17	-	2	-	2	11,55	22,88	7,61
S18	-	2	-	2	8,71	17,42	4,66
S19	1	1	-	2	13,27	26,54	4,49
S20	-	5	-	5	18,88	37,76	3,76

V vzorcih smo našteli od 1 do 73 semen vrst iz rodu *Ambrosia* (5,04 mg do 387,23 mg) na 500 g ptičje krme. Kot smo že zapisali je pelinolistna ambrozija najpogostejša vrsta iz rodu *Ambrosia* v Evropi, precej manj pogoste so druge vrste, na primer trikrpata ambrozija (*A. trifida* L. in trajna ambrozija (*A. psilostachya* DC. (*A. coronopifolia* Torr.&Gray)). Semena vseh vrst so si podobna, zato se zgolj z mikroskopsko preiskavo ni dalo izključiti možnosti, da katero izmed odbranih semen ne pripada kakšni drugi in ne najpogostejši vrsti. Pri označevanju našega izbora semen smo torej uporabili širši pojem semena ambrozij (*Ambrosia* spp.), čeprav je večina semen zelo verjetno izvirala od pelinolistne ambrozije. Naj navedemo še podatek, da so alergene vse vrste iz rodu *Ambrosia* (D'amato in sod., 2007). Podatkov o pojavljanju semen drugih vrst ambrozij v ptičji krmi na območju srednje Evrope je izredno malo. V literaturi je tudi za druge vrste možno najti navedbe, da se lahko širijo s ptičjo krmo (Comtois, 1998; Karnkowski, 1999a, b; Bechet, 2004; Follak in sod. 2013). Možnosti za pojav drugih vrst v ptičji krmi so povezane z izvorom surovin, iz katerih se pripravljajo krmne mešanice (Nawrath in Alberternst, 2012). V primeru proizvajalcev, ki kot surovino uporabljajo ostanke pri čiščenju pošiljk semen iz drugih kontinentov (npr. Argentina, Brazilija, Kanada, ZDA, Južna Afrika, …) ali pa iz območij, kjer so kmetijske površine že zapleveljene z drugimi vrstami ambrozij (npr. Poljska, Ukrajina, Španija, Izrael, Ruska Federacija, Kitajska, …) obstaja povečana verjetnost, da bi krmne mešanice lahko vsebovale semena drugih vrst.

Največ semen smo odbrali v srednji frakciji (1,6 mm - 4 mm). V frakciji, večji od 4 mm, smo našli eno seme, v frakciji manjši od 1,6 mm pa smo ugotovili v sedmih vzorcih od 1 do 13 semen. V vzorcu S11 jih je bilo 9 od skupaj 36 najdenih semen. V vzorcu S13 pa jih je bilo 13 od skupaj 41 najdenih semen. V vzorcu M12 smo našli le 3 semena manjša od 1,6 mm, ki so tehtala 6,88 mg (13,76 mg/kg krme).

Povprečna masa semena v srednji frakciji je bila 4,908 mg, v najmanjši frakciji pa 2,600 mg. Povprečna masa semena, če upoštevamo semena obeh frakcij, je bila 4,548 mg.

Po protokolu mikroskopske metode bi bilo sicer dovolj, če bi preiskali samo frakcijo semen velikosti 1,5 do 4 mm. Vendar pa smo pri našem delu ugotovili semena vrst iz rodu *Ambrosia* v vseh treh sejanih frakcijah (<1,6 mm, 1,6-4,0 mm in >4,0 mm), skupno v 20 vzorcih. Če bi obdelali samo srednjo frakcijo, bi kot pozitivne določili 19 vzorcev, kar se ne razlikuje bistveno od prej omenjenega rezultata. Vendar pa dejstvo, da smo v zadnji frakciji našli semena ambrozije v 7 vzorcih, nikakor ni zanemarljivo. Število semen v tej frakciji je nihalo od 1 do 13. Večina avtorjev sicer meni, da semen, manjših od 1,5 mm ni in da so večja od 3 mm zelo redka (Frick in sod., 2011). Glede na naše rezultate menimo, da sta za preiskavo vzorcev pomembni srednja in najdrobnejša frakcija. Ker majhnost semen še ne pomeni, da ta niso kaljiva (Fumanal in sod., 2007a, b) bi bilo strokovno ustrezno obravnavati vse frakcije. Tudi zelo drobna semena lahko omogočajo razširjanje.

Predpisi EU navajajo, da lahko krma za živali, ki vsebuje nezmleta žita, vsebuje do 50 mg semen vrst iz rodu *Ambrosia*/kg krme, izraženo pri 12 % vlagi v vzorcu (UL L 290/54). Rezultate smo zato preračunali na 12 % vlago ter ugotovili, da vrednost 50 mg/kg pomeni približno 9-10 semen v kilogramu krme. Dovoljeno mejo je presegalo 5 vzorcev (12,5 %). Bolj onesnaženi so bili vzorci sončničnih semen. Med njimi je bil tudi vzorec, ki je vseboval 774,46 mg semen (Preglednica 1 in 2).

V skladu z zgoraj navedenimi predpisi se v Sloveniji izvaja letni nadzor krme, vendar je predvideno število uradnih vzorcev majhno (trije vzorci) oziroma premajhno, da bi bil nadzor verodostojen.

Na Bavarskem so leta 2008 ugotovili, da so verjetno 42 % populacije pelinolistne ambrozije tja zanesli z onesnaženo krmo za sobne ali prostoživeče ptice (Vitalos in sod., 2008). V Nemčiji kontaminacija komercialne ptičje krme niha od 0 do 34 semen vrst iz rodu *Ambrosia* na kilogram krme. Povprečne vrednosti so 23,8 semen v kilogramu, največ pa so našteli 170 semen (Thibaudon in sod., 2012). V Franciji je Chauvel s sod. (2006) določal vsebnost semen vrst iz rodu *Ambrosia* v krmi iz sončničnih semen. Ugotovili so, da je možnost širjenja vrst iz rodu *Ambrosia* s sončničnimi semeni, namenjenimi za prehrano ptic

za približno 10 % večja od možnosti širjenja z drugimi semeni, namenjenimi za prehrano živine. To potrjuje tudi raziskava raziskovalcev Strgulc-Krajšek in Novak (2013). Rezultati naše raziskave so podobni rezultatom pridobljenim v okviru uradnega nadzora krme v nekaterih drugih državah, na primer iz Švice. Švicarski podatki za obdobje 2009-2013 so dostopni na spletu (admin.ch, 2013).

Tudi v Sloveniji smo v preteklih raziskavah ugotovili, da je semena vrst iz rodu *Ambrosia* vsebovala več kot polovica pregledanih vzorcev komercialno dostopne hrane za prostoživeče ptice (Ujčič-Vrhovnik in sod., 2008). Povprečno je kilogram krme za prostoživeče ptice vseboval 86 semen. V naši raziskavi je bilo to število dosti manjše, vendar pa smo v obeh raziskavah prišli do enakega sklepa: semena sončnic so bolj ter pogosteje onesnažena s semeni vrst iz rodu *Ambrosia* kot mešanice semen. Sončnice so poljščina, kjer imamo težave pri kemičnem zatiranju ambrozije. Ambrozija in sončnica (*Helianthus sp.*) sta sorodna rodova in pripadata isti družini, zato nimamo na voljo velike izbire učinkovitih herbicidov. Izjema so gensko spremenjeni hibridi in hibridi selekcionirani na odpornost na posamezne herbicide (npr. IMI sončnice). Izhodiščna surovina za pripravo ptičje krme lahko vsebuje zelo velike količine semen ambrozije, kar ustrezno pojasni prejšnje podatke (EFSA, 2010).

V Švici mora biti od marca 2005 vsa komercialno dostopna ptičja hrana, tako domača kot iz uvoza, prosta semen vrst iz rodu *Ambrosia.* Med 40 vzorci komercialno dostopne krme za prostoživeče ptice smo pregledali tudi dva vzorca, ki sta imela napis, da ne vsebujeta semen ambrozije. V teh vzorcih semen vrst iz rodu *Ambrosia* nismo našli. V tujini imajo izkušnje, da se tudi v pakiranjih, označenih z oznako ″prosto ambrozije″ večkrat najdejo kaljiva semena (LUGV, 2011). Iz teh razlogov so večkrat izpostavljene zahteve po termičnem obdelovanju krmnih mešanic. Ker te predstavljajo pot vnosa tudi za druge neželene invazivne plevele (npr. iz rodov *Panicum*, *Phalaris*, *Bidens*, *Iva*, *Sorghum*, *Setaria*, *Polygonum*, *Eleusine*, *Amaranthus*, …) ponekod uporabnikom mešanic svetujejo termično obdelavo doma (kar v kuhinjski pečici) in sistematično opazovanje mest, kjer krmila nastavljajo pticam. Osveščeni ljubitelji ptic potem redno spremljajo, katere rastline se pojavljajo ob krmiščih in neželene pravočasno odstranijo.

4 SKLEPI

Vrste iz rodu *Ambrosia* so močno alergene rastline, ki se lahko širijo tudi s krmo za prostoživeče ptice. S pregledom 40 vzorcev takšne krme smo ugotovili:

1. da so bila semena vrst iz rodu *Ambrosia* v 50 % vzorcev, kar je veliko. Največjo dovoljeno vsebnost semen ambrozij, ki jo predpisuje evropska zakonodaja je sicer presegalo 5 vzorcev, vendar pa bi se vrste lahko širile z vsemi vzorci, ki so vsebovali njihova semena.

2. Pelinolistna ambrozija (*A. artemisiifolia*) se ja na ozemlju RS v zadnjih desetletjih tako razširila, da njeno izkoreninjenje več ni možno. Kljub temu je sistematične preiskave krme za prostoživeče ptice glede vsebnosti semen ambrozij smiselno nadaljevati, ker se s ptičjo krmo lahko širijo tudi druge vrste, ki pa jih na ozemlju RS še nimamo (npr. *A. trifida* L., *A. tenuifolia* Sprengl, *A. psilostachya* DC., *A. grayi* (A. Nels) Shinners in *A. confertiflora* DC.). V primeru najdb teh vrst v ptičji krmi pa je potrebno preventivno ukrepati in sprejeti pravočasne ukrepe za preprečitev širjenja.

3. Preiskave krme za prostoživeče ptice je smiselno nadaljevati v čim večjem obsegu in s tem sodelovati pri omejevanju širjenja ambrozij.

4. Z rezultati smo potrdili možnosti širjenja vrst iz rodu *Ambrosia* s ptičjo krmo tudi v Sloveniji. Ljudi bi lahko bolj osveščali, da s hrano za prostoživeče ptice, ki vsebuje semena vrst iz rodu *Ambrosia* v svojo okolico lahko zanesejo to nevarno rastlino. Potrošnike ptičje krme bi bilo dobro opozoriti, da naj opazujejo, katere rastline se pojavljajo v bližini krmišč prostoživečih ptic in naj neželene odstranijo preden oblikujejo seme.

5. Pri mikroskopski preiskavi predlagamo pregled tudi srednje in najmanjše frakcije.

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6 VIRI

Admin.ch – Bundesverwaltung berichte, 2013. Amtlichen Futtermittelkontrolle - Tabelle 1: Ergebnisse der Ambrosiauntersuchungen 2009 - 2013. (http://www.news.admin.ch/message/index.html?lang=de&msg-id=48259)

Alberternst B., Nawrath S., Hussner A., Starfinger J. 2008. Auswirkungen invasiver Arten und Vorsorge - Sofortmaßnahmen und Management am Beispiel von vier unterschiedlich weit verbreiteten Neophyten. Natuur und Landschaft, 83: 412-417

Baskin J.M., Baskin C.C. 1977. Dormancy and germination in seeds of common ragweed with reference to Beal's buried seed experiement. American Journal of Botany, 64, 9: 1174-1176

Bassett I.J., Crompton C.W. 1975. Biology of Canadian weeds. Ambrosia-Artemisiifolia l and A-Pilostachya DC. Canadian Journal of Plant Science, 55: 463-476

Bassett I.J., Crompton C.W. 1982. The biology of Canadian weeds. 55. Ambrosia trifida L. Canadian Journal of Plant Science, 63: 1003-1010

Behcet L. 2004. A new record for the flora of Turkey: *Ambrosia tenuifolia* Spreng. (Compositae). Turkish Journal of Botany, 28: 201-203

Bohren C., Delabays N., Mermillod G., Keimer C., Kündig C. 2005. *Ambrosia artemisiifolia* in der Schweiz - eine herbologische Annäherung. Agrarforschung, 12: 7-78.

Buttenschøn R.M., Waldispühl S., Bohren C., Simončič A., Lešnik M., Leskošek R. Navodila za zatiranje in preprečevanje širjenja pelinolistne ambrozije (*Ambrosia artemisiifolia*). Projekt EUPHRESCO – strategies for *Ambrosia* control 2008-2009. 47 str. http://www.ruse.si/data/upload/ambrozija_navodila_za_zatiranje.pdf (28. maj 2013)

Chauvel B., Dessaint F., Cardinal-Legrand C., Bretagnolle F. 2006. The historical spread of *Ambrosia artemisiifolia L.* in France from herbarium records. Journal of Biogeography, 33: 665-673

Comtois P. 1998. Ragweed (Ambrosia sp.): The Phoenix of allergophytes. V: F. T. M. Spieksma (Ed.), Ragweed in Europe. The 6th International congress on aerobiology, Symposium Proceedings, Perugia, Italy, 3–5

D'amato G., Cecchi L., Bonini S. et al. 2007. Allergic pollen and pollen allergy in Europe. Allergy, 62: 976-990

Dahl Å., Standhede S., Wihl J. 1999. Ragweed - an allergy risk in Sweden? Aerobiologia, 15: 293-297

EFSA. 2010. Scientific opinion on the effect on public or animal health or on the environment on the presence of seeds of *Ambrosia sp*. in animal feed. EFSA Journal, 8, 6: 1566

Essl F., Dullinger S., Kleinbauer I. 2009. Changes in the spatio-temporal patterns and habitat preferences of *Ambrosia artemisiifolia* during its invasion of Austria. Preslia, 81: 119–133

Follak S., Dullinger S., Kleinbauer I., Moser D., Essl F. 2013. Invasion dynamics of three allergenic invasive *Asteraceae* (*Ambrosia trifida*, *Artemisia annua*, *Iva xanthiifolia*) in central and eastern Europe. Preslia, 85: 41-61

Frick G., Boschung H., Schulz-Schroeder G. et al. 2011. Ragweed (*Ambrosia sp.*) seeds in bird feed. Biotechnology, Agronomy, Society and Environment, 15, 1: 39-44

Fumanal B., Chauvel B., Bretagnolle F. 2007a. Estimation of pollen and seed production of common ragweed in France. Annals of Agricultural and Environmental Medicine, 14: 233-236

Fumanal B., Chauvel B., Sabatier A., Bretagnolle F. 2007b. Variability and cryptic heteromorphism of *Ambrosia artemisiifolia* seeds: what consequences for its invasion in France?, Annales of Botanyyy, 100: 305-313

Hanson C., Mason J. 1985. Bird seed aliens in Britain. Walsonia, 15: 237-252

IAG-Method 5. Method for the Determination of *Ambrosia* (*Ambrosia artemisiifolia L.)* in non-pelleted Animal Feedingstuff. International Association of Feedingstuff Analysis. http://www.iag-micro.org/files/iag-a5_ambrosia.pdf (28 maj 2012)

Karnkowski W. 1999a. Pest Risk Analysis on *Ambrosia* spp. for Poland. Main Inspectorate of Plant Protection Monograph .08-14124 PRA, 54 s.

Karnkowski, W., 1999b. Quarantine weeds and parasitic plants occurring in the plant material imported to Poland in 1996-1999, Ochrona Roślin**,** 43: 15-16

Kofol Seliger A. 2001. Rod ambrozija (žvrklja). Proteus, 63, 6: 276-278

LUGV - Landesamt für Umwelt, Gesundheit und Verbraucherschutz Brandenburg, 2011. Amtliche Futtermittelkontrolle nach Ambrosiasamen - Ergebnisse der Amtlichen Vogelfutteruntersuchungen aus 2009 und 2010. (http://www.lugv.brandenburg.de/cms/detail.php/bb1.c.331423.de)

Nawrath S., Alberternst B. 2012. Forschungsvorhaben Beifuß-Ambrosie in Bayern FOBAB II-Studie. Bayerischen Staatsministeriums für Umwelt und Gesundheit, Friedberg / Hessen, Endbericht, 203 s.

Simard M.J., Benoit D.L. 2011. Effect of repetitive mowing on common ragweed (*Ambrosia artemisiifolia L.*) pollen and seed production. Annals of Agricultural and Environmental Medicine, 18, 1: 55-62

Strgulc-Krajšek S., Novak M. 2013. Achenes of common ragweed (*Ambrosia artemisiifolia)* in packages of sunflower achenes for outdoor birds. Acta biologica Slovenica, 56, 1: 3-9

Šilc U. 2006. Vsiljiva škodljivka iz Severne Amerike. Proteus, 69, 2: 81-83

Thibaudon M., Colonnello C., Basancenot J.P., Toloba Y., Francois H., Caillaud C. 2012. Can birdfeed contribute to the spread of Ragweed? Journal of Investigational Allergology and Clinical Immunology, 22, 3: 215-235

Ujčič-Vrhovnik I., Jakovac-Strajn B., Vengušt A. 2008. Mikroskopska preiskava krme. V: 17. Mednarodno znanstveno posvetovanje o prehrani domačih živali: Zadravčevo-Erjavčevi dnevi. Radenci: Kmetijsko gozdarska zbornica Slovenije: 23-30

Vitalos M., Karrer G. 2008. Distribution of *Ambrosia artemisiifolia* L.: Is birdseed a relevant vector? Journal of Plant Diseases and Protection: 345-347

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Tla ali prst ? Prispevek k razpravam o rabi izrazov 'tla' in 'prst' v slovenskem poljudnem in strokovnem izrazoslovju

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IZVLEČEK

Skrb za natančno in bogato izrazoslovje, tako poljudno in predvsem strokovno, je nujen in pomemben prispevek k razvoju in pestrosti materinega jezika. Pri rabi strokovnih izrazov se pogosto soočamo z različnimi interpretacijami in nepotrebno pestrostjo izrazov. To praviloma ne prispeva h kakovosti jezika in prej kaže na premalo skrbno izrazoslovje, na pomanjkljive oz. strokovno neustrezne opredelitve in/ali spregledan izvorni pomen posameznih izrazov. Strokovni izrazi morajo biti nedvoumni in ne smejo dovoljevati različne interpretacije, pomena. Raba in uvajanje poljudnih izrazov v strokovno izrazoslovje je prisotno tudi v izobraževanju. Primer takih zadreg v pedologiji je tudi raba besed 'tla' in 'prst'. Na prvi pogled sicer obrobna tematika ima širšo in pomembnejšo dimenzijo. Zmeda v strokovnem jeziku se namreč pojavlja tudi v prevodih evropske zakonodaje. Tako slovenske verzije nekaterih EU dokumentov mestoma zaradi neustreznih in pomensko dvoumnih oz. zgrešenih prevodov ne odražajo pravega pomena izvornih besedil. To predstavlja zadrego, ki jo je potrebno urediti. Prispevek predstavlja terminološke nedoslednosti v pedološkem izrazoslovju, pojasnjuje in utemeljuje razloge za rabo osnovnih izrazov s področja tal, osvetljuje ljudski pomen besed 'prst' in 'zemlja', primerja s stanjem v drugih jezikih ter nakazuje ustrezno rabo nekaterih ključnih izrazov v strokovnih/znanstvenih besedilih, za potrebe prevajanja in pedagoškega procesa.

Ključne besede: tla, prst, zemlja, jerina, ilovica, pedologija

ABSTRACT

A CONTRIBUTION TO THE DEBATE ON THE USE OF THE TERMS 'TLA' AND 'PRST' IN SLOVENIAN COLLOQUIAL AND PROFESSIONAL TERMINOLOGY

Correct and rich professional and scientific terminology is an important contribution to the development and richness of the national languages. Within the scientific and professional terminology we can often find incorrect, missed or misinterpreted use of professional terms. In general, such ‘diversity’ of terms does not contribute to the quality and the development of the professional terminology. On the contrary, it demonstrates the lack of precision in scientific terminology, presence of definitions without scientific background and/or overlooked original etymology of individual terms. We are witnessing the attempts of replacement of terms and changes in professional terminology without scientifically sound arguments. Moreover, even at the university level the inappropriate use of technical / professional terms can be detected. An example of such embarrassments in Slovenian language is the use of the words' tla' and 'prst' in professional language. Terminological dispute can be considered as peripheral theme, even unnecessary, however it has a much broader and more important dimension. Namely, confusion in the technical language is also appearing in translations of the European legislation. The Slovenian versions of some EU legal documents are semantically ambiguous and inadequate and, thus do not reflect the correct meaning of source texts due to missed translations. The latter can evolve in a problem and has to be adjusted. The paper presents the soil science terminological inconsistencies, explains and arguments the reasons for use of selected basic soil science terms in Slovenian language. Additionally, it recalls the etymology of the popular terms ‘prst’ and ‘zemlja’, compares the situation in number of European languages and, most importantly, suggests the appropriate use of terms in professional/scientific language, in education and for translation purposes.

Key words: soil, fine earth, earth, terra rossa, loam, pedology

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1 UVOD

V medijih lahko občasno zasledimo polemike o potrebnosti in nujnosti izobraževanja na univerzah tudi v tujem jeziku. Poleg ostalega, pomembnega a nebistvenega za ta prispevek, so avtorji zapisali sicer redko izraženo mnenje, da je slovensko strokovno izrazoslovje pomembno in da ga je potrebno vzdrževati in razvijati. Drži, strokovnjaki in učitelji morajo skrbeti za dobro, natančno, pestro in bogato izrazoslovje, tako poljudno kot strokovno in na ta način prispevati k razvoju in pestrosti materinega jezika. Vendar se pri rabi strokovnih izrazov neredko soočamo s problemi, ko v strokovnem izrazoslovju obstajajo različne interpretacije in pestrost izrazov. To praviloma ne prispeva k bogastvu strokovnega jezika; prej kaže na premalo skrbno izrazoslovje, na etimološko pomanjkljive oz. strokovno neustrezne opredelitve in/ali spregledan izvorni pomen posameznih izrazov.

Primer takih zadreg je raba besed 'tla' in'prst', sicer osnovnih izrazov v pedologiji. Izraza izvirata iz dveh različnih »šol«. Natančneje, gre za geografsko 'prst' in pedološka 'tla' (Repe, 2009). 'Prst' je v uporabi v okviru pedogeografije in posledično celotne geografije (Lovrenčak, 1994). Izraz 'tla' uporablja matična pedologija oz. biotehnika, povzele so jo druge vede, državna uprava in zakonodaja. Kljub temu prihaja v zadnjih letih do poizkusov spreminjanja izrazov v pedoloških avtorskih besedilih in predlogov zamenjave izraza 'tla' z izrazom 'prst'. Včasih smo priča slabim kompromisom (Jamnik in sod., 2009), ki v resnici vnašajo zmedo v strokovni in pogovorni jezik. Ravno tako pri poimenovanju vede 'pedologija' prihaja do nadomeščanja s 'pedogeografijo' (Kladnik, 1999). Raba besede 'prst' se širše pojavlja v medijih, posameznih publikacijah in pogosto napačno v delih diplomantov Biotehniške fakultete (Fabjan, 2006; Hafner, 2007; Drašler, 2008; Jamšek, 2009; Bremec, 2011; Kunšek, 2011; Drofenik, 2012).

Problem bi lahko uvrstili med nesmiselne terminološke razprave, vendar se v zadnjih letih neustrezna raba besed 'prst' pojavlja tudi v prevodih evropske zakonodaje. Tako slovenske verzije nekaterih dokumentov mestoma ne odražajo pravega pomena izvornih besedil (Evropska komisija [European Commission], 2006, 2011, 2012), kar pa presega akademski terminološki problem.

Menim, da neustrezna raba izrazov krni strokovno izrazoslovje, vnaša zmedo v zakonodajno izrazoslovje in siromaši pogovorni jezik. Namen besedila ni uvajanje novitet pač pa prispevati k urejanju terminoloških nedoslednosti na podlagi izvornih razlogov, predvsem na podlagi zgradbe in geneze tal. Želi (ponovno) predstaviti in utemeljiti strokovne razloge za rabo izraza 'tla'; osvetliti ljudski pomen 'prsti' in 'zemlje' in nekaterih drugih izrazov; predstaviti primerjavo v drugih jezikih. Predvsem pa želi besedilo nakazati ustrezno in nedvoumno rabo izrazov v strokovnih besedilih in zakonodaji.

1.1 Tla, veda o tleh

Tla so tvorba na površini zemlje z logično zgradbo, ki se razvija in spreminja v prostoru in času. Sestavljena so iz horizontov (gradnikov) zelo različnih kemijskih in fizikalnih lastnosti ter pojavnih oblik. Prisotnost in lastnosti posameznih horizontov (globina pojavljanja, barva, struktura, tekstura, kislost, vsebnost organske snovi in hranil…) in njihova razporeditev v profilu, določajo skupne lastnosti tal (skupna globina, rodovitnost, erodibilnost, propustnost, …). Glede na lastnosti horizontov, zgradbo, skupne lastnosti in lego v profilu izkazujejo tla različno kakovost za posamezne vrste rabe ter s tem različno primarno primernost, uporabnost oz. namembnost. Med mnogimi lastnostmi, ki jih tla v svojem razvoju pridobijo (in tudi izgubijo), smo v kmetijstvu in biotehniki izpostavljali predvsem rodovitnost - temeljno in za obstoj življenja v kopenskih ekosistemih najpomembnejšo lastnost. Zato pridelava hrane/biomase že dolgo velja za primarno funkcijo tal. V zadnjem času postajajo (enako ali bolj?) pomembne ključne ekosistemske oz. okoljske funkcije tal in storitve (npr. nevtralizacija in imobilizacija škodljivih snovi, filtriranje voda, ponor/vir atmosferskega ogljika, kroženje biogeogenih prvin - hranil, uravnavanje pretoka energije in kroženje snovi,… itd.). Intenzivnejše raziskovanje vloge tal v kopenskih ekosistemih, vpliv na habitate, sposobnosti izvajanja okoljskih funkcij in storitev tal…, je v veliki meri posledica degradacije okolja v preteklih

desetletjih in hkrati povečanih potreb po hrani ob zmanjševanju obsega kakovostnih kmetijskih površin. 'Tla' so torej naravno telo v vseh svojih razvojnih stopnjah, ki poleg prvotne nosilne funkcije skozi pedogenezo pridobijo in izgubijo mnoge druge lastnosti, med katerimi je rodovitnost pomembnejša.

2 ETIMOLOGIJA IZRAZOV

2.1 Izraz 'prst'

Izraz 'prst' v slovenskem jeziku označuje rahlo, drobljivo, lahko tudi sipko zemljino, ki je največkrat dobro humozna in predvsem dobro rodovitna. 'Prst' je ljudski izraz, ki opredeljuje predvsem zemljino zgornjega oz. zgornjih horizontov ali zgornje obdelovalne plasti tal; ta je največkrat strukturna, založena s hranili, rodovitna, oz. največkrat v rabi kot *prst*. 'Prst' uporabljamo tudi za mineralne a rahle in ustrezno strukturne kambične Bv horizonte in za bogat substrat antropogenega nastanka kot je npr. *prst za lončnice*. Z ljudskim izrazom 'gozdna prst' največkrat označujemo tanek in zelo organski A ali Ah horizont ali celo Oh horizont, ki so značilni za tla gozdov. Tega svetujejo kot dodatek vrtnarski zemlji za izboljšanje rasti (Urbančič in sod.,).

V ljudskem pojmovanju 'prst' ne uporabljamo za drugo vrsto talnih horizontov, ki so značilni gradniki profilov nekaterih vrst tal. Kot nazoren primer lahko izpostavimo močno ilovnat ali glinast, gost, zbit ter nepropusten Bg horizont, ki bistveno vpliva na skupne lastnosti in v temeljih določa talni tip *psevdoglej*. Isto velja npr. za Bt horizont v spranih pokarbonatnih tleh. Ljudsko poimenovanje horizontov s takšnimi lastnostmi je *ilovka*. Ravno tako 'prst' ne uporabljamo za druge horizonte v talnem profilu, ki niso rahli, strukturni, drobljivi…, so npr. preveč skeletni (B/C in C horizont) ali za matično podlago (C, R horizonta), ter za horizonte bolj ali manj nasičene z vodo (Go, Gr) v hidromorfnih tleh.

Ljudskemu pojmovanju so sledili tudi prvi pedologi in strokovnjaki s kmetijskega področja, ki so postavljali temelje pedološkemu izrazoslovju v Sloveniji. Tako prof. Vovk dosledno uporablja izraz tla in zemlja (npr. »…ni v vsakih tleh enako hrane…«; »v vsaki zemlji so navadno vse rudninske snovi…«) (Vovk, 1955, 1959, 1966). Prof. Sušin v svojem Pedološkem terminološkem slovarju (1983) opredeli izraz 'prst' kot »s humusom bogata tla, drug izraz za tla« in v oklepaju doda »v pedologiji se ta izraz ne uporablja«. Prof. Adamič v Kmetijskem tehniškem slovarju opredeli izraz 'prst' kot "zgornja, rodovitna plast zemlje" in izraz primerja z izrazi "humus, apnena prst, kisla prst, barska prst, črna prst, humusna prst in kisla prst" (Ritz, 1973). Izpeljani izrazi se tako nanašajo na neko specifično ali bolj izraženo lastnost posameznega horizonta, vendar za vse te izpeljanke velja, da gre za koherentno in strukturno zemljo. Geografsko izobraženi razumejo izpeljanke predvsem v smislu celotnega talnega profila (kisla prst, barska prst,) medtem ko so ti izrazi za pedologe presplošni, dvoumni oz slabo opredeljeni.

V sodobnem pogovornem jeziku in brez izjeme med narečji, je 'prst' zelo redko uporabljena beseda; uporabljajo jo predvsem mlajši ljudje in to takrat, ko želijo svojim besedam dati nekaj več šolskega ozadja. Ljudje pa govorimo predvsem o zemlji. *'zakopal je v zemljo…'*,. V preteklosti sicer pogosteje uporabljana 'prst' v dandanašnji pogovornem jeziku zveni arhaično.

V pedologiji v skladu z izvorno rabo izraza uporabljamo izpeljanke korena 'prst' za opisovanje in ločevanje organskih in mineralnih horizontov. Npr. 'prhnina', 'prhninasta sprstenina' in 'sprstenina' so izrazi za opisovanje razvitosti in organo-mineralnega kompleksa posameznega horizonta v talnem profilu. Tako na podlagi vsebnost, oblike organske snovi in razvitosti organsko-mineralnega kompleksa A horizonta ločujemo sprsteninasto rendzino (A horizont je sprstenina z dobro razvitim organo-mineralnim kompleksom) in prhninasto rendzino (A horizont vsebuje pretežno slabo razgrajeno organsko snov, organomineralni kompleks ni razvit).

S pedogenetskega stališča se je težko strinjati s poimenovanji izvedenimi iz besede 'prst'**.** Kot značilen primer je možno izpostaviti neskladnost ljudskega pojmovanja in izrazov, ki izhajajo iz uvedbe besede 'prst' v slovensko klasifikacijo tal.

Tako je bil s strani geografov npr. uveden izraz »*litosolna prst*«. Ta označuje talni tip z golim R ali C horizontom lahko mestoma prekrit s tankim in slabo ali celo nerazvitim A horizontom v začetni stopnji razvoja. Gre za talni tip kamnišča oz. litosola.

Izraz 'prst' ne označuje tal kot celovito naravno tvorbo z zaporedjem zelo različnih horizontov oz. plasti od površine do matične podlage.

2.2 Zemlja

Zemlja je izraz v najširši rabi. Poleg planeta v pogovornem jeziku predstavlja mineralno-organsko preperino v kateri lahko uspevajo rastline, zemljišče. Izraz 'zemlja' so v pravem pomenu tal v strokovni literaturi uporabljali v predvsem prvi polovici 20. stoletja. V knjigi Kmetijska kemija ing. agr. Iva Zobca (1930) v poglavju 'Zemlja' razloži osnove vede o 'zemlji'. V uvodu »Kaj je zemlja?« razloži: »Zemljo ali prst imenujemo zgornjo plast Zemlje, ki daje rastlinstvu hrano in bivališče…«. V odstavku »Kako se tvori prst?« nadaljuje z »Voda, mraz in toplota neprestano drobijo kamenje v grušč pesek, prod, grez in blato…«. V poglavju »Vrste zemlje«, ko obravnava teksturne lastnosti, govori o »peščeni zemlji«, »glinasti zemlji«, »humozni zemlji«. Besedo 'prst' poveže samo z izrazom ilovnata zemlja, ki jo opredeli kot zmes približno 40 % gline in 60 % peska, je "rodovitna prst in je primerna za vse kulturne rastline. Najrodovitnejša prst sploh je ilovnata prst s humusom in apnom«. V poglavju »Lastnosti zemlje« obravnava osnovne fizikalne lastnosti tal kot so kapilarnost, vodna kapaciteta, absorpcija, … itd. Pri tem uporablja besedo zemlja v zvezah kot so »lastnosti zemlje«, »kakovost zemlje zavisi od njene strukture«. Besedo 'prst' v nadaljevanju knjige praktično ne uporablja več, temveč dosledno uporablja izraz 'zemlja'. V besedilu se kot zamenjava za izraz 'zemlja' pojavlja tudi izraz 'tla' npr. »bakterije najbolje uspevajo na toplih tleh, slabo pa v kisli, težki zemlji'. Menim, da avtor v uvodni definiciji uporabi izraz 'prst', da tematiko približa bralcu. 'Prst' v nadaljevanju nekajkrat uporabi v povezavi s strukturno, koherentno, humozno in rodovitno sprstenino. Besedo 'zemlja' Zobec dosledno uporablja kot strokoven izraz v povezavi s posameznimi z morfološkimi lastnostmi, pri razlikovanju vrst tal ter v povezavi s kemijskimi in fizikalnimi procesi v tleh kot so »denitrifikacija«, »kemijska adsorpcija«, »predstavniki anorganskih koloidov v zemlji«, »vodne kapacitete zemlje« do »gnojenja zemlje«. Ing. Zobec uporablja izraz 'zemlja' tako, kot sedaj pedologi uporabljamo besedo 'tla'.

Izraz 'zemlja' uporabljamo še sedaj v nekaterih agrokemičnih laboratorijih kot matriks – talne vzorce, pogosto za rastne substrate – mešanice mineralne in organske komponente z dodatki (hranila, umetne snovi, ki povečajo kapaciteto za vodo, mineralne in organske snovi, ki povečajo sposobnost vezave hranil ali strukturnost in drugo, kar poveča rodovitnost).

2.3 Ilovka, ilovica, jerina, jerovica

'Ilovica' oz. ponekod pogovorno 'ilovka' je v pogovornem jeziku močno in povsod prisoten izraz. Slovenci ga pomensko in brez izjeme med narečji uporabljamo za težko drobljivo, gosto, ilovnato ali glinasto zemljo, v suhem stanju trdo in zbito ter v mokrem gnetljivo ali celo mazavo mineralno preperino. Strokovni izraz 'ilovica' uporabljamo za poimenovanje teksturnega razreda in finejšega materiala, ki ga glede na teksturno sestavo uvrščamo v tla s približno enakim deležem peska, melja in gline. Ob povečanem deležu gline ali melja postanejo težje (meljasto glinasta ilovica, meljasta ilovica), oz. ob povečanem deležu peska lažje teksture (peščeno meljasta ilovica). Težje ilovice so goste in lahko tudi zbite; v sušnih razmerah trde in največkrat praktično nedrobljive. V vlažnem stanju so bolj ali manj plastične in celo gnetljive ter v mokrem stanju mazave. Ilovnat je tudi Brz horizont, ki je diagnostični gradnik skupine pokarbonatnih tal. Izraz označuje tudi druge z vodo premeščene in odložene rezultate pedogenetskih procesov, ki so osnovni gradniki tipov tal, ki so zastopani v pedosekvenci na glinah in ilovicah.

'Jerina' je avtohton izraz slovenskega Krasa, ki označuje izrazito rdeče obarvana tla in/ali izrazito rdeče obarvan Brz horizont, ki pa za razliko od drugih rdečih tal (*Terra rossa*) vsebuje veliko kremenovega skeleta. Ta posebnost botruje nekaterim nacionalnim atributom kot je vino teran, ki je zaradi tega zaščiteno tudi v evropskem pravnem redu. 'Jerovica' je sinonim, ki ga je uporabljala slovenska pedološka literatura, a ga Kraševci zvračajo; poznajo 'jerino'.

2.4 Izraz 'tla'

V prvi četrtini dvajsetega stoletja v pedologiji v celoti prevladalo spoznanje, da tla nastajajo in se razvijajo v skladu z delovanjem pedogenetskih dejavnikov. To je v svojem za razvoj stroke pomembnem delu ustrezno predstavil Hans Jenny (1941). Med procesom pedogeneze se razvijajo in spreminjajo fizikalne in kemijske lastnosti, ki so, gledano dosledno, večinoma prisotne v vseh fazah razvoja tal, pa čeprav v minimalnem, lahko komaj zaznavnem obsegu. Predstavimo to na primeru skupne in kompleksne lastnosti tal, t.j. rodovitnosti. Gola površina kamnine je v tehničnem smislu ravno tako rodovitna. Lišaju nudi oporo in hranila ter s tem omogoča njegov razvoj in obstoj. Tla kljub svoji plitvosti in inicialnem razvojnem stadiju opravljajo poleg funkcije nosilnosti tudi še funkcijo preskrbe lišaja, predvsemalge(cepljivkes hranili – torej rodovitnosti. Glive v tej fazi z izločki raztapljajo rešetke mineralov in tako fotobiont (algo/cepljivko) v simbiozi preskrbujejo s hranili. Z razvojem lišaja poteka proces biološkega preperevanja in pedogeneze - spreminjanja kamnine v sprstenino. Sam lišaj, ko odmre, funkcionira kot plitva humusno akumulativna tla vendar na mikro ravni. Plast lišaja, ki je sicer globoka do nekaj milimetrov, bi lahko označili z (A) horizontom, ki leži neposredno na R horizontu; gre torej za A-R profil tal, ki je analogen npr. prhninasti rendzini). Seveda tak strokovno-tehnični pogled ni v skladu s poljudno prepoznano rodovitnostjo kot jo premorejo tla s kambičnim B horizontom. Rodovitnost talnega profila je v opisanem primeru marginalna, za ne-pedologa nepoznana, nepomembna in v praksi neobstoječa. Strogo znanstveno so utemeljitve izrazov na podlagi rodovitnosti na sploh problematične. Ali so npr. tla distričnih psevdoglejev nižinskih gozdov rodovitna? Bližnji kmet bi rekel, da te 'slabe zemlje' ne obdeluje, ker je nerodovitna. Na takih tleh raste rdeči bor, dob in pravi kostanj. Za rast gozda so tla rodovitna, ne pa dovolj za pšenico, krompir, koruzo. Oz. na ravni posamezne rastline: najboljše njive na evtričnih rjavih tleh (strukturna sprstenina, bogata s hranili in kalcijem, ...) so manj rodovitna za borovnico. V kolikor jo sadimo, bo v letu, dveh propadla. Podobno velja za druge talne lastnosti, funkcije in storitve tal. Tako npr. tudi gruščmelišč filtrira grobe delce padavin ali nevtralizira (še posebej apnen) kisle padavine oz. nekatera onesnažila v njih.

Skozi pedogenezo se tla razvijajo, horizonti nastajajo, se diferencirajo in poglabljajo. Do določene razvojne stopnje (recimo temu srednja leta) tla pridobivajo na rodovitnosti. S staranjem, in čeprav s poglabljanjem, pa tla sicer izgubljajo rodovitnost, a ohranjajo in celo pridobivajo nekatere druge funkcije (npr. filtrirne sposobnosti ali sposobnost zadrževanja vode). Vzemimo na primer serijo tal, ki se razvije na pretežno apnenem produ in pesku. V fazi plitve rendzine (A-C profil) so tla predvsem zaradi plitvosti in skeletnosti slabo rodovitna. Njihova rodovitnost se povečuje z nastankom in poglabljanjem Bv horizonta (evtrična rjava tla na produ in pesku A-Bv-C profila) in se z nadaljnjim staranjem prične zmanjševati (sprana tla na apnenem konglomeratu A-E-Bt-C/R profila) do faze zelo starih spranih in močno glinastih distričnih rjavih tal (npr. A-E-Bt_1-Bt_2-… - Bt_9 profila). Taka tla izjemne starosti, tja do 1,5 milijona let, imamo v Sloveniji na starih terasah prodnega zasipa Zgornje Savske doline (Jaecks Vidic, 1994).

Zaradi doslednosti je potrebno ugotoviti, da tla niso samo naravno telo. Obstajajo tudi antropogena tla, ki nastajajo pod močnim vplivom posegov človeka in tehnogena tla, ki so substrat umetnega nastanka (mešanja, dodajanja, kompostiranja, obdelave, …). Tako antropogena kot tehnogena tla so lahko zelo rodovitna, torej imajo svoje 'osnovne' lastnosti in izvajajo funkcije in storitve. Ravno tako je lahko umetnega izvora matična podlaga iz katere tal nastajajo. Beton je mešanica mineralov in tako kot naravne kamnine izpostavljen vsem trem načinom preperevanja - fizikalnem, kemičnem in biološkem. Čez skromnih 30-40.000 let se bodo v primernih razmerah razvila npr. '*evtrična rjava tla na betonu'* (ugotovitev je glede na izgubo tal zaradi pozidave sicer optimistična, a le v kolikor zanemarimo, da potreben čas krepko presega dobo naše civilizacije). In spet obratno, ko se duripan (trda naravna tvorba v tleh) zaradi erozije pojavi na površini tal, ima podobne lastnosti kot beton in bo podobno prepereval.

Za obravnavano tematiko prispevka je pomembno, da se tla v svojem razvoju spreminjajo in vsebujejo tako po kemijskih kot fizikalnih lastnostih zelo različne horizonte, ki smo jim v jezikih poiskali ustrezne izraze. Med stanjem tal v fazi gole kamnite površine z lišajem in evtričnimi rjavimi tlemi so desettisočletja, med njimi in globokimi

spranimi tlemi pa milijon let in več postopnega razvoja in spreminjanja kamnine. S tega vidika je argumentacija *'kamen'* → *nerodovitno* → *t.j. 'tla' po katerih hodimo* in *'rodovitno'* → *'prst',* neustrezna, nedosledna in vsebinsko pomanjkljiva. Glede na rahlo in strukturno zemljo oz. 'prst', ki je rezultat pedogeneze, je izraz 'tla' mnogo širši. Tla zajemajo tako 'gozdno prst' (Oh, Ah, A horizont), sprstenino (i.e. E, Bv) kot 'ilovko' (e.g. spodnji Bt, težji Brz horizonti); vse tako po fizikalnih kot po kemijskih lastnostih zelo različne tvorbe.

Če sklenemo: 'tla' je vsebinsko širši in strokoven izraz za tisto po čemer tudi hodimo, a se pod našimi nogami razvija in spreminja, pridobiva in izgublja mnoge fizikalne in kemijske lastnosti. Tla izvajajo mnoge funkcije in storitve v vseh fazah svojega razvoja pri čemer so pomembne razlike predvsem v obsegu in intenzivnosti procesov. Rodovitnost je sicer najbolj prepoznana, a le ena izmed mnogih pomembnih lastnosti, ki zagotavljajo delovanje kopenskih ekosistemov.

3 RABA IZRAZOV 'PRST' IN 'TLA' V STROKI

Izraz 'tla' zajema vse plasti / horizonte od površine do podtalja/matične podlage. Lastnosti tal so funkcija (in ne seštevek) lastnosti posameznih horizontov. Posamezni horizonti in njihove lastnosti opredeljujejo talni tip - so diagnostični. Spranih tal ni brez E horizonta, pokarbonatnih ne brez Brz horizonta in hidromorfnih ne brez G horizontov.

Sušin v Kmetijskem tehniškem slovarju - Nauk o tleh (Sušin, 1983), tako kot Jenny, izčrpneje opredeli *tla* in ga poveže s pedogenetskimi dejavniki. Ta *tla* pravi, da so "prirodna tvorba na površini zemeljske skorje, ki je nastala in se razvijala pod vplivom tlotvornih dejavnikov: matične podlage, klime, organizmov, reliefa in časa; fizikalne in kemične, biološke in morfološke lastnosti se razlikujejo od matične podlage, iz katere so nastala tla; prirodno okolje za rast rastlin neposredno na površini zemeljske skorje". V nadaljevanju opredeli devetinštirideset besednih zvez, ki se nanašajo na *tla*; začne z "aconalna tla" in zaključi z "zrela tla".

Adamič je v Kmetijskem tehniškem slovarju - sadjarstvo (Ritz, 1973) precej obširen a nedoločen pri opredelitvi izraza *tla*. So "zemeljska površina kot podlaga, trda plast pod zemeljsko površino in vrhnji del plasti, ki omogoča rast rastlin", ter v nadaljevanju primerja s "prst, zemlja: propustna tla, apnena tla, prhka tla, lapornata tla, rodovitna tla, gozdna tla, kraška tla, težka tla".

V učbeniku *Geografija prsti in rastja*, *skripta za geografe* profesorja Svetozarja Ilešiča (1960), se izraza *tla* in *prst izmenjujeta*. Prvi stavek uvoda avtor prične z »Geografija tal ali prsti je eno najvažnejših poglavij prirodne geografije.« in nato uporablja »Opredelitev prsti ali tal«, »Pojem prsti ali tal«, »Osnovni pojmi iz kemije tal«, »Dovajanje in premeščanje mineralnih elementov v tleh«, »Organske snovi v prsti« (torej gornji horizonti tal), »Osnovni pojmi iz fizike tal«, »tekstura tal«, Voda in zrak v tleh«,«Barve prsti«, »Tipi prsti«, itd. Poznejši avtorji geografi poskušajo izraz 'prst' bolj dosledno uporabljati vendar ponovno selektivno. V istih besedilih govorijo o prsti in nato o rabi tal oz. o propustnosti tal in podtalnici.

Izraz 'tla' so uporabljali oz. še uporabljajo slovenski pedologi. Že med svetovnima vojnama je bil to prof. dr. Bogdan Vovk (starosta slovenske pedologije in prvi slovenski pedolog z doktorskim nazivom), njegovi sodobniki in poznejši raziskovalci ter učitelji (dr. Dušan Stepančič, prof.dr. Albin Stritar, prof. dr. Jože Sušin, Marija Kodrič, Lojze Briški, prof.dr. Jože Furlan, prof.dr. Marjan Ažnik, in drugi). Izraz dosledno uporabljamo raziskovalci in učitelji pedologije. Bibliografija pedologov je v glavnem razširjena v okviru biotehniških strok in zato, žal, premalo znana diplomantom ne-biotehniških študijev.

4 POMEN IN RABA IZRAZOV V DRUGIH JEZIKIH

Boden je beseda v nemščini, ki jo uporabljajo tudi kmetije za svoja tla, zemljišča. Z besedo *Bodenkunde* označujejo vedo o tleh, pedologijo. *Bodentyp, Bodenprofil, Bodenhorizont, Bodenfunktionen*, … so izrazi, ki jih uporabljajo v strokovnem izrazoslovju. Koren Erde ohranjajo v nekaterih imenih talnih tipov (*Braunerde*) in *Feinerde* za delce manjše od 2 mm. *Boden* je tudi izraz za tla po katerih hodijo, ne glede na material (*Holcboden*). Čeprav je nekaj razlik. Ko jim na severu Nemčije nekaj pade na tla, uporabijo *Erde*, na bavarskem in Avstriji pa *Boden*. Izraz *Erde* uporabljajo pogovornem jeziku v kontekstu zemlje, prsti; npr. doma za lončnice (Lehman, 2012; Schad, 2013). Tako izraz *Erde* tudi prevajamo v slovenščino (Debenjak in sod., 2001). V poljščini v strokovnem izrazoslovju kot 'tla'/*soil* uporabljajo *gleba*. To velja tudi za talni profil, talni tip, ipd. *Ziemia* uporabljajo v pogovornem jeziku za rahel in drobljiv talni horizont, za zemljo oz. substrat za lončnice. *Ziemia* je poljskim kmetom tudi njihovo zemljišče, njihova zemlja (Sabielec, 2013). Slovaki v vedi *pôdoznalectvo* strogo ločijo med strokovnimi izrazi *pôda* – 'tla', *pôdny typ, pôdny profil, pôdne funkcie*, … itd. ter pomenom izraza *zem* za sprstenino oz. strukturno zemljo oz. kot pogovorni izraz za zemljo kot posest in teritorij. Zanimivo je to, da uporabljajo izraz *krajina* za angleški *landscape*. Slovaški kmet uporablja oba izraza *pôda* in *zem* v enakih pomenih kot pedološka 'tla' in 'zemlja' (Sobocka, 2012). Ruski strokovni in v znanosti uporabljan izraz je *počva* /почва, medtem ko je *zemlya*/земля široko uporabljan izraz za zemljišče, posestvo, rusko zemljo kot nacionalni teritorij, … itd. *Zemlya* je ime planeta (Stolbovoy, 2012). Hrvatje in Bosanci v strokovnem izrazoslovju dosledno uporabljajo *tlo* za tla, posebej še v kontekstu tretje dimenzije (torej talni profil). *Zemljište* uporabljajo za površino, torej zemljišče v dvodimenzionalnem pomenu (Čustović, 2012). Hrvatje imajo hektar *zemlje* in pripeljejo tovornjak *zemlje*, *Zemlja* je planet. Srbi za razliko od svojih sosedov uporabljajo v pedološkem strokovnem izrazoslovju izraz *zemljište* za 'tla' in se v tem ločijo od Hrvatov (Bašić, 2012). Makedonci v okviru *nauke za počvite* uporabljajo *počva* za *'tla'* medtem ko ima poljuden izraz *zemlja* enak pomen kot v prej omenjenih južnoslovanskih jezikih (Mukaetov, 2012).

Čeprav v pogovornem jeziku Italijani uporabljajo *suolo* tudi za tla po katerih hodijo in za nacionalni teritorij, je to strokovni izraz v *scienca del suolo*. Izraz *terreno,* ki označuje zemljišče kot posest, italijanski pedologi za razliko od agronomov ne uporabljajo. V italijanščini torej ločijo med strokovnim *suolo* in *terra* za zemljo, substrat za lončnice, material s katerim se igrajo otroci in nekaj v kar nas na koncu zakopljejo (Ajmone Marsan, 2012). *Terra* z veliko začetnico je planet. Izraz *suolo* je v slovarju (Šlenc in sod., 2006) preveden v 'tla'. Glede na sorodnost z italijanščino je francoščini raba izrazov enaka. *Sol* uporabljajo v *science du sol*, ko strokovno govorijo o tleh, profilih talnih tipih, funkcijah tal,… itd. *Terre fine* je zemlja z delci < 2mm. *Terre* je v pogovornem jeziku drobljiva strukturna, lahko organska zemlja ali substrat za lončnice. Terre je planet. Francoski kmet uporablja za svojo zemljo oba izraza, *sol* in *terre* (Arrouays, 2012). Enako loči španska *ciencia del suelo* izraza *suelo* in *tierra*.

V angleščini uporabljajo besedo *soil* za tla, zemljo, zemljišče (*soil types*, *soil map…)*. V ameriški angleščini pogovorno uporabljajo *dirt* za zemljo, blato, nesnago, umazanijo, prah (Grad in sod., 1995). Starejša in zelo splošna FAO opredelitev za angleščino namesto besede *soil* uporablja *solum* "the part of the earth's crust influenced by climate and vegetation (usually A and B horizons) (FAO, 1954). V najnovejšem ameriškem Glossary of Soil Science Terms je izraz *soil* opredeljen s kmetijskega vidika kot "i. *the unconsolidated mineral or organic material on the immediate surface of the earth that serves as a natural medium for growth for plants.*" in bolj pedogenetska opredelitev, ki začenja z"ii. *The unconsolidated mineral or organic matter on the surface of the earth that has been subjected to and shows effects of environmental factors of material..."* (Soil Science Society of America, 2008). Zanimivo v istem viru ni opredelitev izrazov "*fine earth*", *Earth in earth*". Izraz *earth* prevajamo kot zemlja, *fine earth* pa kot prst in *Earth* kot planet Zemlja. V smislu opisovanja lastnosti posameznega horizonta, npr. vzorčenja in analitike tal, angleško govoreči avtorji uporabljajo izraz *fine earth* za sprstenino oz. delce < 2 mm.

Na podlagi tega kratkega pregleda lahko sklenemo, da v evropskih jezikih prevladuje strokovni izraz 'tla' (*sol, suolo, boden, soil, tlo, zemljište, počva, pôda, počva*) in ter poljudni oz. pogovorni izraz 'zemlja' (*terra, tiera, terre, erde, earth, zem, zemlja, zemlya*), ki ima več pomenov, ki pa se med jeziki praktično ne razlikujejo. Izraz 'prst' nima svojega analoga in ga pomensko lahko prevajamo v 'zemljo', ne pa v 'tla'. Ločenost strokovnih in ljudskih izrazov torej ni lastna samo slovenskemu jeziku. Pomenska razlikovanja v izrazih 'tla' in 'zemlja' so pomembna tudi v drugih evropskih jezikih.

5 IZRAZOSLOVJE V IZOBRAŽEVALNEM PROCESU

Izraz 'tla' uporabljamo okviru predmeta Pedologija, ki jo poslušajo predvsem študentje biotehniških študijev. Tako na Oddelku za agronomijo Biotehniške fakultete Univerze v Ljubljani (UL), predavajo predmete Pedologija, Raba in varstvo tal ter Ekopedologija (UL-BF, 2012a); na Oddelku za gozdarstvo predmeta Pedologija z mikrobiologijo tal in Pedologija z osnovami geologije (UL-BF, 2012b), na Oddelku za krajinsko arhitekturo Pedologijo in osnove geologije (UL-BF, 2012c), ter na Oddelku za zootehniko predmet Splošno poljedelstvo s pedologijo (UL-BF, 2012d). Slušatelji na Fakulteti za kmetijstvo in biosistemske vede Univerze v Mariboru poslušajo predmete Pedologija, Raba in varstvo tal in Ekologija tal (FKBV, 2012a; b). Študentje geologije Naravoslovno tehniške fakultete UL poslušajo predmet Pedologija (UL-NTF, 2012), medtem ko v okviru študija arheologije (Oddelek za arheologijo, Filozofska fakulteta UL) poučujejo predmet Geoarheologija s pedologijo (UL-FF, 2012a). Na Visoki šoli za varstvo okolja v Velenju poslušajo predmet Raba in varstvo tal, ki vključuje pomemben delež pedoloških vsebin (VSVO, 2012). Kemijo in biologijo tal poslušajo na Visoki šoli za vinogradništvo in vinarstvo Univerze v Novi Gorici (UNG, 2012). V navedenih študijih na prvostopenjskih in magistrskih bolonjskih študijih uporabljamo izraz 'tla'. Izraz 'prst' so uvedli na Oddelku za geografijo Filozofske fakultete UL in ga poučujejo v okviru predmetov Pedogeografija in biogeografija (UL-FF, 2012b). Podobno velja tudi za Univerzo v Mariboru. V slovenski prostor ga predvsem v okviru osnovnošolskega in srednješolskega izobraževanja in predmetov Zemljepis oz. Geografija, širijo učitelji - geografi.

V slovenskem izobraževalnem sistemu izraz 'prst' prevladuje na osnovnošolski in srednješolski ravni v okviru zemljepisa oz. geografije. Na šestih drugih univerzah in visokih šolah oz. v okviru devetih različnih študijskih usmeritev na dodiplomskem in podiplomskem nivoju slušatelji spoznajo 'tla' najpogosteje že v začetku študija.

6 PEDOLOGIJA, PEDOGEOGRAFIJA IN 'SOIL SCIENCE'

Pedologija je naravoslovna oz. biotehniška veda, ki proučuje tla. Prvi pravi začetki poglobljenega raziskovanja tal segajo v konec 19. stoletja. Prvi svetovni pedološki kongres je potekal aprila 1909 v Budimpešti. Metode raziskovanja lastnosti in procesov v tleh so se razvijale v okviru fizike, kemije, geologije in drugih naravoslovnih ved. Primarni vzroki raziskovanja tal so bile povečane potrebe po hrani in drugi biomasi. Veda je torej tradicionalno umeščena na področje kmetijstva in gozdarstva. Zato je pedologija temeljni predmet biotehniških ved oz. *life sciences*. Hkrati je pedologija temeljna veda o okolju. Pedološko izrazoslovje se je v vseh jezikih intenzivneje razvijalo šele v prvi polovici dvajsetega stoletja; po I. in predvsem po II. svetovni vojni. Slednje velja tudi za slovensko pedologijo. Razvoj izrazoslovja še ni zaključen, ne pri nas, ne v svetu.

'Pedogeography' se v primerjavi s 'pedology' in 'soil science' v tujini izjemno redko pojavlja. Opredeljen je kot »veja geografije, ki zajema proučevanje geografske porazdelitve tal« (Unabridged Meriam Webster, 2012). Izraz 'pedogeografija' je nepoznan v šestih splošnih slovarjih, 'pedologija' pa v treh (Macmillan Dictionary, GS Soil Thesaurus, 2012; ISJ ZRC SAZU:, 2012; Longman dictionary, 2012; Merriam-Webster Dictionary, 2012; Oxford Dictionaries, 2012). Zanimiva izjema je slovenski

Leksikon geografije in podeželja (Kladnik, 1999), ki samostojnega gesla 'pedologija' ne pozna, kljub temu, da 'pedologijo' v opredelitvah drugih gesel nekajkrat omenja. Kot nadomestek pomensko uporablja 'pedogeografija', kar je v nasprotju tudi s slovenskimi geografi (Ilešič, 1960; Lovrenčak, 1994) in pedologi (Sušin, 1983; Stritar, 1991).

V svetu uporaba imena vede ni v celoti poenotena. 'Pedologija' je ponekod izraz za vejo širše 'vede o tleh' (*soil science*). Opredeljena je kot npr. »znanstveno proučevanje tal in njihovih profilov« (Soil Science Society of America, 2008) oz. »se osredotoča na nastanek, morfologijo in klasifikacijo tal kot naravne tvorbe v naravnih krajinah« (Utah State University, 2012). Podobnih primerov opredelitve pedologije kot veje vede o tleh je več. '*Soil science*' je bolj celovito opredeljena kot »znanost o tleh kot naravnem viru na površini Zemlje in samo po sebi vključuje nastanek, klasifikacijo, kartiranje, fizikalne, kemijske in biološke lastnosti vključno z rodovitnostjo, ter teh lastnosti v povezavi z rabo in ravnanju s tlemi« (Soil Science Society of America, 2008). Pregled opredelitev '*pedology*' in '*soil science*' pa narekuje zaključek, da »je pedologija dobesedno veda o tleh« (Soil Science Society of America, 2012), V slovenščini torej ni potrebe po nadomeščanju izraza 'pedologija' z 'veda o tleh' in za 'pedologijo' vsebinsko veljajo celovite opredelitve kot je to v primeru SSSA.

7 SKLEPI IN PRIPOROČILA

Potrebno je negovati in ohranjati bogastvo slovenskega jezika in uporabljati poljudne izraze 'prst', 'ilovica', 'ilovka', 'glina', 'zemlja,' 'jerina', … in druge, v njihovem izvornem oz. lokalnem pomenu. Kadar ni strokovnih zadržkov in gre za sopomenke naj strokovni jezik prevzame ljudska poimenovanja.

Izraz '**prst**' uporabljajmo za sprstenino oz. za posamezne horizonte, ki so rezultat pedogeneze. Označuje preperino primerne strukture (največkrat gre za sferične strukturne agregate) in drobljivosti, lahko oz. pogosto z večjo vsebnostjo organske snovi. Pomen besednih zvez 'zajeti prgišče prsti', 'napolniti lonec s prstjo' je jasen tudi glede lastnosti materiala. 'Prst' lahko označuje zemljo omenjenih fizikalnih lastnosti, ki ima hkrati še dobre kemijske lastnosti (kislost, vsebnost hranil). Te se izrazijo v nadpovprečni rodovitnosti in to ustreza pojmovanju ljudske besede 'prst' (vrtna prst, prst za ločnice). Izraz '**zemlja**' je pomensko enak 'prsti' in je tako v pogovornem jeziku tudi najpogosteje uporabljan. Tako kot pri drugih jezikih izraz ga uporabljamo za ime planeta in predvsem v pomenu za zemljišče z mejami, za zemljo kot lastnino in v teritorialnem pomenu. Izraz '**ilovica**' v pogovornem in strokovnem jeziku uporabljamo za teksturno finejši, v svežem stanju gost in zbit, v sušnem trd in zelo težko drobljiv ter v vlažnem stanju gnetljiv material. Dodatno je v strokovnem jeziku ilovica izraz za teksturni razred, ki po USDA opredelitvi vsebuje med 7 in 27 % gline, med 28 in 50 % melja ter med 22 in manj kot 52 % peska. Pedološko '**jerovico**' zamenjamo z avtohtono kraško '**jerino**'.

'**Tla**' uporabljamo v strokovnih in poljubnih besedilih ter drugje v javni rabi in zakonodaji za a) naravno, antropogenizirano in tudi v celoti antropogeno tridimenzionalno tvorbo na kopenski površini; b) za talne tipe kot osnovne enote klasifikacije tal, ter c) v povezavah z rabo zemljišč (raba tal). 'Tla' uporabimo posebej takrat, ko govorimo o naravni ali logični razvrstitvi horizontov različnih kemijsko-fizikalnih lastnostih od površine v globino do matične podlage, ne glede na stadij razvoja tal in ne glede na obseg izvajanja funkcij ali kompleksnih lastnosti talnega profila kot celote (rodovitnost).

Izraz '**pedologija**' uporabljamo kot sopomenko za '**vedo o tleh**' in je tudi ustrezno najširše opredeljena. Geografska veda '**pedogeografija**', je pomensko ožji, saj se v skladu s sodobnimi tujimi in starejšimi opredelitvami slovenskih geografov osredotoča na geografsko-conalno porazdelitev tal na kopnem.

Prispevek obravnava sicer ključne terminološke zadrege s področja pedologije, vendar je podobne primere zaslediti tudi v drugih strokah (geologija). Ustrezna in dosledna uporaba izrazov koristi poljudnemu in strokovnemu jeziku, posebej pomembna pa je za vsebinsko korektne in pomensko nedvoumne prevode evropske zakonodaje.

8 ZAHVALE

Sodelavci s Kmetijskega inštituta Slovenije, Biotehniške fakultete, Fakultete za kmetijstvo in biosistemske vede, Filozofske fakultete – Oddelka za geografijo, Znanstveno raziskovalnega centra SAZU so pripomogli k nastanku prispevka z v preteklosti izraženimi mnenji. Mag. T. Prusu, dr. M. Muršec, mag. T. Verniku in J. Sušinu se zahvaljujem za pregled prispevka in pripombe. Pri pregledu stanja in primerjavi s tujimi jeziki so s pojasnili izrazov pripomogli dr. D. Arrouays, prof.dr. F. Ajmone Marsan, dr. A. Lehman, prof.dr. P. Strauss, prof.dr. J. Sobocka, prof.dr. F.Bašić, prof.dr. H. Čustović, prof.dr. O. Čukaliev, prof. dr. D.Mukaetov, dr. V. Stolbovoy, dr. G. Sabielec in drugi, za kar se jim najlepše zahvaljujem.

9 VIRI

Ajmone Marsan F. 2012. Soil terminology: Suolo, Terra (written correspondence).

Arrouays D. 2012. Soil terminology: Sol, Terre (written correspondence).

Bašić F. 2012. Soil terminology: Tlo, Zemljište, Zemlja (written correspondence).

Bremec K. 2011. Vplivi načina rabe nekaterih bohinjskih planin na vegetacijo in rastlinsko vrstno pestrost. Diplomsko delo, Ljubljana, Slovenija [Slovenia], Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo: 36 str.

Čustović H. 2012. Soil terminology: Tlo, Zemlja (written correspondence).

Debenjak D., Debenjak B., Debenjak P. 2001. Veliki nemsko-slovenski slovar [Grosses deutsch-slowenisches Wörterbuch]. Ljubljana, DZS:

Drašler V. 2008. Stanje in možnosti razvoja sadjarstva v občini Hoče - Slovnica. Diplomsko delo, Ljubljana, Slovenija [Slovenia], Univerza v Ljubljani, Biotehniška fakulteta; Oddelek za agronomijo: 39 str.

Drofenik U. 2012. Gorsko kolesarstvo v zavarovanih območjih na primeru Triglavskega narodnega parka. Diplomsko delo, Ljubljana, Slovenija [Slovenia], Univerza v Ljubljani, Biotehniška fakulteta; Oddelek za agronomijo: 67 str.

Evropska komisija [European Commission]. 2006. [COMMUNICATION FROM THE COMMISSION TO THE COUNCIL AND THE EUROPEAN PARLIAMENT on Thematic Strategy on the Urban Environment]. 2006,: 61

Evropska komisija [European Commission]. 2011. Časovni vir za Evropo, gospodarno z viri [Roadmap to a Resource Efficient Europe] (COM(2011)571). COM(2011)571,

Evropska komisija [European Commission]. 2012. Smernice o najboljši praksi za omejevanje, blažitev ali nadomestitev pozidave tal [Guidelines on best practice to limit, mitigate or compensate soil sealing] (COM(2012)1010 final/2. SWD(2012)101 final/2,: 61

Fabjan M. 2006. Vpliv spremembe gojitvene oblike na rastni in kakovostni potencial sorte „refošk“. Diplomsko delo, Ljubljana, Slovenija [Slovenia], Univerza v Ljubljani, Biotehniška fakulteta; Oddelek za agronomijo: 44 str.

FAO. 1954. Multilingual vocabulary of soil science. Revised edition 1960. Rome, Italy, UN FAO: 430 str.

FKBV. 2012a. Študijski programi I. stopnje na FKBV. http://www.fk.uni-mb.si/fkbv/index.php/component/content/article/41/1295-studij-1-stopnja (dec 2012)

FKBV. 2012b. Študijski programi II. stopnje na FKBV. http://www.fk.uni-mb.si/fkbv/index.php/component/content/article/41/1294-studij-2-stopnja (dec 2012)

Grad A., Skerlj R., Vitorovic N. 1995. Veliki angleško-slovenski slovar = English-Slovene dictionary. Ljubljana, DZS:

GS Soil Thesaurus. 2012. GS Soil Thesaurus. https://secure.umweltbundesamt.at/soil/en/alphabetical_concepts/a.html (jan 2013)

Hafner P. 2007. Zgradba in razvoj gozdnih ekotopov v Udin Borštu. Diplomsko delo, Ljubljana, Slovenija [Slovenia], Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za gozdarstvo: 76 str.

Ilešič S. 1960. Geografija prsti in rastja (skripta za geografe). Ljubljana, Naravoslovna fakulteta v Ljubljani: 145 str.

ISJ ZRC SAZU: 2012. Slovar slovenskega knjižnega jezika. http://bos.zrc-sazu.si/sskj.html (jan 2013)

Jaecks Vidic N. 1994. Pedogenesis and soil-age reationships of soils on glacial outwash terraces in the Ljubljana Basin. Doktorska dizertacija [Doctoral thesis], Boulder, Colorado, USA, Univesity of Colorado; Department of Geological Sciences: 179 str.

Jamnik B., Smrekar A., Vrščaj B. 2009. Vrtičkarstvo v Ljubljani. Ljubljana, ZRC SAZU: 224 str.

Jamšek P. 2009. Opredelitev geogenih parametrov terroir-ja sorte malvazija v Vipavski dolini. Diplomsko delo, Ljubljana, Slovenija [Slovenia], Univerza v Ljubljani, Naravoslovnotehniška fakulteta; Oddelek za geologijo: 102 str.

Jenny H. 1941. Factors of Soil Formation, A System of Quantitative Pedology. New York and London, McGraw Hill Book Company: 281 str.

Kladnik D. 1999. Leksikon geografije podeželja. Ljubljana, Inštitut za geografijo: 318 str.

Kunšek B. 2011. Mineralna gnojila v ekološkem kmetijstvu. Diplomsko delo, Ljubljana, Slovenija [Slovenia], Univerza v Ljubljani, Biotehniška fakulteta; Oddelek za agronomijo: 17 str.

Lehman A. 2012. Soil terminology: Boden, Erde (written correspondence).

Longman dictionary. 2012. Longman English Dictionary Online. http://www.ldoceonline.com/ (jan 2013)

Lovrenčak F. 1994. Pedogeografija. Ljubljana, Univerza v Ljubljani, Filozofska fakulteta, Oddelek za geografijo: 185 str.

Macmillan Dictionary. Macmillan Dictionary and Thesaurus. http://www.macmillandictionary.com/ (jan 2013)

Merriam-Webster Dictionary. 2012. Merriam-Webster Dictionary. http://www.merriam-webster.com/dictionary/pedogeography (jan 2013)

Mukaetov D. 2012. Soil terminology: Počva, Zemlja (written correspondence).

Oxford Dictionaries. 2012. Oxford Dictionaries Online. http://oxforddictionaries.com/ (jan 2013)

Repe B. 2009. Prst ali tla. Al prav se piše kaša ali kasha? Polet; Delo, 2009, 1

Ritz J. 1973. Poljoprivredni rječnik: Englesko hrvatski ili srpski hrvatski ili srpskio engleski. Zagreb, Hrvaška [Croatia], Universitas Studiorum Zagrabensis: 419 str.

Sabielec G. 2013. Soil terminology: Gleba, Ziemia (written correspondence).

Schad P. 2013. Soil terminology: Boden, Erde (written correspondence).

Sobocka J. 2012. Soil terminology: Pôda, Zem (written correspondence).

Soil Science Society of America. 2008. Glossary of soil science terms 2008. Madison, Wis., Soil Science Society of America: 88 str.

Soil Science Society of America. 2012. S-5 Pedology|. https://www.soils.org/membership/divisions/s05 (jan 2013)

Stolbovoy V. 2012. Soil terminology: Počva, Zemlya (written correspondence).

Stritar A. 1991. Pedologija (kompendij). Ljubljana, Bootehniška fakulteta, Agronomski oddelek: 126 str.

Sušin J. 1983. Gradivo za Pedološki slovar. Ljubljana, Univerza Edvarda Kardelja v Ljubljani, Biotehniška fakulteta, VTOZD za agronomijo:

Šlenc S., Kocjancic P., Mulej B., Mikolic T. 2006. Veliki slovensko-italijanski slovar = Grande dizionario sloveno italiano. Ljubljana, DZS:

UL-BF. 2012a. Biotehniška fakulteta, Univerza v Ljubljani: 1. Bolonjska Stopnja - Univerzitetni Študiji / Kmetijstvo - Agronomija / Predmetnik. http://www.bf.uni-lj.si/dekanat/studijski-programi/1-bolonjska-stopnja-univerzitetni-studiji/kmetijstvo-agronomija/predmetnik/ (dec 2012)

UL-BF. 2012b. Biotehniška fakulteta, Univerza v Ljubljani: 1. Bolonjska Stopnja - Univerzitetni Študiji / Gozdarstvo In Obnovljivi Gozdni Viri / Predmetnik. http://www.bf.uni-lj.si/dekanat/studijski-programi/1-bolonjska-stopnja-univerzitetni-studiji/gozdarstvo-in-obnovljivi-gozdni-viri/predmetnik/ (dec 2012)

UL-BF. 2012c. Biotehniška fakulteta, Univerza v Ljubljani: 1. Bolonjska Stopnja - Univerzitetni Študiji / Krajinska Arhitektura / Predmetnik. http://www.bf.uni-lj.si/dekanat/studijski-programi/1-bolonjska-stopnja-univerzitetni-studiji/krajinska-arhitektura/predmetnik/ (dec 2012)

UL-BF. 2012d. Biotehniška fakulteta, Univerza v Ljubljani: 1. Bolonjska Stopnja - Univerzitetni Študiji / Kmetijstvo - Zootehnika / Predmetnik. http://www.bf.uni-lj.si/dekanat/studijski-programi/1-bolonjska-stopnja-univerzitetni-studiji/kmetijstvo-zootehnika/predmetnik/#c540 (dec 2012)

UL-FF. 2012a. Oddelek za arheologijo Filozofske fakultete; Novi bolonjski programi.

http://arheologija.ff.uni-lj.si/studij/bolonja.html (dec 2012)

UL-FF. 2012b. Oddelek za geografijo; 1. stopnja: GEOGRAFIJA. http://geo.ff.uni-lj.si/1-stopnja-geografija (dec 2012)

UL-NTF. 2012. Naravoslovnotehniška fakulteta; Predmetnik bolonjskega programa OG. http://www.ntf.uni-lj.si/og/index.php?page=static&item=855 (dec 2012)

Unabridged Meriam Webster. 2012. Unabridged Merriam-Webster Dictionary. http://www.merriam-webster.com/dictionary/pedogeography (jan 2013)

UNG. 2012. Univerza v Novi Gorici; Študijski program prve stopnje; Vinogradništvo in vinarstvo. http://www.ung.si/si/studijski-programi/5100/ (dec 2012)

Urbančič M., Simončič P., Prus T., Kutnar L. Atlas gozdnih tal. Zveza gozdarskih društev Slovenije, Gozdarski vestnik in Gozdarski inštitut Slovenije: 100 str.

Utah State University. 2012. Pedology - Plants, Soils, & Climate. http://psc.usu.edu/htm/research/research-groups/pedology/ (jan 2013)

Vovk B. 1955. Gnoj, gnojila in gnojenje [Manure, fertilizers and fertilization]. Ljubljana, Slovenija [Slovenia], Kmečka knjiga: 62 str.

Vovk B. 1959. Stanje travniških in pašniških kultur v Sloveniji ter možnost za povečanje njihove proizvodnje. Ljubljana, Slovenija [Slovenia], Fakulteta za agronomijo, gozdarstvo in veterinarstvo: 3-34 str.

Vovk B. 1966. Določanje stroncija v tleh Slovenije. Ljubljana, Slovenija [Slovenia], Biotehniška fakulteta, Inštitut za tla in prehrano rastlin:

VSVO. 2012. Visoka šola za varstvo okolja; Predmetnik. http://www.vsvo.si/sub.php?cid=2_19_59 (dec 2012)

Zobec I. 1930. Kmetijska kemija. Ljubljana, Jugoslovanska tiskarna v Ljubljani: 157 str.

CONTENT ANALYSIS OF THE PAPERS IN THE ACTA AGRICULTURAE SLOVENICA

VSEBINSKA OBDELAVA PRISPEVKOV V ACTA AGRICULTURAE SLOVENICA let. 101 št. 2

Tomaž BARTOL[a], Karmen STOPAR[b],

SUBJECT INDEX BY AGROVOC DESCRIPTORS
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VSEBINSKO KAZALO PO SKUPINAH ZNANJA (PREDMETNIH KATEGORIJAH)

NAVODILA AVTORJEM

(letniki z liho številko - rastlinska proizvodnja)

Prispevki

Sprejemamo izvirne znanstvene članke s področja agronomije, hortikulture, rastlinske biotehnologije, raziskave živil rastlinskega izvora, agrarne ekonomike in informatike ter s sorodnih področij - **letniki z liho številko** (npr. 97, 99) - v slovenskem in angleškem jeziku; pregledne znanstvene članke samo po poprejšnjem dogovoru. Objavljamo tudi izbrane razširjene znanstvene prispevke s posvetovanj, vendar morajo taki prispevki zajeti najmanj 30 % dodatnih originalnih vsebin, ki še niso bile objavljene. O tovrstni predhodni objavi mora avtor obvestiti uredniški odbor. Če je prispevek del diplomske naloge, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Navedbe morajo biti v slovenskem in angleškem jeziku.
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