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Some plant extracts retarde nitrification in soil

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ABSTRACT

An incubation experiment was conducted to evaluate the effect of aqueous extracts of 17 plant materials on nitrification inhibition of urea- N in soil as compared with chemical inhibitor Dicyandiamide (DCD). Plant materials used in study were collected from different areas of Basrah province, south of Iraq. Aqueous extracts were prepared at ratio of 1:10 (plant material:water) and added at conc. of 0.05, 0.10 and 0.20 ml g^{-1} soil to loamy sand soil. DCD was added to soil at rate of 50 µg g^{-1} soil . Soil received urea at rate of 1000 µg N g^{-1} soil. Treated soils were incubated at 30^{O}C for 40days. Results showed that application of all plant extracts, except those of casuarina, date palm and eucalyptus to soil retarded nitrification in soil. Caper, Sowthistle ,bladygrass and pomegranate extracts showed highest inhibition percentage (51,42,40 and 40% ,respectively) and were found to be more effective than DCD (33%). Highest inhibition was achieved by using those extracts at conc. of 0.1 ml g^{-1} soil after 10 days of incubation . Data also revealed that treated soil with these plant extracts significantly increased amount of NH_4^+–N and decreased amount of NO_3^-–N accumulation in soil compared with DCD and control treatments. Results of the study suggested a possibility of using aqueous extracts of some studied plants as potent nitrification inhibitor in soil.

Key words: nitrification inhibitor, plant extract , inorganic nitrogen

IZVLEČEK

NEKATERI RASTLINSKI IZVLEČKI UPOČASNJUJEJO NITRIFIKACIJO V TLEH

V inkubacijskem poskusu je bil ovrednoten vpliv 17 vodnih rastlinskih izvlečkov na inhibicijo nitrifikacije dušika v urei primerjalno s kemijskim inhibitorjem diciandiamidom (DCD). Uporabljen rastlinski material je bil nabran na različnih območjih province Basrah v južnem delu Iraka. Vodni izvlečki so bili pripravljeni v razmerju 1:10 (rastlinski material:voda) in dodani v koncentracijah 0.05, 0.10 in 0.20 ml g^{-1} ilovnato-peščenim tlem. DCD je bil dodan tlem v razmerju 50 µg g^{-1} tal, urea pa v razmerju 1000 µg N g^{-1} tal. Tretirana tla so bila inkubirana pri 30^{O} C 40 dni. Rezultati so pokazali, da je uporaba rastlinskih izvlečkov upočasnila nitrifikacijo v tleh, razen pri izvlečkih kazaurine, dateljeve palme in evkalipta. Izvlečki kaprovca, škrbinke, trave (*Imperata cylindrica* (L.) P.Beauv) in granatnega jabolka so se izkazali za bolj učinkovite inhibitorje (51, 42, 40 in 40 % inhibicija) kot DCD (33 %). Največja inhibicija je bila dosežena z uporabo izvlečkov v koncentraciji 0.1 ml g^{-1} tal, pri inkubaciji 10 dni. Rezultati so tudi pokazali, da se je v tleh obravnavanih s temi izvlečki značilno povečala količina NH_4^+–N in zmanjšala količina NO_3^-–N v primerjavi s tlemi, obravnavanimi z DCD in kontrolo. Rezultati te raziskave nakazujejo možnost uporabe izvlečkov preučevanih rastlin kot potencialnih inhibitorjev nitrifikacije v tleh.

Ključne besede: inhibitorji nitrifikacije, rastlinski izvlečki, anorganski dušik

1 INTRODUCTION

Prilled urea is the main source of N fertilizer applied to soil. In tropical agriculture, it accounts for about 49% of total fertilizer N use (Byrnes and Freney , 1995). Urea applied to soil, is hydrolyzed by urease enzyme to form NH_4^+ which is subsequently converted to nitrate (NO_3^-)through nitrification process (Kiran and Patra,2003) . The NO_3^- is subject to losses either through percolation of soil water or as nitrogen gases or nitrogen oxides through denitrification process (Mikkelsen et.al.,1978;Katyal et al.,1985). Excessive loss of N due to NO_3^- leaching or loss through

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denitrification in addition to other ways of N losses from soil environment results in very poor recovery of applied nitrogen (Yadav and Mohan,1982). To increase nitrogen fertilizer use efficiency, several approaches have been tried. These include: use of slow release fertilizers (Malhi et al., 2003), addition of salts and acids with urea (Sloan and Anderson , 1995) and use super granules urea (Shah and Wolfe,2003). In addition to that, several chemicals such as N–serve(nitrapyrin), dicyandiamide(DCD) and many other chemicals have been applied to retard urea hydrolysis or nitrification in soil (Kiran and Patra,2003) . In spite of the encouraging results obtained with the use of these chemicals in retarding urea hydrolysis and nitrification their use is limited to experimental one due to high cost, and risk of adverse effect on beneficial soil micro flora (Vyas et al.,1993) and risk of soil and water pollution(Kiran and Patra,2003). Erickson et al. (2000) reported that plant in mature stages produce numerous organic compounds that can inhibit autotrophic nitrifiying organisms, even at low concentration in soils. Other workers reported experimental evidence for roles of root exudates and leachates of plants under climate vegetation inhibit nitrification in soil (Paavolainen et al., 1998; Jafari and Kholdebarin,2002). On the other hand, Purchase(1974)and Johnson and Edwards(1979)found no evidence of nitrification inhibition from root exudates or variety of plant extracts. Literatures reviewed above showed inconsistent results of the effect of plants extract on nitrification in soil. Hence, a comprehensive study was conducted to investigate effect of aqueous extracts of 17 natural plant materials on urea N transformations in soil as compared with the synthetic chemical nitrification inhibitors (i. e. DCD). The purpose of this paper is to report effect of these extracts on nitrification of urea – N.

2 MATERIALS AND METHODS

Soil and plant materials :

Soil used in the experiment was loamy sand collected from tomato field, located at AL-Burjsia area, Basrah province, south of Iraq. The soil classified as Entisol; Typic Torripsamment. Soil samples were collected from surface layers (0–30cm), air dried and sieved (2 mm). Some physical and chemical properties of the soil were determined following procedures described in Page et al. (1982) and presented in table (1).

Plant materials used in study were collected from different areas from Basrah province and described in in table (2). Selected plant materials were cleaned, air dried and grounded to pass 1mm sieve then kept in plastic bags at room temperature (25°C) and humidity (35%) until use. To get aqueous extract, 10 g of ground dry material was mixed with 100 ml of distilled water and horizontal shake for six hours. The homogenate was filtered through tissue paper to separate large particles, and then the filtrate was filtered further using Whatman filter paper No. 1. This process was repeated several times to collect enough quantity of extract. The filtrate was used as stock solution.

Table 1. Some physical , chemical and biological properties of soil used.

Prop.	Symbol		Value
pH (1:1 in water)	———		8.05
E. C.	dS m^{-1}		2.30
$CaCO_3$	g kg^{-1}		75.00
CEC	Cmole ($^{+}$) kg^{-1}		3.40
P (NaHCO3)	mg kg^{-1}		5.60
Total N	g kg^{-1}		0.03
Organic C	g kg^{-1}		0.40
Organic matter	g kg^{-1}		0.70
C:N Ratio	———		13.3
Urease activity	µg NH_4^+/g Soil/2h		2.3
NH_4^+ – N	µg g^{-1}		1.57
NO_3^- – N			0.61
NO_2^- – N			0.00
Ca^{+2}	m *M* L^{-1}		5.40
Mg^{+2}			3.00
Na^+			6.50
K^+			1.02
HCO_3^-			2.00
$SO_4^=$			8.50
Cl^-			7.00
$CO_3^=$			0.00
Loamy Sand	Sand	g kg^{-1}	866.00
	Silt		51.96
	Clay		82.04

Table 2. : Plants used in study

Common name	Latine name	Sampling part	Sampling date
Zizyphus	*Ziziphus mauritiano* Lam. CV. Zaitoni	leaves	Oct.
	Ziziphus spina – christi (L.) Willd.	leaves	Oct.
Colacynth	*Citrullus colocynthis* (L.) Schrod.	Fruits	Nov.
Caper	*Capparis spinosa* L.	Seeds	Oct.
Casuarina	*Casuarina equisetifolia* L.	Stem bark	Jan.
Bead tree	*Melia azedarach* L.	Fruits	Oct.
Pomegranate	*Punica granatum* L.	Peels	Jan.
Cotton	*Gossypium herbaceum* L.	roots	Jan.
Bermudagrass	*Cynodon dactylon* (L.) Pers.	rhizomes	Feb.
Bladygrass	*Imperata cylindrica* (L.) Beauv.	rhizomes	Feb.
Sowthistle	*Sonchus oleraceus* L.	Total shoot	Mar.
Wheat	*Triticum aestivum* L.	bran	Jul.
Date palm	*Phoenix dactylifera* L.CV. Zehdi	Leaves Fiber	Dec. Feb.
Oleander	*Nerium oleander* L.	leaves	Apr.
Eucalyptus	*Eucalyptus camaldulensis* Dehnh.	leaves	Apr
Myrtus	*Myrtus communis* L.	leaves	Apr.

Nitrification inhibition :

Sample of sieved soil was washed with enough 0.01 *N* KCl to leach out the inorganic forms of nitrogen present in soil. Leached soil was air dried, then 5 g of dried soil was placed in plastic containers (capacity 20ml). Soils in plastic containers were treated with solutions contain urea(at rate of 1000 µg N g^{-1} soil) and test solutions (at rates of 0.05, 0.10 and 0.20 ml gm^{-1}soil) . To compare the effect of plant extracts on nitrification on soil with synthetic chemical inhibitor (i. e. dicyandiamide, DCD), set of containers was treated with solution contain 250 µg of DCD (50 µg DCD gm^{-1} soil) and urea at rate of 1000 µg N g^{-1} soil.

Soil of control treatments was treated with solution contains only urea at the same rate as that of other treatments. The moisture content of all treatment was maintained at field capacity during the study period. Treatments were triplicated and incubated at 30°C. Set of samples was withdrawn at 10 days and other at 40 days after amendment of extracts and urea . Soils were extracted with 2 *M* KCl , then the extracted amount of NH_4^+ , NO_2^- and NO_3^- were determined following procedure of Bremner and Edwards(1965).Percentage inhibition of nitrification was calculated according to Bremner and McCarty (1988) :

$$\% \text{ inhibition} = \frac{C - T}{C} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$T = NO_2^- + NO_3^-$ in treated soil
$C = NO_2^- + NO_3^-$ in control soil

E.C. and pH determination

To reveal the effect of plant extracts on soil electrical conductivity(E.C.) and acidity (pH), fifty grams of soils amendment with plant extracts(that showed most effect on nitrification) and urea at rate of 1000 µ N g^{-1} were placed in plastic containers, then incubated at 30° c for 40 days. Soil moisture was adjusted to field capacity during incubation periods. Set of samples was withdrawn after 2,4,8,10,25,and 40 days after incubation and soil E.C. and PH were determined.

Statistical analysis :

The experiment was designed as factorial experiment with three variables (plant extract × extract concentration × incubation period) with three replicates. The results were analyzed using analysis of variance carried out by $SPSS_{11}$ (Agyrous,2005). Differences among means were compared using revised LSD test.

3 RESULTS

Nitrification inhibition :

Data in table (3) show that application of all plant extracts, except those of casuarina, date palm and eucalyptus, to soil reduced nitrification of urea – N during incubation periods of 10 and 40 days. However, the persistence of the inhibitory effect of these extracts on nitrification decreasing with increasing incubation time from 10 to 40 days. Data indicated that degree of nitrification inhibition in soil differs with source and concentration of the extracts used . Retardation of nitrification caused by extracts of caper, sowthistle, bladygrass and pomegranate were higher than that of DCD treatment. The highest retardation was achieved by using extracts of the plants at concentration of 0.1 ml g^{-1} after 10 days of incubation. The inhibition percentages were 51,40 ,40 and 42% for caper, pomegranate, bladygrass and sowthistle, respectively as compared to 33% for DCD treatment . Statistical analysis of treatments is shown in table (4).

Inhibition effects of other plant extracts were either lower (Zizyphus, bermudagrass and oleander) or did not significantly differ (sowthistle, colacynth, bladygrass and bead tree) from that of DCD treatment.

Table 3: (%) inhibition of nitrification in soil treated with different concentrations of plant aqueous extracts after 10 and 40 days of incubation.

Conc. (ml gm $^{-1}$ soil) / Plant	After 10 days of incubation				After 40 days of incubation			
	0.05	**0.10**	**0.20**	**mean**	**0.05**	**0.10**	**0.20**	**Mean**
Zizyphus (CV. Zaitoni)	0	15	30	15	0	0	18	6
Zizyphus (Willd)	0	39	38	25.6	0	10	15	5
Colacynth	26	34	34	31.3	21	16	5	14
Caper	46	51	46	47.6	5	21	16	14.00
Casuarina	0	0	10	3.3	5	0	5	3.3
Bead tree	36	31	32	33	26	16	5	15.6
Pomegranate	34	40	14	29.3	26	32	32	30.0
Cotton	0	36	38	24.6	0	16	5	7
Bermudagrass	39	24	31	31.3	6	16	21	17.6
Bladygrass	39	40	40	39.6	0	10	16	8.6
Sowthistle	40	42	36	39.3	0	5	0	1.6
Wheat	0	37	30	22.3	0	0	0	0
Date palm (leaves)	0	35	29	21.3	0	0	0	0
Date palm (fiber)	0	0	0	0	0	0	0	0
Oleander	0	20	28	16.0	5	10	5	6.6
Eucalyptus	0	6	0	2	0	0	5	1.6
Myrtus	36	35	33	34.6	5	0	5	3.3
DCD			33	33	10	10	10	10
Mean	18.2	28.7	27.8	24.9	6.6	9.0	9.05	8.21

L.S.D. 0.01

plant extracts(p) =2.81, Conc. of extract(c) =1.14, incubation time (t) =*
p×c = 4.87, p×t = 3.98, c×t = 1.62, p×t×c = 6.89

C.V = 9.25

Inorganic N accumulation :

Table (4) shows the amount of inorganic N (NO_2^-, NO_3^- and NH_4^+) accumulated in soils treated with DCD or aqueous extracts of caper, sowthistle, bladygrass and pomegranate after 10 and 40 days of incubation as compare to control treatment. Data in the table indicated that treated soils with these plant extracts significantly increased amount of NH_4^+- N accumulated in soils comparing with that of DCD. Caper, sowthistle, bladygrass and pomegranate maintained 220.97, 207.57, 193.84 and 212.14 mg NH_4^+- N kg $^{-1}$ soil as compared with 182.14 mg NH_4^+-N kg $^{-1}$ soil at DCD treatment after 10 days of incubation. The amount of $NH4^+$-N accumulated in control soil at that time was 149.73 mg NH_4^+ - N kg $^{-1}$ soil. However, the amount of NO_3^- – N produced in soils treated with these plant extracts was lower than these of DCD or control treatments. Plant extracts or DCD effects on NO_3^- – N produced was much lower at 40 days than 10 days of incubation . No NO_2^- – N was detected at any of the treatments involved in the study.

E.C. and pH :

The effect of caper , sowthistle , bladygrass and pomegranate extracts on soil E.C. and pH as compared with control are presented in (Fig. 1 and

Fig.2). Fig. (1) shows that plant extracts increased E. C. of treated soils from 2dSm^{-1} at control treatment to about 4dSm^{-1} at early period of incubation. However, effect of all plant extracts on E.C. decreased as time of incubation increased. Fig. (2) shows that soil pH of all treatments were close to that of control treatment during the incubation period (40 days) and was in the range of 7.9 to 8.3.

Table 4: NH_4^+, NO_2^- and NO_3^- (mg Kg $^{-1}$ soil) released from soil treated with plant aqueous extracts after 10 and 40 days of incubation .

After 10 days of incubation				**After 40 days of incubation**		
treatments	**NH_4^+–N**	**NO_2^- – N**	**NO_3^- – N**	**NH_4^+–N**	**NO_2^- – N**	**NO_3^- –N**
Control	149.73	0	46.64	21.45	0	17.72
Caper	220.97	0	22.95	24.66	0	13.99
Sowthistle	207.57	0	27.05	22.39	0	16.79
Bladygrass	193.84	0	27.99	22.39	0	15.86
Pomegranate	212.14	0	27.98	23.63	0	12.12
DCD	182.14	0	31.25	21.45	0	15.86

RLSD0.01 C.V

NH_4^+ -N = 8.16 15.40

NO3- N = 2.3 12.33

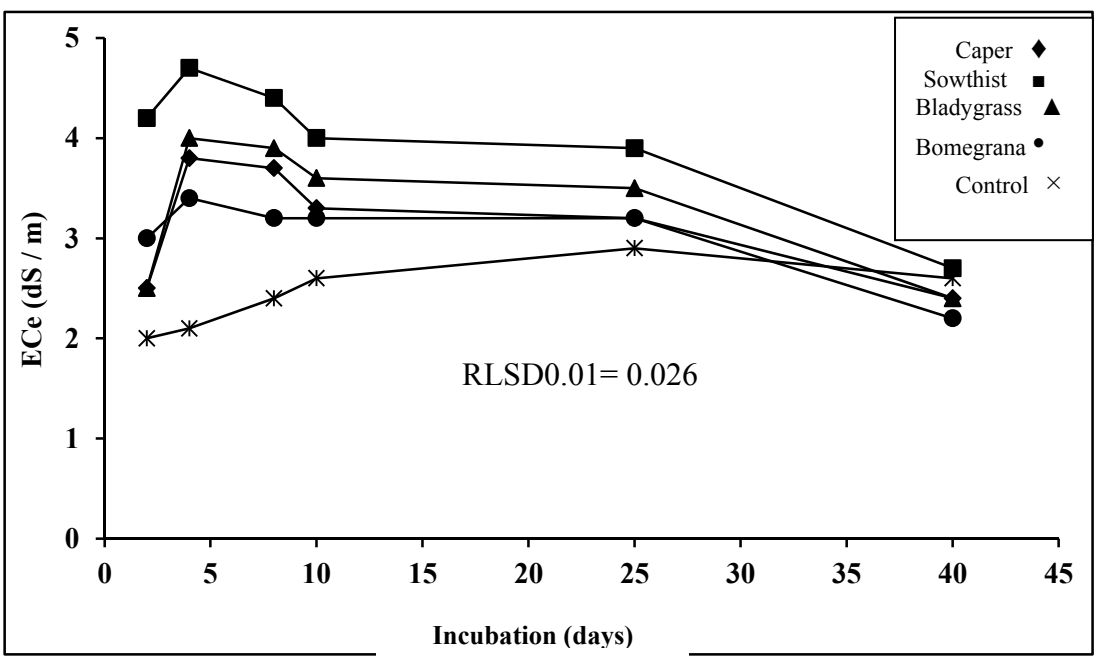

Figure 1: Effect of plant aqueous extracts on soil E.C. at different incubation periods.

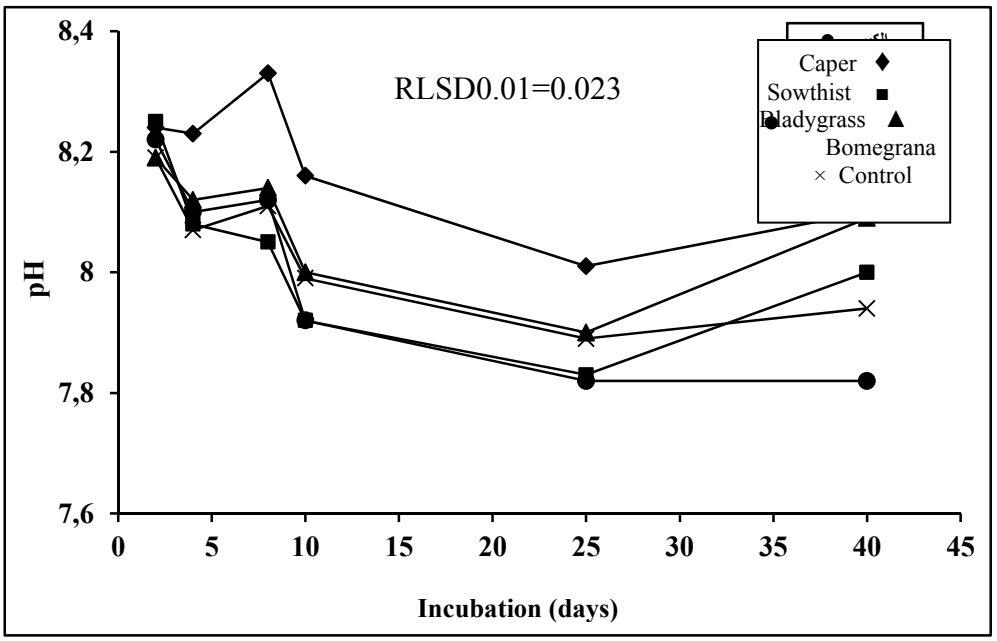

Figure 2: Effect of plant aqueous extracts on soil pH. at different incubation periods. (L.S.D 0.01 = 0.023)

4 DISCUSSION AND CONCLUSION

Nitrification inhibition and inorganic N accumulation:

Several chemical such as DCD , N–serve(DowElanco, USA) , and other have been tried to reduce urea hydrolysis or nitrification in soil in order to increase N–fertilizer efficiency . However, use of such chemicals may have adverse influence on soil micro flora and soil and water pollution(Trenkel,1997) . As early as 1952, Steiven reported presence of naturally occurring substances mostly in higher plants when introduced into soil delay nitrification. Since then interest in using of organic compounds produced and released by plants to control nitrification in soil has increased. However, inconsistent results of effect organic compounds on nitrification have been reported . In this study, selected plants or parts of plants were tested for their effect on nitrification of urea–N in soil. Results of the study showed application of most of studied plants indicated possibility of retarding nitrification of urea–N and the persistence of the inhibitory effect decreased with the time, however, degree of nitrification inhibition differs with source and concentration of the extracts used . Data of Hardy and Sevasithamparam (1989) showed negative effects of added eucalyptus bark on soil microorganism decreased with time. Organic compounds in soil could be volatilized, leached, or converted to non–toxic products as time elapse (Alexander , 1985) . Comparing with chemical nitrification inhibitor (i. e. DCD) retardation of nitrification caused by extracts of plants under study were higher/lower than, or did not differ from that of DCD.

Nitrification inhibitory properties of plant materials such as Karenj (*Pongemia glebra*) neem (*Azadirachta indica*) and tea *(Camellia sinensis*) have been reported (Kiran and Patra ,2003). White (1991) and Paavalainen (1998) reported that introducing water or ethanol extracts of plants contain phenolics, monoterpenes, and other organic compounds into soil exert allelopathic effect on nitrification in soil . Ito and Ichikawa (1999) suggested that *D. adscendens* roots release substances that inhibit not only the growth of other plants and Rhizobium nodulation, but also the nitrifiers activities in soils .On other hand, Kholdebarin and Oertli (1992) and Bremner and McCarty (1988,1993) revealed that any decrease in the amount of NO_3^-- N produced

during nitrification in the presence of cotyledon powder or climax vegetation could be due fixation, volatilization and immobilization of nitrification substrate by organic materials such as phenolics or other compounds released into soil , rather than to their effect on nitrifying bacteria . Whatever, might be the mechanism of their action aqueous extracts of caper , sowthistle , bladygrass and pomegranate plant used in this study showed higher inhibitory effect on urea – N nitrification in soil than that of chemical inhibiter (i.e. DCD). Data in table (5) supported this conclusion by showing that the amount of NH_4^+-N accumulated in soil treated with these plant extracts was significantly higher and the amount of NO_3^-- N accumulated was significantly lower than those of DCD or control treatments at early period of incubation .

E.C. and pH:

Since soil E.C. and pH are among factors controlling nitrification in soil (Alexander, 1985) , the effect of studied plant extracts on these parameters as compared with control were studied (Fig. 1 and Fig. 2) . Results in Fig. (1) Showed that, treating soils with plant extracts increased soil E.C. during early periods of incubation, however, this effect decreased as time of incubation increased. Data in table(3) showed that persistence of the inhibitory effect of plant extracts used in this study decreased with increasing incubation time from 10 to 40 days. Hence, inhibitory effect observed in this study could partly due to increased salinity of treated soils caused by plant extracts during early periods of incubation. Kumar and Wagenet (1985) and Jarallah (1998) reported negative correlation between salinity and nitrification in soil. On other hand, other studies showed that increasing soil salinity from 3 to 12 dSm^{-1} (Jabari,1989; Al-Rashdi et al.,1991) and increased salt concentration up to 0.01 M (Agrawal et al., 1971) or 0.22% (Laura, 1979) increased nitrification in soil.

In conclusion, the results obtained in this study suggested a possibility of using aqueous extracts of some plants as potent substitute for chemical compounds to retard nitrification process in soil thereby, reducing the risk of environmental pollution associated with using chemical compounds as nitrification inhibitors.

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Soil acidification and liming in grassland production and grassland soil fertility in Slovenia

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ABSTRACT

This paper reviews the evidences on grassland soil acidity and liming in relation to soil processes and herbage production. There is also an outline of the present state of soil acidity and acidity-related traits – contents of organic matter (OM), phosphorus (P) and potassium (K) in Slovene grassland. In grassland, soil acidification is an ongoing process under humid climate conditions. It is mainly driven by leaching of nutrients, net loss of cations due to retention in livestock products, use of physiologically acid fertilizers, acid rain and N_2 fixation. This process is reduced by strong pH buffering capacity of the soil and by physiologically basic fertilizers. Acid grassland soils in Slovenia are widely distributed in spite of the fact that 44% of the total land has developed from a carbonate parent material. Of the 1713 grassland soil samples analysed during 2005-2007 45% were regarded as acid ones (pH < 5.5; in KCl), 57% as soils with very low P status (< 6 mg P_2O_5/100 g soil) and 22% as soils with very low K status (< 10 mg K_2O/100 soil). Increased content of soil organic matter was identified for alpine pastures (> 10% OM in 44% of samples), mainly as a result of low decomposition rate. Liming of acid grassland soils did not always reflect in a higher herbage yield. The cause for this inefficiency is plant composition of grassland. Thus, many grassland plants with relatively high production potential have adapted to acid soil conditions. To illustrate the inconsistent liming effect three researches are reviewed. In the first two researches liming along with fertilizer application did not increase the yield comparing to the fertilized control while in the third research the increase amounted 26%. Liming improves considerably botanical composition of the acid grassland (e.g. sward where Common Bent – *Agrostis tenuis* Sibth. – prevails) and thus indirectly affects palatability and nutritive value of herbage. Grassland liming has a weak direct effect on herbage quality – it usually increases content of Ca and sometimes decreases Mg in herbage. The latter effect is rare. In Slovenia, ameliorative liming is advised for grassland soils with pH < 5.0 and maintenance liming for grassland soils with pH < 6.0 (pH in KCl or $CaCl_2$).

Key words: grassland, soil acidity, liming, herbage yield, Slovenia

IZVLEČEK

ZAKISANJE TAL IN APNJENJE V TRAVNIŠTVU TER RODOVITNOST TRAVNIŠKIH TAL V SLOVENIJI

Pregledni članek obravnava kislost travniških tal in apnjenje v povezavi s procesi v tleh in pridelavo travniške krme. Dodan je zgoščen prikaz kislosti ter s tem povezane vsebnosti organske snovi, fosforja in kalija v travniških tleh v Sloveniji. Zakisanje travniških tal je v vlažnem podnebju stalen proces, ki je odvisen predvsem od izpiranja hranil, negativne bilance hranil zaradi odvzema, uporabe fiziološko kislih gnojil, kislega dežja in biotske fiksacije N_2. Zakisanje tal po drugi strani pomembno zmanjšujejo puferski mehanizmi v tleh in nekatera gnojila. Razširjenost kislih travniških tal v Sloveniji je velika, kljub temu, da ima 44 % zemljišč karbonatno podlago. Od 1713 vzorcev travniških tal je imelo 45 % pH vrednost pod 5,5 (v KCl), 57 % zelo malo fosforja (< 6 mg P_2O_5/100 g tal) in 22 % zelo malo kalija (< 10 mg K_2O/100 g tal). Vsebnost organske snovi je povečana na planinskih pašnikih (> 10 % OS v 44 % vzorcev) predvsem zaradi slabe razgradnje. Apnjenje kislih travniških tal vedno ne poveča pridelka krme. Razlog za to je v travniških rastlinah, ki dobro prenašajo kisla tla, a imajo obenem razmeroma velik rastni potencial. V zvezi s tem so predstavljene tri raziskave, v dveh apnjenje skupaj z gnojenjem ni povečalo pridelka v primerjavi z gnojenimi kontrolami, v enem pa je pridelek povečalo za 26 %. Apnjenje pomembno izboljša botanično sestavo acidofilne travne ruše (npr. ruša s prevladujočo lasasto šopuljo – *Agrostis tenuis* Sibth.) in s tem posredno vpliva na okusnost in hranilno vrednost krme. Neposredni vpliv apnjenja na kakovost krme je razmeroma majhen – običajno poveča vsebnost Ca v krmi, lahko pa tudi zmanjša vsebnost Mg. Slednji vpliv je redek. Za Slovenijo stroka priporoča meliorativno apnjenje travniških tal s pH < 5,0 in vzdrževalno apnjenje tal s pH < 6,0 (pH v KCl ali $CaCl_2$).

Ključne besede: travništvo, kislost tal, apnjenje, pridelek zelinja, Slovenija

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1 INTRODUCTION

Soil acidity expressed as a pH value can importantly influence plant growth in grassland. Much of agricultural plants actively grow in the pH range between 4.0 and 8.5 but not at the same rate throughout the interval (Whitehead, 2000). Optimal pH value for grassland soil has much narrower interval and differs among grassland species. In spite of these differences, there is a uniform optimal pH value for grassland which differs among countries. Different methods for pH determination represent the key reason for this. Therefore it should be taken into account when dealing with a pH soil value. Table 1 shows some recommended soil pH values for grassland in particular countries. The extraction media used for soil pH determination are also denoted.

Grassland soils with non-optimal pH value are usually acid and this is generally true for the situation in Slovenia.

Table 1: Optimal and target pH value for grassland soils in Slovenia and in some other countries.

	pH (in KCl or $CaCl_2$)	pH (in water)	Source
Slovenia	5.0-5.5 (6.0)	–	Leskošek, 1987
Austria	5.0-6.0	–	BMLFUW, 2006
Swiss	–	5.5-6.7	GRUDAF, 2009
Germany	4.7-6.1	–	Wendland in sod., 2011
United Kingdom	–	6.0	DEFRA, 2010
Ireland	–	6.2 (6.5)	Tunney in sod., 2010
New Zealand	–	5.5-6.3	Sparling in sod., 2008

Note: Soil pH values measured in solution of potassium chloride or calcium chloride are very similar, while those values measured in water are higher than the formers. There is no linear correlation between the results of the first two methods and the third one.

2 GRASSLAND SOIL ACIDIFICATION

Acid soils occur mainly in areas with humid climate and are the most widespread in forest and grassland habitats. After von Uexkülla and Muterta's (1995) estimation, the world's area with acid soils counts 3950 million ha. Of this, 67% is covered by forest, 18% by grassland vegetation and 4.5% by arable crops. In addition to climate development of acid soil is also affected by the parent materials. Soils with silicate parent materials are usually more acid than those with carbonate ones. Acidity of the latter soils is of secondary origin and results mainly from leaching of base cations through soil profile. Soil acidification results from mass flow and interrelated biochemical processes in grassland ecosystems. The most important flows consist in one hand of loss of mineral nutrients due to leaching and yield removal and in the other hand of input of matters from fertilizers, rainwater and lithological basis. Chemical processes involved in soil acidifications are mainly occurring during the nitrogen cycling and less importantly during carbon and sulphur cycling.

In general soil acidification is more pronounced on grassland than on arable land. In Europe the main reasons for this accelerated acidification is connected with geographic position, soil properties and management of grasslands. Grassland here prevails in uplands and mountain areas, i.e. the altitude belt between 700 and 1900 m a.s.l. Meadows and pastures in these areas are usually sloping and have porous and shallow soils. It causes these soils to be prone to soil erosion and leaching of plant nutrients (e.g. NO_3^-, Ca^{2+}, Mg^{2+}) through soil profile, thus leading to soil acidification. These shortcomings can also occur in grassland of low-laying areas in Europe.

Soil acidification in the extensive grassland production is partly caused by insufficient fertilizer application. Increased removal of mineral cations with grassland fodder, which is not matched by

fertilizer application, leads to the excess of H^+ ions in the soil and consequently lowering the pH value (de Klein *et al*., 1997). Fertilizer application influences on soil pH value when using physiological acid or alkaline fertilizers. The former type (e.g. ammonium sulphate and urea) acidifies the soil while the latter one (e.g. Thomas phosphate) has the reverse effect. Soil acidification may also result from acid rain (pH < 5.6) containing SO_4^{2-}, NH_4^+ in NO_3^-. Among these ions, the ammonium ion is the critical acidification input (Bini and Bresolin, 1998). Austrian experimental results on liming and fertilizer application (Schechtner, 1993) show the importance of these three groups of matter flows for grassland soil pH value. In a 2-cut grassland experiment soil pH ($CaCl_2$) fell from 5.8 to 4.7 in 44 year period under zero fertilizer treatment. In another 4-cut experiment, which was fertilized with recommended amounts, the physiological neutral PK fertilizer and physiological acid fertilizer (N in the form of urea) declined soil pH from original 5.6 to 4.5 and 4.2 respectively in 17 year period.

In addition to mass flow, soil pH affecting processes occur during the cycling of carbon, nitrogen and sulphur (Bolan *et al*., 1991). In the case of the carbon cycle, the source of H^+ ions is carbonic acid, formed by the reaction of CO_2 with water, and carboxylic acids. However, these two sources of soil acidification are less important if the soil is aerated to allow diffusion of CO_2 through air-filled pores (Marschner, 1986). Of the other two cycles, nitrogen cycle is much more important for soil acidification because its cycling within an ecosystem is roughly ten times faster than the sulphur cycling. Besides, NO_3^- for soil acidification compared to SO_4^{2-} is more important due to its higher leaching loss in some soils. Basic cations which leach together with NO_3^- are replaced by H^+ ions on colloids and the soil solution. Hydrogen ions are generated in a ratio 1 H^+ to 1 N in the metabolism of urea to NH_4^+ (ammonification) and then to NO_3^- (nitrification). Metabolism of urea is important factor for higher tendency to acidification of grassland soil compared to the arable one (Whitehead, 2000). It generally occurs to a greater extent under grassland than under arable crops. Important factor for potential acidification of the soil is also biological nitrogen fixation where legumes and rhizobia are involved. In this process assimilation of NH_3 into amino acids (aspartate and glutamate) generates H^+ by their dissociation. In a case study, Bolan et al (1991) calculated the acidification rate for two farms in New Zealand and one in Australia which was caused mainly by biological nitrogen fixation. First two farms grazed their livestock herds on ryegrass-white clover pastures, and the third one did the same on Verano stylo monoculture (*Stylosanthes hamata* L.). Proportion of biologically N_2 fixed in the systems ranged from 91 to 95%. Their results show the net input of H^+ ion into the surface soil in the order of 8 kmol/ha and per year for the first two farms and 1 kmol/ha and per year for the third one. Taking into account a short term pH buffering capacity of 30 kmol/ha for two soil types for Australia it may take 12 years to cause a drop in pH of one unit from 6 to 5 for the New Zealander farms and 30 years for the Australian farms. The importance of biological N_2 fixation is shown by the experimental results of Mengel and Steffens (1982). In their pot experiments, the soil pH value (in KCl) under red clover (*Trifolium pratense* L.), which was completely supplied with N by biological fixation, dropped from 7.2 to 4.5 in 14 month period. In the control, where perennial ryegrass (*Lolium perenne* L.) grew with the addition of ammonium nitrate (NH_4NO_3), the soil pH value remained unchanged. Hydrogen cations required for the decreasing of soil pH value under red clover derived from the plants to the extent of 60%.

In relation to the latter experiment, it should be noted that the proportion of legumes in the sward and N_2 fixation rate are different in grassland practices. In the case when this proportion and/or N_2 fixation are low, the impact on soil pH can be negligible. Also, pH buffering capacity in the soil under field conditions is usually much higher than that in the case of pot experiments. Therefore, the effect of legumes on the soil pH reported by Mengel and Steffens (1982) is hardly probable to occur in the real situation. An example of very high buffering activity can be found in the article of Murphy *et al*. (2005). The results of 32 year field experiment about the impact of slurry application on the cation balance of the grassland soil in Northern Ireland show that the use of extremely high amount of slurry (200 m^3/ha and per year; 4,76 % dry matter, 0,27 % N) did not decrease the soil pH value. Instead, it even slightly increased compared to unfertilized control. In this

case, buffering activity of both soil and applied slurry surpassed acidifying activity of nitrogen in excess of crop need in the system.

The ammonia volatilization and denitrification also affect the pH value of the soil. In the first process H^+ ions in the soil increase while in the second one they decrease. The importance of NH_3 volatilization and denitrification for soil pH value is generally difficult to define due to large diversity of measured values obtained experimentally. Much information on these two soil pH factors can be found in the monograph of Whitehead (1995).

3 THE PH VALUE AND RELATED PROPERTIES OF GRASSLAND SOILS IN SLOVENIA

Published data of the Agricultural institute of Slovenia were used for the assessment of grassland soil fertility in Slovenia on the basis of key indicators (Sušin, 2008a, 2008b, 2008c). In a comprehensive survey of soil fertility in Slovenia 1713 grassland soil samples were analysed in the period from 2005 to 2007. Of which 1215 samples were taken from meadows, 323 samples from lowland and hilly pastures and 139 samples from mountain pastures. All of these samples were taken on the depth of 0 to 6 cm. A large number of samples taken and a good dispersion of sampling sites allowed generalized conclusions that refer to the entire country.

As expected, acid grassland soils prevail in Slovenia (Figure 1). Taking into account that the optimal pH (in KCl) for the grassland soil is between 5 and 6 and that good growth of sward occurs also in neutral soils (pH 6.6 to 7.2), it can be concluded that over quarter of mountain pastures (pH < 4.6) and less than one fifth of meadows and of lowland and hilly pastures have an inadequate pH value. All grassland soils placed in the lower part (pH 4.6 to 5.0) of the acid soil class have also an inadequate pH value, but their proportion is not clear from the published data. Increased soil acidity is emphasized in mountain pastures as a result of the geographic position of these pastures, shallowness and permeability of the soil and the low use of fertilizers.

The content of organic matter in grassland soil of Slovenia is mostly within the normal limits (4 to 10%; Figure 1). Mountain pastures are an exception to this, because the organic matter content of almost half of them is more than 10%. The increased organic matter content is associated with drought conditions and cold weather as well as the soil acidity and excreta of grazing livestock. Accumulation of the root material together with other dead plant material in the soil reduces the productivity of sward. The importance of the soil pH for the mineralization of organic matter is showed by Silvertown *et al*. (2006). They found 3% of carbon in the soil layer from 0 to 23 cm in the case of unfertilized sward and sward fertilized with sodium nitrate ($NaNO_3$), where the soil pH was between 5.0 and 5.6. Sward fertilized with ammonium sulphate ($(NH_4)_2SO_4$) with the soil pH between 4.1 and 3.6 contained from 4.8 to 6.6% of carbon, respectively.

Soil acidity in Slovene conditions is a synonym for the poor supply of the soil with plant nutrients. This is especially true for grassland soils (Figure 2). Analysed soil samples were very poorly supplied with available phosphorus, i.e. as many as 58% of samples contained less than 6 mg P_2O_5 per 100 g of soil (AL method). Acidity of the soil contributes significantly to this poor supply of the soil with phosphorus. It is well known that the availability of phosphorus in the acid soil is reduced due to high amount of aluminium cations (Al_3^+) adsorbed to clay participles (Whitehead, 2000).

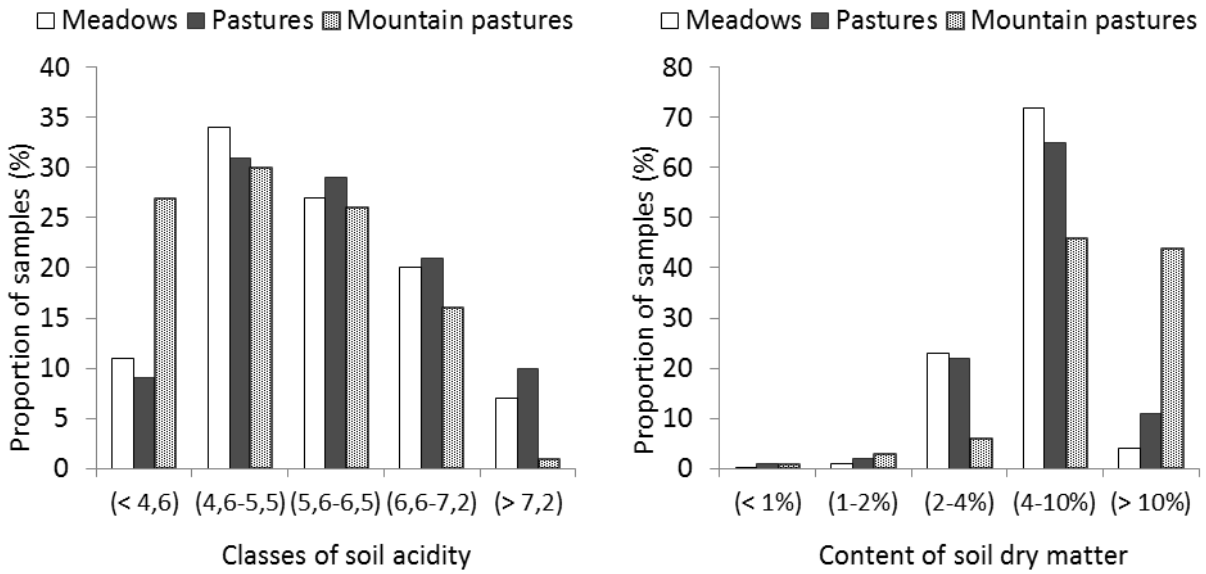

Figure 1: Distribution of soil samples from grassland into 5 categories according to the acidity (pH value) and content of organic matter. The pH classes: very acid, acid, moderately acid, neutral and basic soil. The samples were taken during 2005-2007, n = 1713 (1251, meadows; 323, pastures; 139, alpine pastures; Sušin, 2008a, 2008b, 2008c).

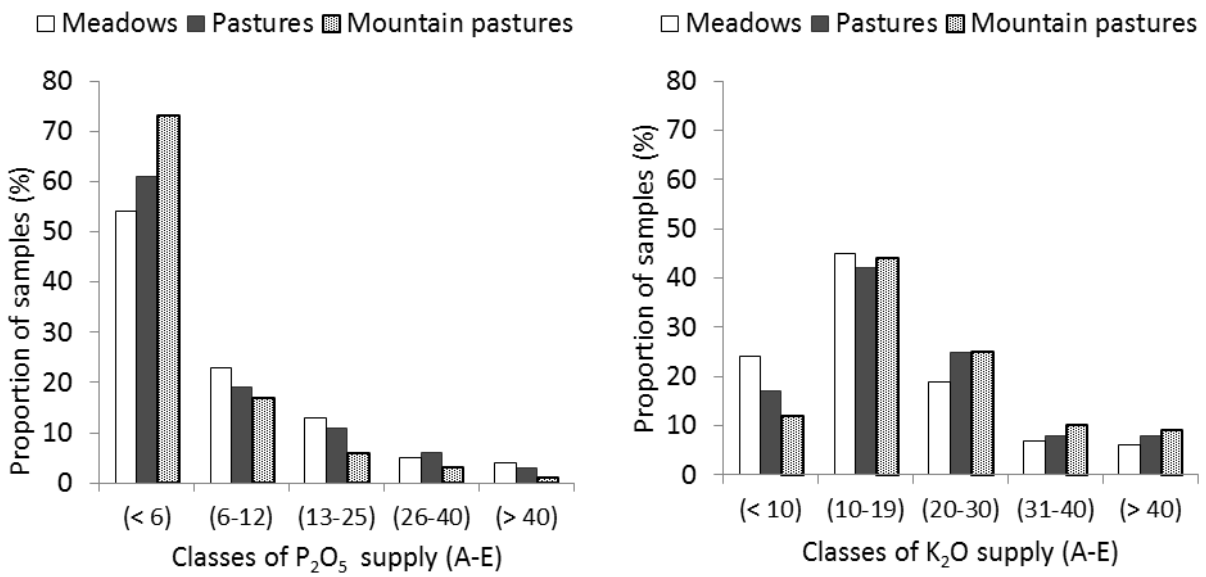

Figure 2: Distribution of soil samples from grassland into 5 categories according to the status of phosphorus (P_2O_5) and potassium (K_2O). The classes: A – low, B – medium, C – optimal, D – excessive, E – extreme. The samples were taken during 2005-2007, n = 1713 (1251, meadows; 323, pastures; 139, alpine pastures; Sušin, 2008a, 2008b, 2008c).

In Slovenia, grassland soils are also relatively poorly supplied with available potassium (Figure 2). Of the analysed soil samples, 21% is arranged in the class of low content of potassium and 43% in the class of medium content. Despite these discouraging findings, the lack of potassium in the soil of Slovene grassland is much less severe than the lack of phosphorus. This is also confirmed by the fact that the uptake of potassium by yield of herbage is higher than one would expect taking into account fertilization and analytically defined potassium content in the soil.

4 INFLUENCE OF LIMING ON GRASSLAND PRODUCTION

Soil liming is the amelioration measure used to improve soil pH value and its structure as well as plant and animal calcium supply. Despite this general benefit grassland liming has variable impact if being judged on the basis of herbage yield (Edmeades *et al*., 1984). The reasons for this uncertainty can be found in the complexity of the interactions between the ground limestone ($CaCO_3$) or lime ($Ca(OH)_2$) and the soil in which the sward plants have important role. Liming increases pH value of soil which affects its physical, chemical and biological characteristics. Soil pH *per se* does not have an important impact on grassland plants. Wheeler *et al*. (1992) established that various pasture species are not sensitive to pH (water) 4.5 with the exception of Harding grass (*Phalaris aquatica* L.) and lucerne (*Medicago sativa* L.). These results had been derived from many solution culture experiments with two pH treatments at zero aluminium concentration (pH 4.5 vs. 5.5; e.g. Edmeades *et al*. 1991).

More important problem for meadow plants growing on acid soils is toxicity of aluminium cations (Al^{3+}) on one hand, and the lack of phosphorous ($H_2PO_4^-$), calcium (Ca^{2+}) and magnesium (Mg^{2+}) on the other. Growth disturbance of grassland plants may also occur in conjunction with other nutrients but is less common. Toxicity of Al^{3+} in grassland soil begins to emerge at pH < 5.5 (measured in water; Wheeler and O'Connor, 1998). Whitehead (2000) states that the threshold of soil pH value for this toxicity is 4.5 (a measuring method is not indicated). Toxic influence of Al^{3+} on grassland plants is reflected in the same manner as in other cultures, i.e. in poorer growth of roots and thus the whole plants. The increased content of Al^{3+} in the soil minimizes the availability of phosphorus to plants, which is often the main negative effect of acid soil on the growth of grassland plants. It seems that this is also the main problem of acid grassland soil in Slovenia.

Liming of acid grassland soil eliminates Al^{3+} toxicity and improves the supply of plants with calcium and phosphorus. But this cannot replace grassland fertilization with phosphorus. Liming accelerates the mineralization of organic matter in soil and consequently improves the plant supply with nutrients, especially nitrogen. Wheeler and O'Connor (1998) report that the total net amount of nitrogen mineralized in the first two years after ground limestone application of 5 and 10 t/ha on the mowing trials was 32 and 68 kg N/ha, respectively.

4.1 Herbage yield

The positive impact of liming on the herbage yield can be expected up to pH (water) 6, as reported by Edmeades *et al*. (1985). However, such positive impact has not been always confirmed even at much lower pH values (e.g. Leskošek, 1987). The results of three experiments are stated bellow in order to illustrate the variable impact of liming on the yield of permanent grasslands.

Leskošek (1987) performed two experiments that involved different fertilization treatments and found that liming along with PK or NPK fertilizers applications had week effect on the herbage dry matter yield compared to non-limed controls (Figure 3). The reason for poor performance of liming is attributed to relatively high pH value compared to the recommendations for grassland soils. This value was before the start of the research, in both experiments, 4.9 (in KCl). At the same time the concentration of exchangeable Al^{3+} ions in both experiments was small. It was 0.96 and 0.25 $mmol_c/100$ g soil in Dragatuš and in Brezje near Ljubljana, respectively.

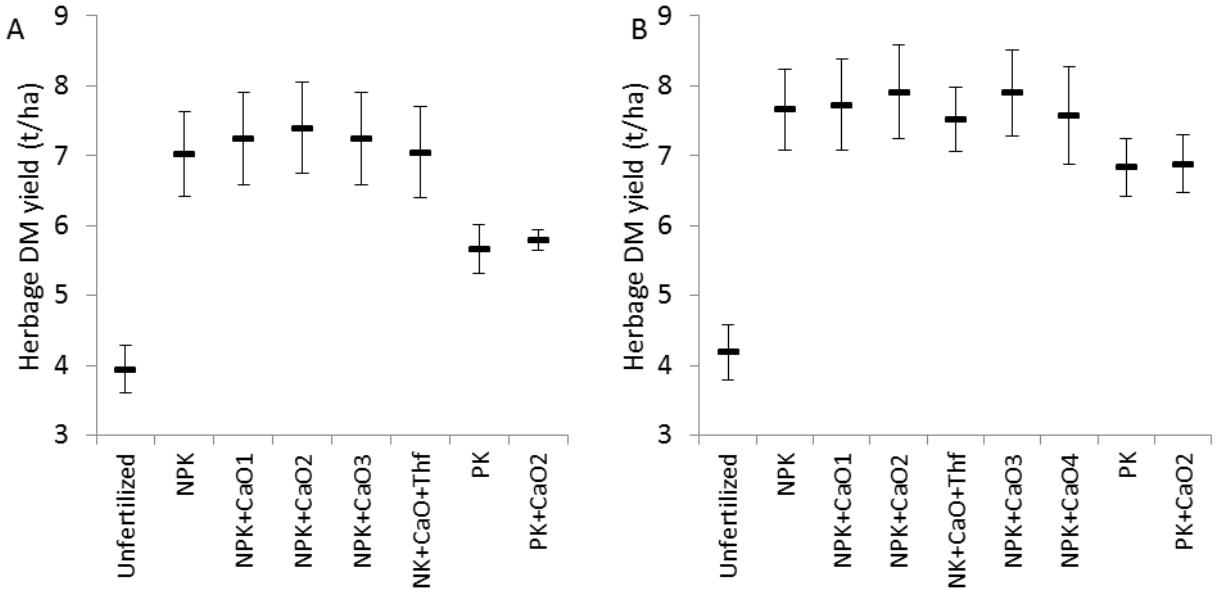

Figure 3: Influence of fertilizer application and liming on the herbage dry matter yield on two 2-cut meadows. The figure shows the annual yields averaged over 5-year period with standard errors of means (Leskošek, 1987).

A, trial at Dragatuš, silt loam, pH_{KCl} 4.9	B, trial at Brezje, silty clay loam, pH_{KCl} 4.9
Unfertilized = zero fertilizers, no liming	Unfertilized = zero fertilizers, no liming
NPK = 40+40 N, 80 P_2O_5, 110 K_2O (all in kg/ha/year,variant b)	NPK = 40+40 N, 80 P_2O_5, 110 K_2O (all in kg/ha/year,variant b)
NPK+CaO_1 = b + 1000 kg CaO/ha (mixed lime)	NPK+CaO_1 = b + 1500 kg CaO/ha (groun limestone)
NPK+CaO_2 = b + 2000 kg CaO/ha (mixed lime)	NPK+CaO_2 = b + 2000 kg CaO/ha (mixed lime)
NPK+CaO_3 = b + 3000 kg CaO/ha (mixed lime)	NK+CaO+Thf = NK as at b + 1500 kg CaO/ha and Thomas phosphate (80 kg P_2O_5/ha/year)
NK+CaO+Thf = NK as at b + 2000 kg CaO/ha and Thomas phosphate (80 kg P_2O_5/ha/year)	NPK+CaO_3 = b + 2000 kg CaO/ha (quick lime)
PK = as at b	NPK+CaO_4 = b + 2000 kg CaO/ha (ground limestone)
PK+CaO_2 = as at b + 2000 kg CaO/ha (mixed lime)	PK = as at b
	PK+CaO_2 = as at b + 2000 kg CaO/ha (mixed lime)

Schechtner (1993) also reports about the poor performance of liming. He conducted two experiments on a high-mountain pasture – *Nardetum* association. In one he compared the effect of different amounts of mixed lime within the PK fertilization and in the other the effect of different amounts of mixed lime within the NPK fertilization (Figure 4).

The influence of liming was expressed only when compared the PK fertilization to the implication of the maximum amount of mixed lime (990 kg CaO/ha every second year) during the period from the fifth to the ninth year of the trial. As for all other comparisons, the increase of herbage yield due to liming was very low regardless the period of comparison. There were two reasons for poor impact of liming: the first one was the grassland community itself, which was difficult to improve and the second reason was the methodological approach since they used Thomas phosphate for phosphorus fertilization that contents calcium in the form of salt and oxide. For this reason both controls (PK and NPK) as well as other treatments received 135 kg CaO/ha per year from Thomas phosphate, meaning that it masked almost all liming influence. This disturbance in trial caused by Thomas phosphate was obvious also in the development of pH values in soil. The implemented amount of 135 kg CaO/ha finally increased pH (KCl) value on around 5 which already provided relatively favorable conditions for growth of grassland species.

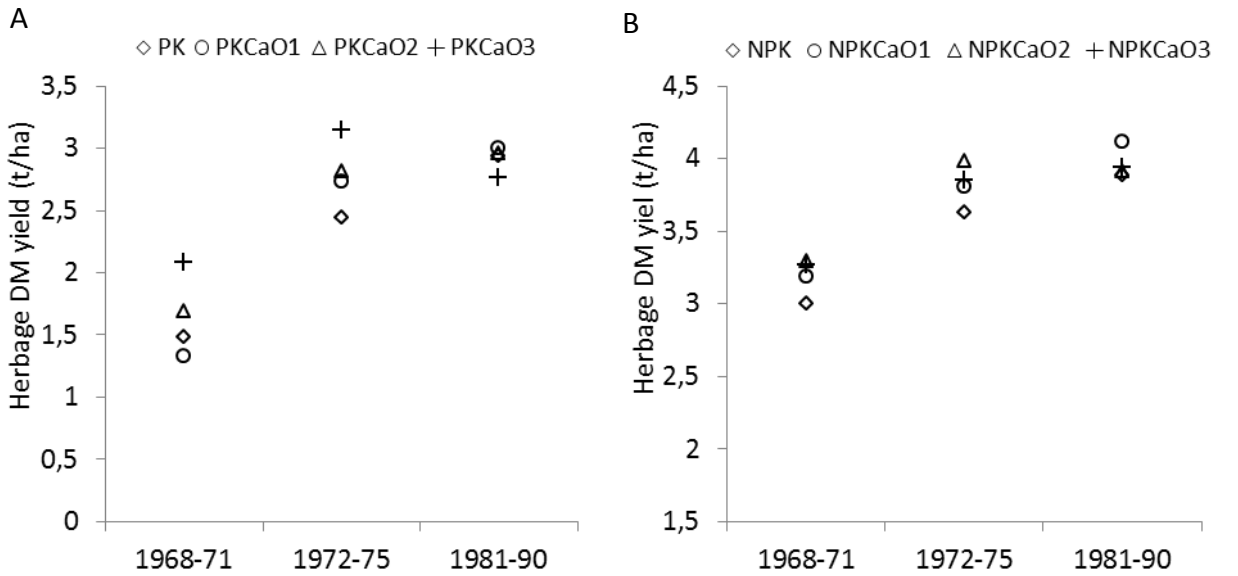

Figure 4: Influence of fertilizer application and liming on the herbage dry matter yield on a poor pasture belonging to the *Nardetum* association (locality: Zachenschöberl, 1300 m a.s.l.). The figure shows the mean annual yield for three periods (duration of the experiment: 1964-1984; Schechtner, 1993).

PK = 300 kg Thomas phosphate (45 % CaO) + 200 kg /ha/year potassium chloride (40 % K_2O); PK application is the same in all treatments
CaO_1, CaO_2, CaO_3 = 500, 1000, 1500 kg/ha (mixed lime, application each second year)
Rates of CaO/ha/year: 135 kg (PK), 300 kg (CaO_1), 465 kg (CaO_2), 630 kg (CaO_3)
N = 2 × 40 kg/ha (KAN, application in spring and after the first cutting)

Unlike the two above described trials, Grundler and Voigtländer (1979) report on good impact of liming using physiologically acid NPK fertilizer (Figure 5). Using this fertilizer liming increased average hay yield in the whole period (1954–1974) for 1.8 t/ha per year i.e. 26 %. At the same time the final soil acidity decreased indicating high liming activity of slag. The research shows that implementation of lime to physiologically acid fertilizer had strong effect on botanical composition of sward. Physiologically alkaline NPK fertilizer had similar effect. The original sward with prevailing red fescue (*Festuca rubra* L.), thread rush (*Juncus filiformis* L.), quaking grass sedge (*Carex brizoides* L.) and Yorkshire fog (*Holcus lanatus* L.) changed due to the impact of above stated factors into a sward with prevailing meadow foxtail (*Alopecurus pratensis* L.), meadow and red fescue (*F. pratensis* Huds.) and Yorkshire fog.

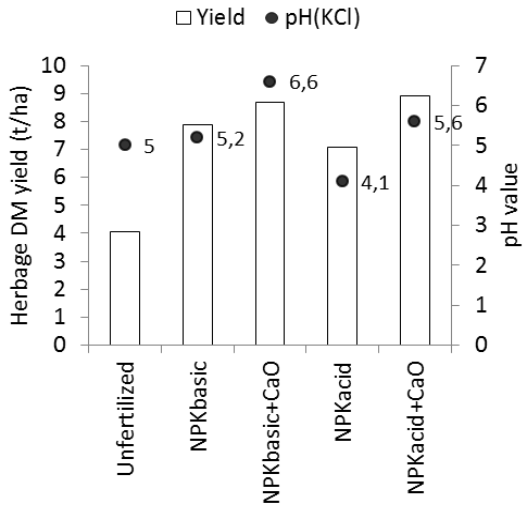

Figure 5: Influence of fertilizer application and liming on the herbage dry matter yield on a permanent pasture at Niedersteinnachu (soil type: gley; pH_{KCl} 4,2; Grundler in Voigtländer,1979). The figure shows the annual yield averaged over 1954-1974. Till 1967, 2-cut system was applied after that it was changed to 3-cut system. pH shown in the figure refers to 1974.

Unfertilized = zero fertilizers, no liming
NPKbasic = basic NPK (rate per ha: 30 kg N/cutting + 80 kg P_2O_5/year + 160 kg K_2O/year), variant b
NPKbasic+CaO = b + 9 applications of CaO (1955-1972, annual rate 1 t CaO/ha), variant c
NPKacid = acid NPK, rate as at b
NPKacid+CaO = acid NPK + CaO as at c

4.2 Herbage yield quality

In acid soil (pH ≈ 4; in KCl) liming together with fertilizer application improves the sward botanical composition and consequently nutritional value of herbage. Both ameliorating measures are needed to obtain optimal growth conditions for grassland plants that do not strive well on acid and poor soil. Example of such improvement is stated in the previous paragraph (Figure 5). Based on a 109 years long field trial in Rothamsted, that is still going on, Thurston (1969) reports about major differences in sward botanical composition connected to the soil pH value. Fertilizer application of physiologically acid ammonium sulphate ($(NH_4)_2SO_4$) caused soil acidity (pH ≈ 3.8; in water). In the sward with this treatment, prevailing species was common bent (*Agrostis tenuis* Sibth.) followed by less abundant red fescue and two other acidophilic species common sorrel (*Rumex acetosa* L.) and creeping cinquefoil (*Potentilla reptans* L.) which were rare. In lime-treated sward with soil pH value of about 5.3 higher quality species throve, especially the most abundant meadow fox tail. There was also a lot of common dandelion (*Taraxacum officinale* F. H. Wigg). Even though liming together with fertilizer application improved sward species composition in many experiments it did not have major influence on the proportion of legumes in swards (e.g. Grundler and Voigtländer, 1979; Leskošek, 1984; Edmeades in *et al*., 1990; Schechtner, 1993).

Liming also affects directly the quality of herbage from grasslands but to a lesser extent. It can increase phosphorus uptake and content in plants due to increased mineralization of organic matter in soil and better availability of mineral bound phosphorus in soil (Wheeler and O'Connor, 1998). Root growth is improved due to decrease of Al^{3+} ions caused by liming. This consequently improves phosphorus supply to the plant. Liming usually increases calcium content in herbage. This is reported by several sources (e.g. Whitehead, 2000; Wheeler, 1998). In two field trials on grassland with original pH value 5.1 and 5.5 (in water) application of limestone at rate of 8 t/ha increased herbage calcium content from 5.0 to 6.1 g/kg of dry matter (Stevens and Laughlin, 1996). But this is not very important, because the control value

itself is already high. Liming can have also the negative impact on grassland forage quality. Wheeler (1998) noted the negative impact of liming (5 and 10 t of ground limestone/ha) on content of exchangeable magnesium in soil and on magnesium content in herbage, but this lower content was still appropriate. He also cites the finding of an increase in grazing tetany due to late autumn liming.

5 CONCLUSIONS

Soil acidity in meadows and pastures is often too high for good growth of grassland vegetation. The reason for this is not pH *per se*, but increased content of Al_3^+ in the soil which has negative impact on plants. The content of Al_3^+ is also linked to the restricted availability of phosphorus to plants. Other disturbances in the growth of grassland plants on acid soils are less important. Considering the soil pH value, it is necessary to know the method of its measuring, because it influences on the results considerably. Methods of measuring pH values in the soil represent the key reason for the differences among the optimal values cited in literature.

Grassland soils in Slovenia are often too acidified and very poorly supplied with phosphorus. The situation is the worst in mountain pastures due to management of the grassland as due to adverse pedo-climatic conditions. The latter increase the loss of plant nutrients from the rhizosphere and upper soil layers.

Liming has a number of beneficial effects on soil fertility and also contributes to the better supply of plants with calcium. Nevertheless, liming of acid grassland soils (pH ≈ 5; in KCl) does not always increase the yield of herbage, which has been confirmed by many researches. The reason for this is in particular grassland plants which tolerate the acid soil conditions and have quite a high production capacity as well. In such situation, the herbage yield can be increased simply by adequate fertilizer application to 7 to 9 t dry matter/ha which is quite good. Here, the quality of herbage is also improved in terms of increased proportion of better grasses in the sward. On grassland with very acid soil (pH < 4.5; in KCl) liming is required to improve botanical composition of sward which does not meet yield and quality standards in the managed grassland. At the same time liming of very acid soil improves the supply of plants with some nutrients, especially with phosphorus and nitrogen.

Direct impact of liming of grassland soils on herbage quality is small. Normally, this measure increases the content of calcium in herbage, but it is generally less problematic in the diet of livestock. Due to dilution effect the content of other mineral nutrients in herbage is changed slightly by liming although their uptake is increased. Liming can even worsen the supply of animals with magnesium, although this risk is low.

In Slovenia, liming of grasslands is recommended on acid soil with pH below 5.0, but also on soils wit pH below 6.0 (measured in KCl or $CaCl_2$). In the first case, liming increases the yield and quality of herbage and in the second case it prevents the acidification of soil. Liming also improves the soil structure which may reduce soil compaction on grasslands due to animal trampling and use of heavy machinery. For liming, ground limestone has the advantage before lime and ground dolomite.

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Agris category code: f50, f60, f04

Response of leaf area and dry matter of crop, weeds and cover crops to competition and fertilizer resources

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ABSTRACT

Plasticity of plants to allocate leaf area and dry matter to upper layer of canopy play important role in canopy architecture and competition. In order to study the vertical distribution of leaf area and dry matter of corn (*Zea mays* L.), cover crops and weeds canopy in different fertilizer condition and competition, a randomized complete block design experiment with 8 treatments and 3 replicates was conducted at Sari Agricultural Sciences and Natural Resources University in 2012. Treatments were included corn with soybean (*Glycine max* (L.) Merr.) as cover crop without fertilizer application, corn with soybean as cover crop with chemical fertilizer application, corn with soybean as cover crop with compost fertilizer application, corn with wheat (*Triticum aesitivum* L.) as cover crop without fertilizer application, corn with wheat as cover crop with chemical fertilizer application, corn with wheat as cover crop with compost fertilizer application and corn monoculture both in weedy and weed free conditions. The results showed that weed infestation reduced total leaf area and dry matter of corn. Corn distributed more leaf area and dry mater of canopy to the upper layer in weedy conditions. Between cover crops, soybeans allocated corn leaf area and dry mater to the higher layers of canopy than wheat. Also, soybean reduced leaf area and dry mater production of weeds more than wheat. Soybean as cover crop with the use of compost treatment was more efficient in reducing of weed biomass and corn yield production.

Key words: corn, compost, dry matter allocation, soybean**,** weed biomass, wheat

IZVLEČEK

ODZIV LISTNE POVRŠINE IN SUHE SNOVI POLJŠČIN, VMESNIH POSEVKOV IN PLEVELOV NA KOMPETICIJO IN IZRABO GNOJIL

Sposobnost rastlin za premeščanje listne površine in suhe snovi v zgornje plasti krošnje ima pomebno vlogo v njihovi zgradbi in tekmovalnosti. Za preučevanje vertikalne razporeditve listne površine in suhe snovi v krošnji koruze (*Zea mays* L.), njenega vmesnega posevka in plevelov v različnih razmerah gnojenja in tekmovalnosti je bil v letu 2012 izveden naključni bločni poskus z osmimi obravnavanji in tremi ponovitvami na Sari Agricultural Sciences and Natural Resources University. Obravnavanja so obsegala koruzo in njen vmesni posevek sojo (*Glycine max* (L.) Merr.) brez uporabe mineralnih gnojil, koruzo s sojo kot vmesnim posevkom gnojeno s kompostom, koruzo s pšenico (*Triticum aesitivum* L.) kot vmesnim posevkom brez gnojenja, koruzo s pšenico kot vmesnim posevkom, gnojeno z mineralnimi gnojili, koruzo s pšenico kot vmesnim posevkom gnojeno s kompostom in monokulturo koruze v razmerah z in brez plevelov. Rezultati so pokazali, da so pleveli zmanjšali listno površino in vsebnost suhe snovi pri koruzi. Koruza je premestila več listne površine in suhe snovi v zgornje plasti krošnje v razmerah zapleveljenosti. V razmerah z vmesnimi posevki je koruza premestila več listne površine in suhe snovi v zgornje plasti z vmesnim posevkom sojo kot pa s pšenico. Podobno je soja bolj zmanjšala listno površino in suho snov plevelov kot pšenica. Soja je kot vmesni posevek ob uporabi komposta bolj učinkovito zmanjšala biomaso plevelov kot tudi pridelek koruze.

Ključne besede: koruza, kompost, alokacija suhe snovi, soja**,** biomasa plevelov, pšenica, vmesni posevek

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1 INTRODUCTION

Weeds are one of the major threats to crop production in cropping systems. The risk of weeds not only reducing crop yields but also decreasing the commercial quality and the feeding palatability of main crops and enhance the soil seed bank of weeds, which may cause continuous weed infestation of field crops (Uchino *et al*., 2012).

The primary goal of weed management approaches is reducing the negative effects of weeds on crop. Herbicides have developed to be a reliable and highly effective method for weed control. However, demand for safe food products and food that have been produced with a minimum application of chemical inputs increased. Therefore, farmers interested in weed management have to rely on other control approaches (Hollander *et al*., 2007). An alternative weed control method is the use of cover crops (Uchino *et al*., 2009), which can suppress the growth of weeds by competition for light, soil moisture and nutrients, and also by producing allelopathic compounds (Compigla *et al*., 2010). Cover crops have been successfully integrated into conservational agriculture systems in many parts of the world. Jedrszczyk and Poniedziałek (2007) reported cover crops increased the content of corn dry matter in comparison to monoculture of corn in weedy condition. Uchino *et al.,* (2009) resulted in increased coverage of corn, soybean and cover crop, declined weed dry matter. Weeds were suppressed effectively and stably without yield reductions of main crops by inter-seeded cover crops with sufficient fertilization in organic farming systems (Uchino *et al*., 2012).

In competition, height and leaf area index are two important, so that species with greater leaf area and height are more successful (Vazin *et al*., 2010; Rezvani *et al*., 2013). Plant ability to allocate green shoot to upper layer is one of the main traits in competition (Agha-Alikhani *et al*., 2009). Because, the canopy structure impact on the absorption of radiation, evaporation and transpiration, canopy and dry matter accumulation and yield (Rezvani *et al*., 2010). Agha-Alikhani *et al*. (2009) indicated that in weed free corn pure stand, 30.36 % of the maximum leaf area was distributed in 90-120 cm layer of canopy, but when corn was grown with weed, the maximum leaf area were established in the upper.

The objective of the research was investigating leaf area and biomass profile in corn, cover crops and weeds under different treatments of cover crops and fertilizer resources. Also, yield of corn and performances of applied weed managements were evaluated.

2 MATERIALS AND METHODS

The experiment was carried out at Sari Agricultural Sciences and Natural Resources University, Sari. The soil was a silt-clay soil with 7.34 pH, 2.53 % organic matter, 0.23 % total N, 38.74 ppm P and 400 ppm K. Field preparation was consisted of a deep tillage in previous fall and a vertical disk in spring.

The experiment was established in a randomized complete block design with four replicates. Corn was considered as main crop and soybean and wheat were the cover crops. Treatments were included corn with soybean as cover crop without fertilizer application, corn with soybean as cover crop with chemical fertilizer application, corn with soybean as cover crop with compost fertilizer application, corn with wheat as cover crop without fertilizer application, corn with wheat as cover crop with chemical fertilizer application, corn with wheat as cover crop with compost fertilizer application, corn monoculture both in weedy and corn monoculture in weed free conditions. Natural weed population of all plots except corn monoculture in weed free treatment maintained in all growth stages. Weed free corn monoculture treatment was weeded in all growth stage. Varieties of corn, wheat and soybean were NS-640, Milan and Sari, respectively.

Corn planted in 75 cm row spacing with 20 cm between plants in the same row. Each plot was

included 5 rows corn. Crops were planted on 26 May 2012.

Cover crop inter-seeded simultaneously in the main crop. Chemical fertilizer treatment was used according to the soil analysis. 400 kg ha^{-1} N-fertilizer as Urea, 200 kg ha^{-1} K-fertilizer as Potassium sulfate and 150 kg ha^{-1} P-fertilizer as Triple superphosphate were applied. A total of 20 ton ha^{-1} mushroom compost was used as an organic fertilizer resource. The chemicals properties of compost were as 6.9 pH, 1.8 % N, 1.8 % P and 1.6 % K. At planting, 200 kg ha^{-1} N-fertilizer and total P and K-fertilizer and compost was incorporated into the soil. Other 200 kg ha^{-1} N-fertilizer top dressed in early flowering stage of corn.

At the corn canopy closure stage, a vertical card board frame marked in 30-cm increments was used in the field as a guide to cut standing plants including corn, cover crops and weeds. In each vertical layer of canopy, leaves and stem samples were separated. The leaf area both crops and weeds were measured with a leaf area meter LICOR-3000A (LI-COR, Lincoln, NE, USA). Stem and leaf samples oven dried.

Weed biomass production of treatments evaluated at the corn harvest time. Also, Corn yield was measured by mechanically harvesting both middle rows and adjusting to 13% moisture.

2.1 Statistical analysis

Corn yield and weed biomass data subjected to analysis of variance (ANOVA) using the SAS (ver. 9.2). Means were compared with LSD test at $P=0.05$. The vertical distribution of leaf area and dry matter were plotted by Grapher (ver. 9) software.

3 RESULTS AND DISCUSSION

3.1 Vertical changes of corn and cover crops leaf area

The maximum leaf area of corn cultivated with soybean as cover crop both in compost and chemical fertilizer treatments were placed at 90-120 cm layer (Figs. 1a). In all treatments that wheat used as cover crop, corn allocated the leaf area in lower layers of canopy that those of treatments that corn growth with soybean as cover crop (Figs. 1a).

Figure 1b indicates vertical distribution of corn monoculture both in weedy and weed free conditions. Results showed that in weedy condition the most leaf area (24.01 %) placed in layer of 150-180 cm corn canopy. But, in weed free condition the maximum leaf area of corn (22.33 %) established in the layer of 90-120 cm (Fig. 1b).

Between cover crops, soybeans compared to wheat expanded corn leaf area to the upper layers of the canopy. Large proportion of the soybean leaf area was established at both layers of 60-90 cm and 90-120 cm (Fig. 2), while the major part of the wheat leaf area was allocated to the layer of 0-30 cm of the canopy in all corn with wheat as cover crop treatments. Soybean leaf area was expanded to the upper layers of the canopy than wheat, which can cause superiority of soybean plants in competition with weeds (Fig. 2).

Corn allocated more leaf area to the upper layer in presence of weeds. In response to competition, plants transfer their leaf area to upper layers of canopy; through preventing light penetration to the bottom layers, to increase their competitive abilities (Safahani-Langerodi *et al.,* 2008). Saadatian *et al.* (2011) also reported increasing the ration of the upper layer of wheat canopy in interference conditions with wild mustard.

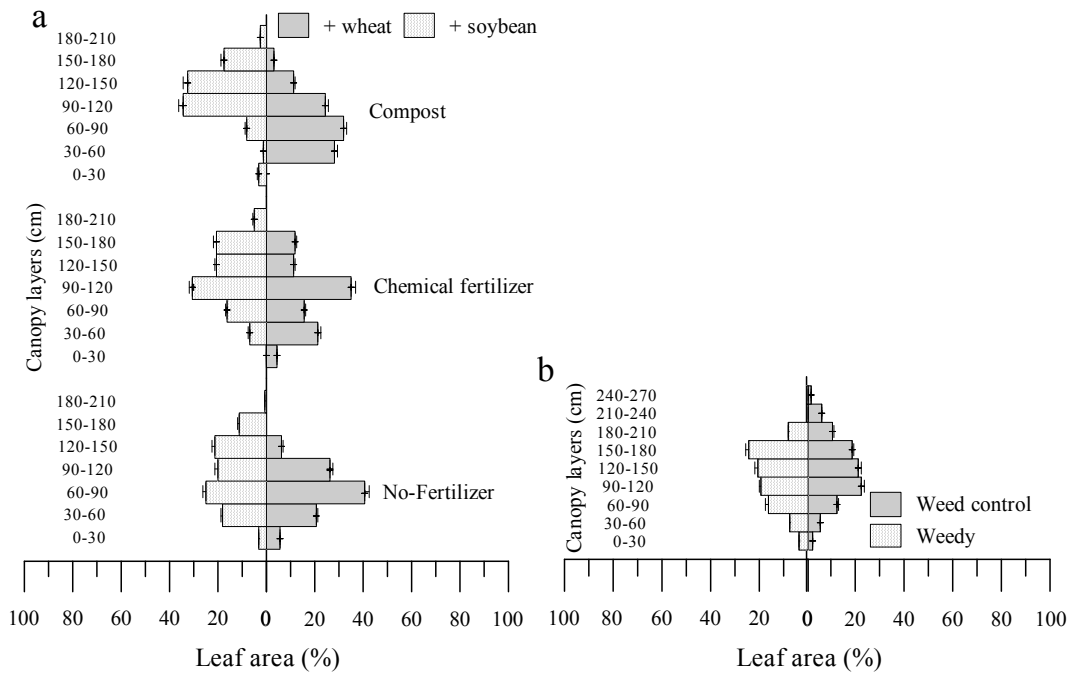

Figure 1: Vertical distribution of corn leaf area under presence of cover crops (a) and corn monocropping (b). Vertical bars represent *Se* of the means.

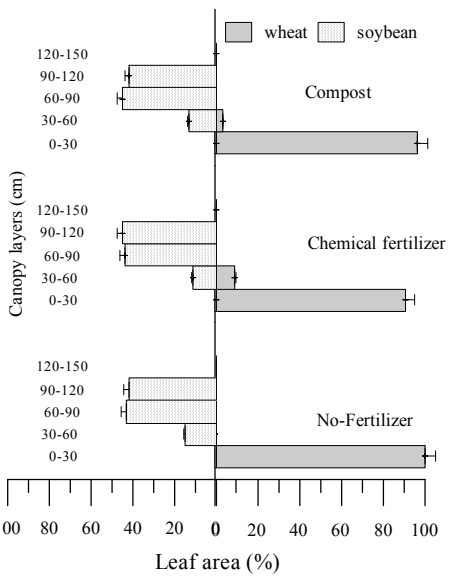

Figure 2: Vertical distribution of cover crops leaf area. Vertical bars represent *Se* of the means.

3.2 Vertical changes of weed population leaf area

The dominant weed species were velvetleaf (*Abutilon theophrasti* Medic.), Johnson grass (*Sorghum halepense* (L.) Pers.), wild melon (*Cucumis melo* var. agrestis) and giant foxtail (*Setaria glauca*) in the experimental field.

Velvetleaf leaf area was expanded to the upper layers of the canopy in wheat used as cover crop compared with soybean used as cover crop treatment. But, in soybean was as cover crop with

compost treatment, velvetleaf expanded all its leaf area to 30-60 cm layer (Fig. 3a).

Velvetleaf in weedy monoculture of corn expanded its leaf area to 90-120 cm layer and the maximum proportion of the leaf area allocated to 60-90 cm layer (Fig. 3f). Generally, in presence of cover crops conditions weeds allocated leaf area to the lower layers. But, in no fertilizer treatment due to competition for nutrients, weeds allocated the major part of leaf area at 90-120 cm layer (Fig. 3a).

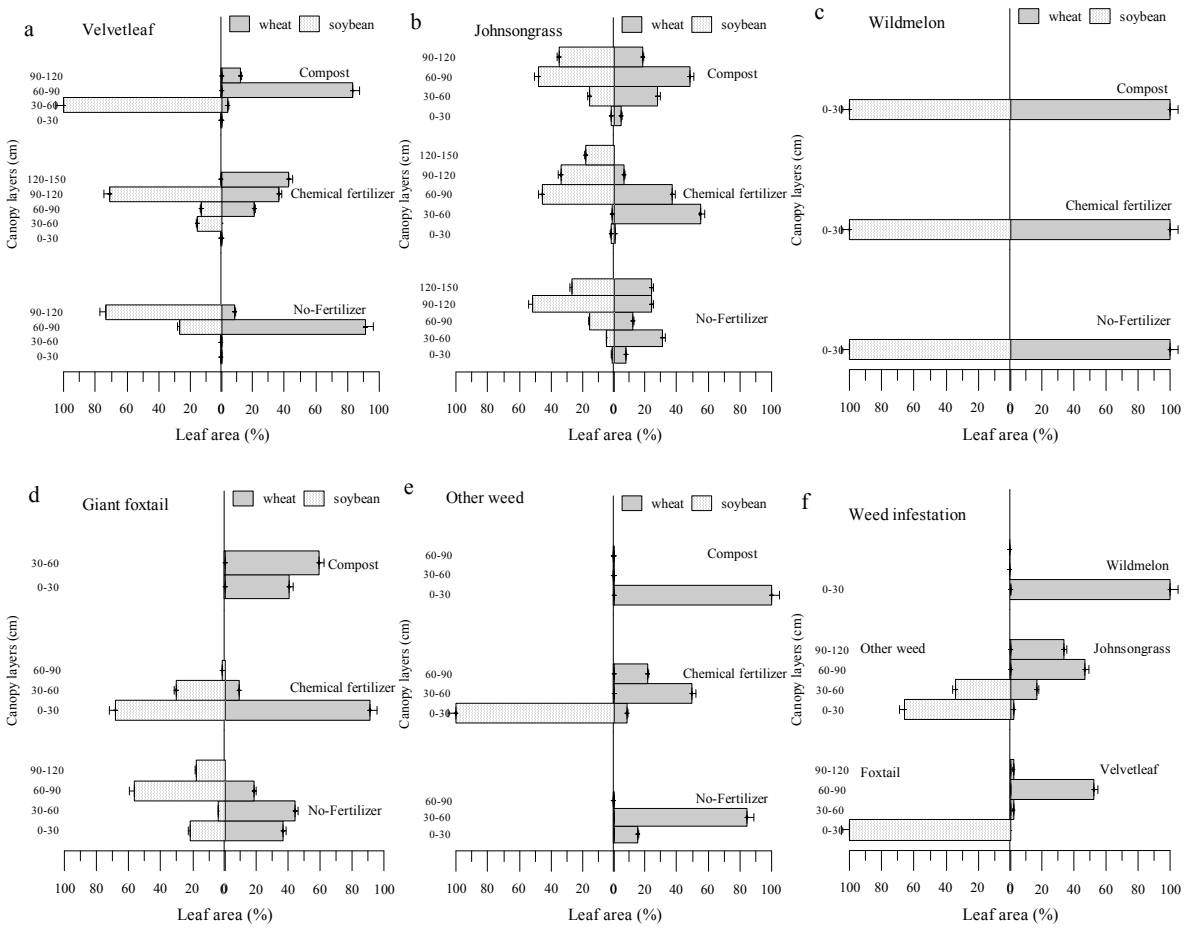

Figure 3: Vertical distribution of leaf area of velvetleaf (a), johnson grass (b), wild melon(c), giant foxtail (d) and other (e) under presence of cover crops and vertical distribution of weeds leaf area in monocropping of corn under weed infestation. Vertical bars represent *Se* of the means.

Johnson grass in soybean with cover crop was more successful in competition than wheat with cover crop treatments, because of the ability to expand the most leaf area to the upper layers of canopy height. The maximum leaf area Johnson grass in soybean cover crop with compost, chemical fertilizer and no fertilizer treatments were placed at 60-90 cm, 60-90 cm and 90-120 cm layers of canopy, respectively (Fig. 3b). Johnson grass leaf area in no fertilizer allocated to higher layer of canopy than other fertilizer treatments. The maximum leaf area of Johnson grass in monoculture of corn was observed at 60-90 cm layer (Fig. 3f).

Leaf area of Wild melon in all treatments expanded only at 0-30 cm canopy layer. Giant foxtail completely suppressed and controlled with treatments of compost and soybean used as cover crop (Fig. 3d). Soybean as cover crop was more successful than wheat in giant foxtail control. Other weeds of fields in treatments of soybean

cover crop were observed only in chemical fertilizer treatments at 0-30 cm layer (Fig. 3e) and two other fertilizer treatments were free of other weeds. In wheat used as cover crop treatments other weeds were presented in all three fertilizer treatments and in chemical fertilizer treatment compared to other treatments, expanded their leaf area to the upper layers of the canopy. In corn monoculture weedy condition treatments, other weeds allocated the maximum leaf area Layer to 0-30 cm (Fig. 3f).

Uchino *et al.* (2012) with study of the possibility of suppressing weeds in corn by cover crops reported that the maximum total leaf area of weeds was observed in no cover crops treatments.

3.3 Vertical changes of corn and cover crops dry matter

Corn in soybean cover crop treatments compared to wheat cover crop allocated dry matter to the upper layer of canopy. The layer of the maximum corn dry matter in soybean cover crop treatments with compost and chemical fertilizer were observed at 90-120 cm. But, in no fertilizer treatments the layer was formed at 60-90cm layer of canopy. In wheat cover crop treatments the maximum layer of corn dry matter accumulated at 30-60 cm, 90-120 cm and 60-90 cm layers in plots with compost, chemical fertilizer and no fertilizer, respectively (Fig. 4a). Ahmadvand *et al.* (2006) who worked on wheat and wild oat (*Avena fatua* L.) canopy structure, reported allocation of total dry matter and leaf area of wild oat to the upper layers increased by enhancement of density.

In the monoculture of corn with weed infestation due to competition between corn and weed, observed a reduction in dry matter of corn than in weed free (Data did not shown). The maximum amount of corn dry matter in weed free treatment was established in layer of 120-150 cm (Fig. 4b). Corn in competition with weeds, translocated the most percentage of dry matter to the upper layers of canopy. The changes in distribution of dry matter pattern may be due to more light achievement. Study of rice (*Oryza sativa* L.) canopy structure showed that rice allocated more leaf area and dry matter to the upper layers in competition with barnyard grass (*Echinochloa cruss-galli*) because of competition over nutrient and light sources (Aminpanah *et al.*, 2009).

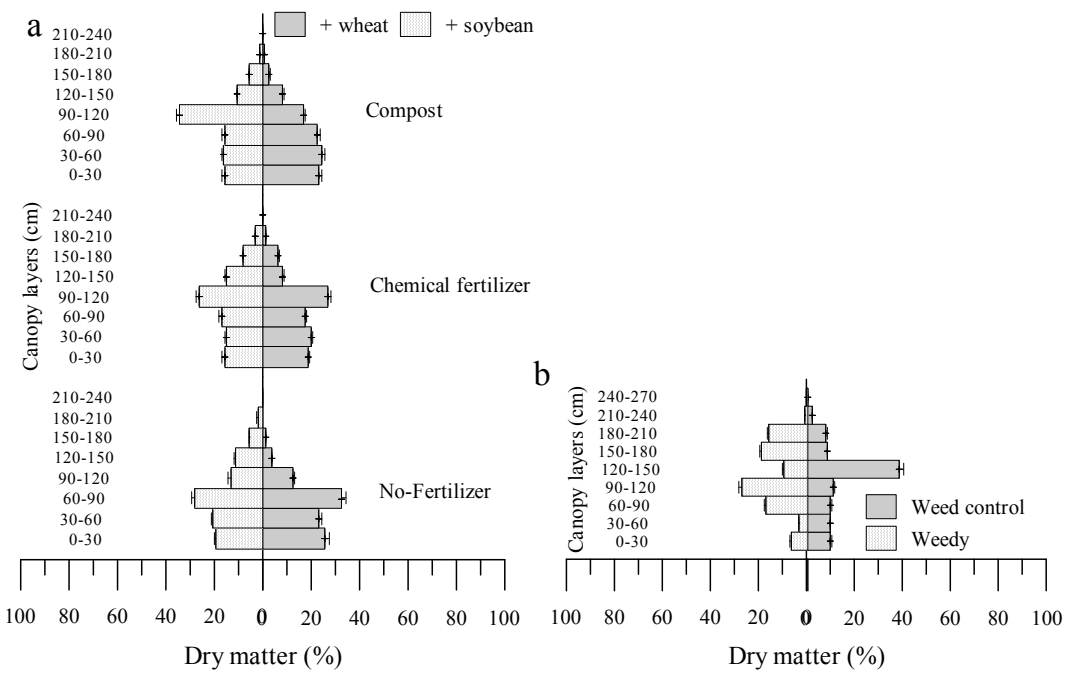

Figure 4: Vertical distribution of corn dry matter under presence of cover crops (a) and monocropping of corn (b). Vertical bars represent *Se* of the means.

Both compost and chemical fertilizer with soybean used as cover crop treatments dry matter of corn allocated to the upper layers of the canopy than no fertilizer ones. In treatment of compost with soybean as cover crop allocated more dry matter to higher layer (90-120 cm) than chemical fertilizer (Fig. 4a). All dry matter of wheat cover crop accumulated at 0-30 cm layer in no fertilizer treatment while in compost and chemical fertilizer treatments 9.12 % and 6.59 % of dry matter transferred to the 30-60 cm layer, respectively (Fig. 5). Also wheat dry matter transferred to the upper layer in compost application treatment was more than chemical fertilizer ones (Fig. 5). The maximum dry matter of soybean in compost and chemical fertilizer treatments were allocated to the upper layer of canopy than no fertilizer ones (Fig. 5).

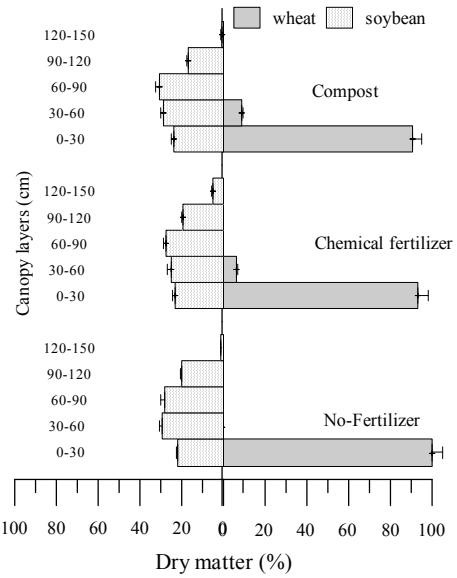

Figure 5: Vertical distribution of cover crops dry matter. Vertical bars represent *Se* of the means.

3.4 Vertical changes of weed population dry matter

In soybean used as cover crop with compost velvetleaf allocated whole dry matter to the 0-30 cm and 30-60 cm layers (Fig. 6a). In monoculture of corn velvetleaf transferred its dry matter to the upper layers (120-150 cm) (Fig. 6f). Haj-Seydhady *et al.* (2007) reported allocation of redroot pigweed (*Amaranthus retroflexus*) and lambsquarters (*Chenopodium album* L.) biomass to the upper layers of the canopy in order to compete with potato (*Solanum tubersum* L.) to absorb more light. Soybean in control of Johnson grass was not as successful as wheat and transferred the dry matter to the upper layers of canopy in compared to soybean. Study of profiles of weeds dry matter distribution in the treatments showed that, when corn and soybean competed with Johnson grass, accumulated the most percentage of dry matter to the upper layers. The main characteristics that allowed this weed to compete against a strong competitor such as corn and soybean were its height plasticity, canopy architecture, concentrated leaves in the upper part of the plant and higher light extinction coefficient (Agha-Alikhani *et al.*, 2009).

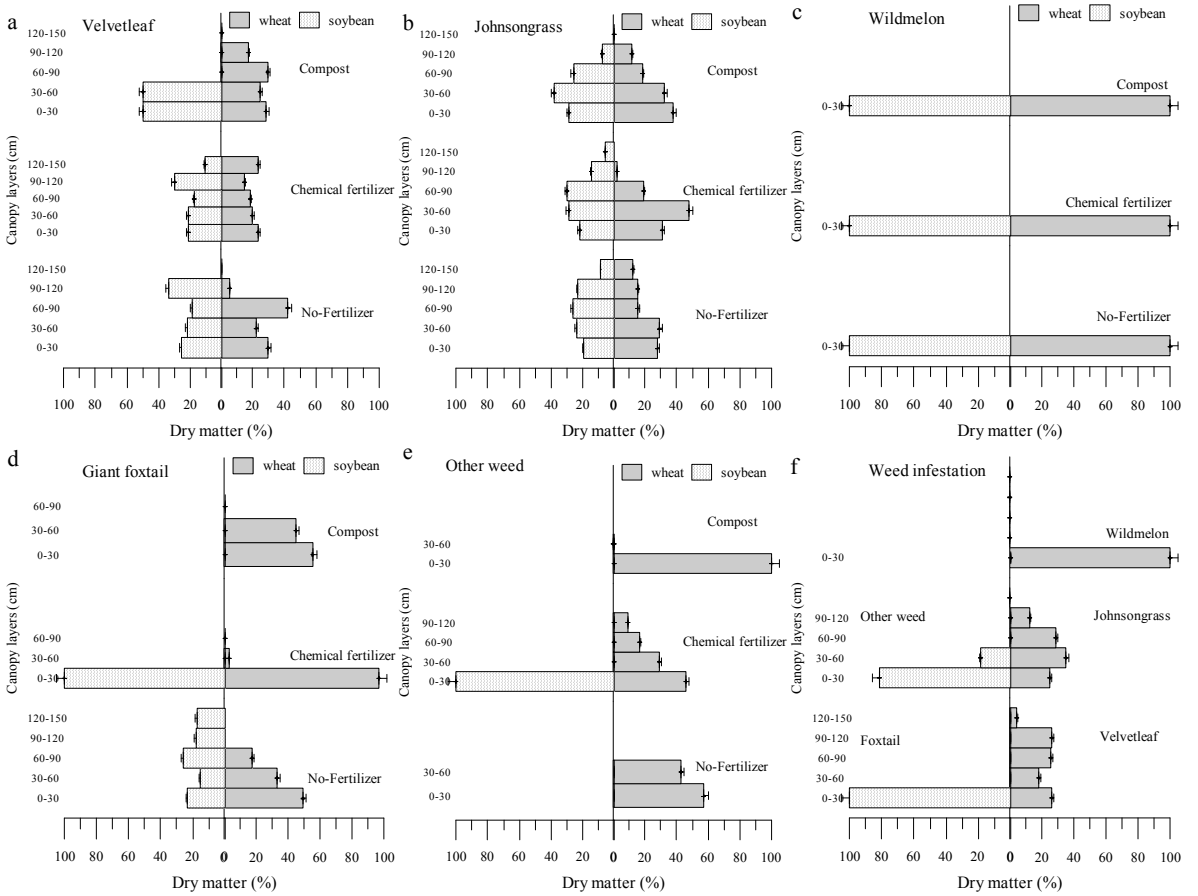

Figure 6: Vertical distribution of dry matter of velvetleaf (a), johnson grass (b), wild melon (c), giant foxtail (d) and other (e) under presence of cover crops and vertical distribution of weeds dry matter in moonocropping of corn under weed infestation. Vertical bars represent *Se* of the means.

Wild melon because of vegetative growth form allocated dry matter to the 0-30 cm layer (Fig. 6c). Giant foxtail growth completely suppressed by the soybean cover crop both in compost and chemical fertilizer application treatment (Fig. 6d). Other weed in the soybean cover crop were observed only in chemical fertilizer application treatments and in wheat cover crop with compost was observed only at 0-30 cm layer, but in other treatments were placed at the upper layers (Fig. 6e and 6f). Previous studies showed that use of hairy vetch (*Vicia villosa*) (Czaper *et al.,* 2002), Italian ryegrass (*Lolium multiflorum*) (Faget *et al.,* 2012), red clover (*Trifulium pratense*) and rye (*Secale montanum*) (Altentorbert *et al.,* 1996) as cover crops reduced biomass, density and diversity of weeds.

3.5 Weed biomass and Corn yield

Use of cover crops decreased weed biomass. In treatments of soybean with compost and also wheat with compost produced the minimum weed biomass. Generally, soybean as cover crop was more successful than wheat to inhibit weed growth (Table 1). There is a wide agreement in the researches conclusion that living cover crops will suppress weeds successfully. Barnes and Putnam (1983) also reported a living mulch of spring-planted rye reduced early season biomass of common lambsquarters, large crabgrass [*Digitaria sanguinalis* (L.) Scop.], and common ragweed (*Ambrosia artemisi-ifolia* L.) compared to controls. Effect of cover crops in reducing weed biomass previously reported by Ngoguajio *et al.* (2003) Samarajeewa *et al.* (2006). The suppressive effect of cover crop could be due to inhibition of weed seed germination through effect on the radiation and chemicals environment of seeds. Also

continuous suppressive effect of cover crop could reduce seed production by weeds (Brennan and Smith, 2005).

Results of analysis of variance indicated treatments had significant effect on corn economic yield (Data not shown). Corn monoculture in weed free condition produced the maximal yield (Table 1). In the cover crop treatments including soybean with compost and soybean with chemical fertilizer had the highest economic yield (Table 1). All wheat used as cover crop treatment was not as successful as soybean in grain yield production (Table 1). There are reports that cover crops can suppress cash crops growth through the competition for resources. But, simulative effect of legume cover crops on cash crops through enhancement of nitrogen availability was reported by Sarrantonio and Gallandt (2003) and Calegari *et al.* (2005) and promotion of genes that delay senescence and enhance disease resistance (Kumar *et al.*, 2004).

Table 1: Mean comparison of corn yield and weed biomass.

Treatments		Economic yield (kg/ha)	Weed biomass (g/m^2)
Corn monoculture	Weed free	12124.00a	-
	Weedy	2733.30d	33.92a
Corn+soybean	No-fertilizer	3884.70cd	21.4c
	Chemical fertilizer	7769.70b	19.52c
	Compost	8351.30b	5.45d
Corn+wheat	No-fertilizer	3795.00cd	27.53b
	Chemical fertilizer	4903.00bcd	25.55b
	Compost	4089.70c	12.58cd

In each column, numbers with the same letter are not significantly different at 5 % level.

4 CONCLUSIONS

According to our research corn allocated more leaf and dry matter to upper layers in response to competition to cover crops and weeds to light absorption. Compost application and use of soybean as a cover crop was a successful management in weed growth suppression. Results showed use of legume as cover crop especially with organic fertilizers can be an alternative approach for herbicides and are more effective than others. However, further studies are required on cover crops species, seeding rate and growth pattern and their nutrition management such as amount and type of fertilizer.

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Physico-chemical and sensory characteristics of jellies made from seven grapevine (*Vitis vinifera* L.) varieties

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ABSTRACT

Jellies of seven grapevine varieties were physico-chemical and sensorial characterized for the first time. Jellies differed significantly in moisture and ash contents, colour, pH, acidity and antioxidant activities. 'Tinta Carvalha' was the darkest and redness jelly, showing the highest antioxidant activity. Regarding sensory characteristics, no significant differences in the appearance, taste, sweetness, acidity and global assessment were observed among jellies. Nevertheless, these attributes were positively evaluated. In conclusion, the production of different jellies will allow the valorisation of grapevine varieties with less potential for wine production, helping to preserve biodiversity, and be an economic alternative to grape producers who may elaborate an enjoyable product with interesting bioactivity.

Key words: grapevine, jellies, physico-chemical characterization, antioxidant activity, sensory analysis

IZVLEČEK

FIZIKALNO-KEMIČNE IN SENZORIČNE LASTNOSTI ŽELEJEV NAREJENIH IZ SEDMIH SORT GROZDJA (*Vitis vinifera* L.)

Fizikalno-kemične in senzorične lastnosti sedmih sort grozdja so bile prvič analizirane. Želeji so se značilno razlikovali v vsebnosti vode in pepela, v barvi, pH, kislosti in antioksidativni aktivnosti. Žele pripravljen iz sorte 'Tinta Carvalha' je bil najtemnejši in najbolj rdeč. V senzoričnih lastnostih med želeji ni bilo značilnih razlik v izgledu, okusu, sladkosti, kislosti in celotni oceni, vendar so bile te lastnosti pozitivno ovrednotene. Sklepamo, da bo izdelava različnih želejev omogočala ovrednotenje tistih sort vinske trte, ki imajo manjši potencial za pridelavo vina, kar bo prispevalo k ohranjanju raznolikosti žlahtne vinske trte in bo ekonomska alternativa vinogradnikom za izdelavo koristnega izdelka z zanimivo bioaktivnostjo.

Ključne besede: grozdje, želeji, fizikalno-kemično vrednotenje, antioksidacijska aktivnost, senzorična analiza

1 INTRODUCTION

Portugal has great tradition in wine production. The Northeast region is not exception and it is known for the different grapevine (*Vitis vinifera* L.) varieties that grow there. The quality and specificity of these varieties are the result of their diversity and adaptation to different agro-climatic conditions. Through the knowledge on these varieties and the processes that may contribute to their preservation, the development of differentiated products may be a great opportunity to increase market competitiveness of this sector, besides preserving biodiversity. Furthermore, consumers are increasingly demanding for natural and healthy products, being products rich in

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antioxidants a good example. Grape meets these requirements, since it has a high antioxidant potential, being part of this due to its skin (Bekhit et al., 2011; Katalinic et al., 2010; Poudel et al., 2008). Several studies report that grape skin is rich in phenolic compounds, whose profile varies with variety, maturation stage and origin of the fruit (Bekhit et al., 2011). In this way, different grape varieties may exhibit different chemical compositions that may consequently influence their antioxidant potential, both in fresh and grape sub products (Abe et al., 2007).

Jelly production is a good example of a product easy to produce and consume, with long storage periods that continue to have the value of fruit. Moreover, jelly production allows the employment of underused fruits, such as secondary quality (e.g. small) and over-ripe grapes that are often not desired by consumers and, hence, are generally wasted.

According to the Portuguese law (Law-Decree No. 230/2003), jelly is defined as a product sufficiently gelled that result from the mixture of sugar and juice and/or aqueous extract of one or more types of fruit. Unlike other jellies of other fruits, of our knowledge few studies have been conducted to date on grape jellies. The works performed until now have addressed the effect of using different gelling agents (Gaspar et al., 1998), the role of the enzymatic activity, namely peroxidase and polyphenol oxidase (Freitas et al., 2008), and the antioxidant activity of two jellies produced from grapes of *V. labrusca* and *V. vinifera* (Falcão el al., 2007).

In order to obtain more valuable data on this subject, in this study grape jellies of seven varieties of *V. vinifera,* namely, 'Periquita', 'Touriga Nacional', 'Cornifesto', 'Tinta Barroca', 'Marufo', 'Trincadeira Preta' and 'Tinta Carvalha', were produced and characterized in terms of physico-chemical properties, antioxidant activity and sensory characteristics.

2 MATERIALS AND METHODS

2.1 Grape samples

Seven red grapevine varieties, namely, 'Periquita', 'Touriga Nacional', 'Cornifesto', 'Tinta Barroca', 'Marufo', 'Trincadeira Preta' and 'Tinta Carvalha', were harvested at their technological ripening time in Valpaços, Northeast of Portugal. After harvest, the grapes were transported under refrigeration and on their arrival at the laboratory the fruits were washed with ultra-pure water (Milli-Q system, Merck Millipore, Massachusetts, USA). A grapes portion (ca. 900 g) was used to formulate jellies, while other portion (ca. 100 g) was used to separate the skins. The skins proportion for each variety was determined after separating them from the pulp and by fruit and skin weighting. Grapes and skins were packed properly at -18 °C until further use.

2.2 Jellies production

For jellies production the ingredients used were fresh grapes and sucrose. At the beginning 500 g of crushed grapes of each variety were macerated at room temperature (ca. 20 °C) for approximately 5 minutes and the mixture was heated to boiling for 10 minutes. Subsequently, the mixture was filtered through a strainer and the sugar added at a ratio of 1:2 (1 g of sugar per 2 ml of liquid). Afterwards, the solution was again taken to boiling until a Total Soluble Solids (TSS) value between 65-70 °Brix (Lago et al., 2006; Lago-Vanzela et al., 2011), measured on an Abbe refractometer (Optic Ivymen System, Madrid, Spain). Before cooling the mixture were poured into glass jars with 250 g capacity and closed with metal caps. Then the jars were placed in a hot water bath (100 ºC) for 15 minutes (Granada et al., 2005) and left to cool at room temperature (ca. 20 °C).

2.3 Physico-chemical analysis

Before jellies processing, the TSS values of the juices of the seven grapevine varieties were measured.

The following parameters were evaluated in the jellies: colour, pH, moisture, ash and acidity. Colour was measured with a CR-400 colorimeter (Konica Minolta, Tokyo, Japan) in the CIELab colour space, through the coordinates: L*, a* and b*, using the Spectra Magic Nx software (version

CM-S100W 2.03.0006, Konica Minolta, Tokyo, Japan). The instrument was always calibrated with a standard white tile before analysis. Illuminant C and 2º standard observer were used. pH was measured directly (Jenway potentiometer, model 370, Jenway, Essex, United Kingdom). Moisture and ash contents were determined by weight loss at 105 °C until constant weight and 550 °C for at least 4 hours (AOAC, 1999), respectively. Acidity was determined by titrimetric analysis, consisting of a titration with 0.10 mol l^{-1} NaOH, being the values reported in % of tartaric acid. Due to colour of the jelly, it was not possible to detect clearly the end point of the titration when using phenolphthalein as indicator. So, the pH of the solution was monitored continuously in order to obtain the titration curve. The pH at the equivalence point was established as 8.1, as indicated in the Portuguese rule NP-1421 (1977). The titratable acidity (*TA*) was calculated by Equation 1, according to NP-1421 (1977):

$$TA\ (\%\ tartaric\ acid) = \frac{c \times v \times MM}{2 \times m} \times 100 \qquad (1)$$

Where *c* is NaOH concentration (mol l^{-1}), *v* is NaOH volume spent at the titration (l), MM the molar mass of tartaric acid (150.087 g mol^{-1}) and *m* the sample mass (g).

The TSS contents (ºBrix) of jellies were measured with an Abbe refractometer (Optic Ivymen System, Madrid, Spain).

All reagents were p.a. (pro analysis) and were purchased to Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA).

2.4 Antioxidant Activity

2.4.1 Grape jelly and skin extracts

Extracts were prepared by mixing 5 g of sample (grape jelly or skins) with 20 ml of methanol. In case of grape skins, these were previously deep-frozen and grounded. The solutions were placed under stirring for one hour. Subsequently the solutions were filtered through Whatman No. 2 filters (Whatman, Kent, United Kingdom) to round bottom flasks previously weighed. In order to evaporate the solvent, the flasks were placed on a rotary evaporator RE300DB (Stuart, Stone, United Kingdom) and afterwards in the oven UNB500 (Memmert, Schwabach, Germany) at 40-45 °C. The extracts were redissolved in methanol to an extract concentration of 50.0 mg ml^{-1}.

2.4.2 Total Reducing Capacity

Total reducing capacity (TRC) of the extracts was determined by the Folin-Ciocalteu's assay (Singleton and Rossi, 1965). To 100 µl of the extract solutions, 7.90 ml of deionized water and 500 µl of Folin-Ciocalteu reagent were added. The blank was prepared in a similar way, replacing the extract solution by methanol. After 3 to 8 minutes, 1.50 ml of sodium carbonate saturated solution was added. After two hours the absorbance values were read at 765 nm (Genesys 10UV, Thermo Scientific, Madrid, Spain). Gallic acid was used as standard, being the results expressed in g of gallic acid equivalents (GAE) per kilogram of extract.

2.4.3 DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

DPPH radical scavenging activity was determined by the procedure described by Delgado et al. (2010) with some modifications. DPPH assay evaluates the ability of the grape extracts to scavenge this free radical. To 0.30 ml of extract solutions (5.00 mg extract ml^{-1}) were added 2.70 ml of DPPH methanol solution (6.09×10^{-4} mol l^{-1}). After 1 hour at room temperature (ca. 20 °C) in the dark, the absorbance was read at 517 nm (Genesys 10UV, Thermo Scientific, Madrid, Spain). DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity}\ (\%) = \frac{A_{DPPH} - A_{Sample}}{A_{DPPH}} \times 100 \qquad (2)$$

Where A_{DPPH} is the absorbance of the DPPH solution and A_{Sample} the absorbance of the solution when the sample extract was added. The blank was made with methanol.

2.4.4 Reducing Power

The reducing powers of the extracts were determined by the procedure described by Delgado et al. (2010). Extract solutions at different concentrations were prepared from the stock solution of 50.0 mg extract ml^{-1}. To 1.00 ml of each solution were added 2.50 ml of 0.20 mol l^{-1} phosphate buffer (pH 6.6) and 2.50 ml of 10 g l^{-1} $K_3[Fe(CN)_6]$. After stirring, the mixture was

incubated at 50 °C for 20 minutes. Afterwards, 2.50 ml of 100 g l^{-1} trichloroacetic acid was added to the test tubes. 2.50 ml of the mixture were transferred to another test tube, to which 2.50 ml of distilled water and 0.50 ml of 1 g l^{-1} $FeCl_3$ were added. The absorbance values were read at 700 nm (Genesys 10UV, Thermo Scientific, Madrid, Spain). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance versus extract concentration.

2.5 Sensory analysis

In order to evaluate the acceptability of the grape jellies a consumer panel was used, following the methodology of Lago et al. (2006). The sensory analysis took part at the University on two consecutive days due to the high number of samples, being four jellies analysed each day. Since there were seven grape jellies, one of these ('Tinta Barroca' grape jelly) was repeated in both days but identified with a different number. Around twenty-gram samples of jelly at room temperature were presented in white plastic plates labelled with three-digit random codes. A glass of water was offered to the consumers to rinse their mouths. To prevent biases related to the serving order, this was determined by random permutation. After a brief explanation of how to perform the sensory analysis, the consumers were asked to evaluate the samples according to a 9-point hedonic scale: 1 - Dislike extremely, 2 - Dislike very much, 3 - Dislike moderately, 4 - Dislike slightly, 5 – Neither like nor dislike, 6 - Like slightly, 7 - Like moderately, 8 - Like very much, 9 - Like extremely. The attributes evaluated were the appearance, colour, taste, acidity, sweetness and global assessment.

2.6 Statistical analysis

The statistical analysis was performed using the SPSS software (SPSS, Chicago, Illinois, USA), version 20.0. When analysing the physico-chemical properties and antioxidant activity data, the normality and homogeneity of variance were always checked by the Shapiro-Wilk and Levene Tests, respectively. When both conditions failed the nonparametric Kruskal-Wallis test was applied, followed by multiple comparison of order means. On contrary, when normality and homogeneity of variances were observed, an ANOVA followed by Tukey post-hoc test was used. Regarding the sensory analysis data, the nonparametric Kruskal-Wallis test was applied because ordinal variables were used. To check whether there were differences between the first and second days for the jelly that was repeated ('Tinta Barroca' grape jelly), it was used the Wilcoxon-Mann-Whitney test.

3 RESULTS AND DISCUSSION

3.1 Physico-chemical characterization of grape jellies

The juices of the seven grapevine varieties studied in the present work had different total soluble solid contents, varying between 19.1 ºBrix and 33.5 ºBrix for 'Touriga Nacional' and 'Trincadeira Preta', respectively. These values indicated differences in grapes sweetness.

Concerning grape jellies, their physico-chemical characterization is shown in Table 1. The moisture contents ranged from 38.6% ('Periquita') to 45.0% ('Touriga Nacional') and ash levels between 0.4% ('Cornifesto') and 0.7% ('Marufo'), suggesting a higher mineral content in this jelly. Regarding colour and in particular lightness (L*), differences on jellies colour were found. 'Periquita' jelly was the one with the highest L* value (33.44), indicating that it was the clearest jelly, while 'Tinta Carvalha' was the darkest (31.84) jelly. Concerning a* ($^{-}$green-red^{+}) and b* ($^{-}$blue-yellow^{+}) parameters, the highest values were obtained for 'Tinta Carvalha' and 'Marufo' jellies, respectively, whereas 'Tinta Barroca' and 'Tinta Carvalha' jellies presented the lowest values. Accordingly, the differences found in grape jellies colour depended on grape variety. Regarding pH and acidity, significant differences between jellies were also observed. pH varied between 3.60 ('Marufo') and 3.74 ('Touriga Nacional') and acidity between 0.7% ('Tinta Barroca') and 1.0% ('Touriga Nacional'). These results indicated that jellies prepared from some grapevine varieties were significantly different in colour because they had different CIELab parameters, as well as in flavour, due to differences on pH and acidity values,

allowing the production of a wider range of products that may meet different consumer's tastes and wishes.

Table 1: Physico-chemical parameters of the jellies prepared in the present work from seven grapevine varieties

Variety	Moisture (%)	Ash (%)	Colour			pH	Acidity (% tartaric acid)
			L*	a*	b*		
Cornifesto	42.00±0.07^{a}	0.40±0.06^{a}	32.42±0.08^{b,d}	0.05±0.01^{b,c}	0.88±0.02^{a,b}	3.64±0.01^{b}	0.85±0.03^{a,b,c}
Marufo	41.04±0.06^{a,b}	0.69±0.08^{a}	32.40±0.19^{a,b,d}	0.27±0.03^{a}	1.00±0.07^{a}	3.60±0.06^{a,b}	0.73±0.00^{a}
Periquita	38.59±0.14^{b}	0.56±0.02^{a}	33.44±0.18^{c}	0.09±0.03^{b}	0.90±0.04^{a}	3.68±0.01^{a}	0.82±0.01^{c}
Tinta Barroca	43.49±0.06^{d}	0.51±0.00^{a}	32.22±0.01^{d}	-0.09±-0.04^{c}	0.88±0.01^{a}	3.71±0.02^{a,b}	0.72±0.01^{a}
Tinta Carvalha	43.90±0.43^{d}	0.58±0.14^{a}	31.84±0.05^{a}	0.41±0.04^{a}	0.77±0.04^{a,b}	3.62±0.01^{b}	0.75±0.14^{a,b,c}
Touriga Nacional	44.97±0.29^{c}	0.56±0.10^{a}	32.31±0.02^{b,d}	0.09±0.01^{b,c}	0.81±0.05^{b}	3.74±0.01^{a}	1.01±0.00^{b}
Trincadeira Preta	42.93±0.44^{d}	0.51±0.02^{a}	32.43±0.04^{b}	0.04±0.01^{b,c}	0.86±0.02^{a,b}	3.66±0.01^{b}	0.74±0.01^{a}

*Different letters in the same column indicate significant differences ($p < 0.05$)

3.2 Antioxidant activity

3.2.1 Total Reducing Capacity (TRC)

TRC was measured according the Folin-Ciocalteau's assay. This method is currently used and the results are usually expressed in total phenols content; however, once different chemicals react (reducing saccharides, proteins, etc…) with this reagent (Singleton et al., 1999), the value of total phenols is overestimated. So, the use of total reducing capacity is more accepted.

The TRC of the extracts prepared from the jellies produced in the present work and from the berry skins are given in Figures 1A and 1B, respectively. The TRC of the jellies extracts were much lower than those of the berry skin extracts. These results were expected, since jellies were prepared from grape pulp and skins, as well as sucrose, being the skins the richest constituent in total phenols.

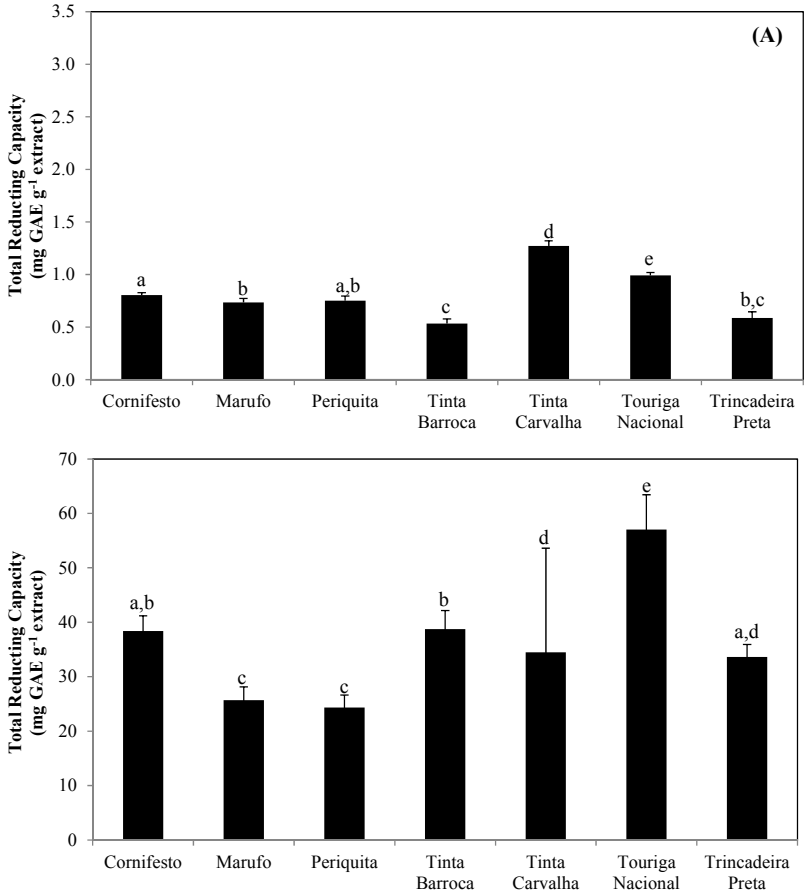

Figure 1: Total Reducing Capacity (mg GAE g^{-1} of extract) of jellies (A) and skins (B) of seven grapevine varieties.

When comparing grapevine varieties, significantly differences on TRCs of the jellies and skin extracts were found. The TRC of the jellies varied between 0.54 and 1.27 g of GAE kg^{-1} of extract of 'Tinta Barroca' and 'Tinta Carvalha', respectively. Nevertheless for berry skin extracts, higher TRC values were found and varied between 24.36 g of GAE kg^{-1} of extract ('Periquita') and 57.05 g of GAE kg^{-1} of extract ('Touriga Nacional'), respectively, showing that the grapevine varieties with skins with the highest TRCs were not those that originated the jellies with the highest antioxidant potentials.

After expressing the TRC in g of GAE kg^{-1} of jelly (Table 2), the values varied between 0.356 and 0.874 g of GAE kg^{-1} of jelly for 'Tinta Barroca' and 'Tinta Carvalha' varieties, respectively. The total phenol contents of the grape jellies produced in the present study were much lower than those reported by Falcão et al. (2007) for a model system of a jelly prepared from grapes of 'Isabel' variety, namely 63.4 g of GAE kg^{-1} of jelly and 95.1 g of GAE kg^{-1} of jelly when acetone and ethanol at 70% (v/v) were used as extraction solvents. However, even though the extraction solvents used were different to that employed in the present study, the production method of the jelly was much more complex, including the use of gelling agents, citric acid and anthocyanin extracts, which explain the higher antioxidant activity of the jelly produced by Falcão et al. (2007) than ours.

Taking into account the fruit quantity used in jelly production, the TRC values expressed on g of GAE kg^{-1} of fruit weight were determined (TRC_{fruit}). Then, through the skins proportion determined previously for each grapevine variety, the TRCs expected from the berry skins were determined and named by $TRC_{skin\ (expected)}$, expressed on g of GAE kg^{-1} of skin. After comparing these concentrations with the TRCs

determined for the skins before processing (*in natura*) ($TRC_{skin\ (real)}$), recovery yields between 14.6% ('Tinta Barroca') and 62.3% ('Periquita') were found, indicating that during jelly production the diffusion rate of phenolic compounds for sucrose solutions or the loss of such compounds due to heating, depended on grapevine variety.

In general terms and considering the TRC obtained for all varieties (Table 2 and Figure 1A), 'Tinta Carvalha' seemed to be the most suitable grape variety for jelly production with the highest content of bioactive compounds (0.874 g kg^{-1} of GAE by jelly weight).

Table 2: Total Reducing Capacity (TRC) (mg GAE g^{-1}) of the jellies and berry skins of seven grapevine varieties studied in the present work.

Variety	TRC_{jelly} (mg GAE g_{jelly}^{-1})	TRC_{fruit} (mg GAE g_{fruit}^{-1})	$m_{(fruit)}$ (g)	$m_{(skin)}$ (g)	$TRC_{skin\ (real)}$ (mg GAE g_{skin}^{-1})	$TRC_{skin\ (expected)}$ (mg GAE g_{skin}^{-1})	Recovery Yield TRC skins (%)
Cornifesto	0.567	0.85	1.92	0.37	4.41	16.3	27.1
Marufo	0.516	0.77	3.24	0.41	6.12	12.0	50.8
Periquita	0.588	0.88	3.05	0.34	7.92	12.7	62.3
Tinta Barroca	0.356	0.53	2.79	0.56	2.66	18.2	14.6
Tinta Carvalha	0.874	1.31	2.77	0.44	8.26	14.8	55.7
Touriga Nacional	0.679	1.02	2.27	0.29	7.97	19.6	40.6
Trincadeira Preta	0.417	0.63	2.24	0.25	5.60	15.7	35.7

This jelly was the one that also showed the darkest colour (lowest L*) and the highest proportion of red pigments (highest a*), suggesting the presence of a high amount of anthocyanins. Nevertheless, these results also suggest that in the future, it will be of great interest to optimize the process of jelly production in order to extract a high number of phenolic compounds of the skins and increase their recovery yields.

3.2.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

The antioxidant activity determined by the DPPH method for the extract concentration of 5 mg ml^{-1} (Table 3) showed that the seven grape jellies had different DPPH radical scavenging activities, varying from 9.8% ('Periquita') to 60.0% ('Tinta Carvalha'). The extracts with the highest blocking effect on DPPH radicals were again those of 'Tinta Carvalha' jelly, in line with the TRC results. Regarding berry skins, similar values were obtained within grapevine varieties, ranging between 84.9% ('Tinta Barroca') and 89.9% ('Periquita'). Once again it was found that the skins showed higher antioxidant potential than jellies, since the processing may affect the bioactive compounds present in vegetable products (Marquina et al., 2008).

Table 3: DPPH radical scavenging effect (%) for the concentration of 5 mg extract ml^{-1} of jellies and berry skins of seven grapevine varieties.

Variety	Jelly	Skins
Cornifesto	$31.8\pm1.0^{d,e}$	$88.9\pm0.4^{b,c}$
Marufo	22.5 ± 0.5^{a}	88.3 ± 0.8^{a}
Periquita	9.8 ± 0.4^{a}	$89.9\pm0.3^{d,e}$
Tinta Barroca	59.0 ± 1.2^{e}	84.9 ± 0.7^{e}
Tinta Carvalha	60.0 ± 0.2^{c}	$89.7\pm0.2^{b,c}$
Touriga Nacional	42.3 ± 0.8^{b}	87.7 ± 0.4^{b}
Trincadeira Preta	$28.1\pm0.4^{c,d}$	$88.9\pm0.6^{c,d}$

*Different letters in the same column indicate significant differences ($p < 0.05$).

3.2.3 Reducing Power

The reducing power of the jellies and skins extracts of the seven grapevine varieties studied in the present work increased with the extract concentration (Figure 2).

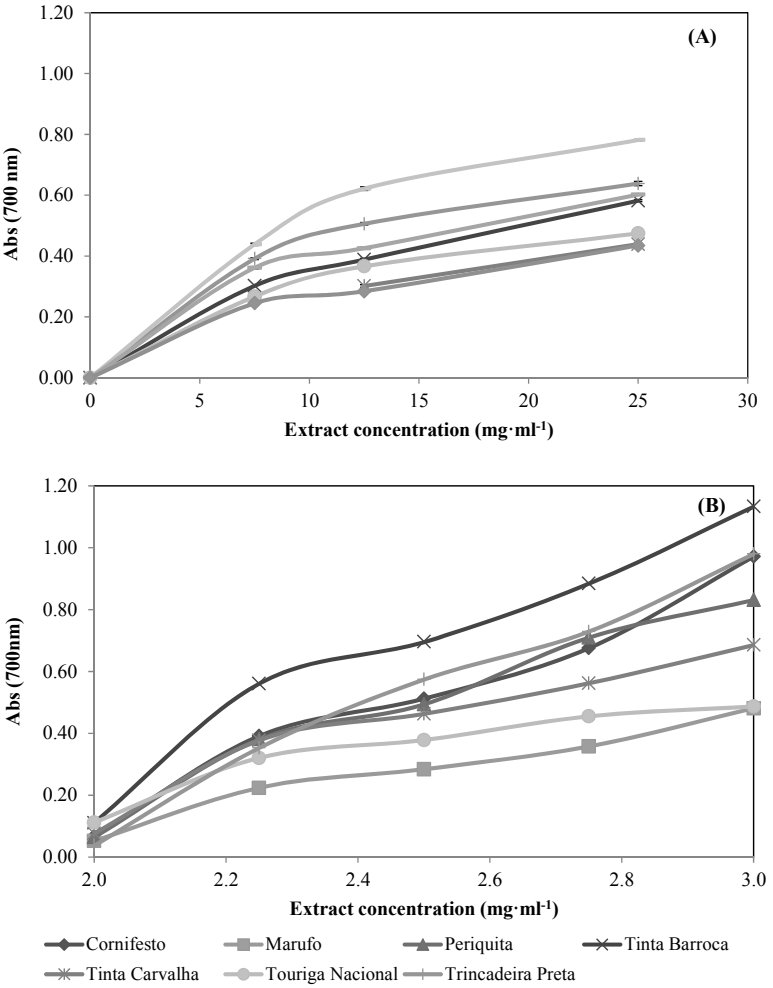

Figure 2: Reducing power (Abs 700 (nm)) versus extract concentration of jellies (A) and berry skins (B) of seven grapevine varieties.

However, it should be referred that similar reducing powers were obtained with solutions of skin extracts ten times more diluted than those of grape jellies. These results explained the EC_{50} values obtained (Table 4), being the lowest values determined for the skins (2.19 mg ml^{-1} to 2.60 mg ml^{-1}). Jellies presented higher EC_{50} values, varying between 9.17 mg ml^{-1} and 41.28 mg ml^{-1} for 'Tinta Carvalha' and 'Periquita' grape varieties, respectively. These results were expected since the EC_{50} value is inversely proportional to the antioxidant potential, demonstrating that the skins were richer in antioxidants than jellies. Moreover, the jelly produced from 'Tinta Carvalha' variety showed again the lowest EC_{50} value, suggesting a high antioxidant potential. Our results are in line with Abe et al. (2007) who stated that grapes with darker colour had a higher content of antioxidants. Indeed, as indicated earlier, 'Tinta Carvalha' was the variety that showed the darkest grape berries (lowest L* value) (Table 1) and redness colouration (highest a* value), suggesting the highest anthocyanins concentration, which may led to a jelly with the highest total phenol content (1.27 g kg^{-1} of GAE), the highest DPPH radical scavenging activity (60.0%) and the lowest EC_{50} value for the Reducing Power assay (9.17 mg ml^{-1}).

Table 4: EC_{50} values (mg extract ml^{-1}) determined on the reducing power assay of jellies and berry skins of seven grapevine varieties.

Variety	Jelly	Skins
Cornifesto	19.70±0.38	2.24±0.00
Marufo	26.93±3.76	2.60±0.08
Periquita	41.28±0.70	2.28±0.00
Tinta Barroca	12.22±0.03	2.19±0.00
Tinta Carvalha	9.17±0.17	2.35±0.00
Touriga Nacional	17.78±0.46	2.54±1.11
Trincadeira Preta	31.97±1.32	2.25±0.00

3.3 Sensory analysis

The test for acceptability was carried out by 54 consumers, 34 females and 20 males. The age of consumers ranged from 12 to 55 years.

Firstly, we started to compare the results obtained for the jelly analysed in both days. It was observed that most of the parameters evaluated for the 'Tinta Barroca' jelly did not present significantly different scores at $\alpha = 0.05$ on both days and no significant differences were detected between genders. In more detail, no significant differences on the appearance ($p = 0.088$), taste ($p = 0.054$), sweetness ($p = 0.309$), acidity ($p = 0.323$) and global assessment ($p = 0.077$) were observed. However, in terms of colour significant differences were determined ($p = 0.003$). One possible explanation was that 'Tinta Barroca' jelly was tested simultaneously with other jellies that had different colours, being 'Tinta Barroca' jelly colour judgment influenced by the colour of the other jellies. In fact, if jellies with colour that the consumer liked more were presented on the second day, the panellist would rate lower the colour of the repeated jelly.

When comparing the seven grape jellies, no significant differences on the attributes appearance ($p = 0.442$), taste ($p = 0.607$), sweetness ($p = 0.870$), acidity ($p = 0.911$) and global assessment ($p = 0.652$) were observed. On contrary, significant differences on colour ($p = 0.001$) were again observed. Observing Figures 3 and 4, it can be seen that all evaluated attributes presented medians above the 5 point scale (indifferent), being most of the cases close to 7 (like moderately). In terms of global evaluation, all jellies presented a sensory profile almost totally situated in the region of acceptance (>5.00). Regarding colour, 'Tinta Carvalha' and 'Marufo' jellies were the worst rated. On contrary, 'Tinta Barroca' was the preferable jelly mainly on the 2nd day, followed by 'Touriga Nacional' on the same day, and 'Cornifesto' and 'Trincadeira Preta' on the 1st day.

Generally, these results indicated that the seven grape jellies will have good acceptance by consumers, revealing good perspectives to broaden the application of grapes in food industry.

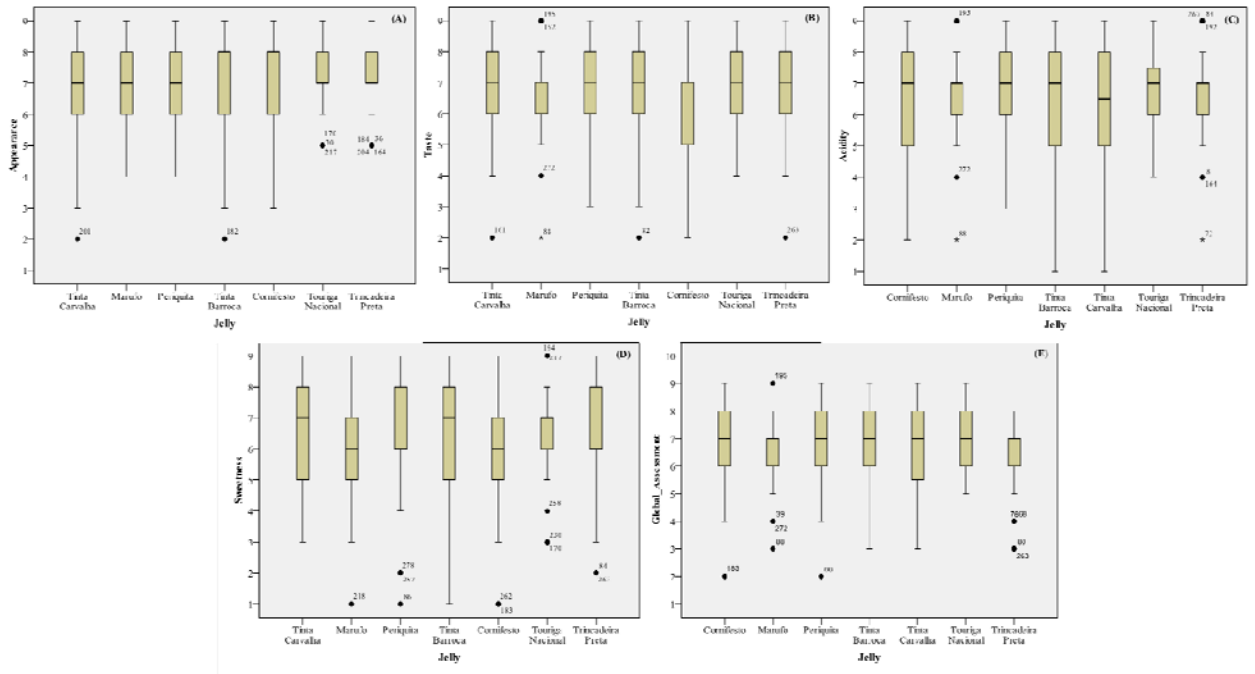

Figure 3: Box-plots obtained for the appearance (A), taste (B), acidity (C), sweetness (D) and global assessment (E) of jellies produced from seven grapevine varieties. (Not significant at $\alpha = 0.05$)

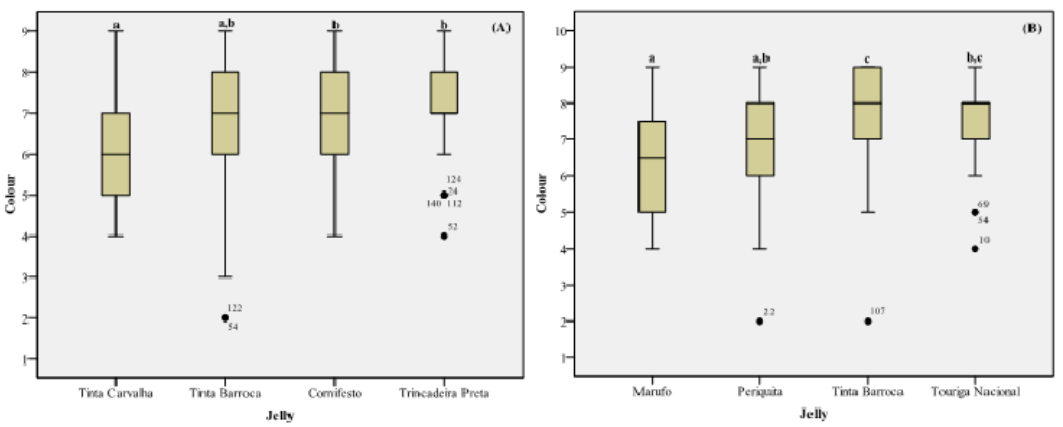

Figure 4: Box-plots obtained for the colour evaluation of the grape jellies on the first (A) and second (B) days of sensory analysis.

4 CONCLUSIONS

The present work showed that jelly production using different grapevine varieties seems to be a good option for small grape farmers and industrials and it will allow the valorisation of grapevine varieties with less potential for wine production and the preservation of grape biodiversity. After performing the physico-chemical characterization of the jellies significant differences were found in colour, pH, ash content and acidity, indicating that the production of grape jellies with different characteristics is possible in the future, meeting the wishes of a greater number of consumer types. 'Tinta Carvalha' jelly was the one that showed the highest amount of bioactive compounds, being also the darkest and redness jelly. Even though processing may cause the loss of some antioxidant activity, our results showed that jellies still have antioxidant potential and may emerge as an

interesting product for the market, as they continue to maintain bioactive properties of the fresh fruit. Regarding sensory analysis, no significant differences were found among the seven jellies studied for most of the parameters, except colour. Regarding the overall assessment, consumers classified them as enjoyable.

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Agris category code: f60, p35

Cultivation of three medicinal mushroom species on olive oil press cakes containing substrates

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ABSTRACT

Olive oil press cakes (OOPC) represent a waste that has a negative impact on environment. OOPC have little or no use and because of that solutions for their alternative use are sought after. In our experiments we investigated substrate mixtures composed of different proportions of OOPC, wheat bran, crushed corn seeds and beech sawdust for cultivation of *Ganoderma lucidum*, *Lentinula edodes* and *Grifola frondosa* fruiting bodies. The increasing amount of OOPC in fruiting bodies cultivation substrates resulted in decreasing production of fruiting bodies. Results show, that although OOPC in small portion can be successfully used as a medicinal mushroom fruiting bodies cultivating substrate, their use is rational only, if no other substrate composing materials can be found or when OOPC usage solves the problem of its deposition.

Key words: *Ganoderma lucidum, Grifola frondosa, Lentinula edodes*, mushroom cultivation, olive oil press cakes

IZVLEČEK

GOJENJE TREH VRST MEDICINSKIH GOB NA SUBSTRATIH VSEBUJOČIH OLJČNE TROPINE

Oljčne tropine (OT) predstavljajo odpadek s škodljivim vplivom na okolje in omejenimi možnostmi uporabe. OT imajo malo ali nobene uporabne vrednosti, zaradi česar se išče načine za njihovo alternativno uporabo. V naših poizkusih smo preizkušali substrate iz različnih deležev OT, pšeničnih otrobov, zdrobljenega koruznega zrnja in bukove žagovine za gojenje trosnjakov gliv *Ganoderma lucidum*, *Lentinula edodes* in *Grifola frondosa*. S povečevanjem deleža OT v substratu smo opazili trend zmanjševanja biološke učinkovitosti obroda. Rezultati kažejo, da čeprav lahko OT v manjših deležih uspešno uporabimo za substrat za gojenje trosnjakov, je to smotrno le v primerih, ko ni na voljo drugih primernejših sestavin substrata ali takrat, ko je dodatek OT namenjem preprečavanju negativnih vplivov teh odpadkov na okolje.

Ključne besede: *Ganoderma lucidum, Grifola frondosa, Lentinula edodes*, gojenje gob, oljčne tropine

1 INTRODUCTION

Olive oil press cakes (OOPC) represent a waste with a great negative impact on environment in Mediterranean countries, where many olive oil producing plants are located. OOPC have little or no use and solutions for their alternative use are sought after. It is well known that mushrooms can be cultivated on broad assortment of organic matter, including sawdust, straw, weeds, husks, compost and others. There were also reports of successful OOPC usage as a mushroom cultivating media (Soler-Rivas et al., 2006; Ruiz-Rodriguez et al., 2010; Zervakis et al., 2013).

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All experiments were performed at the mycology laboratory of MycoMedica d.o.o., Podkoren 72, 4280 Kranjska Gora, Slovenia

Ganoderma lucidum (Curtis) P. Karst., *Grifola frondosa* (Dicks.) Gray and *Lentinula edodes* (Berk.) Pegler are popular medicinal mushrooms with immune system strengthening, anticholesterolemic, antitumor, hepatoprotective, antidiabetic and other medicinal properties (Wasser, 2010; Powel, 2010). Successful cultivation of these species on OOPC containing substrates would have multiplicative positive effects, firstly on environment being polluted by OOPC and secondly on humans ingesting the cultivated fruiting bodies and medicinal substances they contain.

In our experiments we tested substrate mixtures composed of different portions of OOPC, wheat bran and beech sawdust for cultivation of *Ganoderma lucidum*, *Lentinula edodes* and *Grifola frondosa*. We aimed to analyze how medicinal mushroom cultivation substrates containing variable OOPC concentration influence the production of *G. lucidum*, *L. edodes* and *G. frondosa* fruiting bodies.

2 MATERIALS AND METHODS

Lentinula edodes strain No. 4080, *Ganoderma lucidum* strain Gal5 from culture collection of Zavod za naravoslovje (Institute for natural sciences), Ljubljana, and *Grifola frondosa* strain Gf3 from fungal collection of Wood Science and Technology department, Biotechnical Faculty, University of Ljubljana, Slovenia were used. Cultures were maintained on potato dextrose agar (Difco) at 24 ºC.

Substrates were composed of variable proportions of OOPC (Torklja, Koper, Slovenia), beech sawdust (BS) (Gorazd Rant s.p., Železniki, Slovenia), wheat bran (WB) (Mlin Katić, Velika vas pri Krškem, Slovenia) and gypsum (Rigips Austria GmbH, Saint-Gobain, Austria) (Table 1). Substrate components were mixed and water content adjusted to 65 %. Substrate was filled into polypropylene bags with breathing filters (3.5 kg for *L. edodes* and *G. lucidum* and 3.0 kg for *G. frondosa*) and sterilized for five hours at 121 ºC. At least four replicates were prepared for each substrate mixture.

Table 1. Substrate mixtures used for *Lentinula edodes, Grifola frondosa* and *Ganoderma lucidum* cultivation

Olive oil press cakes (OOPC) (%)	Wheat bran (WB) (%)	Beech sawdust (BS) (%)	Gypsum (%)
80	18	0	2
60	18	20	2
40	18	40	2
20	18	60	2
0	18	80	2

After the sterilization and cooling process substrates were inoculated with 100 g of *Ganoderma lucidum*, *Lentinula edodes* or *Grifola frondosa* mycelium, cultivated on rye grains, mixed by hand and incubated at 24 ± 1 ºC in a dark growth chamber. When the surface of the overgrown substrate became dark brown (*L. edodes*) or when primordia started to form (*G. frondosa* and *G. lucidum*), bags were moved into cultivation room with 17 ± 2 ºC, 10 hours of light daily and 80 % relative humidity. Fruiting bodies were harvested after they fully matured and their fresh weight determined. Biological efficiency (BE), being fresh fruiting bodies weight divided by weight of fresh substrate, multiplied by 100, was calculated (Royse and Sanchez-Vasquez, 2003). Because *G. lucidum* fruiting bodies are usually used and sold in dry form, BE for this species was calculated using dry weight of fruiting bodies after drying at 60 ºC for 48 hours (to constant weight).

All experiments were conducted in a mycological laboratory of MycoMedica d.o.o. company, Podkoren (Slovenia).

3 RESULTS

Lentinula edodes mycelium tends to grow slower and in some cases ceases to grow, if the substrates contained 80 % OOPC (Figure 1). Also there was a negative impact of OOPC on the growth of *L. edodes* mycelia and also its maturation. Substrates containing higher proportions of OOPC had a tendency to mature (change of color) later than substrates with lower OOPC share (Figure 1).

Figure 1. Substrates inoculated with *Lentinula edodes* mycelia containing (from left to right column) 80 %, 60 %, 40 %, 20 % or 0 % olive oil press cakes (OOPC).

Highest BE (38 %) of *L. edodes* fruiting bodies was calculated on substrates composed of 0 % OOPC, 80 % BS, 2 % gypsum and 18 % WB. Biological efficiency of fruiting bodies decreases in correlation to increasing proportions of OOPC in the growing substrates. When substrate contained 80 % OOPC, fruiting bodies ceased forming completely (Figure 1).

Figure 2. Deformed *Ganoderma lucidum* fruiting bodies emerging from substrate containing 80 % olive oil press cakes.

Figure 3. Non-deformed *Ganoderma lucidum* fruiting bodies emerging from substrate containing without olive oil press cakes.

Biological efficiency of *Grifola frondosa* fruiting bodies was reduced with increasing share of OOPC in the cultivating substrate. With *G. frondosa* higher fruiting bodies yields (62 %) were obtained on substrates not containing OOPC (Figure 4).

Ganoderma lucidum fruiting bodies yields also decreased with increasing portions of OOPC in the growth substrate (Figure 4). At higher OOPC content (60 % and 80 %) in the substrate fruiting bodies tended to have slight deformations in the shape (Figure 2), and were more sensitive to mold and bacterial infections. Development of *G. lucidum* fruiting bodies on 0 % OOPC containing substrates was not hindered (Figure 3).

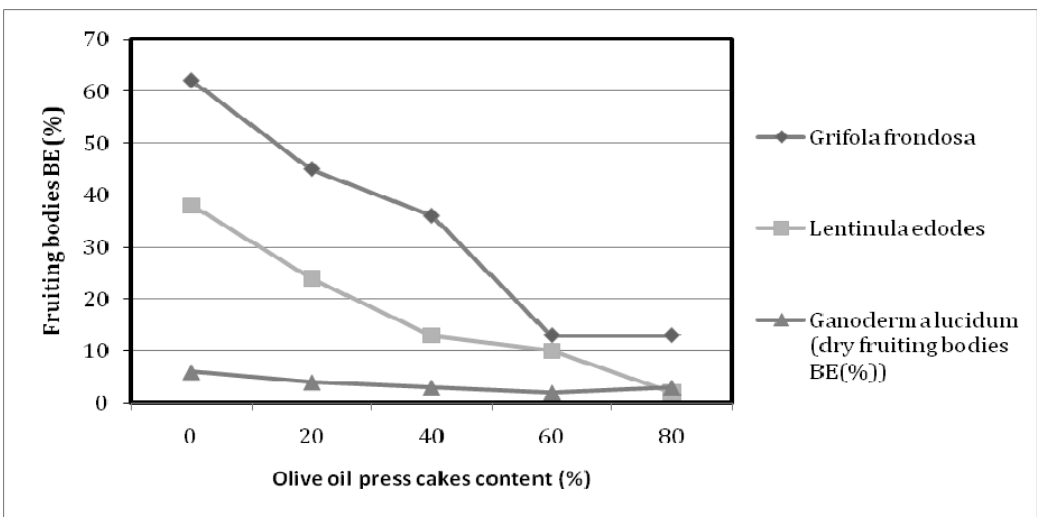

Figure 4. Biological efficiency (BE (%)) of *Lentinula edodes, Ganoderma lucidum* and *Grifola frondosa* fruiting bodies cultivated on olive oil press cakes containing substrates. (BE (%) of *Ganoderma lucidum* was calculated for dry fruiting bodies.)

4 DISCUSSION

Higher proportions of OOPC contained in cultivation substrates hindered the formation of *Lentinula edodes, Ganoderma lucidum* and *Grifola frondosa* fruiting bodies (Figure 4) and as well caused their deformation (Figure 2). With substrates not containing OOPC no yield reduction and fruiting bodies deformation was noticed (Figure 3). With *L. edodes* hindered mycelial growth was noticed immediatelly after inoculation (when mycelia ceased to grow completely), and during the substrate incubation period (when mycelia was maturing slower) compared with other substrates (Figure 1).

Hindered mycelia growth during incubation period as well as decrease of fruiting bodies yield on OOPC containing substrates could be the consequence of polyphenols contained in OOPC (Lakhtar et al., 2010; Zervakis et al. 2013). Beside polyphenolic compounds, a low porosity of OOPC and consequently lower substrate aeration and low water retaining capacity could be the reason for slower mycelial growth and lowered yields of fruiting bodies. It was found out that aeration greatly influences mycelial overgrowth and *L. edodes* fruiting bodies yields (Kalberer, 1995; Donoghue and Deninson, 1995). On the other hand fungal species and strains ability to utilize OOPC, or exposition to higher content of polyphenolic compounds in the substrate could have a significant influence on fruiting body yields. Strain characteristics tend to strongly influence mycelial growth as well as quantity and quality of produced fruiting bodies (Diehle and Royse, 1986; Royse and Bahler, 1986). Reduction of fruiting bodies yields on higher proportions of OOPC containing substrates is in accordance to the findings of other authors, who tested OOPC as a substrate component for cultivation of *Pleurotus ostreatus* (Ruiz-Rodriguez et al., 2010), *Pleurotus pulmonarius* (Soler-Rivas et al., 2006) as well as other *Pleurotus* and *Agrocybe cylindracea* species (Zervakis et al., 2013).

Nevertheless, the results show that OOPC in small proportions can be successfully used as a supplement to the medicinal mushrooms cultivating substrate. This application is reasonable only, if no other substrate composing materials are available, or when OOPC usage solves the problem of its deposition. Zervakis and coworkers (2013) found that composting of olive mill waste greatly increases the BE of produced fruiting bodies. This method could be used also with OOPC, potentially reducing its negative effect on mycelium growth and mushroom yields.

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The characterisation of *Vitis vinifera* 'Refošk' with AFLP and SSR molecular markers and ampelographic traits

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ABSTRACT

The genetic diversity and ampelographic variability of autochthonous red wine cultivar 'Refošk' (*Vitis vinifera* L.) grown in Slovenia were evaluated with AFLP molecular markers and OIV descriptors, respectively. SSR molecular markers were employed to confirm cultivar identity of analysed samples. Eight AFLP primer combinations, one was monomorphic, produced 16 polymorphic markers in 41 out of 113 samples, what classified samples into monomorphic and polymorphic group. Dendrogram constructed with simple matching coefficient and unweighted pair-group method analysis presented genetic diversity within polymorphic group. Refošk biotypes from monomorphic and polymorphic groups were evaluated with 22 OIV descriptors related to bunch, berry and must, but on the basis of ampelographic characterization samples were not differentiated among two major groups obtained with AFLP analysis. Results of genetic analysis indicated that 'Refošk' originated from closely related plants that are phenotypically very similar. With regard to low observed genetic diversity more attention should be dedicated to the selection in order to conserve remaining genetic diversity.

Key words: AFLP, genetic diversity, SSR, cultivar identity, morphological traits, germplasm, grapevine, Refošk, Refosco

IZVLEČEK

KARAKTERIZACIJA ŽLAHTNE VINSKE TRTE (*Vitis vinifera* L.) SORTE 'REFOŠK' Z AFLP IN SSR MOLEKULSKIMI MARKERJI IN AMPELOGRAFSKIMI LASTNOSTMI

Z AFLP molekulskimi markerji in z OIV deskriptorji je bila ovrednotena genetska variabilnost in ampelografska raznolikost avtohtone sorte 'Refošk' (*Vitis vinifera* L.) v Sloveniji. Sortna pristnost analiziranih vzorcev je bila potrjena z mikrosatelitskimi markerji. Pri 41 vzorcih od skupno 113 smo z uporabo osmih parov začetnih selektivnih oligonukleotidov, od katerih je bila ena kombinacija monomorfna, odkrili 16 polimorfnih markerjev. Na podlagi rezultatov AFLP analize smo vzorce razvrstili v dve skupini in sicer v monomorfno in polimorfno skupino. Dendrogram, narejen na podlagi koeficientov enostavnega ujemanja in z metodo netehtanih parnih skupin z aritmetično sredino prikazuje genetsko variabilnost znotraj polimorfne skupine. Trse iz različnih genetskih skupin smo ovrednotili z 22 OIV deskriptorji, ki se nanašajo na grozd, jagode in mošt, vendar se na podlagi ampelografske karakterizacije niso razvrstili v skladu z razvrstitvijo pri AFLP analizi. Rezultati nakazujejo na izvor sorte 'Refošk' iz sorodnih, fenotipsko zelo podobnih starševskih rastlin. Glede na nizko število dobljenih polimorfnih AFLP markerjev bi morali intenzivneje delati na selekciji sorte 'Refošk' z namenom ohranitve obstoječe genetske variabilnosti.

Ključne besede: AFLP, genetska variabilnost, SSR, sortna pristnost, morfološke lastnosti, dednina, vinska trta, Refošk, Refosco

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1 INTRODUCTION

The red wine cultivar 'Refošk' (*Vitis vinifera* L.), in Italy known as Refosco del Carso, Refosco d'Istria or Terrano d'Istria, and in Croatia as Refošk istarski or Teran is a member of the Refosco family. In Slovenia it is cultivated mainly in the Kras and the Slovenska Istra winegrowing districts where it presents 73 % and 45 % of the vineyards area, respectively (MOP, 2011), totalling 1.200 hectares. In the Karst region the produced wine is Karst Teran with high lactic acid and mineral iron contents in comparison with Refošk wine produced from the same cultivar in Slovenian Istria, due to pedoclimatic factors. Content of anthocyanins in Refošk grapes is similar to that in Cabernet Sauvignon (Vrhovesk et al., 2002). 'Refošk' represents one of the earliest cultivated cultivars in this region and due to several biotypes, the ampelographers are still not in agreement on the basic traits of the cultivar. However, it is already known that Italian types of Refosco (e.g. Refosco dal peduncolo rosso, Refoscone, Refosco nostrano, Refosco di Rauscedo) are morphologically and genetically different from 'Refošk' grown in Slovenia (Cipriani et al., 1994; Plahuta and Korosec-Koruza, 2009). In 1989 a collection vineyard in Komen (the Karst district) was established with the aim to choose appropriate clones and to preserve the old local Refošk biotypes. Since only one clone of 'Refošk' is officially certified, there is great need to promote further selection process.

Ampelography is essential in order to obtain information about viticultural performance of cultivars and clones included in selection. This method is based on phenotypic traits that are heavily influenced by different environmental conditions as well as nutritional state and health (Mannini, 2000; Sefc et al., 2001), thus DNA analysis approaches are frequently used in the characterisation of grapevine germplasm (Barth et al., 2009; María Ortiz et al., 2004). Kozjak et al. (2003) tested some selected accessions from the collection vineyard in Komen with 6 microsatellite loci, also known as simple sequence repeats (SSR), and found that two Refošk samples are probably different from cultivar Refošk, showed different patterns, while other accessions revealed identical SSR allelic profiles. The insufficient clone discrimination ability of SSR molecular markers was also stated in other papers (Imazio et al., 2002; Laucou et al., 2011), although microsatellite markers have been widely used for grapevine cultivar identification, defining synonyms and homonyms, and for pedigree reconstruction (Cipriani et al., 2010; Laucou et al., 2011; Rusjan et al., 2012). Molecular markers that have been used on grapevine in several studies to detect intravarietal variability are the inter simple sequence repeats (ISSR) (Regner et al., 2000), amplified fragment length polymorphism (AFLP) (Cervera et al., 1998; Fanizza et al., 2003; Imazio et al., 2002; Konradi et al., 2007; Meneghetti et al., 2012), selective amplification of microsatellite polymorphic loci (SAMPL) (Cretazzo et al., 2010; Meneghetti et al., 2012), microsatellite amplified fragment length polymorphism (M-AFLP) (Cretazzo et al., 2010; Meneghetti et al., 2012) and specific sequence amplified polymorphism (S-SAP) (Carrier et al., 2012; Stajner et al., 2009). Identifying and preserving rare genetic diverse plant material is highly recommended in order to maintain the existing genetic variability within a cultivar to allow a good response to the natural selection pressure (new pests, environmental and management changes, etc.) and to enhance the quality and complexity of wines (Mannini, 2000).

The objectives of this work were to assess the genetic variability of the Refošk cultivar planted in the collection vineyard in Komen and in production vineyards in the Kras and Slovenska Istra winegrowing districts with AFLP markers. Microsatellite markers were employed to confirm the cultivar identity of analysed samples. Ampelographic characters of Refošk biotypes chosen on the basis of AFLP results were describe with OIV described.

2 MATERIAL AND METHODS

2.1 Plant material

Refošk samples were taken from: a collection vineyard established in Komen (N45 48.917 E13 44.692) (biotypes No. 1-35, 37-54, 56, 58-67, 69, 70, 73, 74 and 76, all together 69 samples); thirteen production vineyards randomly chosen in the Kras and Slovenska Istra winegrowing districts (41 samples) and three vines from Merče, Šepulje and Marezige, each more than 150 years old, were also included in analysis (Table 1). A total of 113 samples were included in the analysis.

2.2 DNA isolation

Genomic DNA for SSR and AFLP analysis was extracted from young leaves of shoot tips using the modified cetyltrimethylammonium bromide (CTAB) method described by Kump and Javornik (1996). The DNA was quantified by fluorometric determination using the Quant-iT^{TM} dsDNA Broad-Range (BR) Assay Kit by the QubitFluorometer (Invitrogen, Darmstadt, Germany).

Table 1. List of Refošk samples used for SSR and AFLP analysis from production vineyards, together with old Refošk vines

Origin	Code	Year of planting	Coordinates	Winegrowing district
Križ (a)	1s, 3s, 7s, 8s, 10s	2002	N45 44.566 E13 51.905	Kras
Križ (b)	12s, 13s, 17s, 18s, 19s, 20s	2005	N45 44.566 E13 51.905	Kras
Tomaj	21s, 25s, 26s, 28s, 29s, 30s	1970	N45 45.220 E13 51.077	Kras
Godnje	37s, 38s, 39s, 40s	1990	N45 45.399 E13 50.434	Kras
Dutovlje (a)	45s, 46s, 47s	2002	N45 45.203 E13 49.805	Kras
Dutovlje (b)	54s, 58s	1958	N45 45.204 E13 49.805	Kras
Krajna vas	63s	1999	N45 45.870 E13 48.119	Kras
Šepulje	72s	app. 1780	N45 45.080 E13 52.191	Kras
Merče	73s	app. 1700	N45 42.071 E13 54.047	Kras
Prade	3k, 5k, 10k	1998	N45 32.903 E13 46.909	Slovenska Istra
Pobegi (a)	11k, 12k, 13k, 15k	1980	N45 32.266 E13 47.156	Slovenska Istra
Marezige	18k	app. 1880	N45 30.383 E13 48.143	Slovenska Istra
Truške	19k, 20k, 21k, 22k	2000	N45 29.674 E13 48.960	Slovenska Istra
Boršt	33k	1980	N45 28.647 E13 46.903	Slovenska Istra
Izola	36k	2001	N45 31.745 E13 40.288	Slovenska Istra
Pobegi (b)	41k	2003	N45 32.081 E13 47.396	Slovenska Istra

2.3 Microsatellite analysis

To prove cultivar identity, six previously described microsatellite loci were analysed: VVMD5, VVMD7 (Bowers et al., 1996); VVMD27, VVMD32 (Bowers et al., 1999); ssrVrZAG62 and ssrVrZAG79 (Sefc et al., 1999). Amplifications were made with the economic method described by Schuelke (2000) where the loci specific primer was elongated for M13 sequence and four M13 primers fluorescently labelled with dye phosphoramidites (6-FAM, VIC, PET or NED) were used in PCR as well. In a total volume of 15 µl the PCR reaction mixture contained 20 ng of genomic DNA, 1 x Taq Buffer with $(NH_4)_2SO_4$, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer (Integrated DNA Technologies, Leuven, Belgium), 0.25 µM of M13 fluorescent primer (Applied Biosystems, Cheshire, UK) and 0.375 U of *Taq* DNA polymerase. All chemicals were supplied by Fermentas/Thermo Fisher Scientific, MA, USA. PCR reactions were carried out in a 2720 thermal cycler (Applied Biosystems, Darmstadt, Germany) with a two-step PCR protocol started with initial touchdown cycle: 94 °C for 5 min, followed by five cycles of 30 s at 94 °C, 30 s at 60 °C, which was lowered by 1 °C each cycle, 90 s at 72 °C, followed by 30 cycles with annealing temperature at 55 °C and ending with an 8-min extension step at 72 °C. PCR products were multiplexed as shown in Table 2 and separated by capillary electrophoresis on an Applied Biosystems 3130

Genetic Analyser, using GeneScan™ -500 LIZ® (Applied Biosystemsh, Cheshire, UK) as size standard.

2.4 AFLP analysis

AFLP analysis was performed on 113 samples according to Vos et al. (1995) with the modifications described below. Each 500 ng sample of genomic DNA was digested with *Tru*1I (*Mse*I iso-schizomer) and *Pst*I (3 U each) restriction endonucleases for 120 min at 37 °C (*Pst*I incubation temperature) and 120 min at 65 °C (*Tru*1I incubation temperature) in a 40 µl volume in the presence of 1x Buffer R. After restriction 10 µl of ligation mix, including 50 pmol of *Mse*I adapters, 5 pmol *Pst*I adapters, 2 µl 10 mM ATP, 1µl 10x T4 DNA ligase buffer and 1 U T4 ligase was added to restriction reaction. Adapters were prepared by adding equimolar amounts of both strands (Integrated DNA Technologies, Leuven, Belgium). Ligation was performed at 22 °C for 60 min, followed by the final step at 65 °C for 10 min to inactivate enzymes. The pre-amplification of DNA templates (50 ng) was performed in 50 µl volume with non-selective *Pst*I and *Mse*I primers in a final concentration 0.2 µM, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1x *Taq* Buffer with $(NH_4)_2SO_4$ and 1.25 U *Taq* DNA polymerase.

Selective amplifications were performed in a volume of 10 µl containing the following components: 2 µl 10-times diluted pre-amplification products, 1x *Taq* Buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM with fluorescent dye (6-FAM, VIC, PET) labelled *Pst*I primer (Applied Biosystems, Cheshire, UK), 0.2 µM unlabelled *Mse*I primer and 0.25 U *Taq* DNA polymerase. Selective amplifications were performed using a total of seven primer combinations with two or three selective nucleotides (Table 3). Primer pairs were chosen based on previous testing of 56 combinations on 8 samples (*Pst*I primers with selective nucleotides: ATA, AAC, AGA and ACA; *Mse*I primers with selective nucleotides: AG, CG, CA, AC, CC, CTT, CAT, CAA, CAG, CAC, CTG, CTA, CTC, ACC) with the aim of obtaining an optimized number of scorable bands for every primer combination (data not shown). PCR protocols were as described by Vos et al. (1995), except preamplification was performed with the initial step of 2 min at 72 °C. PCR products were multiplexed (as shown in Table 3), and separated by capillary electrophoresis with GeneScan™ -500 LIZ® (Applied Biosystemsh, Cheshire, UK) as internal size standard on an Applied Biosystems 3130 Genetic Analyser. All accessions were analysed twice (DNA restriction, pre-amplification and selective amplification) to test the reproducibility of the AFLP profiles.

2.5 Ampelographic Analysis

Twelve Refošk biotypes, chosen on the basis of AFLP results, grown in the collection vineyard were described with 22 OIV descriptors related to bunch, berry and must (2nd edition of the OIV descriptor list for grape varieties and Vitis species) (O.I.V., 2009). Descriptions were performed on 10 shoots of 3 to 5 vines per biotype. Vines are grafted on rootstock SO4 (*Vitis berlandieri* x *Vitis riparia*), trained as double guyot and cultivated following the instructions of integrated pest management. The vineyard was permanently green covered. Each biotype has 3 to 35 vines planted in the block.

2.6 Data analysis

SSR and AFLP electropherograms were analysed and sized with Gene Mapper software version 4.1 (Applied Biosystems, Chesire, UK). AFLP electropherograms were scored for the presence or absence of bands and expressed in binary data, while microsatellite alleles were presented in the amplification lengths. For AFLP, only reproducible, clear bands falling within the range of 50 - 500 bp were considered for analysis. The total number of fragments and percentage of polymorphic fragments were assessed for every primer combination and in the total set. The genetic similarity among clones was calculated using simple matching (SM) genetic distance. A dendrogram was constructed using the unweighted pair group method average (UPGMA) clustering of the NTSYSpc software package, version 2.02i (Rohlf, 1998). Average gene diversity over loci was calculated based on Nei (1987) formula using the Arlequin program (Excoffier and Lischer, 2010).

The observed mean values of ampelographic characters were transformed to numerical scales according to the OIV descriptors (O.I.V., 2009).

The dendrogram was drawn using UPGMA method and distance (DIST) coefficient for interval measure (quantitative) data. Calculations were performed with NTSYSpc 2.02i software (Rohlf, 1998).

3 RESULTS

3.1 Molecular Analysis

The microsatellite analyses confirmed the cultivar identity of all tested Refošk vines. All 113 samples had the same fingerprint at 6 microsatellite loci (Table 2).

AFLP analysis, conducted on 113 samples, using eight different primer pairs, produced 208 scorable fragments, 16 of which were polymorphic. One combination generated only monomorphic markers, while 7 combinations were informative. Polymorphic fragments and percentage of polymorphism varied from 1 to 5 loci and from 2.3 to 18.8 % per primer combination, respectively (Table 3). The size of polymorphic amplified products ranged from 100 bp to 397 bp. The AFLP analysis was repeated at least twice and all polymorphic bands were reproducible. In general samples included in analysis could be classified into monomorphic group and polymorphic group since 72 samples showed no polymorphisms compared to other 41 samples that showed polymorphisms in terms of gaining new bands compared to the monomorphic group (Figure 1). Average gene diversity over loci for all samples was 0.0294 with standard deviation 0.0155.

Twenty two out of 41 samples had identical fingerprints, while other 19 samples were more diverse, with 78 to 93.8 % genetic similarity compared with the main identical group.

Table 2: SSR allele length (alleles in bp) at 6 microsatellite loci performed on all 113 Refošk samples, fluorescently labelled M13 primer labelled with different dyes for different SSR markers and multiplexing combinations after PCR. Combinations analysed in the same electrophoresis run are marked with the same letters (A and B).

SSR marker	Dye of M13 primer	Electrophoresis multiplex	Genotype
VVMD5	NED	A	241:243
VVMD7	6-FAM	A	262:264
VVMD27	VIC	B	208:208
VVMD32	VIC	A	266:289
VrZAG62	6-FAM	B	210:212
VrZAG79	PET	A	256:268

Table 3: The number of total scorable and polymorphic AFLP markers generated by the selected primer combinations, where "P" and "M" are *Pst*I and *Mse*I primers, respectively. Combinations analysed in the same electrophoresis run are marked with the same letters (A, B and C).

Primer combination	Total bands	Polymorphic markers	Polymorphism (%)	Electrophoresis multiplex
6-FAM-P-AGA/M-CTT	43	1	2.3	A
VIC-P-AAC/M-CTG	29	2	6.9	A
6-FAM-P-AGA/M-CAT	23	3	13.0	B
VIC-P-AAC/M-AG	34	5	14.7	B
PET-P-ATA/M-CAA	16	3	18.8	B
6-FAM-P-AGA/M-AG	22	1	4.5	C
VIC-P-AAC/M-CTC	16	1	6.25	C
PET-P-ATA/M-CTT	25	/	/	A
Total	208	16	7.7	

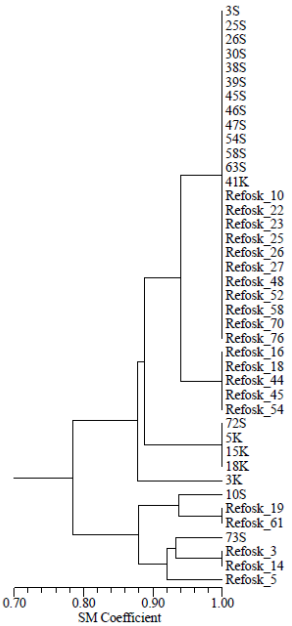

Figure 1: UPGMA-derived dendrogram of genetic similarity based on the SM coefficient among the 41 Refošk samples

3.2 Ampelographic characterization

Twelve Refošk biotypes grown in the collection vineyard were selected on the basis of AFLP analysis, 4 from the monomorphic group and 8 from the polymorphic group, and were evaluated for 22 OIV descriptors (Table 4). Traits, showing variability among Refošk biotypes, were: bunch density (OIV 204) varied from loose to medium; observed length of peduncle of primary bunch (OIV 206) varied from 46.2 to 72.5 mm; peduncle was lignified up to about the middle or more than the middle (OIV 207); the number of wings of the primary bunch (OIV 209) varied from 1 to 5; berry shapes (OIV 223) were either globose or ellipsoid; mean weight of a single bunch (OIV 502) varied from 371 to 696 g; mean weight of 30 typical berries of 5 bunches (OIV 503) varied from 2.77 to 4.30 g; observed anthocyanin coloration of flesh (OIV 231) varied from none to medium; and must traits: sugar (OIV 505) and total acid content expressed as tartaric acid equivalents (OIV 506), varied from 18.2 to 23.3 % and from 10 to 11.8 g l^{-1}, respectively. However, Refošk biotypes did not show any similar distribution with regards to AFLP monomorphic and polymorphic group, since Refošk biotypes 31, 39, 40 and 43, presenting the monomorphic group, were distributed among different clusters (Figure 2).

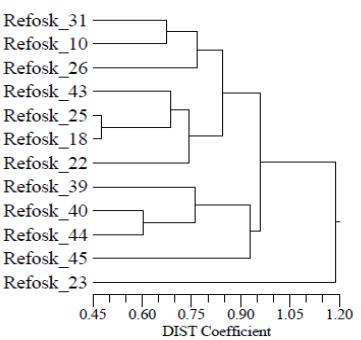

Figure 2: Dendrogram based on ampelographic characterization of the 12 Refošk biotypes from the collection vineyard in Komen (Kras winegrowing district, Slovenia), constructed with UPGMA method and distance (DIST) coefficient

Table 4: Scoring results of 22 OIV codes of the 12 Refošk biotypes from collection vineyard in Komen (Kras winegrowing district, Slovenia). Observations were performed on 10 shoots on 3 to 5 vines per biotype.

OIV code	Characteristic	Refošk biotypes											
		31	39	40	43	10	22	23	18	25	26	44	45
202	Bunch length with peduncle excluded	5	5	5	5	5	5	5	5	5	5	5	5
203	Bunch width	5	5	5	5	5	5	5	5	5	5	5	5
204	Bunch density	5	3	3	5	5	5	5	5	5	5	3	5
206	Length of peduncle of primary bunch	3	3	3	5	5	3	3	5	5	3	3	3
207	Lignification of peduncle	5	5	5	7	7	5	5	5	5	7	5	5
208	Bunch shape	2	2	2	2	2	2	2	2	2	2	2	2
209	Number of wings of the primary bunch	3	2	3	4	2	2	4	2	2	3	3	4
220	Berry length	5	5	5	5	5	5	5	5	5	5	5	5
221	Berry width	5	5	5	5	5	5	5	5	5	5	5	5
222	Uniformity of berry size	2	2	2	2	2	2	2	2	2	2	2	2
223	Berry shape	2	3	3	2	3	2	3	2	3	2	3	2
225	Berry skin colour	6	6	6	6	6	6	6	6	6	6	6	6
226	Uniformity of berry skin colour	6	6	6	6	6	6	6	6	6	6	6	6
231	Intensity of the anthocyanin coloration of flesh	3	1	1	1	3	1	5	1	3	3	1	3
233	Must yield	5	5	5	5	5	5	5	5	5	5	5	5
238	Length of pedicel	3	3	3	3	3	3	3	3	3	3	3	3
240	Ease of detachment from pedicel	3	3	3	3	3	3	3	3	3	3	3	3
502	Weight of a single bunch	5	3	5	5	5	5	3	5	5	7	5	3
503	Single berry weight	3	3	3	3	3	5	3	3	3	5	5	3
505	Sugar content of must	7	7	5	7	7	7	9	7	7	7	5	5
506	Total acid content of must	5	5	5	7	5	7	5	7	7	5	7	7
508	Must specific pH	3	3	3	3	3	3	3	3	3	3	3	3

4 DISCUSSION

The genetic diversity of cultivar and the presence of several biotypes are of great importance because of their adaptation to different climate conditions as this can contribute to typical characteristics of vine. In the present study, the cultivar identity was confirmed with SSR markers and genetic diversity of Refošk was assessed with AFLP molecular markers. The amplification of six SSR loci revealed the same allele patterns in all 113 accessions and confirmed the genetic identity of the cultivar. Kozjak et al. (2003) distinguished samples labelled with number 7 and 50 from other Refošk biotypes with microsatellite analysis, while all other analysed samples had the same microsatellite fingerprint in our research. The discrepancies that were observed between the Refošk samples 7 and 50 by Kozjak et al. (2003) and our results, were probably due to mistakes at planting or collecting stage. No other previous information is available on genetic diversity within cultivar Refošk grown in Slovenia.

The *Pst*I-*Mse*I primer combinations used reveal 16 reproducible polymorphisms out of 208 scorable markers and thus allowed to differentiate analysed samples in polymorphic and monomorphic group. Overall mean value of gene diversity was lower than published for example for Pinot Noir clones and similar as observed for Pinot gris clones (Blaich et al., 2007; Konradi et al., 2007).

The dendrogram presented genetic variability within the polymorphic group (Figure 1). When analysing clonal diversity of grapevine cultivars in other studies, a wide range of obtained AFLP polymorphisms and power of discrimination have been reported. For example, Fanizza et al. (2003) did not manage to differentiate 4 clones of the table grapevine cultivar Italia, although 3880 markers had been produced with 49 primer combinations; Filippetti et al. (2005) discriminated only 3 polymorphic clones out of 26 using 9 primer combinations; Konradi et al. (2007) revealed 72 polymorphic markers of total 375 among 32 Pinot clones exhibiting up to 5 % dissimilarity, on the other hand Anhalt et al. (2011) obtained 135 polymorphic markers out of 305 with 10 primer combinations when studying 86 Riesling clones, but most clones showed none, one or two mutations over all primer combinations

Discrimination of samples in monomorphic and polymorphic groups could indicate that Refošk grown in Slovenia originated from different, but genetically and morphologically very similar plant material. A possible explanation for this phenomenon is provided by Filippetti et al. (1999) who demonstrated that seedlings from a single self-pollinated vines were morphologically similar, but at the DNA level could be differentiated. The results show that genetically different plant material is equally represented in production vineyards, indicating that vine nurseries propagate genetically mixed material. Due to low detected variability (only 10 different AFLP fingerprints) it is necessary to continue with the analysis to determine as much genetic variability as possible for efficient and proper conservation.

Results of ampelographic description showed that Refošk biotypes differ in several traits. However, correlation between ampelographic characters and either monomorphic or polymorphic group according to AFLP results were not observed. Since traits including berry weight, number of wings of the primary bunch, lignification of the peduncle, and the must traits of sugar and total acid content could vary from year to year, due to different crop level and other biotic and abiotic factors, multiple years of ampelographic observations should be considering for comparison.

5 CONCLUSION

Genetic diversity is needed for efficient adaptation of cultivars to environmental changes and to be more resilient to environmental shocks. ‘Refošk’ is cultivated across a relatively large region but AFLP genetic analysis showed that very little genetic diversity exists within cultivar, which subsequently presents higher production risks. In recent years Refošk was recognized as very susceptible to Grapevine yellows and few vineyards were already grubbed-up. When

selecting morphological appropriate grapevine, genetic analysis should complement ampelography what allows to prevent diminishing of genetic variability. Using AFLP markers we were able to detect greater variability compared to microsatellite molecular markers, where no polymorphisms were discovered and thus provided valuable information for further selection and conservation processes.

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Autografted vines of cultivar 'Refošk' (*Vitis vinifera* L.) reveal symptoms of the rugose wood disease

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ABSTRACT

Rugose wood disease complex is one of the most important graft-transmissible grapevine diseases and it is considered to be a viral disease. With the aim to obtain more information about appearance of rugose wood disease observed on cultivar 'Refošk', 'Refošk' vines from collection vineyard in Komen were used for green grafting on SO4 rootstock and autografts for control were made as well. Rugose wood symptoms were observed on grafts of two 'Refošk' biotypes, which confirmed graft transmissibility. Appearance of rugose wood symptoms on autografts excluded the impact of incompatibility in rugose wood disease, but at the same time it could be proposed that stress caused by grafting has an important role.

Key words: rugose wood complex disease, green grafting, graft indexing

IZVLEČEK

POJAV ZNAMENJ RAZBRAZDANJA LESA NA CEPLJENKAH S SPOJENIMI LASTNIMI DELI TRSOV SORTE *Vitis vinifera*'Refošk'

Kompleks bolezni razbrazdanja lesa je ena od najpomembnejših bolezni vinske trte, ki se prenaša s cepilnim materialom in za katero velja, da naj bi jo povzročali virusi. Da bi pridobili več podatkov o razvoju znamenj bolezni razbrazdanja lesa smo cepiče sorte 'Refošk' s kolekcijskega vinograda iz Komna s tehniko cepljenja zeleno na zeleno cepili na podlago SO4. Za kontrolo smo mladike prerezali in jih ponovno spojili. S pojavom znamenj razbrazdanja lesa na cepljenkah pri dveh biotipih sorte 'Refošk' smo potrdili ugotovitev, da se bolezen prenaša s cepljenjem, medtem ko lahko zaradi pojava znamenj na cepljenkah s spojenimi lastnimi deli mladik sklepamo, da na razvoj bolezni ne vpliva inkompatibilnost cepiča in podlage, temveč bi lahko imel pomembno vlogo stres, ki ga izzove cepljenje.

Ključne besede: kompleks bolezni razbrazdanja lesa, zeleno cepljenje, indeksiranje

1 INTRODUCTION

Rugose wood disease complex is one of the most important graft-transmissible grapevine diseases, however despite numerous studies (Credi, 1997b; Meng et al., 1999; Nakaune et al., 2008) its etiology is still largely unknown. The disease is spread worldwide (Martelli, 1993) and has been found on many grapevine cultivars *V. vinifera* and

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on other species of the genus *Vitis* (Bonfiglioli et al., 1998; Credi, 1997a; Nakaune et al., 2008).

Rugose wood complex is a disease complex usually characterized by modifications of the woody cylinder that is typically marked by pits and/or grooves (Martelli, 1993). The ridges of the cortex consist of hyperthrophied rays extending from the bark into the functional xylem (Martelli, 1993). Anatomical abnormalities originate from the altered behavior of the vascular cambium (Martelli, 1993). The symptoms can appear either on scion, rootstock or on both (Martelli, 1993). The rugose wood complex of diseases are essentially diseases of grafted vines, since appearance of symptoms for non-grafted vines is unusual (Bonfiglioli et al., 1998).

Rugose wood disease is considered to be a viral disease, although this assumption is based only on its graft transmissibility and in part on its vector transmissibility (Martelli, 1993). Rupestris stem pitting, the most widespread disease of the rugose wood complex, is consistently associated with *Grapevine Rupestris stem pitting associated virus* (GRSPaV) (Meng et al., 1999; Nakaune et al., 2008). On the other hand GRSPaV was also detected in asymptomatic vines (Meng et al., 2005; Nakaune et al., 2008). Recently GRSPaV was observed to be closely associated with vein necrosis symptoms (Bouyahia et al., 2005). The exact etiological role of GRSPaV in different diseases remains unknown. *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) are thought to be involved in Kober stem grooving and Corky bark, respectively (Bonavia et al., 1996; Garau et al., 1994) and they are transmitted by mealybug (*Phenacoccus aceris*) (Le Maguet et al., 2012). However, due to the complexity of the disease, a general causal agent has not been identified yet.

Symptoms similar to those of rugose wood were observed and recorded in Slovenia as well. 'Refošk' vines from the collection vineyard in Komen, Slovenia, that have shown diverse rugose wood symptoms, like swelling above the grafted site, deep grooves, pitting, thicker scion vines; were already tested by ELISA, ISEM, Western blot and RT-PCR for the presence of the rugose wood disease related viruses (Petrovic et al., 2003; Tomazic et al., 2005a; Tomazic et al., 2005b; Tomazic et al., 2008) but none of the tested viruses could be correlated with rugose wood disease on the cultivar 'Refošk'.

According to EPPO certification scheme for the production of healthy plants for planting, graft indexing is still a compulsory step, since rugose wood disease can be identified solely on woody differential hosts; in case of *Grapevine virus A* and *Grapevine virus B* molecular testing is recommended (EPPO, 2008). However, it was already shown that biological indexing tests may not be completely reliable, because results can be affected by various elements, such as a possible synergistic effect of various causal pathogenic agents and possible latency of wood disorders (Credi, 1997a). Due to extreme complexity of rugose wood disease complex much more research is needed to enable the use of modern technologies to clarify the etiology of this disease. We believe the work we are presenting in the present paper will contribute to enhanced understanding of the etiology of rugose wood disease complex.

The first objective of this study was to find out if rugose wood symptoms are expressed in green grafts and to determine when they appear after green grafting. Indexing tests are most frequently performed with the dormant chip budding method, which may take two to several years before rugose wood symptoms become visible (Credi, 1997b; Martelli, 1993). The green grafting method has several advantages in comparison with woody grafts: for several of the graft-transmitted diseases, symptoms develop in a matter of weeks instead of months; less space is required for green-grafted indicators and it can be done throughout the growing season (Walter et al., 1990). Green graft indexing is used in Slovenia in grapevine sanitary selection as well. However, Walter et al. (1990) didn't manage to detect stem pitting when using green grafting on the indicators Kober 5BB and *V. rupestris* after 3 months. Besides that, when they compared results of green grafting and dormant budding, they discovered more positive indicators of corky bark on grafts with the dormant budding technique. The second objective of this work was to test if rugose wood symptoms are expressed on autografted 'Refošk' vines to determine if the appearance of rugose wood symptoms could be due to a physiological response of the plant to grafting stress, rather than or in addition to the transmission of a virus or virus-like agents.

2 MATERIAL AND METHODS

2.1 Plant material

Plant material for indexing was obtained from the collection vineyard in Komen (N45 48.917 E13 44.692), established in 1989 when old 'Refošk' vines from the field were propagated by grafting on SO4 (*V. berlandieri* x *V. riparia*) and planted in blocks of 3 to 35 vines per biotype. Vines are trained as double guyot and cultivated according to the instructions of integrated pest management.

Fifteen percent of vines from collection vineyard have shown rugose wood symptoms on rootstocks and/or scions, while 18 % of plants with rugose wood symptoms died in 10 years after planting (Tomazic et al., 2005b). The virus status of 'Refošk' vines used in this study was already reported (Petrovic et al., 2003; Tomazic et al., 2005a; Tomazic et al., 2005b; Tomazic, 2002; Tomazic et al., 2008). Results were summarized in Table 1. However, virus status could be changed in case of transmission.

'Refošk' vines used for indexing are listed in Table 1. As a putative source of rugose wood, vines labeled as 'Refošk' 20, 38, 48 and 51 were used. From vineyard observation 'Refošk' biotypes 38 and 48 develop symptoms on itself, on the other hand 'Refošk' biotypes 20 and 51 induce apparent rugose wood symptoms on SO4 rootstock while scion parts maintain a healthy appearance (Table 3). Regarding the differences in symptoms expression, there could be two different types of rugose wood. 'Refošk' biotypes 43 and 61 never showed symptoms in the vineyard and were used as controls. In June 2009, shoots of 'Refošk' vines were collected in the morning and immediately transported to the Vine Selection Center in Vrhpolje, where green grafting on SO4 rootstock and autografting were carried out.

2.2 Grafting experiment

For the rootstock, shoots of 'Refošk' vines and SO4 were cut on two buds (right below lower bud and in the middle of internode above the next bud), while the scion cuttings had one bud. Leaves on upper rootstock node and on the scion were trimmed to about half of their original size before grafting. The method used for indexing was machine splice grafting. The assembled graft was wrapped with white first aid tape. Rootstocks were treated with naphthalene acetic acid (NAA) (Germon Bewurzelungspuder H per talee legnose, Conc. E. Gerlach GmbH, Germany). Grafts were planted in vermiculite and kept for 34 days in humid chamber on 28 °C and 85 % relative humidity. After that period rooted grafts were transplanted into universal substratum in flowerpots and transferred to the green house to the controlled water table for constant irrigation where they were maintained till September 2012.

The grafts were visually examined monthly for the presence of the rugose wood symptoms on the scion and rootstock parts in 2009 and 2010, while the last two years only at the end of active vegetation at the beginning of September. At final examination all grafts were autoclaved and bark was peeled away.

Table 1. Virus status of 'Refošk' vines from collection vineyard in Komen, Slovenia, as reported in previous studies.

Virus / 'Refošk' vine	20 (IV[d]/110[e])	38 (VIII/44)	43 (VIII/113)	48 (IX/43)	51 (IX/69)	61 (XII/68)
ArMV[a]	-	-	-	-	-	-
GFkV[a]	+	-	-	-	-	-
GFLV[a]	-	-	-	-	-	-
GLRaV-1[a]	-	-	-	-	+	-
GLRaV-2[a]	-	-	-	-	?	+
GLRaV-3[a]	-	-	-	-	-	-
GLRaV-6[a]	+	+	-	-	-	-
GLRaV-7[a]	-	-	-	-	?	-
GVA[a]	-	-	-	-	-	-
GVB[a]	-	-	-	-	-	-
RSPaV-1[a, d]	+[a,b,d]/-[c]	+[a,b,d,c]	+[a,b,d]/-[c]	+[a,b,d]/-[c]	+[a,b,d]/-[c]	+[a,b,d] / -[c]

[a] ELISA testing, [b] PCR testing, [c]ISEM testing, [d]Western blot testing, [d] Raw in collection vineyard, [e] Vine number in raw

? unable to confirm infection since threshold was difficult to determine due to higher background in ELISA testing

3 RESULTS AND DISCUSSION

A major part of research on rugose wood disease on 'Refošk' was done in the field of virology, but no causal agent was identified that could be used in sanitation for rugose wood affected vines (Tomazic et al., 2005a; Tomazic et al., 2005b; Tomazic et al., 2008). In the collection vineyard in Komen it is observationally evident that vines, propagated by grafting from the same mother vines are showing similar rugose wood symptoms, which indicates that rugose wood is transmitted with grafting. This is consistent with rugose wood complex in general (Martelli, 1993).

Indexing for rugose wood associated diseases is usually carried out using standard indicator vines, i.e. *V. rupestris* St. George, LN 33 (Coudero 1613 x *V. berlandieri*) and Kober 5BB (*V. berlandieri* x *V. riparia*). In our study, grafting on rootstock SO4 was performed, since it is frequently used rootstock in Kras and Slovenska Istra winegrowing districts, it develops rugose wood symptoms and it was used in the collection vineyard in Komen.

3.1 Survival rate of grafted material

The results of autografting and grafting 'Refošk' on SO4 are summarized in Table 2. At final monitoring from 27.3 % to 80 % of 'Refošk' autografts were still growing. The highest mortality rate at last monitoring was observed in 'Refošk' 43, 51 and 61, reaching 60 % to 72.7 %. 'Refošk' 43 and 61 biotypes are symptomless in the vineyard. On the other hand only 30 % of 'Refošk' 38 and 20 % of 'Refošk' 48 autografts died, while both biotypes show the most severe rugose wood symptoms in the vineyard.

Considering the results of grafting 'Refošk' on SO4 from 25 % to 40 % of grafts were viable at final monitoring, except the 'Refošk' 51 grafts with survival rate of 60 %.

The survival rate of grafted material could not be explained by different grafting treats, especially due to low number of survived 'Refošk' 43 and 61 autografts, higher number of survived 'Refošk' 38 and 48 autografts and very variable survival rate of 'Refošk' 20 and 51 autografts and grafts; therefore it could be assigned to random effect. In the case that grafts, putatively affected with rugose wood, show lower survival rate, they could be eliminated in early stages of planting material production in vine nursery.

Table 2: Graft success and number of 'Refošk' autografts and grafts on SO4 rootstock at final examination. 'Refošk' vines used for green grafting were from collection vineyard in Komen, Slovenia.

	Autografted 'Refošk' vines					'Refošk' grafted on SO4				
'Refošk' vine	Total number of autografts (12th June 2009)	Successfully rooted autografts at the time of transplanting (17th August 2009)		3rd September 2012		Total number of grafts (12th June 2009)	Successfully rooted grafts at the time of transplanting (17th August 2009)		3rd September 2012	
		n	%	n	%		n	%	n	%
'Refošk' 20 IV/110	11	10	90,9	7	63,6	12	11	91,7	3	25,0
'Refošk' 38 VIII/44	10	9	90,0	7	70,0	10	7	70,0	3	30,0
'Refošk' 43 VIII/113	10	9	90,0	4	40,0	10	8	80,0	4	40,0
'Refošk' 48 IX/43	10	10	100,0	8	80,0	8	7	87,5	3	37,5
'Refošk' 51 IX/69	11	8	72,7	3	27,3	10	9	90,0	6	60,0
'Refošk' 61 XII/68	10	9	90,0	3	30,0	10	8	80,0	3	30,0
Total / Average (%)	62	55	88,9	32	51,8	60	50	83,3	22	36,7
SO4	10	7	70,0	4	40,0	10	8	80,0	3	30,0
Total / Average (%)	72	62	86,1	36	50,0	10	8	80,0	4	40,0

3.2 Expression of rugose wood symptoms on 'Refošk' 38 and 'Refošk' 48 autografts and grafts on SO4 rootstock

The first typical symptoms of rugose wood were observed on 'Refošk' 38 and 48 vines at last examination, i.e. 39 months after grafting. Fine grooving was observed on the scion part of 'Refošk' 38 and 48 vines grafted on SO4 rootstock, while no evident symptoms were seen on SO4 rootstock (Table 3 and Figure 1). When 'Refošk' 38 and 48 were autografted, the symptoms of rugose wood were observed on the scion part of 'Refošk' 38 autografts, while on 'Refošk' 48 autografts symptoms were observed on the scion and rootstock (Table 3 and Figure 1). According to the literature this is the first report of observed rugose wood symptoms on autografts.

Appearance of rugose wood symptoms on grafts and autografts of 'Refošk' 38 and 'Refošk' 48 confirmed that rugose wood is transmitted by grafting. Since the symptoms were observed on autografts then the effect of incompatibility in the development of rugose wood disease could be eliminated. Possible reasons could therefore be reaction of plant as response to stress and wound healing or synergistic effect of stress and pathogen agents. The low number of 'Refošk' 38 and 'Refošk' 48 grafts and autografts with symptoms could be explained in two ways: a) in the case that pathogen agents causing the development of rugose wood are viruses, then it is possible that they were not already transmitted to green canes. It is known that viruses are unevenly distributed throughout the canopy (Fiore et al., 2009; Rowhani and Uyemoto, 1997); b) the second reason is possible latency of symptoms as reported by (Credi (1997a); Martelli, 1993). It should be also taken into consideration that symptoms could become visible if plants would be left in green house for additional one year or if different growing conditions were applied.

Although green-grafting was used, more than three years were needed in order to symptoms of rugose wood became visible what is similar to graft indexing with chip budding method (Credi, 1997b; Martelli, 1993).

Table 3: The results of graft indexing and the results of vineyard monitoring for rugose wood symptoms.

'Refošk' vine	Symptoms observed in collection vineyard		Graft indexing results	
	'Refošk' (scion)	SO4 (rootstock)	Appearance of rugose wood symptoms on autografts	Appearance of rugose wood symptoms on 'Refošk'/SO4 grafts
'Refošk' 20 IV/110	-	+	-/-	-/-
'Refošk' 38 VIII/44	+	-	+/- (2/7)	+/- (1/3)
'Refošk' 43 VIII/113	-	-	-/-	-/-
'Refošk' 48 IX/43	+	-	+/+ (2/8)	+/- (1/3)
'Refošk' 51 IX/69	-	+	-/-	-/-
'Refošk' 61 XII/68	-	-	-/-	-/-

- minus or plus before slash indicates absence or presence of rugose wood symptoms on scion, while sign after slash indicates absence or presence of symptoms on rootstock
- numbers in parenthesis indicate the number of autografts or grafts, out of total number of viable vines observed at last monitoring

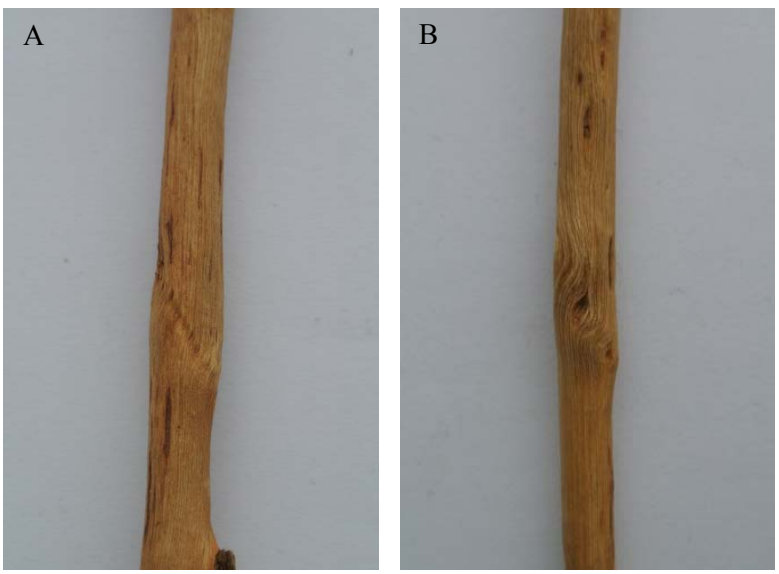

Figure 1: 'Refošk' 48 autograft showing fine grooving on rootstock and on scion part (A); 'Refošk' 38 grafted on SO4 rootstock (B). Symptoms of rugose wood are visible on scion part.

3.3 Symptomless 'Refošk' 20 and 'Refošk' 51 autografts and grafts on SO4 rootstock

The rugose wood symptoms were not detected on 'Refošk' 20 and 51 grafs and autografts (Table 3). Therefore, we were not able to confirm transmittance of rugose wood on SO4 with 'Refošk' 20 and 'Refošk' 51. We proposed that the source of rugose wood should be 'Refošk' vines since infection of rootstock in collection vineyard could be eliminated because rugose wood is present only in specific biotypes and not randomly across vineyard as would be expected in case of the infected rootstock. The possibility of symptomless SO4 could be that a different SO4 clone was used for grafting than the one used in the collection vineyard. Differential sensitivity of clones was observed for example in 'Syrah' clones for Syrah decline, which is also a disease causing

degeneration of the woody cylinder (Renault-Spilmont et al., 2007).

As hypothesized by Credi (1997b) development of rugose wood is dependent also on abiotic factors, as in the case of leafroll where the choice of indicator is based on climatic conditions (EPPO, 2008). For optimum symptoms expression in green grafting effects of temperature and light conditions could be optimized (Walter et al., 1990). This could also improve the development of symptoms in 'Refošk' 38 and 'Refošk' 48 grafts and autografts. However, it is possible, that symptoms would become expressed in the green grafts after one additional year. Walter et al. (1990) did not manage to detect stem pitting disease symptoms on indicators Kober 5BB and *V. rupestris* a few months after green grafting and concluded that perhaps a longer incubation period is required for classic symptoms to develop.

4 CONCLUSIONS

With this experiment we were able to confirm graft-transmissibility of rugose wood. However, graft success of grafts with vines showing rugose wood symptoms in the collection vineyard was not affected, which means that they are not excluded in the process of planting material production. The appearance of rugose wood symptoms on autografts supports the statement that effect of incompatibility is not involved in rugose wood. On the other hand stress caused by grafting could have notably impact on the development of symptoms what makes rugose wood even more complex. Proper growing conditions of graft indexing trials should, therefore, be defined in order to maximize the expression of rugose wood symptoms.

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Influence of chemical and organic fertilizer on growth, yield and essential oil of dragonhead (*Dracocephalum moldavica* L.) plant

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ABSTRACT

Two field experiments were carried out to study the response of *Dracocephalum moldavica* L. to NPK fertilizer and different application techniques of MOG organic fertilizer in two regions of Iran (Piranshahr with cold Mediterranean climate and clay loam soil, Maragheh with cool sub-humid temperate climate and sandy loam soil). MOG is bio-organic fertilizer with plant origin and contains different natural enzymes and amino acids. In current study following treatments have been applied: NPK (a complete NPK 20-20-20, 90 kg fertilizer ha^{-1}); MOG_1 (soil application of MOG organic fertilizer at sowing stage); MOG_2 (foliar application of MOG organic fertilizer at early stage of flowering); MOG_3 (soil application of MOG organic fertilizer at sowing and at 5 to 6 leaf stage); MOG_4 (soil application of MOG organic fertilizer at sowing and at 5 to 6 leaf stage with foliar application at early stage of flowering). Results indicated that all MOG treatments overcome the chemical fertilizers in both locations. However, plants grown in Piranshahr were more responsive to MOG fertilizer treatments than those grown in Mragheh. Overall, it could be concluded that utilization of MOG fertilizer as both soil and foliar application (MOG_4) may increase content and yield of essential oil, which could be suggested as a suitable alternative for chemical fertilizers.

Key words: dry herbage, essential oil yield, flower, Moldavian balm, vegetative growth

IZVLEČEK

VPLIV MINERALNIH IN ORGANSKIH GNOJIL NA RAST, PRIDELEK IN VSEBNOST ETERIČNIH OLJ KAČJEGLAVKE (*Dracocephalum moldavica* L.)

V dveh poljskih poskusih je bil preučevan odziv kačjeglavke (*Dracocephalum moldavica* L.) na gnojenje z NPK in različne tehnike uporabe MOG organskih gnojil na dveh območjih Irana (Piranshahr, s hladnim mediteranskim podnebjem in glineno-ilovnatimi tlemi, Maragheh s hladnim, semi humidnim zmernim podnebjem in peščeno-ilovnatimi tlemi). MOG je biološko gnojilo rastlinskega izvora, ki vsebuje številne naravne encime in amino kisline. V tej raziskavi so bili uporabljeni naslednji tretmani: NPK (NPK 20-20-20, 90 kg gnojila ha^{-1}); MOG_1 (talna aplikacija MOG organskega gnojila ob setvi); MOG_2 (foliarna aplikacija MOG organskega gnojilav zgodnji fazi cvetenja); MOG_3 (talna aplikacija MOG organskega gnojila ob setvi in v fazi 5 do 6 lista); MOG_4 (talna aplikacija MOG organskega gnojila ob setvi in v fazi 5 do 6 lista s foliarno aplikacijo ob začetku cvetenja). Rezultati so pokazali,da so dala vsa obravnavanja z MOG boljše rezultate kot mineralna gnojila na obeh lokacijah. Rastline z območja Piranshahr so bile bolj odzivne na MOG gnojenje kot tiste z območja Maragheh. Zaključimo lahko, da uporaba MOG gnojil, tako talna kot foliarna lahko poveča vsebnost in pridelek eteričnih olj in bi se lahko priporočila kot primerna alternativa gnojenju z mineralnimi gnojili.

Ključne besede: suha zel, pridelek eteričnih olj, cvet, kačjeglavka, vegetativna rast

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1 INTRODUCTION

Dragonhead or Moldavian balm (*Dracocephalum moldavica*) is an annual herbaceous aromatic plant belonging to family of Lamiaceae (El-Baky and El-Baroty, 2007). It is native to central Asia and to eastern and central Europe (Griffiths, 1994). Dragonhead is commonly consumed as a food-related product and as a herbal preparation because of its reputed medicinal properties. In some parts of Iran, distilled aqueous extracts from *D. moldavica* is used as a beverage (Dmitruk & Weryszko-Chmielewska, 2010; Rechinger, 1986). The oil content and its composition showed high variation due to the plant origin (Hussein *et. al*, 2006). In Iran, it distributed in the north and northwestern parts of the country, especially in the western parts of Azerbaijan province, and in the Albourz Mountains (Dastmalchi et al., 2007; Dmitruk & Weryszko-Chmielewska, 2010). The seeds of Moldavian Balm have astringent, carminative and tonic properties. They are used as a demulcent in the treatment of fevers (Dastmalchi et al., 2007). Furthermore, the plant is astringent, tonic and vulnerary (Rechinger, 1986) and has antitumor properties (Chachoyan & Oganesyan, 1996).

Plant nutrition is one of the most important factors affecting quantity and quality of secondary metabolites in plants. In order to meet the ever-increasing demand of medicinal plants need to be identified the best fertilizer application strategies. It is apparent that essential oil content enhance with increasing plant age to reach the maximum values at post flowering stage. The yield of plant fresh herb, the essential oil content and its composition can be influenced by growth stages, ecological and climatic conditions. Several attempts have been made to increase yield potential of medicinal plants (Das et al., 2007; Sharma and Kumar 2011), but they are concerned with use of inorganic fertilizers which may affect biological aspect of soil. Therefore, the use of organics and biofertilizers is gaining more importance for getting higher yield and quality.

Bio-fertilizer as an organic agro-input can promotes plant growth by increasing the supply or availability of macro and micro nutrients through the natural processes (Vessey 2003). Furthermore, bio-fertilizers can be expected to reduce the use of chemical fertilizers. One of the recently introduced organic fertilizer is MOG manufactured by using of some fruits juice and crop residues and contains 18 types enzymes (like as Alkaline Protease, Glucamylase, Lipase, Lipoxigenas, Nitrogenase…), natural form of micro and macro nutrients and vegetable based vitamins. However, the information on the role of MOG organic fertilizer on morphophysiological traits and chemical contents in Dragonhead is little. Hence, there is an urgent need to study the influence of biofertilizer on biochemical, quality and yield components in Dragonhead to boost the productivity potential. The present investigation was performed to study the effect of MOG organic fertilizer and chemical fertilizers on growth and productivity in *Dracocephalum moldavica* in two regions in northwest Iran.

2 MATERIALS AND METHODS

The experiments were conducted at two different locations. The first was agricultural research stations of Piranshahr, West Azarbayejan in the north-west of Iran (36° 40' N, 45° 08' E; 1840 m) with cold Mediterranean climate and a long-term mean air temperature of 17.8°C for the April until August period. In Piranshahr summers are almost dry but rest of year could be considered as wet seasons and soil texture of field was clay loam. Second location was Research farm of University of Maragheh, East Azarbayejan in the north-west of Iran (37° 24' N, 46° 16' E; 1477 m). Maragheh has cool sub-humid temperate climate with relative warm summers and the length of dry season is about 75 days. The soil texture of the field was sandy loam. Meteorological data during the crop growth period at both sites are presented in Table 1.

Table 1. Monthly temperature and precipitation during the growing season in 2012.

Month	Average temperature (°C)						Total precipitation (mm)	
	Minimum		Maximum		Mean			
	Piranshahr	Maragheh	Piranshahr	Maragheh	Piranshahr	Maragheh	Piranshahr	Maragheh
April	10.5	5.6	19.1	21.6	14.8	13.6	95.5	40.5
May	14.7	10.9	23.7	25.3	19.2	18.1	34.1	16.4
Jun	16.7	13.9	29.5	32.7	23.1	23.3	7.2	5.0
July	18.7	3.20	32.7	33.6	26.7	26.5	1.3	0.0
Augest	22.9	2.21	33.3	34.1	28.1	27.2	0.0	1.4

For both locations, composite soil samples were collected two weeks before planting, at a depth of 0–30 cm. The soil was air-dried and crushed before its pH, electrical conductivity (EC), and saturation percentage were evaluated. Then total organic carbon (using the Walkley and Black method, which involves sulphuric acid), total nitrogen (using the Kjeldahl method), available phosphorus (using the Olsen procedure), available potassium after extraction with ammonium acetate and Total Neutralizing Value were determined following the method as described by Jackson (1973) and Tandon (1995) were measured. Details of the soil properties of the both two locations are shown in Table 2.

Table 2: Soil physical and chemical properties in two locations.

Soil properties	Values	
	Piranshahr	Maragheh
Soil texture	clay loam	Sandy loam
Total N (%)	0.103	0.058
Available K (mg kg^{-1})	462	342
Available P (mg kg^{-1})	38.6	5.67
Organic carbon	2.08	0.41
pH	6.85	7.54
EC (ds m^{-1})	0.93	1.96
Total Neutralizing Value (*TNV*)%	49.6	34

The soil characteristics were determined according to Tandon (1995).

The experiment was performed in a randomized block design layout with three replications. Six fertilizer treatments were applied, consist on; Control= no application of fertilizers; NPK= a complete NPK 20-20-20, 90 kg fertilizer ha^{-1}; MOG_1= soil application at sowing stage; MOG_2= foliar application when first flowers was observable; MOG_3= soil application at sowing and at 5 to 6 leaf stage; MOG_4= soil application at sowing and at 5 to 6 leaf stage with foliar application when first flowers was observable. MOG organic fertilizer was provided from Azarabadegan Company, (West Azarbaijan, Iran). In all MOG treatments, organic fertilizer were utilized after dilution to 5% (v/v). The physicochemical properties of MOG organic fertilizer are shown in Table 3. *D. moldavica* seeds were obtained from local market of Bonab, Iran.

Table 3: Chemical characteristics of MOG organic fertilizer.

pH	Total organic carbon (%)	Total N (%)	K_2O (%)	P (%)	Fe (%)	Cu (%)	Enzymes (%)
6.1	25	4	4	1.06	0.42	0.16	13

Each experimental plot was 3 m long and 2 m wide with the spacing of 10 cm between the plants and 40 cm between the rows. There was a space of one meter between the plots and 2 meters between replications. Dragonhead seeds were directly sown by hand on 17 April 2012 in both locations. There was no incidence of pest or disease on dragonhead during the experiment. Weeding was done manually and the plots were irrigated weekly to 70% of field capacity. All necessary cultural practices and plant protection measures were followed uniformly for all the plots during the entire period of experimentation. Harvest time for all investigated traits except 1000-grain weight and harvest index was at 50% flowering. Fresh and dry weight plants were determined with digital weighing scales.

The plants were cut at ground level and samples of plants were dried in the shade and for extracting essential oils were used distillation with water practice and Clevenger device (Yousefzadeh et al., 2013). About 100 g of each dried sample (aerial parts) was separated, triturated and steam-hydro distilled for 2.5 hours. The extraction of oils was carried out according to method of European Pharmacopoeia (1983). The oils were dried over anhydrous sodium sulphate and stored in sealed vials at 2 °C before analysis.

Gas chromatography (GC) analysis was performed using a Thermo-UFM Ultra Fast gas chromatograph equipped with a DB-5 fused silica column (10 m × 0.1 mm i.d., film thickness 0.40 μm). The oven temperature was held at 60 °C for 3 min, and then programmed to increase to 280 °C at a rate of 80 °C min^{-1}. The temperatures of the injector and flame-ionisation detector were held at 285 °C. Helium was used as carrier gas with a linear velocity of 32 cm s^{-1}. The oils were injected manually into the GC instrument without dilution. The percentages of compounds were calculated by using the area normalisation method, without consideration of response factors (Davazdahemami et al., 2008).

Gas chromatography–mass spectroscopy (GC–MS) were carried out using a Varian 3400 GC–MS system equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm). Following injection, the oven temperature was increased from 50 to 240 °C at a rate of 4 °C min^{-1}, the temperature of the transfer line was maintained at 260 °C, and the linear velocity of the helium carrier gas was maintained at 31.5 cm s^{-1}, with a split ratio of 1:60, an ionisation energy of 70 eV, a scan time of 1 s, and a mass range of 40–300 amu. The components of the oils were identified by comparing their mass spectra with those held in a computer library or obtained using authentic compounds. The identities of the components were confirmed by comparing their retention indices, either with those of authentic compounds or with data published in the literature (Adams, 1995).

The statistical analysis including analysis of variance and the Least Significant Differences (LSD) among the means (at the 5% probability levels) were performed Snedecor and Cochran (1990).

3 RESULT AND DISCUSSION

Morphological traits

Plant height significantly influenced by fertilizer treatments and location (Table 4). Plant height comparison between the fertilizer treatments showed that the maximum value is related to plants received organic MOG_4 (90.92 cm), it was followed by organic MOG_2 (82.43 cm) and minimum of that was recorded for control plants (75.71cm). Moreover, the comparison of plant height between two locations revealed that *D. moldavica* plants cultivated in Piranshahr were 25% taller than those grown in Maragheh. The findings of the current study are consistent with those of Mafakheri et al. (2012) who found that concurrent application of organic fertilizer (vermicompost) could significantly improve the height of dragonhead. Numbers of the secondary branches were not affected by fertilizer treatments or by locations. Fertilizer application significantly affected the number of flower per plant, so that the highest number of flower was recorded in plants which experienced the MOG_2 and MOG_4. Furthermore, the number of flowers produced per plant significantly was different between locations, since the number of flowers in plants grown in

Piranshahr was 89% higher than those which grown in Maragheh.

The effects of fertilizer treatments on the chlorophyll content are shown in Table 4. The study showed that, regardless of location and type of fertilizer, the chlorophyll content for the control plants was about 28% lower than plants that received fertilizer. Also results revealed that nutrient source could considerably affect chlorophyll content, since the highest amount observed for MOG_4. These findings confirmed the earlier suggestion that N and Mg can be released by organic fertilizer and then incorporated to porphyrin rings of chlorophyll molecules (Amujoyegbe et al., 2007). Thus, it seems that the higher level of N and Mg could result in developed site of photosynthesis and enhanced plant growth.

Fertilizer treatments had significant effects on the 1000-grain weight and harvest index at 5% probability level. In both locations, the lowest harvest index was recorded in control plants and those grown with chemical fertilizers. The highest 1000-grain weight was observed in plants that received organic MOG fertilizer as both soil and foliar application (Table 4). Obtained results agreed with those of Rahimzadeh et al. (2011) and Mafakheri et al. (2012), they reported that, organic and bio fertilizers are rich and slow release fertilizers which usage leads to stimulate and increase of both vegetative and reproductive growth. Khalid et al. (2006) reported that applying liquid compost improved vegetative growth and reproductive characters of sweet basil (*Ocimum basilicum* L.) plants.

Dry herbage yield

Result revealed that dry herbage yield of dragonhead plants grown in Piranshahr was significantly higher (41%) than Maragheh. In addition, significant differences observed in dry herbage yield among fertilizer treatments (Table 4). In both locations, the MOG_4 treatment gave the highest dry herbage yield (7947 and 6127 kg ha^{-1} in Piranshahr and Maragheh, respectively). In addition, control plants produced the lowest dry herbage yields, with 3942 kg ha^{-1} in Piranshahr and 3206 kg ha^{-1} in Maragheh. Differences between locations can be attributed to soil and climatic conditions. It seems that environmental circumstances in Piranshahr were quite suitable for dragonhead plants. These results are in agreement with those obtained by Abdelaziz et al. (2007) on *Rosmarinus officinalis* and Rahimzadeh et al. (2011) on *Dracocephalum moldavica*. In this respect, it is possible that the favourable effect of organic fertilizer on dry herbage yield will be due to their ability to enhance the physiological, biochemical, and biological properties of the soil.

Essential oil content

The results showed that essential oil content affected by the location and fertilization treatments, and the interaction between both factors (Table 4). Mean comparison between fertilization treatments revealed that the highest essential oil content (0.77%) there is in plants grown in Piranshahr and received MOG organic fertilizer through both soil and leaves (MOG_4). This trend was also observed in Maragheh with a slower rate (0.49%). Essential oil content of the plant grown in Piranshahr averagely was 36% higher than those grown in Maragheh. Increases in the percentage oil content following the application of bio and organic fertilizer were observed in medicinal pumpkin (Habibi et al., 2011), *Rosmarinus officinalis* L. (Abdelaziz et al., 2007), dragonhead (Mafakheri et al., 2012; Yousefzadeh et al., 2013). For optimal plant growth, nutrients must be available in adequate and reasonable quantities (Chen, 2006). Intensive agriculture that emphasize heavy chemical application is led to adverse environmental, ecological; and health consequences (Habibi et al., 2011). One of the promising options to reduce the use of chemical fertilizers could be utilization of bio and organic fertilizers. Soils of arid and semi-arid regions often have low organic matter and need organic amendments to recover their characteristics and consequently their productivity and natural fertility. Addition of organic matter, from different resources may through improving physical and chemical properties of soil can affects the growth and development of plant roots and shoots and accumulation of essential oils (Elashry et al., 2008). Our results showed that the main effects of fertilizer treatments and location on yields of essential oils were significant. For plants grown in both locations, the maximum yields of essential oil were recorded in MOG_4 and MOG_2 treatments. The evaluation of the yields of essential oils between locations showed that yields of plants grown in Piranshahr (19.62 kg ha^{-1}) were 21%

higher than those grown in Maragheh (16.27 kg ha^{-1}). In general, provide the required elements for growth increases the yield of essential oil in medicinal and aromatic plants by increasing photosynthesis, chlorophyll content, and Rubisco activity, biomass yield, plant growth, and leaf surface area (Ram et al., 2003; Sekeroglu and Ozguven, 2006; Sifola and Barbieri, 2006).

Table 4: Effects of Fertilizer treatments on some traits of dragonhead plants in two locations.

Location	Fertilizer Treatments (FT)	Plant height	Number of secondary branches	number of flower	chlorophyll content	RWC	Dry herbage yield	Essential oil content	Essential oil yield	1000-grain weight	Harvest index
Piranshahr	Control	80.67^{c}	12.83^{a}	69.40^{fg}	38.49^{f}	74.60^{abc}	3942^{ef}	0.464^{cde}	11.02^{fg}	1.830^{c}	19.02^{e}
	N.P.K	88.54^{bc}	16.33^{a}	124.67^{cde}	43.42^{cde}	68.74^{bc}	5998^{bcd}	0.436^{def}	14.63^{ef}	1.862^{bc}	19.78^{e}
	MOG_1	93.07^{ab}	14.29^{a}	175.92^{bc}	46.66^{bc}	71.26^{bc}	6679^{abc}	0.409^{ef}	13.36^{efg}	1.820^{c}	21.30^{abc}
	MOG_2	96.53^{ab}	15.26^{a}	200.39^{ab}	46.61^{bc}	73.83^{abc}	7312^{ab}	0.664^{b}	24.76^{bc}	1.873^{c}	20.71^{abc}
	MOG_3	91.53^{b}	13.14^{a}	156.5^{bcd}	48.53^{b}	81.55^{a}	5946^{bcd}	0.645^{b}	20.64^{cd}	1.867^{c}	20.30^{bc}
	MOG_4	101.62^{a}	17.07^{a}	260.73^{a}	53.77^{a}	75.06^{abc}	7947^{a}	0.771^{a}	33.46^{a}	1.983^{a}	22.89^{ab}
Maragheh	Control	66.50^{d}	13.33^{a}	37.26^{g}	39.04^{ef}	71.02^{bc}	3206^{ef}	0.422^{d}	9.79^{g}	1.787^{d}	19.25^{e}
	N.P.K	70.83^{d}	14.40^{a}	62.0^{fg}	40.59^{cd}	67.21^{c}	3757^{e}	0.401^{fg}	11.74^{fg}	1.853^{bc}	19.46^{e}
	MOG_1	69.00^{d}	16.30^{a}	91.64^{efg}	43.70^{def}	69.06^{bc}	4087^{e}	0.445^{def}	12.04^{fg}	1.830^{c}	20.93^{abc}
	MOG_2	67.17^{d}	14.67	108.43^{def}	42.01^{b}	70.02^{bc}	4932^{cde}	0.366^{gh}	20.13^{d}	1.820^{bc}	20.70^{abc}
	MOG_3	69.16^{d}	13.33^{a}	86.27^{efg}	48.73^{b}	76.44^{ab}	4602de	0.357^{h}	17.48^{de}	1.824^{bc}	20.86^{abc}
	MOG_4	80.33^{c}	15.07^{a}	138.56^{bcd}	54.62^{a}	75.42^{abc}	6127^{bc}	0.494^{c}	26.48^{b}	1.907^{ab}	23.12^{abc}
LSD		9.42	4.48	63.86	4.43	8.62	1776	0.041	4.33	0.093	2.78
L		**	ns	*	ns	ns	*	**	*	ns	ns
FT		**	ns	**	**	*	**	**	**	*	*
L× FT		ns	ns	ns	ns	ns	ns	**	ns	ns	ns

Values are given as means of three replicates. Means within each column followed by the same letter are not statistically different at $\alpha = 0.05$ by LSD test.

Fertilizer treatments: MOG_1: soil application at sowing stage, MOG_2: foliar application when first flowers were observable, MOG_3: soil application at sowing and at 5 to 6 leaf stage, MOG_4: soil application at sowing and at 5 to 6 leaf stage with foliar application when first flowers were observable.

*Indicate significance at P level of 0.05.

**Indicate significance at P level of 0.01.

Composition of essential oils

The composition of the essential oil with different treatments in both locations was studied (Table 5). Both GC and GC–MS analyses revealed that the major constituents of the oil that was extracted from all six fertilizer treatments in both locations were geraniol, geranial, and geranyl acetate (Tables 5). 3 mentioned compound represented 73.82-91.25% of total detected constituents with different treatments. The unknown compounds representing 0.82-19.53% of total detected constituents. Other reserchers have also reported that the major constituents of essential oils extracted from dragonhead plants are geraniol, geranial, and geranyl acetate (Davazdahemami et al., 2008; Yousefzadeh et al., 2013). However, some investigators have reported inconsistent results, which show that either citral (Nikitina et al., 2008) or linalool (Hussein et al., 2006) are the major constituents in oil from dragonhead plants. It appears that a range of contemplations, including climatic condition, geographic origin, ecological factors, genetic differences, and agricultural practices, could affect the composition of essential oil extracts from medicinal and aromatic plants (Argyropoulou et al., 2007).

Geraniol ($C_{10}H_{18}O$) is a monoterpenoid and an alcohol. The functional group based on geraniol (in essence, geraniol lacking the terminal -OH) is called geranyl. It is important in biosynthesis of other terpenes. It is a by-product of the metabolism of sorbate. The content of this component was reduced in plants that received MOG organic fertilizer as both soil and foliar applications (MOG_4) in comparison with control. Although MOG_4 may be able to increase growth and result in high dry matter production, it contains modest essential oils, which express as dilution effect hypothesis. In plant treated with MOG_3 a significant difference was observed between two locations, since geraniol content in plant grown in Piranshah was two-times more than Maragheh. This is in line with the assertion of Yousefzadeh et

al. (2013) that plants differ in their response to changing soil fertility and environmental conditions.

Geranial (3,7-dimethyl-2,6-octadienal) is a pair of terpenoids with the molecular formula $C_{10}H_{16}O$. The two compounds are double bond isomers. The *E*-isomer is known as geranial or citral. It also has strong antimicrobial qualities and pheromonal effects in insects (Onawunmi, 1989). The application of chemical fertilizer (N.P.K) induced a slight increase in geranial content. Mean comparison of the locations revealed that plants grown in Piranshahr had a higher content of this compound. Although, the application of MOG organic fertilizer in some case reduced the percentage of essential oils, but it increased in growth and yields of essential oils can compensate the previous loss. Geranyl acetate (3,7-Dimethyl-2,6-octadiene acetate; $C_{12}H_{20}O_2$) is a natural organic compound that is classified as a monoterpene. Geranyl acetate is a natural constituent of more than 60 essential oils, including in different vegetables. Geranyl acetate and p-cymene also presented some antioxidant effects. Comparison of this organic compound between the locations showed that plants grown in Maragheh had a higher content of Geranyl acetate. The highest percentage of this compound was recorded in plant grown in Maragheh and treated with MOG_3.

Table 5: Essential oil composition of *Dracocephalum moldavica* L. influenced by different fertilizer regimes in two locations.

Fertilizer Treatments		**Control**[a]		**N.P.K**		**MOG_1**		**MOG_2**		**MOG_3**		**MOG_4**	
compound	RI[b]	*Pira*[c].	*Mara.*[d]	*Pira.*	*Mara.*	*Pira.*	*Mara.*	*Pira.*	*Mara.*	*Pira.*	*Mara.*	*Pira.*	*Mara.*
Sabinene	977	0.17	0.30	0.23	0.35	0.26	0.18	0.22	0.24	0.24	0.20	0.12	0.29
β-Pinene	989	1.77	1.30	1.93	1.82	1.29	0.89	2.24	1.62	2.13	0.87	2.03	1.26
(E)-β-ocimene	1041	0.24	0.13	0.21	0.22	0.22	0.20	0.26	0.17	0.24	0.14	0.29	0.16
γ-terpinene	1056	0.89	0.51	0.85	0.819	0.18	0.74	0.98	0.65	0.92	0.45	1.11	0.31
Linalool	1109	0.60	0.98	0.76	0.98	0.78	1.12	0.85	0.89	0.82	1.00	0.65	0.85
cis Limonene oxide	1167	1.10	0.87	0.99	0.92	0.62	0.64	1.05	0.92	1.11	0.53	1.05	0.53
Citronellal	1171	0.33	0.27	0.39	0.26	0.19	0.20	0.31	0.28	0.38	0.15	0.37	0.21
Trancelimonene oxide	1180	1.73	1.38	1.73	1.50	0.93	1.00	1.67	1.43	1.69	0.83	3.03	0.75
Neral	1245	0.25	0.38	0.32	0.32	0.37	0.37	0.33	0.38	0.36	0.38	0.22	0.36
Geraniol	1267	34.18	36.03	35.14	34.06	37.93	33.23	35.71	34.37	36.28	16.81	30.84	32.11
geraNial	1289	30.91	27.77	33.92	28.01	30.05	26.78	27.84	27.32	29.34	24.60	29.32	25.82
Neryl acetate	1361	1.52	1.78	1.43	2.02	1.61	2.35	1.46	2.06	1.36	2.14	1.50	3.66
Geranyl acetate	1376	25.43	27.45	21.04	27.88	23.16	30.17	26.19	28.80	24.21	32.43	28.43	29.03
E-Caryophyllene	1482	0.23	0.20	0.24	0.21	0.14	0.10	0.24	0.25	0.26	0.14	0.24	1.45
Total		99.35	99.35	99.18	99.369	97.73	97.97	99.35	99.38	99.34	80.67	99.2	96.79

[a] Fertilizer treatments: MOG_1: soil application at sowing stage, MOG_2: foliar application when first flowers were observable, MOG_3: soil application at sowing and at 5 to 6 leaf stage, MOG_4: soil application at sowing and at 5 to 6 leaf stage with foliar application when first.
[b]RI, retention indices in elution order from DB-5 column.
[c]The fist location: Piranshahr.
[d] The second location Maragheh.

4 CONCLUSION

Our results suggested that using liquid MOG organic fertilizer as both soil and foliar applications could result in better vegetative and reproductive growth, dry herbage yield, and essential oil yield. The results confirm that application of MOG_4 fertilizer treatment provides an appropriate substitute to the use of chemical N.P.K fertilizers and can lead at the end to improving the productivity of this plant. Because organic fertilizer may improve the use efficiency of essential mineral elements and reduced the amount of chemical fertilizers application, also prevented the environment contamination from widespread application of chemical fertilizers. Results revealed that in region with cold Mediterranean climate the production of dragonhead plants will be more successful than cool sub-humid temperate climate.

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The impact of plasmid on regeneration and expression efficiencies of *gfp* gene in tobacco (*Nicotiana tabacum* L.)

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ABSTRACT

Tobacco leaf explants were transformed by bacteria *Agrobacterium tumefaciens* (*A. t.*) and plasmid pBIN mgfp5-ER, which has a single copy of the green fluorescent *gfp* gene and *A. t.*-pART27 2mgfp5-ER, which has two copies of the *gfp* gene. Both plasmids have a built-in selection *nptII* gene for resistance to the antibiotic kanamycin. The presence of the green fluorescent mGFP-ER protein was detected with the epifluorescent microscope in the individual cells 3 days after transformation with *A. t.*-pART27 2mgfp5-ER and after 6 days in cells transformed with *A.t.*-pBIN mgfp5-ER. After infection by *A. t.*-pART27 2mgfp5-ER, in most cases the regeneration was direct, without intermediate stages of callus and faster, as the first globular structures were formed 10–12 days after transformation and a 204 % regeneration was achieved, while the first globular structure, after infection with *A. t.*-pBIN mgfp5-ER, occurred after 18 days and formed more callus and the regeneration was only 78.4 %. The duplex PCR analysis, performed on all 149 resulting regenerants, confirmed the presence of fragments of length 650 bp specific to the selection *nptII* gene and length of 422 bp specific for *gfp* marker gene.

Key words: *Nicotiana tabacum,* marker gfp gene, selection nptII gene, transformation efficiencies, transgene exspression, DNA analysis

IZVLEČEK

VPLIV PLAZMIDA NA USPEŠNOST REGENERACIJE IN IZRAŽANJA *gfp* GENA V TOBAKU (*Nicotiana tabacum* L.)

Listne izsečke tobaka smo transformirali z bakterijo *Agrobacterium tumefaciens* (*A. t.*) in plazmidom pBIN mgfp5-ER, ki ima eno kopijo zeleno fluorescentnega *gfp* gena in *A. t.*-pART27 2mgfp5-ER, ki ima dve kopiji *gfp* gena. Oba plazmida imata vgrajen še selekcijski *nptII* gen za odpornost na antibiotik kanamicin. Prisotnost zeleno fluorescentnega mGFP-ER proteina smo z epifluorescentnim mikroskopom zasledili v posameznih celicah 3 dni po transformaciji z *A. t.*-pART27 2mgfp5-ER in po 6 dneh tudi v celicah transformiranih z *A. t.*-pBIN mgfp5-ER. Regeneracija je bila po okužbi *A. t.*-pART27 2mgfp5-ER v večini primerov direktna, brez vmesne faze kalusa in hitrejša, saj so prve globularne strukture nastale že 10–12 dni po transformaciji ter dosežena je bila 204 % regeneracija. Prve globularne strukture po okužbi z *A. t.*-pBIN mgfp5-ER so se pojavljale šele po 18 dneh, nastalo je več kalusa in regeneracija je bila nižja, samo78,4 %. Pri vseh 149 nastalih regenerantih smo z dupleks PCR analizo potrdili prisotnost fragmentov dolžine 650 bp, značilnih za selekcijski *nptII* gen in fragmentov dolžine 422 bp, značilnih za markerski *gfp* gen.

Ključne besede: *Nicotiana tabacum*, markerski *gfp* gen, selekcijski *nptII* gen, uspešnost transformacije, izražanje transgenov, DNA analiza

1 INTRODUCTION

Biotechnological techniques of genetic transformation represent an integral complement and an appealing alternative to conventional plant breeding methods, since they enable a relatively rapid introduction of desirable traits into selected cultivars. With the possibility to introduce foreign

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DNA into plant cells, it has become possible to modify the expression of plant endogenous genes or to introduce novel genes of agronomical importance. Genetic transformation has become useful in improving plant properties and for the detection of gene functions in plants (Rao *et al*., 2009).

An efficient plant regeneration system is an important prerequisite for a successful transformation procedure. Test or marker genes are genes whose gene product can be visually identified and is location determined. They enable quick identification of transformed tissues. Marker genes that can be detected by other means, such as taste or smell, can also be useful (Witty, 1989).

In most cases, only a small proportion of plant cells transform, so it is necessary to include a selection gene together with the desired gene, by which transformed cells can be distinguished from non-transformed ones. The best known fluorescent protein is the green fluorescent protein (GFP) from the jellyfish (*Aequorea victoria*) (Haseloff and Amos, 1995), which emits green fluorescence under illumination with long-wave UV light. The wild-type *gfp* gene was modified in such a way that it effectively reflects in plants and the spectral properties and fluorescence change and improve (Reichel *et al*., 1996; Haseloff *et al*., 1997).

Genes for the synthesis of fluorescent proteins have advantages over other marker genes because they can be visually detected in living cells without the use of invasive procedures using substrates and products that could diffuse within or between cells. Transformed cells, in which these genes express, can be identified shortly after the transformation and it can be determined whether they are dividing (Harper *et al*., 1999). Fluorescent proteins can also be used to monitor the destiny of transgenes introduced into cultivated plants and their impact on the environment (Stewart, 2005).

Tobacco (*Nicotiana tabacum* L.) has been shown to be a very suitable model plant for genetic transformation because it grows quickly and successfully in tissue culture. Regeneration from leaf explants is fast and efficient (Stolarz *et al*., 1991).

In this study, we monitored the influence of plasmid on regeneration and phenotypic expression of *gfp* fluorescent genes and selection *nptII* gene in tobacco.

2 MATERIAL AND METHODS

2.1 Plant material, plasmids and agrobacterium-mediated transformation

The leave explants of micropropagated tobacco variety Havana 38 were used for transformation with two plasmids. The commercial bacterium *A. t*. strain LBA4404 contains plasmid pBIN mgfp5-ER or plasmid pART27 2mgfp5-ER. Plasmid pBIN mgfp5-ER is a binary vector, it contains the marker green fluorescent *gfp* gene and the plant selection *nptII* gene for resistance to the amino glycoside antibiotic kanamycin for selection of transformed plant tissues. Plasmid pART27 2mgfp5-ER is a binary vector, which contains two repetitions of mgfp5-ER gene from the vector pBIN mgfp5-ER and the same selection *nptII* gene.

Transformation of tobacco leaves with *A. t*. was performed using a slightly modified method for transformation of leaves as suggested Horsch *et al*. (1985) and Fisher and Guiltinan (1995). Tobacco leaves were cut under sterile conditions to explants of about 1 cm^2. For plasmid pBIN mgfp5-ER 60 leaf explants were prepared and for plasmid pART27 2mgfp5-ER 50 explants.

Bacterial suspensions of *A. t*., with the appropriate plasmid included, were incubated and prepared for transformation and co-cultivated according to Oven and Luthar (2013). Then, the leaf explants were transferred onto selective *MSr* medium with the addition of [Fe-Na_2-EDTA 0.1 mg/l, thiamine 0.1 mg/l, 6-benzylaminopurine (BAP) 1.0 mg/l, 1-naphthaleneacetic acid (NAA) 0.1 mg/l, agar 8 g/l; pH 5.8] (Stolarz et al., 1991) without acetosyringone and with the addition of timentin 150 mg/l to prevent the growth of *A t*. bacteria and an appropriate selection antibiotic 300 mg/l of kanamycin for the selection of tobacco transformants after infection both with *A. t*.-pBIN

mgfp5-ER or *A. t.*-pART27 2mgfp5-ER. Explants were cultured in a growth chamber at a 16/8 hour photoperiod and at temperature of 24 ± 1 °C, illuminated with about 40 µmol/m^{2}s. After five weeks, the explants were transferred or sub-cultured on the appropriate fresh selective *MSr* medium. The resulting regenerants were transferred onto *MS* medium with the addition of the selection antibiotic kanamycin, without timentin. After five weeks, the regenerants that had successfully grown were transferred to the appropriate *MS* selective medium.

2.2 Expression of *gfp* gene

Expression of fluorescent marker genes in the explants was observed 3 and 6 days after infection and at the beginning of regeneration in the rising stages of pessaries or inception. Transformed tobacco explants were examined by epifluorescent microscope (Nikkon SMZ 1000) at 20× magnification and appropriate filters for the detection of the green fluorescence *gfp* gene. For the detection of green fluorescent protein mGFP5-ER (both of plasmids pBIN mgfp5-ER or pART27 2mgfp5-ER), which has an excitational maximum at 484 nm and emission maximum at 510 nm, a set of filters with EX 480/40 nm, DM 505 nm and BA 535/50 nm was used.

2.3 Molecular analysis transgenes by PCR method and agarose gel electrophoresis

For determination of the presence of transgenes in 149 tobacco transformed regenerants and non-transformed – negative control, the complete DNA was isolated, according to the method of Kump *et al*. (1992).

The concentration of isolated DNA in solution was measured using a DNA fluorimeter DyNA Quant^{TM} 200 (GE Healthcare), according to the standard method of producer. DNA samples were diluted to 20 ng/µl.

Specific multiplication of *gfp* and *nptII* genes was carried out in duplex PCR reactions using two pairs of primers: GFP1a (forward: 5'-AGT GGA GAG GGT GAA GGT GAT G-3') / GFP1b (reverse: 5'-TTG TGG CGG GTC TTG AAG TTG G-3') and NPTII1a (forward: 5'-GAG GCT ATT CGG CTA TGA CTG-3') / NPTII1b (reverse: 5'-ATG GGG AGC GGC GAT ACC GTA-3'). In a total volume of 25 µl the PCR reaction mixture contained 5 µl of DNA and 20 µl of PCR mixture: 1×PCR buffer [10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40] (Fermentas), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 4×0.5 µM suitable primer and 0.5 units of enzyme Taq DNA polymerase (Fermentas) were added. The PCR reaction was carried out in a cyclical thermostat GeneAmp PCR System 9700 (PE Applied Biosystems, USA) using the modified temperature model (Lakshmi *et al*., 1998): initial denaturation of 5 min at 94 °C; 33 repeated cycles: denaturation of DNA 1 min at 94 °C, annealing of primers 1 min at 58 °C, synthesis of DNA fragments 1.5 min at 72 °C; final incubation 7 min at 72 °C; samples were stored at 12 °C until analysis amplified fragments by agarose gel electrophoresis.

For the separation of DNA fragments, horizontal electrophoresis was used on a 1.4 % gel according Oven and Luthar (2013).

3 RESULTS AND DISCUSSION

3.1 Regeneration of tobacco leaf explants and transgene expression

After three days of *A. t.*-pART27 2mgfp5-ER transformation, some cells expressed the mGFP5-ER protein at the leaf explants and after 6 days, the mGFP5-ER protein expression in the cells transformed with *A. t.*-pBIN mgfp5-ER (Figure 1).

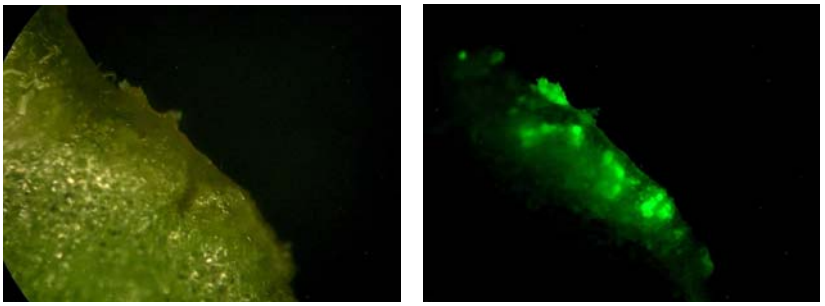

Figure 1: Observation of the mGFP5-ER protein expression after 6 days *A. t.* mediated transformation of tobacco examined under an epifluorescence microscope with white light (left) and with the special filter set for detection of green fluorescence (right)

Germs of the first regenerants occurred after 10-12 days after transformation with *A. t.*-pART27 2mgfp5-ER and after 18 days after transformation with *A. t.*-pBIN mgfp5-ER. After transformation *A. t.*-pART27 2mgfp5-ER the regeneration was mostly direct, without an intermediate callus (Figure 2), as noted by Stolarz *et al*. (1991). After transformation with *A. t.*-pBIN mgfp5-ER we obtained more callus and less regenerants.

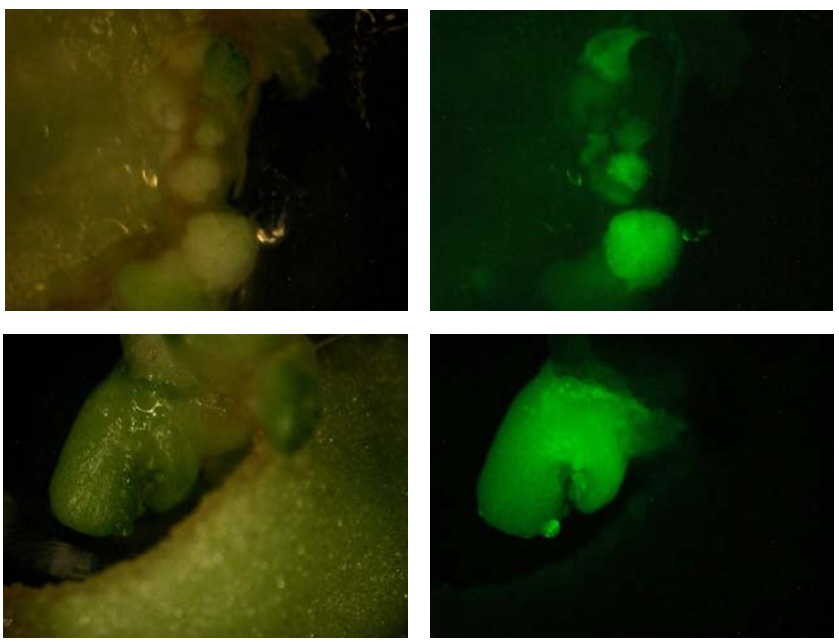

Figure 2: Observation of the mGFP5-ER protein expression in globules and regenerant after *A. t.* mediated transformation of tobacco examined under an epifluorescence microscope with white light (left) and with the special filter set for detection of green fluorescence (right)

After five weeks, a large number of regenerants was observed after transformation with *A. t.*-pART27 2mgfp5-ER and less regenerants after transformation with *A. t.*-pBIN mgfp5-ER. Regenerants from leaf explants, in which phenotypic expression of the inserted fluorescent genes was observed, were transferred onto *MS* medium with the addition of an appropriate selection antibiotic. After next five weeks we obtained new regenerants. In total, 102 regenerants were obtained from 50 explants after transformation with *A. t.*-pART27 2mgfp5-ER and less, only 47 regenerants from 60 explants after transformation with *A. t.*-pBIN mgfp5-ER.

The leaf explants after incubation with *A. t.* and an appropriate plasmid, were co-cultivated on *MSr* medium with added acetosyringone 100 µM, in

order to increase the infection, as described by Sunilkumar *et al*. (1999). In nature, phenolic substances such as acetosyringone, which are released on wounding of plant tissue, trigger the activation of genes for virulence (*vir* genes) in infection with *Agrobacterium* (Gelvin, 2003). We obtained a high percentage of transformed regenerants, which can be attributed to the acetosyringone attached to the *MSr* medium in the period of co-cultivation in the combination with plasmid pART27 2mgfp5-ER.

After the completion of co-cultivation, timentin 150 mg/l was added to the *MSr* medium, which effectively inhibited the growth of the *A. t*. bacteria but did not adversely affect regeneration. The regenerants on the medium with timentin were distinctly dark green. Nauerby *et al*. (1996) reported that timentin in this concentration completely prevented the multiplication of *A. t*. and positively impacted on the regeneration of leaf and cotyledon explants of tobacco. Similarly, Cheng *et al*. (1998) emphasized that timentin is just as effective as carbenicillin and cefotaxime and does not have an inhibitory effect on the regeneration of shoots in tobacco and Siberian elm.

3.2 Molecular analysis of transgenes integration

DNA analysis was performed on all 149 surviving regenerants.

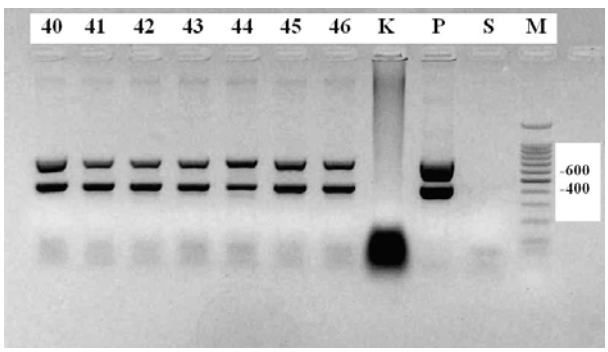

Figure 3: Amplified DNA fragments by duplex PCR with the specific set of primers for the *mgfp-ER* gene (422 bp) and the specific set of primers for the *nptII* gene (650 bp). The figure shows only the 7 regenerants of 149. 40 – 46: transformed tobacco regenerants; K: control, non–transformed tobacco; P: plasmid; S: blind samples; M: size standard.

In all 149 regenerants of tobacco transformed with *A. t*.-pBIN mgfp5-ER (47) and with *A. t*.-pART27 2mgfp5-ER (102) that were grown on selective medium, the presence of fragment length 650 bp (selection *nptII* gen) and fragment length 422 bp (marker *gfp* gene) was released (Figure 3). The transformation efficiencies achieved 204 % after the *A. t*.-pART27 2mgfp5-ER, and 78.4 % after the *A. t*.-pBIN mgfp5-ER-mediated transformation.

4 CONCLUSION

As a result of transformation with *A. t*.-pART27 2mgfp5-ER the regeneration capacity was faster and efficient, mostly direct, without an intermediate callus, while after transformation with *A. t*.-pBIN mgfp5-ER more callus and 2.6 times less regenerants were obtained.

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The effect of land use on phosphorus dynamics in golf course soil

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ABSTRACT

Although, it is usually considered that P applied in fertilizers is taken up by crops or immobilized in the soil, and therefore P losses from agro systems is negligible; recent research indicates that significant P leaching out of the root zone, can occur where certain combinations of land use practice, soil properties and climate condition exist. Therefore special attention was given to dynamics of total P (TP) and plant available P in golf course soils. A field study was carried out to assess how different environmental condition and management practices affect dynamics of TP and plant available P in soil. The proportions of plant available P and TP in the golf rough significantly correlated with precipitation. Since no relationship between precipitation and the P dynamics in soil on the greens and fairways was observed.

Key words: management practices, Technosols, greens, fairways, golf course, molybdate-reactive P, total P

IZVLEČEK

VPLIV RABE TAL NA DINAMIKO FOSFORJA V TLEH GOLFIŠČ

Res je, da se dodani P z gnojenjem porabi za prehrano rastlin ali pa se v procesu imobilizacije močno veže na talne delce, vendar so novejše raziskave pokazale, da se pri določeni kombinaciji rabe zemljišč, lastnosti tal in klimatskih pogojev tudi P lahko izpira v globlje plasti tal. Zaradi spoznanja novejših raziskav o izpiranju P v globlje plasti tal smo v naši raziskavi posebno pozornost namenili dinamiki celokupnega P in rastlinam dostopnega P v tleh igrišč za golf. Z terensko raziskavo smo želeli oceniti, kako različni okoljski dejavniki in raba tal vplivajo na dinamiko celokupnega P in rastlinam dostopnega P v tleh. Rezultati raziskave so pokazali, da obstaja značilna povezava med padavinami in vsebnostjo rastlinam dostopnega P ter celokupnega P v tleh ledine na igrišču za golf. Medtem ko, povezava med padavinami in dinamiko P v tleh zelenic in čistin igrišč za golf ni bila ugotovljena.

Ključne besede: tehnike upravljanja, tehnosoli, zelenice, čistine, golf igrišče, rastlinam dostopni P, celokupni P

1 INTRODUCTION

Golf courses often appear to be some of the most natural environments but, as in the case of well-ordered gardens, they are products of human activity, due to the large use of energy and water required in preparing and maintaining them. The mosaic of “greens” (turfed and carefully mown areas), “bunkers” (unvegetated sandy concave areas), thickets, ponds etc. is obtained through marked modification of the original soils or even their complete removal. The fertilizers required annually to keep greens and fairways, in good condition, can cause serious pollution of soil and waters (Suzuki et al., 1998; Certini and Scalenghe, 2006). Although, it is usually considered that P applied in fertilizers is taken up by crops or immobilized in the soil, and therefore P losses

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from agro systems is negligible; recent research indicates that significant P leaching out of the root zone, can occur where certain combinations of land use practice (over fertilization or excessive manuring), soil properties (sandy subsoil, high organic matter and the presence of preferential flow paths) and climate condition exist (Sims et al., 1998).

Globally, the creation of golf courses is currently one of the most rapidly expanding types of extensive land development (Tanner and Gange, 2005). In recent years, considerable interest in golf course development has also been expressed in the Balkan region, despite the minor golf tradition and low number of domestic golfers. It is therefore expected that a large number of new golf courses will be built over the next few years in the Balkan region. Currently, thirty-two golf courses cover roughly 12 km^2 in the Balkan region and their areas have been increasing in recent years (Petrova, 2005). Slovenia has the highest number of golf courses per capita in the Balkan region (Meglič, 2001).

Slovenia is an agricultural country with a total area of 20 722.77 km^2. The majority of the country is covered by forest (13 577.03 km^2) and agricultural land (5 387.92 km^2) (Yearbook Republic of Slovenia, 2007). Golf courses cover nearly 0.02 % or 3.50 km^2 of the country's total area (Skumavec and Šabić, 2005). A 9-hole golf course requires approximately 0.34 km^2 of land, which is seven times more than the average family farm (0.05 km^2) and six times less than the average area of a commercial agricultural operation (2.20 km^2) in Slovenia (Meglič, 2001). Golf course development in Slovenia typically replaces agricultural or native lands with intensively managed turf grass, and course construction involves modification of the original soil profile and land surface.

Many golf course studies have documented the transport of phosphorus (P) into the groundwater if excessive loading of fertilizer is applied to sandy soil with limited P sorption capacity (Shuman 2005; Shuman, 2003). In a lysimeter study of a golf course, P levels were higher in the leachate than the threshold value for surface water (0.3 mg l^{-1}) (Wong et al., 1998). In a greenhouse study with simulated golf greens, peak P concentrations in the leachate exceeded 20 mg l^{-1} (Shuman, 2001). Very few publications (Bartlett et al. 2008, Devitt et al., 2007, King et al., 2007 and McCoy and McCoy, 2009) have documented the effects of climatic conditions and land use on the P dynamics in golf course soils. The primary objective of this research was to monitor two golf courses (green, fairway and forest as part of golf rough to which no fertilizer or water for irrigation is applied) in Slovenia to assess how different environmental condition (i.e. precipitation) could affect management practices and dynamics of total P (TP) and plant available P in soil. The second objective of the study was to observe the interaction between precipitation and irrigation regimes and dynamics of TP and plant available P, because environmental condition and management practices (irrigation and fertilizer) could affect the TP and plant available P in soil.

2 MATERIALS AND METHODS

An intensive monitoring study was performed on two golf courses in Slovenia, from December 2005 to December 2006. The first golf course is located in Lipica (N: 45°40'34", E: 13°53'04", Height: 320 m.a.s.l., Holes: 9, Total surface: 40 ha), in typical Karst countryside with a Mediterranean climate. The second golf course is located in Bled (N: 46°22'18", E: 14°08'15", Height above sea level: 510 m, Holes: 27, Total surface: 100 ha), on the periphery of the Julian Alps, with a subalpine climate. The choices of location were based on the different soil conditions (Table 3).

During the study period, precipitation was regularly measured at both plots (Lipica and Bled). The amount of rainfall at both locations (1 024 l m^{-2} - Lipica and 947 l m^{-2} - Bled) was much lower than the 30-year historical average (1 420 l m^{-2} - Lipica and 1 400 l m^{-2} - Bled). The average monthly temperature ranges recorded were from 1.5 ºC (January) to 19.8 ºC (July) in Lipica and -2.2 ºC (January) to 18.1 ºC (July) in Bled.

The golf courses operated from March to November, when the bulk of the granular NPK fertilizers were applied. Different fertilization practices and different irrigation scheduling methods were used for each of the two golf courses (Table 1). In Lipica, more water is used for irrigation due to less precipitation and higher temperatures. The total amount of the water applied to the golf greens was 15 000 m^3 ha^{-1} y^{-1} in Lipica and 7000 m^3 ha^{-1} y^{-1} in Bled, while the average amount of water applied to agricultural land in Slovenia in the same period was 2220 m^3 ha^{-1} y^{-1} (Yearbook Republic of Slovenia, 2006).

Table 1. Phosphorus (kg ha^{-1}) and water (m^3 ha^{-1}) annual application rates for the golf courses – Lipica and Bled

Location	Land use	Area (ha)	Phosphorus application			Water application			
			Period of application	Application frequency	Annual application rate (kg ha^{-1})	Period of application	Application frequency	Annual application rate (m^3 ha^{-1})	Sum of annual application rate and rainfall (m^3 ha^{-1})
Lipica	Green	2	February - October	twice a month	17.5	April - October	twice a day	15 000	20 123
	Fairway	12	April - October	twice a year	28.3	April - October	once a day	1 250	6 373
Bled	Green	1.5	April - October	three times during growing season	50	June - September	twice a week	7 000	13 098
	Fairway	35	April August October	three times during growing season	70	June - September	twice a week	557	6 655

We monitored the concentration of plant available P (PO_4-P mg kg^{-1}) and TP (mg kg^{-1}) in the soils of three different land uses (golf green, golf fairway and forest as part of the rough). In order to assess the quantity of plant available P and TP in the soil, fresh soil samples were collected every two weeks, with some gaps, as seen in the results. Five soil samples from five randomly chosen sites from the topsoil (10 cm deep) from each type of land use (green, fairway and rough) at both locations (Lipica, Bled) were collected before the monitoring was performed. All samples were analyzed for their concentrations in plant available P and TP. The results obtained were subjected to statistical evaluation. Evaluated standard deviation (CV %) (Table 2) has been used for further statistical analysis. During the monitoring process, subsamples were randomly collected from the topsoil (10 cm deep) from each type of land use (green, fairway and rough) at both locations (Lipica, Bled) and mixed, to form one composite sample per land. Air-dried soil samples were analyzed using aqua regia extraction methods (SIST ISO 11466, 1995) to assess TP, and ammonium–lactate (AL) extracts, which is the most appropriate method due to the pH of the soil (5.7 - 7.2) (Olsen et al., 1954), to assess plant available P. AL extract was used with the photometric molybdenum blue method (Vajnberger, 1996; Hoffmann, 1991).

Table 2: The samples average concentration (mg kg^{-1}) with standard deviation (SDS) and coefficient of variation in brackets (CV %) of soil phosphorus concentrations (total phosphorus - TP and plant available P) (N=5)

	Lipica		**Bled**	
	plant available P	**TP**	**plant available P**	**TP**
Rough	6.1±2.1 (27.66 %)	920±88 (9.60 %)	4.2±1.7 (40.75%)	368±22 (6.23%)
Green	82.6±9.0 (10.88 %)	407±43 (10.63 %)	26.9±3.3 (12.24%)	115±4 (3.49%)
Fairway	10.8±5.8 (54.58 %)	635±120 (18.98 %)	11.4±2.9 (25.38%)	512±56 (11.09%)

Organic carbon (Table 3) was determined by sulfochromic oxidation without external heating and by titration with 0.5 M $FeSO_4$, following Walkley-Black's method (SIST ISO 14235, 1999) (Jackson, 1962). Soil texture was determined following the particle size limit classification of the U.S. Department of Agriculture (USDA) (Soil Survey Manual, 1993). The cation exchange capacity (CEC) was determined using NH_4OAc extraction methods (Peech et al., 1947). Total soil porosity was calculated from particle (Blake and Hartge, 1986a) and bulk density (Blake and Hartge, 1986b), which were measured using the pycnometer method (50 ml flask) and core method (100 cm^3 Kopecky cylinder), respectively. Soil pH was measured in a suspension of soil in 0.01 M $CaCl_2$ (SIST ISO 10390, 2005). Saturated hydraulic conductivity was determined by the standard method (SIST ISO 17313, 2006). Selected chemical and physical properties of the sampling point are shown in Table 3.

Organic matter concentration in these soils varied from 0.6 - 23.1 % with the highest values in the rough (Lipica 32.1 % and Bled 13.4 %) and the lowest in the green (Lipica 3.2 % and Bled 4.4 %). The pH values for all of the soils were lower than 7.1, except for the individual horizons (green - P2 – pH 7.1 and fairway - B1 – pH 7.3) at the Bled location. The CEC (cation exchange capacity) ranged from 7.3 $cmolc^+/kg$ (Bled – green) to 59.5 $cmolc^+/kg$ (Lipica – rough). In the green and fairway profile, soil porosity was lower than in the rough soil. The soil porosity ranged from 70 % to 76 % in rough soils and from 44 % to 59 % in a fairway and green soils. Those chemical and physical properties make possible the classification of the studied soils. According to the World Reference Base for Soil Resources 2006 (IUSS Working Group WRB, 2006), the studied soils were grouped into three different reference groups; Technosol, Leptosol and Phaeozem (Table 3).

Table 3: Soil characteristics on locations of the golf courses – Lipica and Bled

Location	Land use	Horizon	Depth (cm)	Organic carbon (%)	C:N ratio	Soil texture	CEC (cmolc kg^{-1})	Hydraulic conductivity (m day^{-1})	Plant available water (Vol %)	Soil porosity (%)	Soil pH	WRB Reference Group
Lipica	Green	P2	0-9	1.84	11.3	sand loam	12,8	7.62	5.35	44	6.6	Epileptic Technosol
		P1	0-9	2.41	11.8	silt clay	40.8	0.67	11.13	57	6.9	Protocambic Leptosol (Humic, Eutric)
	Fairway	Apg	9-16	3.96	13.0	silt clay	47.3	0.17	7.42	60	7.0	
		C(B)	16-26	3.56	12.8	silt clay	38.3	0.90	8.78	58	6.8	
	Rough	Ah	0-10	13.26	14.9	silt loam	59.5	89.86	4.71	70	6.5	Rendzic Leptosol
	Green	P1	0-5	2.53	19.2	sand	7.3	22.46	1.12	48	5.7	Technosol (Epiarenic)
		P2	5-28	0.34	15.0	sand	4.6	41.04	2.61	56	7.1	
	Fairway	A1	0-7	4.42	10.7	silt loam	27.6	3.54	8.32	44	6.9	Leptosol (Humic, Eutric)
		B1	7-17	2.76	11.7	loam	32.6	8.99	7.64	56	7.3	
Bled	Rough	A1	0-4	7.69	14.4	silt loam	33.9	27.91	5.6	76	6.0	Epileptic Rendzic Phaeozem (Protosiltic[a])
		B1	4-28	3.78	11.5	silt loam	24.7	15.25	10.6	72	6.0	

[a] Indicating an early stage of development of silt loam feature.

STATGRAF software was used for statistical analysis. Two-sided Pearson's correlations were used to test the relationship between the parameters studied. We used the t-test for dependent samples. The significance level was set at $P < 0.05$. Standard deviation (SDS) and coefficient of variation (CV %) were estimated by examining samples taken before the monitoring was performed.

3 RESULTS AND DISCUSSION

The variation in the quantities of TP and plant available P, which were estimated on five soil samples randomly collected from each of the three studied locations (greens, fairways and rough) at two golf courses (Lipica – 9 holes; 40 ha and Bled – 27 holes; 100 ha), can be attributed to a combination of factors including environmental conditions, vegetation cover and land management practices. Large impact on the quantities of TP and plant available P in different soil can be attributed also to soil sampling procedure. Five soil samples were collected each time but the collected soil samples were then bulked and the chemical analysis of TP and plant available P was conducted on the bulked soil samples. All sampling standard deviations during the P monitoring period were the same as the standard deviations of the five previous samples, collected before the P monitoring work started.

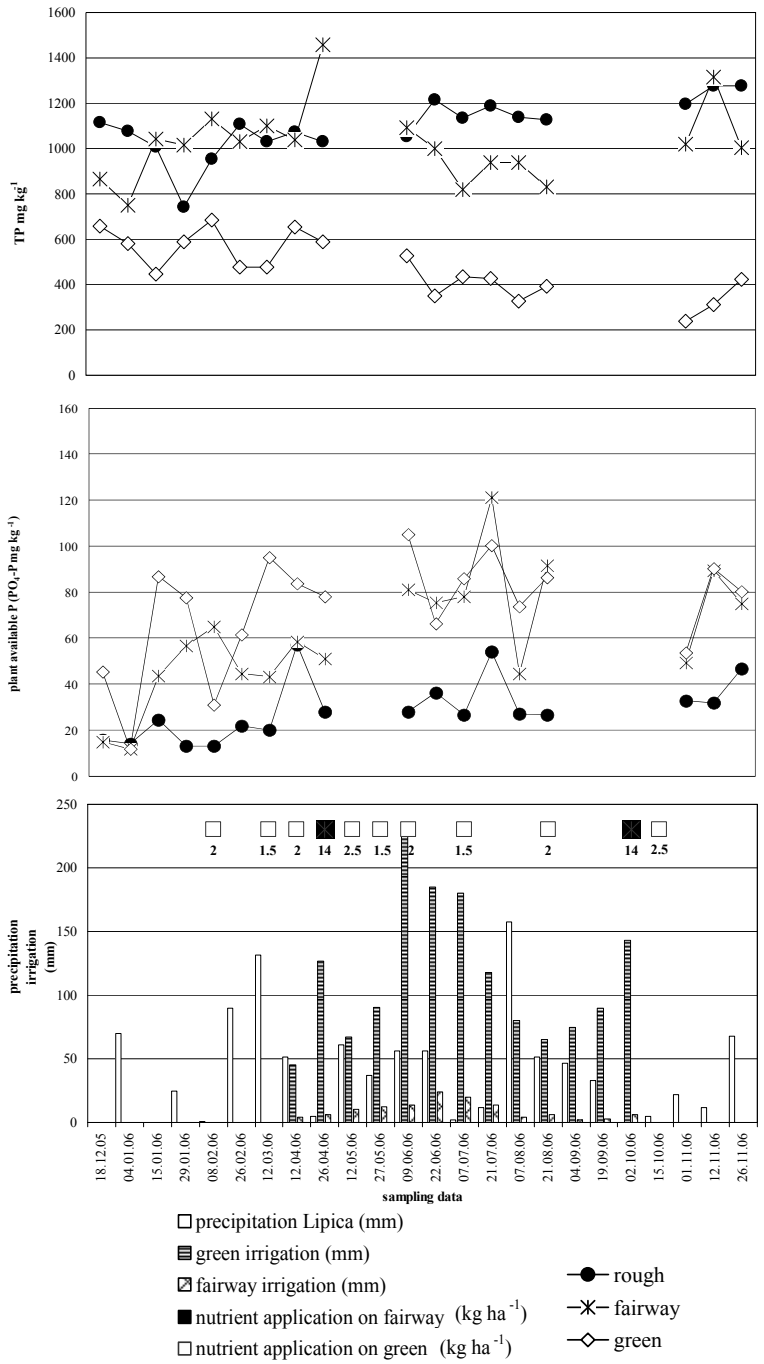
TP and plant available P concentrations

The dynamics of TP concentrations in the soil samples followed different patterns than those for plant available P. Green soils exhibited lower concentrations of TP (average concentration: Lipica - 476±128 mg kg^{-1}; Bled - 187±112 mg kg^{-1}) than rough soils (average concentration: Lipica - 1097±125 mg kg^{-1}; Bled - 740±187 mg kg^{-1}). In contrast, green soils contained a much higher concentration of plant available P (average concentration: Lipica - 73±24 PO_4-P mg kg^{-1}; Bled - 41±17 PO_4-P mg kg^{-1}) than rough soils (average concentration: Lipica - 29±14 PO_4-P mg kg^{-1}; Bled - 26±17 PO_4-P mg kg^{-1}) (Fig. 1 and 2). The results are comparable to a study by Leinweber et al. (1999), in which they found that higher mean concentration of the most labile forms of P tend to occur in arable soils, and more residual (stable) P in soil under fallow or reafforestation. Cultivation and the application of P fertilizers are responsible for the decline in stable P and the higher amount of labile P in the green soil in our study.

The differences in the concentrations of plant available P and TP between areas of rough and green soil mainly originate from the long-term inputs of plant residue and accumulation of organic matter in uncultivated rough areas and fertilizer application on golf greens. Organic matter is a substantial reservoir for P. The P is bound in phosphate esters, phospholipids and nucleic acids and is released into the soil solution when microbes break down organic matter (Tarafdar et al., 2001). Although the organic carbon concentration of green soils (Lipica: 1.84 %; Bled: 2.53 %) was lower than that of rough soils (Lipica: 13.26 %; Bled: 7.69 %), the plant available P concentrations of green soils (average concentration: Lipica - 73±24 PO_4-P mg kg^{-1}; Bled - 41±17 PO_4-P mg kg^{-1}) were higher than those of rough soils (average concentration: Lipica - 29±14 PO_4-P mg kg^{-1}; Bled - 26±17 PO_4-P mg kg^{-1}). The high concentration of organic carbon and low concentration of plant available P in rough soil might be partially due to the slow decomposition of plant litter and mineralization of organic matter. The mineralization of organic matter is the main source of available P for plants in a natural ecosystem (Srivastava and Singh, 1991). In contrast, the main source of plant available P in green soils are fertilizers, because the maintenance of high-quality turf grass on golf greens requires large P fertilizer inputs. According to the study results, organic matter concentration significantly influenced the level of the TP concentration in the rough soils and the high rates of fertilizer P applied to golf course is a possible cause of plant available P accumulation in the green soils.

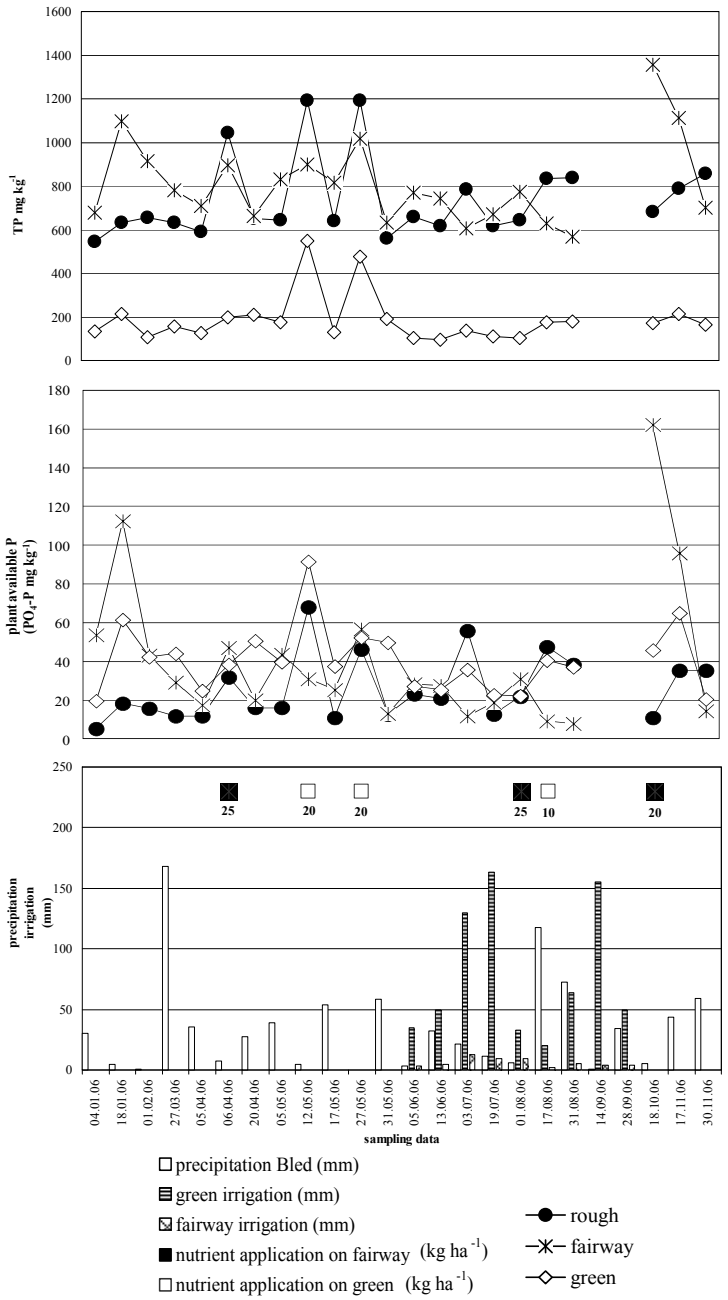
Plant available P concentrations in soil samples from the greens in Lipica (average concentration: 73±24 PO_4-P mg kg^{-1}) exceeded the concentration measured in soil samples from the fairways (average concentration: 61±27 PO_4-P mg kg^{-1}), presumably due to the low P adsorption capacity of sandy soils on the greens. In a greenhouse lysimeter study performed by Wong et al. (1998), the leachate from greens contained significantly higher plant available P than that of fairways. We observed a different pattern for TP concentrations. TP in green soil (average concentration: Lipica – 476±128 and Bled – 187±112 mg kg^{-1}) was significantly lower than in fairway soil (average concentration: Lipica – 1 021±170 and Bled – 811±194 mg kg^{-1}) and rough soil (average concentration: Lipica – 1 097±125 and Bled – 740±187 mg kg^{-1}). The higher TP concentrations in fairway soil are in contrast to the low level of TP in fairway soil measured in the aforementioned lysimeter study (Wong et al., 1998). A possible

reason for the difference is that fairway soil from the lysimeter study probably reflected the interaction between a high frequency of fertilization and low fertilizer application rates. This indicates that the fertilizer application rate on the studied fairways should be reduced and application frequency should change. Such management could be expected to be less harmful to the environment and to reduce the P loading in fairway soils.



Precipitation shown on the figures is the sum of rainfall between sampling dates.

Fig. 1. Dynamics of total phosphorus - TP (mg kg^{-1}) and plant available P concentrations (PO_4-P mg kg^{-1}) in soil samples from rough, green and fairway – Lipica



Precipitation shown on the figures is the sum of rainfall between sampling dates.

Fig. 2. Dynamics of total phosphorus - TP (mg kg^{-1}) and plant available P concentrations (PO_4-P mg kg^{-1}) in soil samples from rough, green and fairway – Bled

Impact of precipitation on P concentrations

It has been reported that climatic conditions also influence P dynamics in the soil (Ronggui, 2001). We found, a statistically significant correlation was found between precipitation, plant available P (Lipica r = 0.73; P = 0.005; Bled r = 0.67; P = 0.001) and TP (Bled r = 0.67; P = 0.001) in the soil of rough areas, except for the TP in the soil of the rough in Lipica (r = 0.15; P = 0.62) (Table 4). The concentrations of plant available P and TP in soil of the rough (except TP concentrations in soil of the rough in Lipica) markedly decreased with rainfall events. No correlations were observed among precipitation, plant available P and TP

dynamics in soil samples from the greens and fairways (Table 4). In addition to precipitation, the application of water also had an important influence on P dynamics in soil and is the governing factor in soil P consumption. In a greenhouse subsurface irrigation study, Wang and Zhang (2009) showed that the percentage of inorganic P, organic P and plant available P to total P in soil could be affected by irrigation schedules. This was confirmed by the findings of our study. A significant correlation was found for both plant available P and TP concentrations and the application of water on greens at Bled (plant available P: r = 0.89, P = 0.01; TP: r = 0.80, P = 0.05) and Lipica (plant available P: r = 0.73, P = 0.05; TP: r = 0.67, P = 0.01). No statistically significant relationship was observed between precipitation and the P dynamics in soil on the fairways (plant available P: Lipica r = 0.42, P = 0.29; Bled r = 0.71, P = 0.10; TP: Lipica r = 0.27, P = 0.51; Bled r = 0.59, P = 0.21). However, further studies should be done in order to assess how irrigation management practices enhance P availability for plants in green and fairway soil.

Table 4: Correlation between the sum of precipitation between two sampling dates and soil phosphorus concentrations (total phosphorus - TP and molybdate-reactive phosphorus – plant available P) on golf courses Lipica and Bled

	Lipica				Bled			
	plant available P		TP		plant available P		TP	
	Multiple correlation coefficient (*r*)	Significance level (*P*)	Multiple correlation coefficient (*r*)	Significance level (*P*)	Multiple correlation coefficient (*r*)	Significance level (*P*)	Multiple correlation coefficient (*r*)	Significance level (*P*)
Rough	0.73	0.0047 [b]	0.15	0.615	0.67	0.001 [b]	0.67	0.001 [b]
Green	0.41	0.11	0.06	0.92	0.27	0.24	0.48	0.03 [b]
Fairway	0.20	0.44	0.14	0.5	0.22	0.34	0.04	0.8

[b] Statistically significance value ($P < 0.05$)

Furthermore, a significant correlation was found between the TP and plant available P concentrations on the golf course in Bled. The TP concentration in soil samples from all the types of land use showed a similar temporal trend as plant available P (Fig. 2). The multiple correlation coefficients between TP and plant available P concentrations are: r = 0.77; P = 0.0001 for greens, r = 0.92; P = 0.0001 for fairways and r = 0.83; P = 0.0001 for roughs.

The dynamics of TP and plant available P in the rough Bled and Lipica reflected the low plant P uptake. Rapid P uptake occurs only with high soil moisture content and that uptake is proportional to the volume of soil brought close to field capacity and the length of time that it remained moist (Simpson and Pinkerton, 1989). On the other hand, the increased moisture content of soils may enhance microbial activity and, consequently, the mineralization of soil organic matter and plant available P. P enrichment in soil solution in topsoil could increase the risk of P leaching into deeper soil layers or even into the groundwater (Sims et al., 1998). This might indicate that the increase of TP and plant available P concentrations after rainfall is due to the leaching of large amounts of plant available P from the topsoil to lower soil layers and reduction in plant P uptake. Unfortunately, no soil sampling was done below the topsoil layer. Since soil water supply (i.e., irrigation) is one of the most important issues on the observed golf courses, it has an important

influence on P dynamics in the soil and is the governing factor in plant P uptake.

Impact of fertilization on P concentrations

In addition to soil moisture, the applied fertilizers also had a significant effect on P dynamics in the soil. Motavalli and Miles (2002) reported that the addition of fertilization, either in the form of fertilizers or manure, significantly influences the TP and plant available P in the agricultural soil. A similar P application effect on TP concentration was also observed in the golf course soil in Lipica and Bled (Fig. 1 and 2). A significant correlation was found between the TP concentrations and the application of P fertilizer on greens at Bled ($r = 0.77$; $P = 0.0002$) and fairways at Lipica ($r = 0.74$; $P = 0.001$). Although the TP concentrations increased greatly after the application of P fertilizer, no statistically significant relationship was observed between the quantity of applied P fertilizers and the level of plant available P and TP in soil samples from the greens and fairways. There were much higher concentration of plant available P and TP in the green and fairway soils in Lipica (Fig. 1) than in Bled (Fig. 2), despite that less fertilizer was applied to the golf course in Lipica. The average concentrations of plant available P (and TP) in soil from the greens and the fairways in Lipica were 73±24 and 61±27 PO_4-P mg kg^{-1}, respectively (TP: 476±128 and 1 022±170 mg kg^{-1}) while those in the soils from Bled were 41±17 and 41±38 PO_4-P mg kg^{-1}, respectively (TP: 187±112 and 811±194 mg kg^{-1}). The different level of soil P between the golf courses in Lipica and Bled is possibly due to their different management practices in the removal of plant biomass on the golf course and fertilizer – irrigation histories. Despite the different level of plant available P (Lipica green: 16,72 mgP_2O_5 100g^{-1}; Lipica fairway: 13,94 mgP_2O_5 100g^{-1}; Bled green: 9,32 mg$P_2O_5$100g^{-1}; Bled fairway: 9,38 mg $P_2O_5$100g^{-1}) and fertilizer management practices, AL-method placed the soil from golf course Lipica and Bled in B (6-12 mg$P_2O_5$100g^{-1}) and C (13-25 mg$P_2O_5$100g^{-1})P – supplying levels, which represent an medium-optimal supplied soil with P. The level of plant available P in the green and fairway soils in Lipica and Bled probably reflected the high uptake efficiency of turf grass and impact of biomass removal on soil nutrient. As described in Hladnik (2005), annual removal of phosphorus with the biomass of cut grass is 8-13kg/ha.

The impact of golf course management on P concentration

The soil samples from the green Lipica had a higher plant available P concentration (average value: 73±24 PO_4-P mg kg^{-1}) than those of fairway (average value: 61±27 PO_4-P mg kg^{-1}), while the plant available P concentrations from green and fairway Bled were uniform (green 41±17 and fairway 41±38 PO_4-P mg kg^{-1}). The low concentration of plant available P from the fairways Lipica indicated a high P absorption capacity of the fairway soil compared to the sandy soil of the greens. There is probably a potential for P leaching into groundwater and surface water (Wong et al., 1998). On the other hand, soil samples from the greens had a lower TP concentration than soil samples collected from the fairways (Fig. 1 and 2), which might be due to a low concentration of organic carbon in the soil of greens (Lipica: 1.84%, Bled: 2.53%) in comparison with fairways (Lipica: 3.96%, Bled: 2.76%).

4 CONCLUSION

The results of this study confirm that precipitation significantly alters the amounts and proportions of plant available P and TP in rough. Despite observing no significant relationship between precipitation and the P dynamics in soil on green and fairway, the dynamics of P is probably affected by the quantities of applied water. On the basis of these data, we concluded that apart from precipitation, the application of water also had an important influence on P dynamics in soil and is the governing factor in soil P consumption.

Based on the estimation of contribution of precipitation and irrigation to P dynamics in soil, it was also found that the vegetation systems, fertilization management and organic matter content in soil markedly influence the P dynamics in the soil. Relatively undisturbed forest ecosystems on the rough has less plant available P

(average concentration: Lipica - 29±14 PO_4-P mg kg^{-1}; Bled - 26±17 PO_4-P mg kg^{-1}) concentrations than fairways (average concentration: Lipica - 61±27 PO_4-P mg kg^{-1}; Bled - 41±38 PO_4-P mg kg^{-1}) and greens (average concentration: Lipica - 73±24 PO_4-P mg kg^{-1}; Bled - 41±17 PO_4-P mg kg^{-1}). Contrary to this the analyses of the same soil samples, showed that soil from the roughs exhibited significantly greater concentrations of TP (average concentration: Lipica - 1097±125 mg kg^{-1}; Bled - 740±187 mg kg^{-1}) than soils from the greens (average concentration: Lipica - 476±128 mg kg^{-1}; Bled - 187±112 mg kg^{-1}. In forest ecosystems most of the plant available P is supplied by the slow decomposition and recycling of plant residue through microbial processes in the soil, while the higher amounts of plant available P in golf course soils is the consequence of large amounts of fertilizer application.

However, no significant relationship was found between the quantity of applied P fertilizers and the level of total P and plant available P in soil samples of the golf courses, although the total P concentration has increased after fertilizer application. The analyses of soil samples collected from the green and fairway in Lipica showed much higher values of plant available P and TP than the golf course in Bled, despite the fact that a lower quantity of fertilizer was applied to the golf course in Lipica. Despite the different level of plant available and fertilizer management practices the soil form golf course Lipica and Bled belongs to the class of optimal - medium supplied soil with phosphorous.

The result of analyses of soil samples from the golf courses also demonstrated that plant available P concentrations in samples from greens exceeded the concentration measured in samples from fairways, probably due to the low P absorption capacity of sandy soils on the green.

In conclusion, it is obvious that rainfall regime, water supply, organic matter, P absorption capacity and land use appeared to be the most important factors influencing the P dynamic in the soil. Therefore, modified fertilizer management of golf courses which considered all of these factors are needed to ensure better plant uptake, to minimize the risk transportation of P to groundwater and to reduce the risk of eutrophication of water bodies. More investigations need to be done to estimate more accurately the impact of golf courses on groundwater in the Balkan region and to assess the effects of management practices on water quality.

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Genetic diversity of spinach (*Spinacia oleracea* L.) landraces collected in Iran using some morphological traits

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ABSTRACT

Spinach has become an important vegetable crop in most regions of the world and remarkable changes in production amounts have occurred in the past decades due to demand increase in many countries. Fifty-four spinach landraces collected from diverse geographical regions of Iran were evaluated for several qualitative and quantitative traits. Landraces indicated a high variability for measured morphologic characteristics regarding results of variance analysis and descriptive statistics. The first three factors of factors analysis explained 76.8% of variation of spinach landraces. The first extracted factor can be regarded as a leaf property vector; the extracted second factor could be named as yield vector and the third factor was female plants percent vector. The dendrogram of cluster analysis generated from genotypes distance matrices showed that in a distance linkage of 800, the 54 spinach landraces could be agglomerated into sixteen clusters. The number of clusters was verified by multivariate analysis of variance test through Wilks' Lambda statistics. Some spinach landraces such as G10 G13, G38 and G41 were individual cluster and were not similar to the other collected genotypes while some of the spinach landraces were similar to each other and grouped as one cluster such as cluster 9 (C9). The cluster C14 (landrace Karaj 2) was the most favorable genotype due to good performance for most measured quantitative traits. This landrace could be recommended for commercial release after complementary experiments. Also, landraces G1 (Arak) and G3 (Urmia) indicate good potential regarding the measured traits. These landraces could be used directly as commercial cultivars or introduced in spinach breeding programs.

Key words: germplasm, morphological variation, multivariate analysis, spinach

IZVLEČEK

GENETSKA RAZNOLIKOST AKCESIJ ŠPINAČE (*Spinacia oleracea* L.) ZBRANIH V IRANU, DOLOČENA Z NEKATERIMI MORFOLOŠKIMI ZNAKI

Špinača je postala pomembna zelenjadnica v večjem delu sveta in znaten porast njene pridelave se je pojavil zaradi vse večjega povpraševanja v mnogih državah. 54 akcesij špinače, nabranih v različnih delih Irana, je bilo ovrednotenih na osnovi številnih kvalitativnih in kvantitativnih znakov. Akcesije so pokazale veliko variabilnost v merjenih morfoloških znakih glede na rezultate analize variance in opisne statistike. Prvi trije faktorji faktorske analize so pojasnili 76.8 % variabilnosti akcesij špinače. Prvi faktor od teh je bil povezan z lastnostmi listov, drugi s pridelkom in tretji z deležem ženskih rastlin. Dendrogram klasterske analize, generiran na osnovi izračunanih distanc med genotipi je pokazal, da lahko na osnovi distančne povezave 800, 54 akcesij špinače združimo v 16 skupin. Število skupin je bilo potrjeno z multivariatno analizo variance s pomočjo Wilks' Lambda statistke. Nekatere akcesije kot na primer G10 G13, G38 in G41 so bile samostojne skupine in niso bile podobne drugim zbranim genotipom, med tem ko so si bile druge akcesije podobne in so se uvrstile v eno skupino, npr. skupino 9 (C9). Skupina C14 (akcesija Karaj 2) je bila najboljši genotip glede na dobre vrednosti za večino merjenih kvantitativnih znakov. To akcesijo bi lahko priporočili za komercialno uporabo po dopolnih preizkusih. Tudi akcesiji G1 (Arak) in G3 (Urmia) kažeta dober potencial glede na merjene znake. Ti akcesiji bi bili lahko neposredno uporabljeni kot komercialni sorti ali vključeni v žlahtniteljski program špinače.

Ključne besede: genski material, morfološka variabilnost, multivariatna analiza, špinača

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1 INTRODUCTION

Spinach (*Spinacia oleracea L.*) is an edible and annual plant that grows rapidly and has the ability to survive over moderate winter. It is versatile which is used as a salad, a cooked vegetable or as a component of many other cooked meat and vegetable dishes (Sensoy et al., 2011). Leafy vegetables are an important part in the human diet and spinach is one of the dark green leafy vegetables which contains high beta carotene and folate, and is also a good source of vitamin C, calcium, iron phosphorous, sodium and potassium (Dicoteau, 2000; Avsar, 2011). Spinach as dioecious specie with both male and female plants is an herbaceous leafy vegetable in the family of *Amaranthaceae* (Salk et al., 2008) and its leaves are alternate, simple, from ovate to triangular-based, with larger leaves at the base of the plant and small leaves higher on the flowering stem (Vural et al., 2000).

Today, China, the United States, Indonesia, Japan and Turkey are among the largest commercial producers of spinach (FAO, 2011). Iran is the one of the spinach producers with about 105 thousand tons per year based on FAO statistics. The average yield of spinach in Iran is 2096 kg ha^{-1} while world's average yield is 2420 kg ha^{-1} (FAO, 2011). Also, the average yield of spinach in China is 2768 kg ha^{-1}, in the United States is 2360 kg ha^{-1}, Indonesia is 3424 kg ha^{-1}, Japan is 12471 kg ha^{-1}, and Turkey is 9249 kg ha^{-1} (FAO, 2011). Spinach is native to southwest Asia and commonly thought to have originated in Iran (Nonnicke, 1989; Swiader and Ware, 2002) and was first mentioned by the Chinese as the herb of Persia. It was first cultivated in North Africa, came to northern Europe by way of Spain, documented in Germany and then was a common garden vegetable by 1500 in England and France (Dicoteau, 2000; Swiader and Ware, 2002)

Although, hybrids cultivars of spinach were introduced in the 1950's and they have become the major type of spinach cultivars (Morelock and Correll, 2008), but Iranian farmers currently use native spinach landraces, which have good adaptability to different local conditions. The yield performance of these landraces is very low (typically about 2000 kg ha^{-1}) compared with the highest global yields (12471 kg ha^{-1}, produced in Japan; FAO 2011). Therefore, it is essential for Iran to has had spinach breeding program for increasing the genetic potential of yield as well as other important traits. Since Iran is a centre of genetic diversity of many cultivated plants, including wheat, alfalfa, spinach and etc, it is essential to conserve these important resources. Most of the spinach accessions are landraces which are highly adapted to specific environmental conditions and are useful sources of genetic variation (Asadi and Hasandokht, 2007). However, utilization of the genetic potential of different germplasms needs detailed knowledge about these genetic collections (Morelock and Correll, 2008), including characterization, evaluation and classification.

Multivariate procedures are useful for characterization, evaluation and classification of germplasm collections when a large number of accessions are to be assessed for several traits. The usefulness of multivariate procedures for handling morphological variation in plant genetic resources has been proved in many crops; wheat (Damania et al., 1996; Sorghum (Ayana and Bekele, 1999). The generated information of multivariate procedures can be useful for identifying different accessions that have explained traits for crossing, for planning efficient plant improvement program. Also, it is possible to establish core collections for revealing the structure of variation in plant genetic resources and for investigating some aspects of crop evolution (Perry and McIntosh, 1991; Ayana and Bekele, 1999).

Some investigations have been performed in the past on Iranian spinach germplasm collections, but most of them studies are limited with either using only univariate statistics or studying samples from a limited geographical range (Benedictos, 1999; Asadi and Hasandokht, 2007; Eftekhari et al., 2010). The objective of this investigation was to determine the structure of distribution of morphological variation for 10 quantitative traits and 9 qualitative traits in 54 accessions of native Iranian spinach germplasm collections sampled from a wide geographical range of Iran and identify groups of accessions with similar quantitative traits.

2 MATERIALS AND METHODS

2.1 Trial protocol

Fifty-four native Iranian spinach germplasm collections were collected as seed in Iran, and then evaluated in the field in a randomized complete block design (RCBD) replicated four times. Each spinach germplasm was collected as seed multiplied by the farmers. The geographical properties of the 54 sites of the collected spinach landraces are given in Table 1. Field soil was calcareous, loamy structure, low organic matter, and low salt content. Also, it had poor nitrogen and phosphorous and adequate potassium. Fertilization was carried out by spreading 80 kg N ha^{-1} (half of N at sowing stage and half of N at seedling emergence). Sowing was done manually at the rate of 50 kg seed ha^{-1}. Each plot contained six 3 m long rows with 25 cm between rows and plot size was 4.5 m^2. Control by hand weeding was carried out twice when the weed density was high, in the pre-flowering and post-flowering stages. The harvested plot size was 2.5 m^2 (four 2.5 m rows at the center of each plot).

Several quantitative traits consist on leaf length (LL), leaf width (LW), petiole length (PL), petiole diameter (PD), leaf area (LA), leaf numbers in flowering (LN), days to flowering (DF), female plants percent (FP), fresh yield (FY) and dry yield (DY) were measured. Also, various qualitative traits consist on leaf texture (LT, 1=smooth, 2=slight crinkled, 3= crinkled), seed type (ST, 1=smooth, 2=prickly), stem anthocyanin (SA, 1=very low, 3=low, 5=intermediate, 7=high, 9=very high), petiole attitude (PA, 1=erect, 2=semi-spared, 3= spared), vegetative leaf shape (VL, 1=elliptic, 2=broad elliptic, 3=circular, 4=ovate, 5=broad ovate, 6=triangular), reproductive leaf shape (RL, 1=smooth, 2=pointy); leaf edge (LE, 1=smooth, 2= rippler); leaf color (LC, 1=yellow-green, 2=grey-green, 3=blue-green); seed color (SC, 1=yellow-green, 2=grey-green, 3=blue-green) were measured.

2.2 Statistical analysis

The datasets were first tested for normality by Anderson and Darling normality test using MINITAB version 16 (2010) statistical software. Analysis of variance was performed to evaluate differences among measured quantitative traits and the accessions were compared by LSD (least significant differences) criteria. The factor analysis (Cattell, 1965), which consisted of the reduction of a large number of correlated variables to a much smaller number of groups of variables called factors. After extraction, the matrix of factor loading was submitted to a varimax orthogonal rotation, as applied by Kaiser (1958). The array of communality, the amount of variance of a variable accounted by the common factors together, was estimated by the highest correlation coefficient in each array as suggested by Seiller and Stafford (1985). The 54 spinach accessions were clustered using the SPSS 16 (SPSS, 2008), which grouped the accessions into different clusters. The measure of dissimilarity was Euclidean distance and the clustering method was un-weighted pair group method using centroids or UPGMC (Sneath and Sokal, 1973). The number of clusters was determined using multivariate ANOVA via Wilks' lambda statistics.

Table 1: Geographical properties of the 54 locations where spinach landraces are collected

No.	Name	Longitude	Latitude	Altitude (meter)	No.	Name	Longitude	Latitude	Altitude (meter)
1	Arak	49º 41´ E	34 ˚ 05´ N	1755	28	Qum	50º 53 ´ E	34 ˚ 38´ N	930
2	Ardestan	52º 22´ E	33 ˚ 23´ N	1205	29	Gochan	58º 30 ´ E	37 ˚ 06´ N	1240
3	Urmia	45º 04´ E	37 ˚ 33´ N	1340	30	Kashan	51º 27 ´ E	33 ˚ 59´ N	950
4	Esfahan 1	52º 02´ E	32 ˚ 32´ N	1525	31	Karaj 1	50º 97 ´ E	35 ˚ 82´ N	1300
5	Esfahan 2	51º 35 ´ E	33 ˚ 10´ N	1570	32	Karaj 2	50º 85 ´ E	35 ˚ 80´ N	1350
6	Bojnord	57º 19 ´ E	37 ˚ 28´ N	1070	33	Karaj 3	50º 87 ´ E	35 ˚ 86´ N	1230
7	Brojerd	48º 45 ´ E	33 ˚ 53´ N	1580	34	Kerman	57º 05 ´ E	30 ˚ 17´ N	1775
8	Beenab	46º 05 ´ E	37 ˚ 53´ N	1290	35	Kermanshah	47º 65 ´ E	34 ˚ 31´ N	1400
9	Birjand	59º 21 ´ E	32 ˚ 87´ N	1491	36	Lahijan 1	50º 14 ´ E	37 ˚ 26´ N	-11
10	Tabriz	46º 18 ´ E	38 ˚ 04´ N	1366	37	Lahijan 2	50º 11 ´ E	37 ˚ 16´ N	-10
11	Chamkahriz	51º 18 ´ E	32 ˚ 18´ N	1685	38	Langrood	50º 14 ´ E	37 ˚ 19´ N	-25
12	Khoramabad	48º 21 ´ E	33 ˚ 29´ N	1200	39	Mako	44º 55 ´ E	39 ˚ 28´ N	1182
13	Drood	48º 70 ´ E	33 ˚ 40´ N	1326	40	Mobarake	51º 30 ´ E	32 ˚ 21´ N	1900
14	Rahimabad	51º 57 ´ E	32 ˚ 28´ N	1550	41	Maragheh 1	46º 16 ´ E	37 ˚ 21´ N	1477
15	Rahnan 1	51º 36 ´ E	32 ˚ 41´ N	1545	42	Maragheh 2	46º 20 ´ E	37 ˚ 24´ N	1485
16	Rahnan 2	51º 40 ´ E	32 ˚ 42´ N	1525	43	Mashahad	59º 36 ´ E	36 ˚ 18´ N	979
17	Zabol	61º 29 ´ E	31 ˚ 01´ N	475	44	Malekan 1	46º 06 ´ E	37 ˚ 08´ N	1302
18	Zanjan	48º 40 ´ E	36 ˚ 40´ N	1650	45	Malekan 2	46º 09 ´ E	37 ˚ 03´ N	1291
19	Saveh	50º 05 ´ E	35 ˚ 10´ N	998	46	Minandab	46º 06 ´ E	36 ˚ 57´ N	1314
20	Salmas	44º 76 ´ E	36 ˚ 19´ N	1398	47	Mianeh	47º 72 ´ E	37 ˚ 41´ N	1100
21	Sanandaj	46º 89 ´ E	35 ˚ 31´ N	1518	48	Hamadan	48º 31 ´ E	34 ˚ 48´ N	1850
22	Sirjan	55º 40 ´ E	29 ˚ 27´ N	1735	49	Varamin 1	51º 39 ´ E	35 ˚ 19´ N	915
23	Shiraz 1	52º 22 ´ E	29 ˚ 37´ N	1540	50	Varamin 2	51º 38 ´ E	35 ˚ 11´ N	911
24	Shiraz 2	52º 12 ´ E	29 ˚ 17´ N	1320	51	Varamin 3	51º 28 ´ E	35 ˚ 19´ N	923
25	Shirvan	57º 92 ´ E	37 ˚ 40´ N	1492	52	Varamin 4	51º 38 ´ E	35 ˚ 23´ N	918
26	Salehabad	50º 57 ´ E	34 ˚ 31´ N	970	53	Varamin 5	51º 35 ´ E	35 ˚ 19´ N	905
27	Ajabsher	45º 55 ´ E	37 ˚ 28´ N	1330	54	Yazd	54º 21 ´ E	31 ˚ 53´ N	1215

3 RESULTS AND DISCUSSION

All of the quantitative dataset was normal according to Anderson and Darling normality test, and so no transformation was applied for traits (data not shown). Some descriptive statistics including minimum value, maximum value, arithmetic mean and coefficient of variation (CV) for all measured traits (variables) of 54 spinach genotypes are presented in Table 2. For example, the minimum amount of fresh yield was 5949.60 kg ha^{-1}, the maximum amount of fresh yield was 44957.00 kg ha^{-1} and the average fresh yield of studied genotypes was 22151.82 kg ha^{-1}. The maximum leaf length was 15.98 cm; the minimum leaf length was 5.87 cm and the average leaf length was 10.16 cm. The maximum, minimum and average leaf numbers at flowering time were 24, 12 and 16.93, respectively. The maximum percent of female plants was 84 %, the minimum percent of female plants was 20 %, and the average percent of female plants was 54.88 %. Such information can be derived for the other traits from Table 2. Regarding CV values which ranges from 6 (in days to flowering) to 40 % (in fresh yield) in quantitative traits and ranges from 32 (in leaf edge)

to 46 % (in vegetative leaf shape) in quantitative traits, indicates remarkable variation among 54 spinach landraces (Table 2).

The results of factor analysis are given in Table 3. When fitting the factor analysis model, the first three factors explained 76.8 % of variation for spinach landraces. The first factor extracted can be regarded as a leaf property vector (Table 3). It has high loadings for five traits as leaf length, leaf width, petiole length, leaf area and leaf numbers in flowering, which all of them were the related to leaf characteristics. This factor accounted for 50.6 % of the total variation in spinach landraces data set. The extracted second factor could be named as yield vector and accounted for 15.4 % of the total data variability. It has high loadings for days to flowering, petiole diameter, fresh yield and dry yield traits, which petiole diameter, fresh yield and dry yield were the related to yield performance. The third factor is a female plants percent vector (Table 3) which shows this trait had high loadings in this factor and accounted for 10.7 % of the total data variability. It seems that leaf property vector as the most important factor and yield vector are more influent characteristics among nine measured quantitative traits.

Table 2: Descriptive statistics of the measured traits in 54 spinach landraces

Traits	Max.	Min.	Average	CV
Leaf length (cm)	15.98	5.87	10.16	0.17
Leaf width (cm)	10.50	2.61	6.31	0.24
Petiole length (cm)	13.3	4	8.40	0.22
Petiole diameter (mm)	14.6	6	10.62	0.15
Leaf area (cm^2)	118.8	11.2	53.88	0.36
Leaf numbers in flowering	24	12	16.93	0.14
Days to flowering	183	137	162.36	0.06
Female plants percent	84	20	54.88	0.20
Fresh yield (kg ha^{-1})	44957.00	5949.60	22151.82	0.40
Dry yield (kg ha^{-1})	4286.90	526.00	2161.66	0.38
Leaf texture	3	1	1.74	0.43
Seed type	2	1	1.24	0.35
Stem anthocyanin content	9	1	1.52	0.46
Petiole attitude	3	1	1.85	0.37
Vegetative leaf shape	6	1	1.65	0.46
Reproductive leaf shape	2	1	2.31	0.44
Leaf edge	2	1	1.57	0.32
Leaf color	3	1	2.02	0.39
Seed color	3	1	2.39	0.45

LT, Leaf texture (1=smooth, 2=slight crinkled, 3= crinkled); Seed type (1=smooth, 2=prickly); SA, Stem anthocyanin (1=very low, 3=low, 5=intermediate, 7=high, 9=very high); PA, Petiole attitude (1=erect, 2=semi-spared, 3= spared); VL, Vegetative leaf shape (1=elliptic, 2=broad elliptic, 3=circular, 4=ovate, 5=broad ovate, 6=triangular); RL, Reproductive leaf shape (1=smooth, 2=Pointy); LE, Leaf edge (1=smooth, 2= Rippler); LC, Leaf color (1=yellow-green, 2=grey-green, 3=blue-green); SC, Seed color (1=yellow-green, 2=grey-green, 3=blue-green).

Table 3: Factor components loadings of quantitative traits obtained from 54 spinach landraces.

	F1	F2	F3
Leaf length (cm)	**0.66**	0.40	0.27
Leaf width (cm)	**0.92**	0.11	0.04
Petiole length (cm)	**0.86**	0.12	-0.08
Petiole diameter (mm)	0.21	**0.78**	0.10
Leaf area (cm^2)	**0.89**	0.29	0.13
Leaf numbers in flowering	**0.63**	0.41	-0.11
Days to flowering	0.07	**0.66**	0.12
Female plants percent	0.04	0.03	**0.97**
Fresh yield (kg ha^{-1})	0.33	**0.90**	-0.08
Dry yield (kg ha^{-1})	0.27	**0.92**	-0.11
Eigen value	5.1	1.5	1.1
% Variance	50.6	15.4	10.7
% Cumulative variance	50.6	66.0	76.8

To better understand the relationships among the quantitative traits of spinach landraces, the relationships are graphically displayed in a plot of factor 1 and factor 2 (Fig. 1). In this plot, the first factor axis mainly distinguishes the methods of leaf width from the other quantitative traits. The second factor axis separates leaf length and petiole length from the other remained quantitative traits (Fig. 1). Therefore, regarding two factors' loading scores, nine measured quantitative traits could be divided into three groups: leaf width as the first group, leaf length and petiole length as the second group, and petiole diameter, leaf area, leaf numbers in flowering, days to flowering, female plants percent, fresh yield and dry yield.

Cluster analysis is a tool for classifying objects into groups. Agglomerative hierarchical clustering methods use the elements of a proximity matrix to generate a tree diagram or dendrogram. The dendrogram generated from genotypes distance matrices showed to clearly group them (Fig. 2). In a distance linkage of 800, the examined 54 spinach landraces could be agglomerated into sixteen clusters. The number of clusters was verified by multivariate analysis of variance test through Wilks' Lambda statistics (data not shown). The related spinach landraces of each sixteen clusters and their qualitative traits are given in Table 4. Some spinach landraces such as G10 G13, G38 and G41 were individual cluster and were not similar to the other collected genotypes while some of the spinach landraces were similar to each other and grouped as one cluster such as cluster 9 (C9) which consist on G17, G12, G23, G37, G28, G34, G36, G40, and G48.

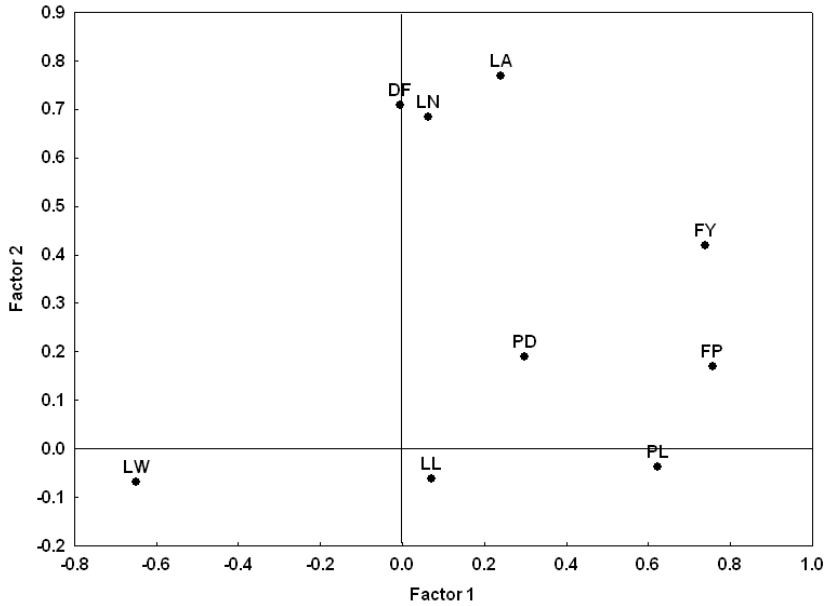

Figure 1: Plot of two first factor analysis of nine traits for the 54 spinach genotypes. LL, Leaf length (cm); LW, Leaf width (cm); PL, Petiole length (cm); PD, Petiole diameter (mm); LA, Leaf area (cm^2); LN, Leaf numbers in flowering; DF, Days to flowering; FP, Female plants percent; FY, Fresh yield (kg ha^{-1}); DY, Dry yield (kg ha^{-1}).

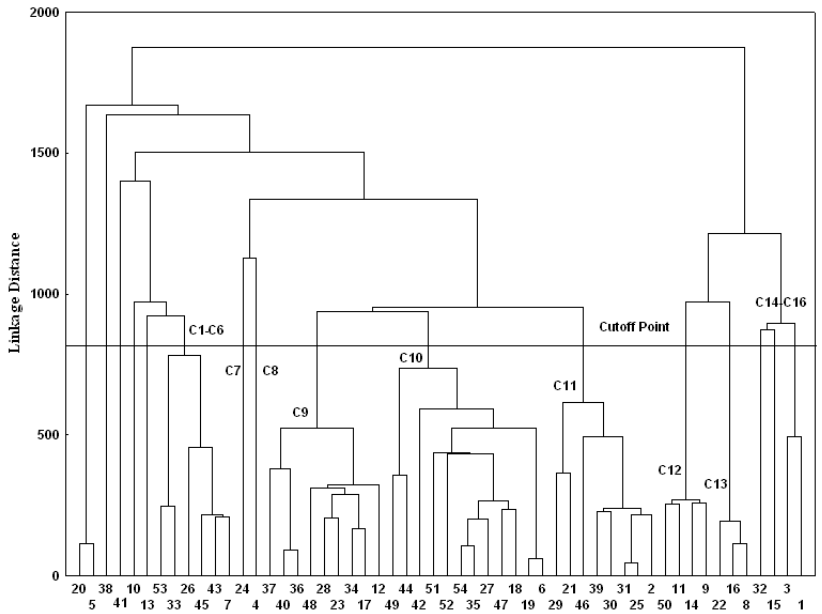

Figure 2: Hierarchical cluster analysis of the 54 spinach genotypes based on Ward's method using measured traits.

The mean and LSD (least significant differences) values of the quantitative traits of sixteen clusters are given in Table 5. The highest leaf length (LL) was belong to cluster C7 (12.57 cm) and the lowest LL was belong to cluster C2 (6.78 cm); and it is clear that, there are good variations in length of spinach landraces. According to the LSD values, sixteen clusters could be divided to six distinct groups based on leaf length. Larger leaves are found at the base of the plant and small leaves are found higher on the flowering stem (LeStrange et al., 1999; Avsar, 2011). The cluster C14 had the largest leaf width (8.18 cm) and the cluster C1 had the smallest leaf width (3.76 cm). According to the LSD values of leaf width, sixteen clusters could be divided to five distinct groups. Both leaf length and leaf width are important traits in spinach yield performance (Asadi and Hasandokht, 2007). Due

to high variability of these two traits which is observed in studied landraces, it is possible to establish a breeding program to increase leaf yield in spinach.

The longest petiole length (PL) was seen in the cluster C11 (9.87 cm), while the shortest PL was seen in the cluster C1 (5.51 cm). The LSD values of petiole length divided the sixteen clusters into three distinct groups. The long petiole length is essential for machinery harvesting and genetic improving for having long petiole length is one of the breeding targets of spinach (Eftekhari et al., 2010). Also, the relative length of petiole is a commercial factor for the producing of spinach canner. The most petiole diameter (PD) as 12.81 mm was observed in the cluster C15 and the low PD as 7.5 mm was observed in the cluster C2 (Table 5). There are not any general correlations among petiole length and petiole diameter, but plants that produce the largest petioles also produce in general the thickest (Pandey and Kalloo, 1993; Avsar, 2011).

The largest leaf area (LA) was belong to cluster C14 (76 cm^2) and the smallest LA was belong to cluster C1 (24.06 cm^2). The largest size of leaf area produces the longest leaf length both in absolute length and relative to petiole length, and conversely, the shortest petioles. This would seem to show that the most of petiole length growth was made relatively in early stages, when conditions favorable for growth occurred; growth in leaf length was more rapid than growth in petiole length. The cluster C14 had the most leaf numbers in flowering time (20 leaf) while the cluster C5 had the lowest leaf numbers in flowering time (12.33 leaf). Harvest of spinach plants of marketable size is depending on leaf number and it is correlated with the length of growing period. Spinach is mainly grown for fresh leaves and both the number of leaves and leaf area determine yield performance. However, a high variance was observed for number of leaf in this study which depicted a broad base of the studied landraces for these traits. This maximizes the scope of selection for these traits in the germplasm assayed. Also, the different environmental conditions influences on leaf numbers production and it seem that leaf production per day to be highest under long-day and moderate temperature conditions (Pandey and Kalloo, 1993).

The early flowering cluster was C4 with 146.67 days to flowering and the late flowering clusters were C7 and C8 with 171 days to flowering (Table 5). The higest percent of female plants (64.67%) was seen in cluster C8 and the lowest percent of female plants (46%) was seen in cluster C4. The high fresh yielding landrace was cluster C16 (36429.50 kg ha^{-1}) and the low fresh yielding landrace was cluster C1 (7452.34 kg ha^{-1}). The LSD values of fresh yield divided the sixteen clusters into nine distinct groups. Finally, the high dry yielding landrace was cluster C15 (3405.66 kg ha^{-1}) and the low dry yielding landrace was cluster C1 (727.97 kg ha^{-1}). The LSD values of dry yield divided the sixteen clusters into six distinct groups. It seems that there are remarkable variation in both fresh and dry yield of 54 spinach landraces and these genotypes could be used for increasing yield in future spinach improvement programs.

Regarding all quantitative traits, it seems that cluster C14 which contain only landrace Karaj 2 was the most favorable genotype due to good performance for most measured quantitative traits. Its leaf texture was smooth and so could accumulate low amounts of nitrate, and it had low amounts of anthocyanin (Table 4). Petiole shapeof landrace Karaj 2 is semi-spared and regarding the long petiole length simply could be used for machinery harvest. This landrace has many of good characteristics for proper performance and could be recommended for commercial release after complementary experiments. The finding of such good spinach landrace in this study at Iran as its origin indicates the high potential of native landraces in origin of plants.

After cluster C14, cluster C16 which consist on landraces G1 (Arak) and G3 (Urmia) indicate good potential regarding the measured traits. The leaf texture of these landraces was moderate (slight crinkled), and their anthocyanin content was acceptable (low). The leaf color of both Arak and Urmia landraces was grey-green which is suitable for frizzing industries (Table 4). However, these landraces could be used directly as commercial cultivars or introduced in spinach breeding programs due to high potential in most measured qualitative and quantitative characteristics. Clusters C12, C13 and C15 had good performances for some important traits such as dry yield and are useful sources of genetic variation for improving

yield performance in spinach. There were landraces G8 (Beenab), G9 (Birjand), G11 (Chamkahriz), G14 (Rahimabad), G15 (Rahnan 1), G16 (Rahnan 2), G22 (Sirjan), G50 (Varamin 2) in these clusters which are collected from different geographical regions of Iran. It seems that these landraces were variable from other aspects which are not measured in this study. Finally every one of the 54 spinach landraces which is used in this investigation maybe had at least one important trait resource and could be enter to different spinach breeding program based on the breeder target(s).

Spinach is a very important source of nutrients and is dispersed throughout Iran as its origin and all over the world. Plant materials of present investigation were chosen because there are not many studies on spinach especially on landraces. A total of 54 spinach landraces were collected from different geographical regions of Iran which provided morphological data for the landraces. The dendrogram of cluster analysis for the dataset showed 16 groups. Multivariate PCA analysis of morphological data was performed for 3 parameters and the analysis showed good separation of the quantitative traits on the plot based on first two PC. This investigation provided suitable information that may be useful to plant breeders who wish to find the most distinct spinach landraces. For germplasm collections, the results of present investigation may aid to conserve more distinct accessions and to eliminate similar accessions to preparing proper spinach gene-bank in Iran. In future studies, a plant breeder may select two distinct accessions and hybridize them to create a new generation and to obtain one or more new cultivars with favorable characteristics such as resistance to biotic and abiotic stresses.

In conclusion, it was seen that characterization of spinach landraces based on the morphological traits was suitable to assess the genetic diversity among collected spinach landraces. Results of this investigation also can aid to define strategies for further collection. Since our results show that the pattern of observed variation is governed by morphological traits, future germplasm collections should aim to investigate genetic variation via different molecular markers. Also, it is essential to explore variation using more landraces which are collected geographically and climatically from different regions, instead of collecting extensively within individual regions. However, a high variability was observed for most measured traits and obtaining more diverse collections especially exotic germplasm is not needed for future breeding in spinach.

Table 4: The genotypes of 16 clusters and their qualitative characteristics

Class	Landraces	LT	ST	SA	PA	VL	RL	LE	LC	SC
C1	G5, G20	2	1	5	2	4	1	1	2	2
C2	G38	1	1	1	3	6	2	2	2	3
C3	G41	3	1	7	2	3	1	1	3	3
C4	G10	3	1	3	1	2	1	2	3	1
C5	G13	1	1	1	2	5	1	2	2	2
C6	G53, G33, G26, G45, G43, G7	2	1	5, 7	2	2,3	1	2	3	3
C7	G24	2	1	7	2	1	1	1	3	3
C8	G4	1	1	9	2	2	1	2	1	2
C9	G37, G40, G36, G48, G28, G23, G34, G17, G12	1	1	3,5, 9	1, 2	3	1, 2	2	2, 3	2
C10	G49, G44, G42, G51, G52, G54, G35, G27, G47, G18, G19, G6	2	1, 2	1, 5	2, 3	1, 2	1	2	2	2, 3
C11	G29, G21, G46, G39, G30, G31, G25, G2	1, 2	1	3, 7	1, 2	1	1, 2	2	1, 2	3
C12	G50, G11, G14, G9	2	2	1	2	1,3	1	2	2	2
C13	G22, G16, G8	1	1	5, 9	2	4	1	1	2	3
C14	G32	1	1	3	2	1	1	1	1	1
C15	G15	1	1	9	3	2	2	1	1	2
C16	G3, G1	2	2	3	2	1	1	1	2	1

LT, Leaf texture (1=smooth, 2=slight crinkled, 3= crinkled); Seed type (1=smooth, 2=prickly); SA, Stem anthocyanin (1=very low, 3=low, 5=intermediate, 7=high, 9=very high); PA, Petiole attitude (1=erect, 2=semi-spared, 3= spared); VL, Vegetative leaf shape (1=elliptic, 2=broad elliptic, 3=circular, 4=ovate, 5=broad ovate, 6=triangular); RL, Reproductive leaf shape (1=smooth, 2=pointy); LE, Leaf edge (1=smooth, 2= rippler); LC, Leaf color (1=yellow-green, 2=grey-green, 3=blue-green); SC, Seed color (1=yellow-green, 2=grey-green, 3=blue-green).

Table 5: The quantitative characteristics of 16 clusters of spinach landraces

Class	LL	LW	PL	PD	LA	LN	DF	FP	FY	DY
C1	7.20	3.76	5.51	8.67	24.06	14.33	150.50	53.17	7452.34	727.97
C2	6.78	4.64	8.05	7.50	31.67	16.67	162.00	53.33	9158.45	973.10
C3	10.73	5.54	7.63	10.63	47.84	18.33	147.67	59.67	10787.81	1109.33
C4	8.89	4.94	7.13	10.43	38.59	14.00	146.67	46.00	15783.36	1534.02
C5	8.08	5.78	7.05	9.50	35.17	12.33	169.33	52.33	14811.47	1546.44
C6	10.01	5.66	7.95	10.07	47.75	15.83	152.72	54.50	13111.50	1365.14
C7	12.57	6.57	8.30	12.57	69.97	17.33	171.00	62.33	28633.95	2729.43
C8	12.50	6.08	8.77	12.77	68.93	18.00	171.00	64.67	27509.96	2669.30
C9	10.64	7.02	8.93	10.51	58.65	17.37	165.27	55.93	23377.59	2302.94
C10	9.98	6.24	8.20	10.37	52.58	16.58	160.94	53.71	20891.73	2057.99
C11	10.54	7.58	9.87	9.97	61.37	16.83	168.00	64.67	24355.39	2131.18
C12	10.45	6.86	9.51	11.67	58.17	19.33	170.33	48.78	33168.25	3240.43
C13	10.90	6.32	9.39	12.20	59.79	18.00	169.56	57.89	31702.52	3103.02
C14	10.63	8.18	9.79	11.81	76.00	20.00	166.00	46.33	35384.93	3167.40
C15	11.01	6.12	9.01	12.81	60.31	17.00	170.67	61.33	34545.36	3405.66
C16	10.88	7.05	7.83	12.29	68.36	19.50	170.17	54.50	36429.54	3291.19
LSD	0.88	0.86	1.03	0.79	11.00	1.34	5.56	7.05	3395.16	314.65

LL, Leaf length (cm); LW, Leaf width (cm); PL, Petiole length (cm); PD, Petiole diameter (mm); LA, Leaf area (cm^2); LN, Leaf numbers in flowering; DF, Days to flowering; FP, Female plants percent; FY, Fresh yield (kg ha^{-1}); DY, Dry yield (kg ha^{-1}).

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Graphic analysis of yield stability in new improved lentil (*Lens culinaris* Medik.) genotypes using nonparametric statistics

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ABSTRACT

Yield stability is an interesting feature of today's lentil breeding programs, due to the high annual variation in mean yield, particularly in the arid and semi-arid areas. The genetic effects including genetic main and genotype × environment (GE) interaction effects for grain yield of eighteen lentil (*Lens culinaris* Medik.) genotypes were studied with fourteen nonparametric stability statistics. Results of five distinct nonparametric tests of GE interaction and combined ANOVA showed there were both additive and crossover interaction types and genotypes varied significantly for grain yield. According to most of the nonparametric stability statistics, genotypes G5, G6, G8 and G18 were the most stable genotypes. Considering mean yield versus stability values via their plotting, indicates that genotypes G2, G11 and G14 following to G5, G16 and G18 were the most favorable genotypes. None of the nonparametric stability statistics were correlated with mean yield and so had static concept of stability. Our results confirmed that rankings of genotypes within environments and using mean yield information permit ease of interpretation of nonparametric results. Finally genotypes G2 (FLIP 92-12L), G11 (Gachsaran) and G14 (ILL 6206) were found to be the most stable and high mean yielding genotype and thus recommended for commercial release. Such an outcome could be used to delineate predictive, more rigorous recommendation strategies as well as to help define stability concepts for lentil and other crops.

Key words: adaptability, dynamic stability, genotype × environment interaction

IZVLEČEK

GRAFIČNA ANALIZA STABILNOSTI PRIDELKA NOVIH IZBOLJŠANIH GENOTIPOV LEČE (*Lens culinaris* Medik.) Z UPORABO NEPARAMETRIČNE STATISTIKE

Stabilnost pridelka je zaradi velikih letnih nihanj, še posebej v aridnih in semi-aridnih območjih, zanimiva lastnost v današnjih žlahtniteljskih programih pri leči (*Lens culinaris* Medik.). Pri 18 genotipih leče smo s 14 neparametričnimi statističnimi testi, ki vrednotijo stabilnost pridelka, preučevali glavne vplive genotipa in interakcije med genotipom in okoljem (GO) na pridelek zrnja. Rezultati petih neparametričnih testov GO interakcij, ter parametrične ANOVA so pokazali, da so se genotipi značilno razlikovali v pridelku zrnja tako v povezanjih kot prekrižanih interakcijah. Gleda na večino neparametričnih testov stabilnosti pridelka so se genotipi G5, G6, G8 in G18 izkazali kot najbolj stabilni. Primerjava povprečnih pridelkov in stabilnosti je pokazala, da so genotipi G2, G11, G14 in G5, G16 ter G18 najbolj primerni. Nobeden izmed neparametričnih testov stabilnosti ni koreliral s povprečnim pridelkom, kar kaže na njihov statičen značaj. Naši rezultati potrjujejo, da rangiranje genotipov po povprečnem pridelku za vsake okoljske razmere posebej omogoča uporabo rezultatov neparametričnih testov. Na koncu so bili genotipi G2 (FLIP 92-12L), G11 (Gachsaran) in G14 (ILL 6206) prepoznani kot najbolj stabilni, z velikim povprečnim pridelkom in priporočeni za komercialno uporabo. Takšni izsledki bi lahko bili uporabljeni za ponazoritev napovedovanj in resnejših priporočil kot tudi pomoč pri določanju stabilnost pridelave leče in drugih poljščin.

Ključne besede: prilagodljivost, dinamična stabilnost, interakcije med genotipom in okoljem

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1 INTRODUCTION

Iran is one of the foremost countries in terms of lentil (*Lens culinaris* Medik.) production and sowing area in the world, and is followed by Canada, Turkey and India. Although, the lentil is the second grain legume crop after the chickpea in Iran but its average yield (489 kg ha^{-1}) is not acceptable for many local farmers (Sabaghnia et al., 2008). According to the latest statistics from The Food and Agricultural Organization of the United Nations, 162000 ha were used for lentil production and 79000 t of production were obtained in 2000 (FAOSTAT, 2010). This low yield performance of the cultivated lentil cultivars in comparison to the highest global yields (14580 kg ha^{-1}, produced in Canada; FAOSTAT, 2010), encouraged Dryland Agricultural Research Institute (DARI) of Iran for performing an important lentil-breeding program in recent years, supported by the International Center for Agricultural Research in Dry Areas (ICARDA).

Like to the other crops, increasing the potential of yield is an important target of lentil breeding programs. The new improved genotypes are evaluated in multi-environment trials to test their performance across different environmental conditions. In most trials, crop yield fluctuates due to suitability of genotypes to different conditions which is known as genotype × environment (GE) interaction (Kang, 1998). In presence of GE interaction, a genotype does not exhibit the same phenotypic characteristics under test environments and various genotypes respond differently to a specific environment. GE interaction exploration and yield stability is an area of current interest and the success of plant breeding efforts depend on the identification of superior genotypes from stability and yield aspects. Exploring, measurement and interpretation of GE interaction can be aided by different statistical modeling and a number of statistics, parametric as well as nonparametric have been proposed for the study of yield stability (Huehn, 1996). These statistical models can be linear formulations (Eberhart and Russell, 1966), multiplicative formulations such as additive main effects and multiplicative interaction (Zobel et al., 1988), or nonparametric procedures (Huehn, 1979).

The use of nonparametric statistics in the assessment of yield stability had several benefits. In this approach, no assumptions about the observations are needed and there is less sensitivity to measurement errors or to outliers (Huehn, 1990a). Also, additions or deletions a few genotypes do not cause distortions and these statistics are useful in situations where parametric statistics fail due to the presence of large non-linear GE interaction (Huehn, 1990b). In most cases the plant breeder is concerned with non-additive (crossover) GE interaction and so yield stability measuring based on rank-information, seems more relevant and usefulness. Therefore, the nonparametric statistics are widely used in the selection of favorable genotypes especially when the interest lies in crossover GE interaction (Nassar and Huehn, 1987; Huehn, 1996; Mut etal., 2009). Although, it is demonstrated that the nonparametric procedures are less powerful than their parametric methods but Raiger and Prabhakaran (2000) have shown that when the number of genotypes is large, the power efficiency of the nonparametric statistics will be quite close to the parametric statistics.

According to both GE interaction types, additive (non-crossover) and crossover (non-additive), several nonparametric tests based on ranks were proposed by different authors. These methods of Bredenkamp (1974), Hildebrand (1980) and Kubinger (1986) for testing of additive GE interaction and methods of de Kroon and van der Laan (1981) and, Azzalini and Cox (1984) for testing of crossover GE interaction were introduced. Also, several nonparametric stability statistics proposed by Huehn (1979), Kang (1988), Ketata et al. (1989), Fox et al. (1990), and Thennarasu (1995) which are identifying genotypes with similar ranking across environments as the most stable genotypes. Nassar and Huehn (1987) developed two distinct statistical tests as Z1 and Z2 for the two first nonparametric stability statistics of Huehn (1979) which known as $S_i^{(1)}$ and $S_i^{(2)}$.

The objectives of present study were to (1) test presence of GE interaction through different nonparametric tests, (2) interpret GE interaction

via ranks obtained by nonparametric stability statistics of 18 lentil genotypes over twelve environments, (3) visually assess how to vary rank statistics versus yield performances based on the plot, (4) determine promising favorable genotype(s) with high mean yielding and good stability, and (5) investigate interrelationships among different nonparametric stability statistics in lentil dataset.

2 MATERIALS AND METHODS

2.1 Plant Material and Field Conditions

The study included 18 lentil genotypes (16 new improved lines and 2 cultivars) that were grown in 4 different locations under rainfed conditions during the 2007-2009 growing seasons. The names of studied lentil genotypes are given in Table 1.

Table 1. Geographical properties and mean yield of the 18 lentil genotypes, studied in 4 locations

Code	Location	Altitude (meter)	Longitude Latitude	Soil Texture	Rainfall (mm)	Yield (kg ha^{-1})
1	Gorgan	45	55° 12′ E 37° 16′ N	Silty Clay Loam	367	767
2	Kermanshah	1351	47° 19′ E 34° 20′ N	Clay Loam	455	1923
4	Gachsaran	710	50° 50′ E 30° 20′ N	Silty Clay Loam	460	1747
5	Shirvan	1131	58° 07′ E 37° 19′ N	Loam	267	384

All trials were arranged in accordance with a randomized complete block design with 4 replicates. The experimental plots consisted of 4 rows, each 4 m in length with 25 cm row spacing. The planted plot size was 4 m^2 and the harvested plot size was about two 3.5 m rows with 1.75 m^2. All trials were fertilized with 20 kg of N ha^{-1} and 80 kg of P_2O_5 during sowing stage. Weeds were controlled by hand twice in the high weed density (pre-flowering and post-flowering stages).

The test locations (Gorgan, Gachsaran, Kermanshah and Shirvan) were selected as sample of lentil growing areas of Iran and to vary in latitude, rainfall, soil types, temperature and other agro-climatic factors. Gorgan in the north-east of Iran is characterized by semi-arid conditions with sandy loam soil. Gachsaran, in southern Iran, is relatively arid and has silt loam soil. Kermanshah in the west of Iran is characterized by semi-arid conditions with clay loam soil. Gachsaran, in southern Iran, is relatively arid and has silt loam soil. Shirvan in the north-east of Iran is characterized by moderate conditions, relatively high rainfall and have clay loam soil. Some of the important properties and the location of the experimental environments are given in Table 2.

Table 2: The name and yield (kg ha $^{-1}$) of 18 lentil genotypes studied in multi-environmental trials

Code	Name	Type	Yield	Code	Name	Type	Yield
G1	FLIP 96-7L	Line	1418.73	G10	ILL 6030	Line	1187.98
G2	FLIP 92-12L	Line	1365.64	G11	Gachsaran	Cultivar	1374.14
G3	FLIP 96-13L	Line	1287.29	G12	ILL 7523	Line	1334.75
G4	FLIP 96-8L	Line	1272.07	G13	ILL 6468	Line	1292.16
G5	FLIP 96-4L	Line	1324.46	G14	ILL 6206	Line	1401.88
G6	FLIP 96-14L	Line	1096.53	G15	ILL 62-12	Line	1307.35
G7	ILL 5583	Line	1304.15	G16	FLIP 82-1L	Line	1272.40
G8	FLIP 96-9L	Line	1191.14	G17	CABRALIA	Cultivar	1203.28
G9	ILL 6002	Line	1329.48	G18	FLIP 92-15L	Line	1314.63

2.2 Nonparametric Statistical Methods

Conventional combined analysis of variance as well as nonparametric tests for presence of GE interaction was done. Three nonparametric tests including Bredenkamp (1974), Hildebrand (1980) and Kubinger (1986) procedures were applied for additive GE interaction and two nonparametric tests including de Kroon and van der Laan (1981) and Azzalini and Cox (1984) procedures were applied for crossover GE interaction. These nonparametric tests have been described in detail by Huehn and Leon (1995) and Truberg and Huehn (2000). For computing of the above mentioned statistics, a SAS-based computer program was used.

Huehn (1979) developed six nonparametric stability statistics, which Kang and Pham (1991) and Kaya and Taner (2002) described only four $S_i^{(1)}$, $S_i^{(2)}$ $S_i^{(3)}$ and $S_i^{(6)}$ statistics. The two other nonparametric statistics are expressed as follows:

$$S_i^{(4)} = \sqrt{\frac{\sum_{j=1}^{n}(r_{ij} - \bar{r}_{i.})^2}{n}}$$

$$S_i^{(5)} = \frac{\sum_{j=1}^{n} | r_{ij} - \bar{r}_{i.} |}{n}$$

for *k* genotypes and *n* environments, the value of *i*th genotype in *j*th environment is x_{ij}, where $i = 1,2,...,k$, $j = 1,2,...,n$, r_{ij} as the rank of the *i*th genotype in the *j*th environment, and $\overline{r_{ij}}$ as the mean rank across all environments for the *i*th genotype. Ketata et al. (1989) proposed plotting mean rank across environments against standard deviation of ranks for all genotypes (σ_r) or plotting mean yield across environments against standard deviation of yields for all genotypes (σ_{my}). The formula for calculating both standard deviations are expressed as:

$$\sigma_r = \sqrt{\frac{\sum_{j=1}^{n}(r_{ij} - \bar{r}_{i.})^2}{n-1}}$$

$$\sigma_{my} = \sqrt{\frac{\sum_{j=1}^{n}(r_{ij} - \bar{x}_{i.})^2}{n-1}}$$

Nonparametric stability statistics as Top, Mid and Low were introduced by Fox et al. (1990) as

nonparametric superiority measure (NSM) using stratified ranking of the genotypes and their ranking was done at each environment separately and the number of environment at which the genotype occurred in the top, middle, and bottom third of the ranks was computed. Kang's (1988) rank-sum is another nonparametric stability statistics where both mean yield and Shukla's (1972) stability variance are used as selection criteria. Thennarasu (1995) proposed the use of the four nonparametric statistics based on the corrected ranks. In other word, the ranks of genotypes in each environment were determined according adjusted values ($x_{ij}^{*} = x_{ij} - \overline{x_{i.}}$). For calculation of these nonparametric stability statistics, SAS-based computer programs of Lu (1995) and Hussein et al. (2000) were used.

3 RESULTS

The residuals mean squares were not correlated to environment mean yield (r = 0.12, $P > 0.05$) thus the data were not transformed. Variances homogeneity test via Bartlett procedure ($\chi^2 = 25.1$, $P < 0.05$) showed that the mean squares of individual environments were homogeny and so the combine analysis of variance could be done. Analysis of variance was conducted to determine the effects of year, location, genotype, and their interactions on grain yield of lentil genotypes (Table 3).

Table 3: Combined ANOVA of lentil performance trial yield data

Source	DF	Mean Squares
Year (Y)	2	8400774^{ns}
Location (L)	3	3962077^{ns}
Y×L	6	4579496**
R (Y×L)	36	38152
Genotype (G)	17	320003**
Y×G	34	80769 ^{ns}
L×G	51	134137*
Y×L×G	102	84021**
Error	612	31713

Genotypes and locations were regarded as fixed effects, while years were regarded as random effects. The main effect of Y, L and Y × L were tested against the replication within environment (R/Y×L). The main effect of G was tested against the G × Y × L interaction and the G × Y × L interaction was tested against error term. The main effects of year (Y) and location (L) were not significant ($P > 0.05$), but their interactions (YL) were highly significant ($P < 0.01$). The main effect of genotypes was significant ($P < 0.01$), the genotype × year interaction (GY) was not significant ($P > 0.05$), the genotype × location interaction (GL) was significant ($P > 0.05$) and three way interactions (GYL) or GE were highly ($P < 0.01$) significant (Table 3). The GE interaction, which arising from the lack of genetic correlation among environments, must be used to understand in breeding program. Analyses of the quantitative traits like grain yield indicate important sources of genetic variation attributed to GE interactions (Gauch et al., 2008). The relative large contributions of GE interaction in grain yield of lentil which found in this study is similar to those found in other multi-environmental trials studies of lentil in rain-fed conditions (Mohebodini et al., 2006; Sabaghnia et al., 2008).

Table 4: Analysis of GE interaction using different non-parametric tests on 18 durum lentil genotypes grown in 12 environments

Nonparametric tests	Nonparametric tests	df	χ^2	P-value
Additive	Bredenkamp	187	894.05	0.00 <
	Hidebrand	187	364.21	0.00 <
	Kubinger	187	385.67	0.00 <
Crossover	de Kroon-van der Laan	187	368.46	0.00 <
	Azzalini-Cox	187	305.31	0.00 <

The results of various nonparametric tests verified the results combined ANOVA. According to chi-squares statistics of Bredenkamp (1974), Hildebrand (1980) and Kubinger (1986) producers, the existence of additive (non-crossover) GE interaction; and based on de Kroon and van der Laan (1981) and Azzalini and Cox (1984) producers, the existence of crossover (non-additive) GE interaction were demonstrated (Table 4). The high significance of GE interactions for lentil grain yield via combined ANOVA and five nonparametric tests indicated the genotypes exhibited both crossover and non-crossover types of GE interaction. In other word, results of nonparametric tests are in agreement with the ANOVA, but provide more specific information about the nature of GE interactions from additive and crossover aspects. Cooper and Byth (1996) explained that the large magnitude of GE interaction due to the more dissimilarity of the genetic systems controlling the physiological processes conferring adaptation to different environments.

The values of the first two nonparametric stability statistics of Huehn (1979), $S_i^{(1)}$ and $S_i^{(2)}$, indicated that genotype G18, followed by G5 and G11 were the most stable genotypes (Table 5). Nassar and Huehn (1987) and Flores et al. (1998) pointed out that the $S_i^{(1)}$ and $S_i^{(2)}$ are associated with the static or biological concept of stability and define stability in the sense of homeostasis. However, the stability property alone is of limited use and for a successful genotype testing program, both stability and mean yield must be considered simultaneously.

Table 5: Nonparametric stability statistics for grain yield of 18 lentil genotypes evaluated in 12 environments

	$S_i^{(1)}$	$S_i^{(2)}$	$S_i^{(3)}$	$S_i^{(4)}$	$S_i^{(5)}$	$S_i^{(6)}$	Top	Mid	Low	RS	$NP_i^{(1)}$	$NP_i^{(2)}$	$NP_i^{(3)}$	$NP_i^{(4)}$	σ_r	σ_{my}
G1	7.61	42.00	73.75	18.81	4.83	12.08	58.33	25.00	16.67	16	5.42	1.806	0.919	0.525	5.67	420.82
G2	6.52	31.24	44.24	15.79	4.30	9.16	58.33	33.33	8.33	9	5.21	1.157	0.743	0.385	4.76	401.19
G3	6.18	28.09	32.99	15.90	3.99	6.24	33.33	25.00	41.67	21	4.54	0.454	0.527	0.290	4.80	375.57
G4	6.15	26.82	39.44	17.69	4.58	6.93	25.00	41.67	33.33	25	4.04	0.385	0.434	0.320	5.33	376.62
G5	4.83	16.57	23.75	12.89	3.29	5.64	33.33	66.67	0.00	9	3.96	0.396	0.388	0.259	3.89	391.36
G6	5.92	25.36	5.58	8.38	2.00	1.90	0.00	25.00	75.00	22	4.46	0.262	0.282	0.087	2.53	319.74
G7	5.86	24.81	34.02	16.26	4.04	6.24	25.00	50.00	25.00	24	4.21	0.411	0.478	0.300	4.90	379.45
G8	6.03	25.55	18.57	14.29	3.63	3.95	8.33	33.33	58.33	23	3.71	0.239	0.335	0.179	4.31	345.34
G9	7.45	41.18	57.60	19.74	5.03	8.93	41.67	33.33	25.00	24	6.04	0.863	0.615	0.414	5.95	392.23
G10	7.74	43.54	38.19	19.35	4.83	5.92	16.67	25.00	58.33	33	5.63	0.388	0.516	0.268	5.83	347.71
G11	5.02	18.27	29.75	12.60	3.22	7.25	41.67	58.33	0.00	9	3.63	0.483	0.533	0.339	3.80	399.98
G12	6.56	30.45	33.77	15.52	3.92	6.59	41.67	33.33	25.00	13	4.58	0.509	0.506	0.310	4.68	391.93
G13	5.26	19.90	30.38	15.26	3.85	6.02	25.00	41.67	33.33	14	3.88	0.456	0.406	0.284	4.60	378.08
G14	6.85	34.42	26.97	11.69	2.83	6.71	50.00	50.00	0.00	15	4.88	0.750	0.792	0.330	3.52	415.54
G15	6.08	27.66	38.11	16.75	4.29	6.99	33.33	41.67	25.00	19	4.71	0.523	0.534	0.325	5.05	387.68
G16	5.76	23.91	31.48	15.74	3.56	5.42	25.00	58.33	16.67	25	3.71	0.371	0.416	0.284	4.75	375.91
G17	7.98	49.30	51.59	21.31	5.83	7.95	41.67	8.33	50.00	32	6.96	0.535	0.549	0.333	6.42	358.98
G18	4.53	14.81	20.64	11.67	2.92	5.30	41.67	50.00	8.33	9	2.88	0.338	0.435	0.254	3.52	385.67

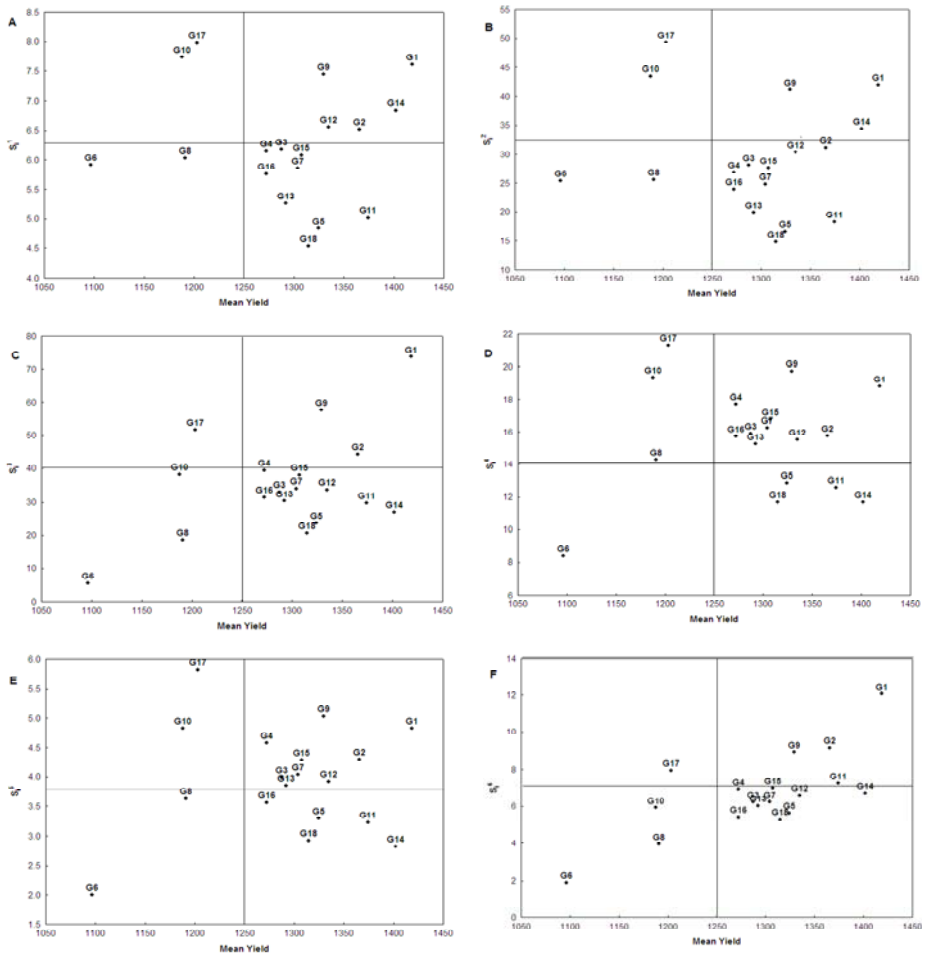

Figure 1: Plot of the mean yield versus Huehn's (1979) nonparametric stability statistics (A) $S_i^{(1)}$, (B) $S_i^{(2)}$, (C) $S_i^{(3)}$, (D) $S_i^{(4)}$, (E) $S_i^{(5)}$ and (F) $S_i^{(6)}$.

Figure 1A represents plot portrayed by mean yield values and $S_i^{(1)}$ nonparametric stability statistic. This figure is divided by grand mean yield and average $S_i^{(1)}$ values into four sections. Therefore studied lentil genotypes are classified as Group I, with stable low yield characteristics; Group II, with high yield stable genotypes; Group III, with unstable low yield properties; Group IV, with unstable high yielding genotypes (Table 6). Among these groups, only Group II is acceptable for recommending as the most favorable genotypes which are consist on G3, G4, G5, G7, G11, G13, G15, G16 and G18 (Table 6). According to Figure 1A, genotypes G2, G3, G4, G5, G7, G11, G12, G13, G15, G16 and G18 were identified as the most stable genotypes regarding both mean yield and $S_i^{(2)}$ nonparametric stability statistic.

Table 6: Grouping of 18 lentil genotypes based on mean yield and nonparametric stability statistics

	Group I	Group II	Group III	Group IV
$S_i^{(1)}$	G6, G7	Remained genotypes	G10, G17	G1, G2, G9, G12, G14
$S_i^{(2)}$	G6, G8	Remained genotypes	G10, G17	G1, G9, G14
$S_i^{(3)}$	G6, G8, G10	Remained genotypes	G17	G1, G2, G9
$S_i^{(4)}$	G6	G5, G11, G14, G18	G8, G10, G17	Remained genotypes
$S_i^{(5)}$	G6, G8	G5, G11, G14, G16, G18	G10, G17	Remained genotypes
$S_i^{(6)}$	G6, G8, G10	Remained genotypes	G17	G1, G2, G9, G11
$NP_i^{(1)}$	G6, G8	Remained genotypes	G10, G17	G1, G2, G9
$NP_i^{(2)}$	G6, G8, G10, G17	Remained genotypes	----	G1, G2
$NP_i^{(3)}$	G6, G8, G10, G17	Remained genotypes	----	G1, G2, G9, G14
$NP_i^{(4)}$	G6, G8, G10	G3, G5, G13, G16, G18	G17	Remained genotypes
σ_r	G6, G8	G5, G11, G14, G18	G10, G17	Remained genotypes
σ_{my}	G6, G8, G10, G17	----	----	Remained genotypes
RS	G5, G12, G13, G18	G2, G11, G14	----	G1, G9, G12
NSM	G17	Remained genotypes	G6, G8, G10	G3, G4, G7, G13, G16

Group I, Stable and low yield; Group II, Stable and high yield; Group III, Unstable and low yield; Group IV, Unstable and high yield

According to $S_i^{(3)}$ and $S_i^{(6)}$ nonparametric statistics, genotypes G6, G8 and G18 were the most stable genotypes while based on $S_i^{(4)}$ and $S_i^{(5)}$ nonparametric statistics, genotypes G6, G14 and G18 were the most stable genotypes (Table 5). Kang and Pham (1991) found that the $S_i^{(3)}$ and $S_i^{(6)}$ nonparametric statistics would be useful tools for selecting simultaneously for yield and yield stability while Ebadi-Segherloo et al. (2008) pointed out that the $S_i^{(4)}$ and $S_i^{(5)}$ nonparametric statistics were similar to the $S_i^{(1)}$ and $S_i^{(2)}$ statistics, and explore GE interaction with the biological concept of stability. Figure 1C showed that all genotypes expect G1, G2, G6, G8, G9, G10 and G17 were the most favorable genotypes based on $S_i^{(3)}$ and mean yield. According to Fig. 1D, genotypes G5, G11, G14 and G18 and according to Fig. 1E, genotypes G5, G11, G14, G16 and G18 were identified as the favorable genotypes with high mean yield and stability. Also, Figure 1F indicated that all genotypes expect G1, G2, G6, G8, G9, G10, G11 and G17 were the most

favorable genotypes based on $S_i^{(6)}$ and mean yield. Finally, according to the most of the nonparametric stability statistics of Huehn (1979), genotypes G5, G6 and G18 were the most stable genotypes while based on the related figures and considering mean yield, genotypes G5, G11, G14, G15, G16 and G18 were the most favorable genotypes. It seems that using graphic presentation of the nonparametric statistics of Huehn (1979) which usually reflect static concept of stability could aid in detecting the most favorable genotypes with high mean yield and stability. Thus, genotypes G11 and G14 following to genotypes G5, G15 and G14 are recommended as the most favorable genotypes.

The nonparametric statistic $NP_i^{(1)}$ showed that genotypes G8, G11, G16 and G18 were the most stable genotypes while based on the nonparametric statistic $NP_i^{(2)}$, genotypes G6, G8, G16 and G18 were the most stable genotypes (Table 5). Many lentil genotypes (except G1, G2, G6, G8, G9, G10 and G17) were grouped in Group II and the most favorable genotypes considering $NP_i^{(1)}$ and mean yield (Figure 2A). Relatively, similar results were observed in Fig. 2B which identified the most favorable genotypes based on $NP_i^{(2)}$ and mean yield. According to the nonparametric statistic $NP_i^{(3)}$, genotypes G5, G6 and G8 were identified the most stable genotypes while the nonparametric statistic $NP_i^{(4)}$ indicated genotypes G6, G8 and G18 as the most stable genotypes (Table 5). Regarding mean yield and $NP_i^{(3)}$ (Figure 2C), all genotypes except G1, G2, G6, G8, G9, G10, G14 and G17 were as the most favorable genotypes while considering $NP_i^{(4)}$ and mean yield (Figure 2D), genotypes G3, G5, G13, G16 and G18 were detected as the most favorable genotypes.

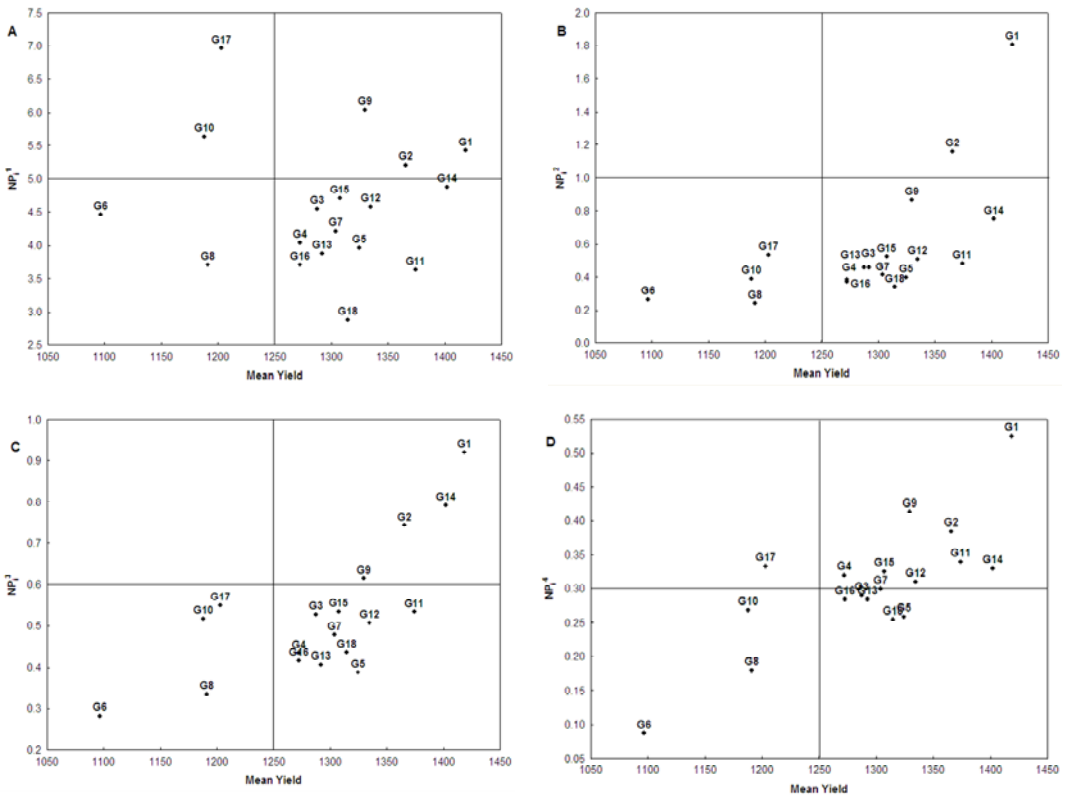

Figure. 2: Plot of the mean yield versus Thennarasu's (1995) nonparametric stability statistics (A) $NP_i^{(1)}$, (B) $NP_i^{(2)}$, (C) $NP_i^{(3)}$ and (D) $NP_i^{(4)}$

According to σ_r statistic of Ketata et al. (1989), genotypes G6, G14 and G18 were the most stable genotypes while based on σ_{my} statistic of Ketata et al. (1989), genotypes G6, G8 and G10 were the most stable genotypes (Table 5). Also, simultaneous considering of mean yield and σ_r statistic (Figure 3A), genotypes G5, G11, G14 and G18 were the most favorable genotypes while based on both mean yield and σ_{my} statistic (Fig. 3B), none of the studied genotypes were the most stable ones. Kang's (1988) rank-sum (RS) uses mean yield and Shukla's (1972) stability variance. According to the rank-sum statistic, G2, G5, G11 and G18 were the most stable genotypes (Table 5). Based on the plot of mean yield versus RS (Figure 3C), genotypes G2, G11 and G14 were the favorable stable genotypes. According to Fox et al. (1990), genotypes G1 and G2 were the most stable because they ranked in the top third of genotype in a high percentage of environments (58.3%), which were the high yield genotypes in this study with 1418.7 and 1365.6 kg ha^{-1}, respectively (Table 2). Considering all Top, Mid and Low statistics of nonparametric superiority measure (NSM), G1, G2 and G14 were the most stable genotypes.

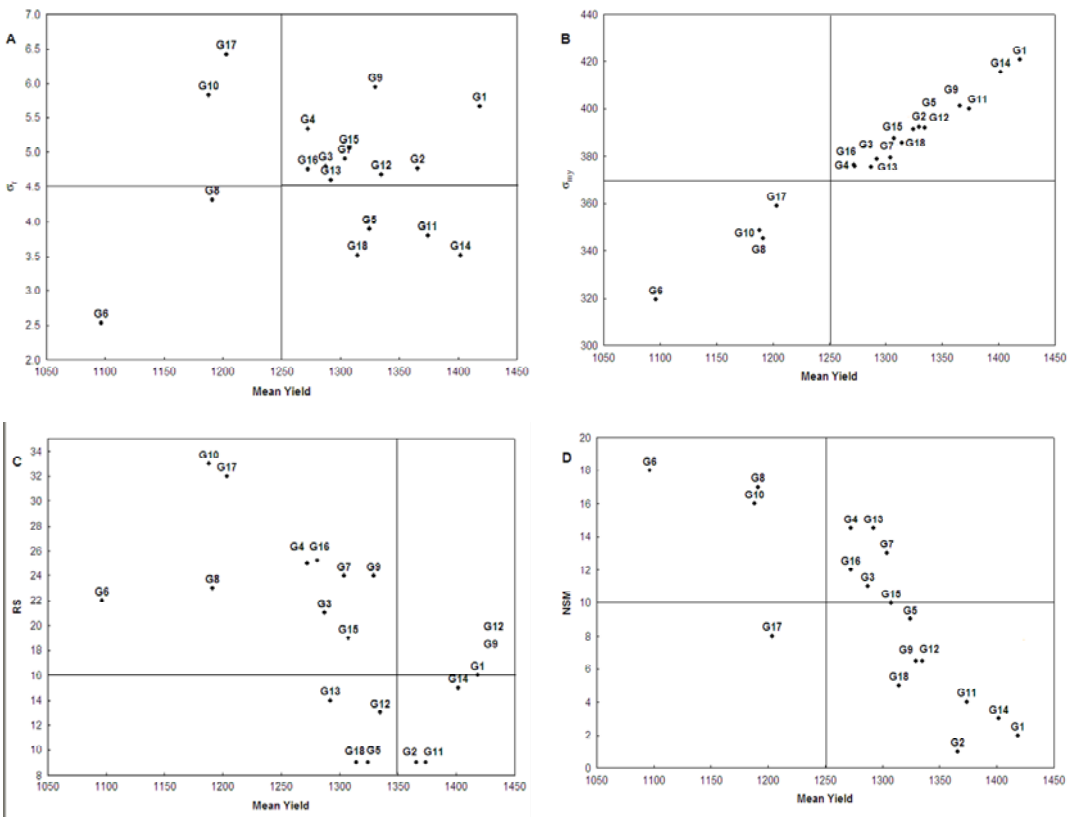

Figure 3: Plot of the mean yield versus nonparametric stability statistics (A) σ_r, (B) σ_{my}, (C) RS and (D) NSM

According to Figure 3D, genotypes G1, G2, G5, G9, G11, G12, G14, G15 and G18 were the favorable stable genotypes due to mean yield as well as Top, Mid and Low statistics of Fox et al. (1990). The rank correlation among the nonparametric stability statistics may indicate if more estimates should be obtained to improve confidence in the prediction of genotype behavior. The nonparametric stability statistics were compared using their ranks for each genotype (Table 7) via calculating Spearman's rank correlation. The rank correlation between the NSM and RS statistics with mean yield (Y) was positive and significant. Selecting the most stable genotypes based on these stability statistics result in high yielding genotypes were selected as the stable genotypes. In contrast, rank correlation

between the $S_i^{(6)}$, $NP_i^{(2)}$, $NP_i^{(3)}$, $NP_i^{(4)}$ and σ_{my} with mean yield (Y) were negative and significant. Therefore, the above mentioned procedures could not introduce the high mean yield genotypes as the most stable genotypes.

Table 7: Spearman's rank correlation coefficients between the nonparametric stability statistics for grain yield of 18 lentil genotypes

NSS¶	MY	$S_i^{(1)}$	$S_i^{(2)}$	$S_i^{(3)}$	$S_i^{(4)}$	$S_i^{(5)}$	$S_i^{(6)}$	NSM	RS	$NP_i^{(1)}$	$NP_i^{(2)}$	$NP_i^{(3)}$	$NP_i^{(4)}$	σ_r
$S_i^{(1)}$	-0.02*													
$S_i^{(2)}$	-0.04	1.00												
$S_i^{(3)}$	-0.21	0.70	0.71											
$S_i^{(4)}$	0.12	0.69	0.69	0.91										
$S_i^{(5)}$	0.06	0.70	0.71	0.93	0.97									
$S_i^{(6)}$	-0.60	0.56	0.58	0.81	0.59	0.64								
NSM	0.89	-0.19	-0.21	-0.33	-0.01	-0.08	-0.68							
RS	0.69	0.47	0.45	0.36	0.63	0.51	-0.07	-0.07						
$NP_i^{(1)}$	-0.10	0.91	0.93	0.73	0.69	0.71	0.61	0.61	0.36					
$NP_i^{(2)}$	-0.71	0.56	0.59	0.68	0.43	0.49	0.90	0.90	-0.23	0.68				
$NP_i^{(3)}$	-0.64	0.65	0.68	0.68	0.47	0.49	0.86	0.86	-0.08	0.68	0.89			
$NP_i^{(4)}$	-0.66	0.53	0.55	0.76	0.53	0.56	0.98	0.98	-0.07	0.56	0.90	0.88		
σ_r	0.12	0.68	0.68	0.91	1.00	0.97	0.58	0.58	0.62	0.68	0.42	0.46	0.52	
σ_{my}	-0.98	0.08	0.09	0.30	-0.03	0.03	0.66	0.66	-0.62	0.17	0.75	0.67	0.71	-0.04

NSS, Nonparametric Stability Statistics
* Critical vales of correlation P<0.05 and P<0.01 (D.F. 16) are 0.47 and 0.59, respectively

According to Table 7, the rank correlations among the six nonparametric stability statistics ($S_i^{(1)}$, $S_i^{(2)}$, $S_i^{(3)}$, $S_i^{(4)}$, $S_i^{(5)}$ and $S_i^{(6)}$) of Huehn (1979) with each other were positive and significant. Similar results were obtained in maize (*Zea mays* L.) by Scapim et al. (2010) and in wheat (*Triticum aestivum* L.) by Kaya and Taner (2002). Also, $S_i^{(1)}$ $S_i^{(2)}$ $S_i^{(3)}$ and statistics show high significant and positive correlations with the other remained nonparametric stability statistics expect NSM, RS, $NP_i^{(4)}$ and σ_{my}. It is interesting that these statistics had positive significant correlations with NSM and RS. The, $S_i^{(4)}$ $S_i^{(5)}$ and $S_i^{(6)}$ stability statistics showed positive significant correlation with $NP_i^{(4)}$ and σ_r (Table 7). In agreement with our results, Flores et al. (1998) found high correlations between $S_i^{(3)}$ and $S_i^{(6)}$ in faba bean (*Vicia faba* L.) and pea (*Pisum sativum* L.) multi environmental trials.

The NSM nonparametric superiority statistic of Fox et al. (1990) had significant positive correlation with mean yield, σ_r and all $NP_i s$ (Table 7). Kang's (1988) rank-sum (RS) statistics indicated significant positive correlation with mean yield, $S_i^{(4)}$ and σ_r. In contrast, Ebadi-Segherloo et al. (2008) found no significant correlations among RS and the other nonparametric procedures. This opposite finding could be result of the different nature of the studied crops, environmental conditions (climatic and edaphic factors) or diverse genetic background obtained from different sources. All four $NP_i s$ except $NP_i^{(4)}$ had significant positive correlation with each other but σ_{my} of Ketata et al. (1989) had significant positive correlation with, $S_i^{(6)}$ NSM, $NP_i^{(1)}$, $NP_i^{(2)}$ and $NP_i^{(3)}$ (Table 7). Sabaghnia et al. (2006) found high correlations between $NP_i^{(2)}$ and $NP_i^{(4)}$ in multi environmental trials of lentil.

4 DISCUSSION

In this investigation, interpretation of the GE interaction was based on nonparametric statistical procedures. The former method (ANOVA) had shown certain deficiencies for determining GE interaction types while nonparametric tests can determine the additive or crossover types of GE interaction. However, both interaction types were observed in lentil multi-environment trials. The presence of GE interaction is expressed either as inconsistent responses of genotypes relative to others due to genotypic rank change or as changes in the absolute differences between genotypes without rank change (Annicchiarico, 2002). In these situations, the risk of selecting inferior genotypes from the use of non-parametric measures is minimal. However, the highly significant GE interaction indicate the necessity for multiple environmental testing if the relative performance of lentil genotypes is to be accurately assessed for a large geographic region (DeLacy et al., 1996; Akcura and Kaya, 2008).

Lentil growing in field can be influenced by genetic, environmental and their interaction effects. The climatic factors were the main causes which could affect the expression of genes for the quantitative traits of lentil such as grain yield under different environments (Sabaghnia et al., 2008). Thus, the GE interaction complicates the interpretation of multi-environment trials in plant breeding programs. Understanding the magnitude of G and GE interaction effects is useful for improving the efficiency of breeding efforts and is helpful for plant breeders to select the better genotypes of lentil which can be steadier in various environments. The results in this study showed that the GE interaction is more important in rain fed condition and it must be paid more attention to the GE interaction during the lentil breeding in arid and semi-arid areas.

An ideal lentil genotype should have a high mean yield combined with a low degree of fluctuation under different environments. There are two important concepts of stability as static and dynamic (Becker and Leon, 1988; Rose et al., 2008). Static stability is analogous to the biological concept or homeostasis and in this concept a stable genotype tends to maintain a constant yield across different environments. In contrast, a stable genotype with dynamic stability concept has a yield response which is parallel to the mean response of the tested genotypes. Most of the nonparametric stability statistics have static or biologic concept of stability and usually introduce low or moderate yielding genotypes as the most stable ones. However, this type of stability is not acceptable to most plant breeders, who would prefer to select the high mean yielding genotypes as the most stable genotypes.

Simultaneous consideration of both mean yield and stability would be useful for selecting the most favorable genotypes (Kang, 1998; Karimizadeh etal., 2012). It seems that plotting mean yield versus each of the nonparametric stability statistics helps in identification of high mean yield and the most stable genotypes. Our results demonstrated the utility of this hypostasis and determined the most favorable genotypes. In each graph, the studied genotypes were classified into four distinct groups which only one group could be regarded as the most favorable genotype (high mean yield and the most stable genotype). According to most of the generated figures, genotypes G2, G3, G5, G11, G14, G16 and G18 were the most favorable genotypes. Among these favorable genotypes, G2, G11 and G14 following to G5, G16 and G18 are good candidates for commercial release. Thus, the stability property alone is of limited use and for a successful genotype testing program, both yield stability and mean yield must be considered simultaneously.

There are different forms to the GE interaction, and the different methods may quantify different components of the GE interaction. Besides being robust to violations of statistical assumptions regarding the dataset distribution, and insensitive to outliers, nonparametric rank-based procedures are of value for elucidating meaningful ways that environments differentially affect the seed yield (Huehn, 1996; Sabaghnia et al., 2012). Using rank-based procedures for GE interaction study and yield stability analysis, there were not consistent rankings of genotypes across environments, and environment affected the rank order of lentil genotypes. Thus, the lentil data analyzed here suggested that differences in yield of genotypes or environmental conditions were relatively great

enough to affect the rank order of genotypes in different environments. Mohebodini et al. (2006) find a significant GE interaction for lentil grain yield based on the different parametric procedures (i.e., normal distribution assumption) analysis, their results are not inconsistent with ours. As stated by Sabaghnia et al. (2008), most of the GE interaction in multi-environment trials appears to result from changes in the magnitude of differences among genotypes across test environment as well as changes in rankings. The rank-based procedures serve as convenient tools to specifically detect situations where the ranks do change with environment. The methods discussed here can be used for any study where the different crops are tested in each of several environments (different locations and/or years). Finally, the following findings can be summarized from this investigation: (1) G2 (FLIP 92-12L), G11 (Gachsaran) and G14 (ILL 6206) were found to be the most stable and high mean yielding genotype and thus recommended for commercial release; (2) the graphic investigation of yield stability using mean yield versus different nonparametric stability statistics was found to be useful in detecting the phenotypic stability of the studied genotypes; and (3) the significant GE interactions suggest a breeding strategy of specifically adapted genotypes in homogeneously grouped environments.

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Sensitivity of field tests, serological and molecular techniques for *Plum Pox Virus* detection in various tissues

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ABSTRACT

Sensitivity of field tests (AgriStrip and Immunochromato), DAS-ELISA, two step RT-PCR and real-time RT-PCR for *Plum pox virus* (PPV) detection was tested in various tissues of apricot, peach, plum and damson plum trees infected with isolates belonging to PPV-D, PPV-M or PPV-Rec, the three strains present in Slovenia. Flowers of apricot and plum in full bloom proved to be a very good source for detection of PPV. PPV could be detected with all tested techniques in symptomatic parts of leaves in May and with one exception even in the beginning of August, but it was not detected in asymptomatic leaves using field tests, DAS-ELISA and partly also molecular techniques. PPV was detected only in some of the samples of asymptomatic parts of the leaves with symptoms and of stalks by field tests and DAS-ELISA. Infections were not detected in buds in August using field tests or DAS-ELISA. Field tests are useful for confirmation of the PPV infection in symptomatic leaves, but in tissues without symptoms DAS-ELISA should be combined or replaced by molecular techniques.

Key words: sharka, *Plum pox virus*, PPV, detection, field tests, DAS-ELISA, RT-PCR, real time RT-PCR

IZVLEČEK

OBČUTLJIVOST HITRIH TESTOV, SEROLOŠKIH IN MOLEKULARNIH TEHNIK ZA DETEKCIJO VIRUSA ŠARKE V RAZLIČNIH TKIVIH

Občutljivost hitrih testov (AgriStrip in Immunochromato), DAS-ELISA, dvostopenjske RT-PCR in RT-PCR v realnem času za detekcijo virusa šarke (*Plum pox virus*, PPV) smo proučevali v različnih tkivih dreves marelice, breskve, slive in cibore, okuženih z izolati PPV-D, PPV-M ali PPV-Rec. Ti trije različki so potrjeno navzoči v Sloveniji. Vzorci cvetov marelice in slive, odvzeti v času polnega cvetenja, so bili zelo primerni za detekcijo PPV. V delih listov z znaki okužbe je bila detekcija uspešna z vsemi tehnikami v maju in z eno izjemo tudi v avgustu. S hitrimi testi, DAS-ELISA in delno tudi z molekularnimi tehnikami nismo uspeli detektirati virusa šarke v listih brez znakov okužbe. S hitrimi testi in DAS-ELISA smo navzočnost PPV potrdili le v delu vzorcev iz asimptomatičnih delov listov z znaki okužbe in iz listnih pecljev ter v nobenem vzorcu brstov v avgustu. Hitri testi so torej primerni le za potrditev okužbe s PPV v listih z znaki okužbe. Če znaki okužbe niso vidni, je potrebno DAS-ELISA kombinirati ali nadomestiti z molekularnimi tehnikami.

Ključne besede: virus šarke, *Plum pox virus*, PPV, detekcija, hitri testi, DAS-ELISA, RT-PCR, RT-PCR v realnem času

1 INTRODUCTION

The introduction of infected plant propagation material is the most important mean of long distance spread of *Plum pox virus* (PPV). PPV is also transmitted by a number of aphid species and by vegetative propagation, including grafting. The length of incubation period is influenced by plant

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species, cultivar, time and mode of infection, vector species and virus strain. The data differ from some weeks to 8 years, but usually the incubation period takes 8 to10 months (Nemeth, 1986). Concentration of virus is low and symptoms are not visible in the early stage of infection. The expression of symptoms also differs considerably among cultivars. In a resistant plant the multiplication of the virus is limited and its spread slow (Polák, 2008), so the virus concentration is low. The concentration of virus varies also during the vegetation period and among tissues. Even in the same leaf there may be infected and virus-free zones (Nemeth, 1986). In the Mediterranean countries sampling of leaves is not recommended in summer months due to the high temperatures (EPPO, 2004) that cause low virus replication. Apart from mature leaves, flowers, small fruits as well as buds and bark in winter period are recommended for sampling by EPPO (EPPO, 2004).

Due to a possibility of low virus concentration, a great sensitivity and accuracy of detection technique is needed for successful and reliable detection. On the other hand cheap methods are desirable, since a lot of samples must be tested to ensure the sharka free status of planting material. Fast results are also needed, especially when testing imported planting material like graft-wood. The method must also be able to detect all the isolates. PPV has been classified into seven strains: PPV-M, PPV-D, PPV-Rec, PPV-EA, PPV-C, PPV-W, and PPV-T (Szathmáry and Palkovics, 2010), which differ in pathogenicity, symptom expression, host range, aphid transmissibility, and geographic distribution.

Sensitivity of different detection methods was therefore tested in different tissues of apricot, plum, damson plum and peach trees infected with isolates belonging to PPV-D, PPV-M or PPV-Rec, the three strains present in Slovenia. In particular suitability of field test for rapid detection was evaluated.

2 MATERIALS AND METHODS

2.1 Plant material

Plant material was collected from the same trees on 3rd of April, 1st of May and 1st of August, 2011 in a small garden in Maribor, NE Slovenia. On 3rd of May, 2011 additional samples were taken in a garden in Ljubljana, central Slovenia. Sampled host plants, the expression of symptoms on the sampled trees at the time of sampling and the tissue types tested are presented in Tables 1-3. Based on data from previous tests it is known that all trees (with exception of the two resistant cultivars ‘Jojo’ nad ‘Katinka’) have been infected for at least five years. For samples of asymptomatic leaves parts of leaves near the petiole (stalk) were used since Myrta *et al*. (2003) detected PPV most frequently in this part. In symptomatic leaves parts with symptoms were sampled separately from parts without symptoms. The tissue used for testing was excised and divided in three sub-samples. Individual sub-samples were used for testing with Immunochromato field test (NIPPON GENE Co., Ltd., Japan), with AgriStrip (BIOREBA AG, Switzerland) and with DAS-ELISA (BIOREBA AG, Switzerland).

2.2 Methods

Testing with AgriStrip (BIOREBA AG, Switzerland) was performed upon producer’s protocol (available on http://www.bioreba.com/). For testing with Immunochromato field test (NIPPON GENE Co., Ltd., Japan), the sample was placed in the supplied extraction bag. Extraction solution (supplied by the producer) was added in 1:20 (w/v) ratio. After grinding, 0.65 ml of the extract was transferred to the sample tube and the test strip was placed in the extract. The results were recorded after 15 minutes.

DAS-ELISA was performed essentially as recommended by the producer (BIOREBA AG, Switzerland). Absorbance was read at 405 nm in a Sunrise Remote Control Reader (TECAN Austria GmbH). Samples were considered positive when the mean absorbance value of a sample exceeded the threshold. The threshold was set as at least three times the mean absorbance value (OD) of healthy controls as recommended by producer (http://www.bioreba.ch/?idpage=6).

Total RNA was isolated from extracts prepared for DAS-ELISA using RNeasy Plant Mini Kit (Qiagen, CA). 250 µl of RLT buffer (Qiagen, Germany) with 1% β-mercaptoethanol was added to 200 µl of extract. The isolation was than performed according to the manufacturer's instructions.

For reverse transcription 3 µl of isolated total RNA was added to 22 µl of reaction mix containing 50 pmol of oligo d(T)-based primer, 5 µl 5X M-MLV RT Buffer (Promega, USA), 5 µl dNTP mix (10 mM), 200 U M-MLV Reverse Transcriptase (Promega) and 20 U RNasin (Promega). The reactions were incubated for 10 minutes at 70 °C, placed on ice for 2 minutes and incubated further at 42 °C for 1 hour.

For the amplification, 47 µl of reaction mix consisting of 75 mM Tris–HCl at pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of each of the primers and 2.5 U Taq DNA Recombinant Polymerase (Fermentas, UAB, Lithuania) were added to each tube containing 3 µl of the cDNA mix. The amplification consisted of an initial denaturation step of 5 min at 94 °C, followed by 35 cycles with a thermal profile of 30 seconds at 94 °C, 30 seconds at 62 °C and 45 seconds at 72 °C and a final elongation step of 10 min at 72 °C. Primer pair P1/P2 (Wetzel *et al.*, 1991) was used for detection of PPV.

For sequencing (Macrogen, The Netherlands) unpurified DNAs obtained in the PCR with P1/P3M or P1/P3D (Wetzel *et al.*, 1991; Candresse *et al.*, 1998) primer pairs were used. 3 µl of the cDNA mix was added to 47 µl of reaction mix containing 10 µl of 5X Colorless GoTaq Flexi Buffer (Promega), 3 µl of $MgCl_2$ (25 mM), 5 µl dNTP mix (10 mM), 20 pmol of each of the primers and 2.5 U of GoTaq Flexi DNA Polymerase. The amplification consisted of an initial denaturation step of 5 min at 94 °C, followed by 40 cycles with a thermal profile of 45 seconds at 94 °C, 30 seconds at 52 °C and 60 seconds at 72 °C and a final elongation step of 10 min at 72 °C.

The isolate type was determined by comparison of Slovenian sequences with sequences from the NCBI GenBank.

Real time RT-PCR was performed upon the protocol described by Mavrič Pleško *et al.* (2009). Ct values over 37 were considered negative.

3 RESULTS AND DISCUSSION

Plum 'Jojo' that possesses a highly reliable hypersensitive type of resistance (Neumüller and Hartmann, 2008) proved to be resistant also under high PPV infection pressure in Slovenia (Tables 1-3). Plum and apricot trees infected with PPV-M or PPV-D are growing in close vicinity and trees are infested with aphids. Since over 20 aphid species are known to be vectors of PPV the probability of transmission is very high. Nevertheless PPV could not be detected even with highly sensitive molecular methods in none of tested samples of this cultivar. Similar results were obtained with samples collected from plum 'Katinka' growing in the same garden. 'Katinka' is considered to be resistant by its breeders (Hartman, 1999), but has shown to be very susceptible to PPV-M by Kamenova and Milusheva (2005). In Maribor, PPV could be detected only in stalks using real time PCR (Table 2), but the Ct value was very low indicating a low concentration of the virus. Apricot 'Tyrinthos' showed a lot of symptoms on the majority of leaves, whereas apricot 'Boccuccia' proved to be much less susceptible with regard to symptoms on leaves. Few symptoms were observed on some leaves of 'Boccuccia' in May, but none in August. Symptoms were newer observed on leaves of plum rootstock. The scion part that has been showing clear PPV symptoms was cut down several years ago and the rootstock is growing as root suckers. On location Ljubljana symptoms were abundantly present on all sampled trees, which were infected with PPV-Rec, PPV-D or PPV-M.

Our results indicate that flowers in full bloom are a good tissue source for detection of PPV. Flowers in full bloom of apricot 'Tyrinthos' and 'Boccuccia', of unknown plum cultivar and of plum rootstock were suitable for detection of PPV, since the infection could be detected with all tested

techniques (Table 1). Negative results were obtained using field test for small closed flowers that gave positive result using DAS-ELISA and molecular techniques. Successful detection of PPV in flowers is in accordance with findings of Adams (1978) who could detect PPV using ELISA in flowers of all three tested varieties of plum. On the other hand Dosba *et al.* (1986) considered detection of PPV by ELISA in apricot and peach flowers as unreliable. Further tests need to be done. Young leaves taken from a rootstock during flowering had a lower concentration (estimated from the Ct value) of virus than flowers (Table 1). Infection could not be detected with field tests. Field tests also failed to detect infection in very small leaves of apricot 'Boccuccia' while DAS-ELISA showed suspiciously elevated OD values. Infection was confirmed using molecular techniques.

Table 1: Results of testing the samples collected on 3rd of April, 2011; Maribor, Slovenia.
Preglednica 1: Rezultati testiranja vzorcev zbranih 3. aprila 2011; Maribor, Slovenija.

Species, cultivar	Isolate	Tested	AgriStrip	Immuno chromato	ELISA (OD values)	RT-PCR	Real time (Ct)
apricot, 'Boccuccia'	D	very small leaves flowers in full bloom	neg. pos.	neg. pos.	susp.(0.092) pos. (0.771)	pos. pos.	pos. (30) pos. (19)
apricot, 'Tyrinthos'	M	flowers in full bloom	pos.	pos.	pos. (3.132)	pos.	pos. (15)
plum, unknown cultivar	M	flowers in full bloom	pos.	pos.	pos. (0.737)	pos.	pos. (21)
plum, 'Požegača' type	D	small closed flowers	neg.	neg.	pos. (0.334)	pos.	pos. (23)
plum rootstock	M	flowers in full bloom young leaves	pos. neg.	pos. neg.	pos. (0.682) pos. (0.134)	pos. pos.	pos. (21) pos. (24)
plum, 'Jojo'		flowers in full bloom	neg.	neg.	neg. (0.048)	neg.	neg.
plum, 'Katinka'		flowers in balloon stage	neg.	neg.	neg. (0.048)	neg.	neg.
		negative control			0.037		

pos. = PPV detected = potrjena okužba s PPV
neg. = PPV not detected = okužba s PPV ni potrjena
susp. = OD suspiciously elevated, but below the threshold

The results show that PPV is not always present in asymptomatic leaves or the amount of virus is very low therefore detection in latently infected trees is not always reliable. Detection of PPV with field tests and DAS-ELISA in mature leaves from infected trees depended much on the presence of symptoms (Tables 2 and 3). In our experiments PPV could be detected with all tested techniques in symptomatic parts of the leaves, even in the beginning of August when the temperatures were high. The only exception was Immunocromato test of symptomatic parts of the leaves taken from apricot 'Tyrinthos' in August. PPV infection was not detected in any of the tested samples from leaves without symptoms using field tests and DAS-ELISA in May and August. These results confirm the findings of several authors (Adams, 1978; Hamdorf, 1982; Myrta *et al.*, 2003), who described ELISA as unreliable when asymptomatic leaves were used. Using RT-PCR the presence of PPV in asymptomatic leaves was confirmed in 4 out of 9 samples from non-resistant trees (i.e. all trees except of 'Jojo' and 'Katinka'). Real time PCR gave somewhat better results. Nevertheless the virus could not be detected in 3 samples of asymptomatic leaves of non-resistant cultivars.

Table 2: Results of testing the samples collected on 1st and 3rd of May, 2011; Maribor and Ljubljana, Slovenia.
Preglednica 2: Rezultati testiranja vzorcev zbranih 1. in 3. maja 2011; Maribor in Ljubljana, Slovenija
Lw = leaves without symptoms = listi brez znakov
La = parts of the symptomatic leaves without symptoms
= asimptomatični deli listov z znaki
Ls = symptomatic parts of the leaves = simptomatični deli listov
Sw = stalks of the leaves without symptoms = peclji listov brez znakov
Ss = stalks of the symptomatic leaves = peclji listov z znaki
Svs = very small stalks = zelo majhni listni peclji

Location	Species, cultivar	Isolate	Symptoms	Tested	AgriStrip	Immuno chromato	ELISA (OD)	RT-PCR	Real time (Ct)
Maribor NE Slovenia	apricot, 'Boccuccia'	D	few symptoms on some leaves	Lw	neg.	neg.	neg. (0.025)	pos.	pos. (25)
				Sw	neg.	neg.	neg. (0.035)	pos.	pos. (24)
				La	pos.	neg.	neg. (0.023)	pos.	pos. (21)
				Ls	pos.	pos.	pos. (0.240)	pos.	pos. (17)
				Ss	pos.	neg.	neg. (0.028)	pos.	pos. (20)
	apricot, 'Tyrinthos'	M	a lot of symptoms on the majority of leaves	Lw	neg.	neg.	neg. (0.022)	neg.	neg. (37)
				Sw	neg.	neg.	neg. (0.027)	pos.	pos. (27)
				La	pos.	neg.	pos. (0.100)	pos.	pos. (18)
				Ls	pos.	pos.	pos. (0.386)	pos.	pos. (14)
				Ss	neg.	neg.	pos. (0.093)	pos.	pos. (21)
	plum, unknown cultivar	M	a lot of symptoms on the majority of leaves	Lw	neg.	neg.	neg. (0.023)	neg.	pos. (26)
				Sw	pos.	weak	pos. (0.110)	pos.	pos. (22)
				La	neg.	neg.	neg. (0.024)	neg.	neg.
				Ls	pos.	pos.	pos. (0.588)	pos.	pos. (13)
				Ss	pos.	weak	pos. (0.671)	pos.	pos. (18)
	plum, 'Požegača ' type	D	a lot of symptoms on the majority of leaves	Lw	neg.	neg.	neg. (0.025)	neg.	pos. (34)
				Sw	pos.	pos.	pos. (0.120)	pos.	pos. (21)
				La	weak	weak	susp. (0.057)	pos.	pos. (21)
				Ls	pos.	pos.	pos. (0.792)	pos.	pos. (13)
				Ss	pos.	weak	pos. (0.121)	pos.	pos. (19)
	plum rootstock	M	no symptoms	Lw	neg.	neg.	neg. (0.026)	neg.	neg. (38)
				Svs	n.t.	n.t.	n.t.	n.t.	n.t.
	plum, 'Jojo'		no symptoms	Lw	neg.	neg.	neg. (0.023)	neg.	neg. (37)
				Sw	neg.	neg.	neg. (0.023)	neg.	neg. (37)
	plum, 'Katinka'		no symptoms	Lw	neg.	neg.	neg. (0.023)	neg.	neg.
				Sw	neg.	neg.	neg. (0.024)	neg.	pos. (33)
Ljubljana, central Slovenia	apricot, unknown cultivar	D	a lot of symptoms on all leaves	La	pos.	neg.	pos. (0.111)	pos.	pos. (25)
				Ls	pos.	pos.	pos. (2.061)	pos.	pos. (18)
				Ss	pos.	pos.	pos. (0.408)	pos.	pos. (24)
	plum, unknown cultivar	Rec	a lot of symptoms on all leaves	La	pos.	neg.	pos. (0.206)	pos.	pos. (22)
				Ls	pos.	pos.	pos. (0.607)	pos.	pos. (18)
				Ss	pos.	pos.	pos. (0.369)	pos.	pos. (24)
	P. insititia = damson plum	Rec	a lot of symptoms on all leaves	La	neg.	neg.	pos. (0.274)	pos.	pos. (23)
				Ls	pos.	pos.	pos. (1.461)	pos.	pos. (16)
				Ss	pos.	pos.	pos. (0.242)	pos.	pos. (23)
	peach, unknown cultivar	M	symptoms on the majority of leaves	La	weak	neg.	pos. (0.305)	pos.	pos. (23)
				Ls	pos.	pos.	pos. (0.983)	pos.	pos. (18)
				Ss	neg.	neg.	susp. (0.073)	pos.	pos. (25)
negative controls							0.021 – 0.028		

n.t. = not tested = ni testirano
pos. = PPV detected = potrjena okužba s PPV
neg. = PPV not detected = okužba s PPV ni potrjena
susp. = OD suspiciously elevated, but below the threshold

Table 3: Results of testing the samples collected on 1st of August, 2011; Maribor, Slovenia.
Preglednica 3: Rezultati testiranja vzorcev zbranih 1. avgusta 2011; Maribor, Slovenija.
Lw = leaves without symptoms = listi brez znakov
B = buds = brsti
La = parts of the symptomatic leaves without symptoms = asimptomatični deli listov z znaki
Ls = symptomatic parts of the leaves = simptomatični deli listov

Species, cultivar	Isolate	Symptoms	Tested	AgriStrip	Immuno chromato	ELISA (OD values)	RT-PCR	Real time Ct
apricot, 'Boccuccia'	D	no symptoms	Lw B	neg. neg.	neg. neg.	neg. (0.049) neg. (0.048	pos. pos.	pos. (26) pos. (30)
apricot, 'Tyrinthos'	M	a lot of symptoms	Lw La Ls B	neg. neg. pos. neg.	neg. neg. neg. neg.	neg. (0.031) neg. (0.037) pos. (0.254) neg. (0.043)	pos. pos. pos. pos.	pos. (25) pos. (26) pos. (21) pos. (27)
plum, unknown cultivar	M	a lot of symptoms	La Ls B	neg. pos. neg.	neg. pos. neg.	susp. (0.087) pos. (1.684) susp. (0.069)	pos. pos. pos.	pos. (22) pos. (16) pos. (24)
plum, 'Požegača'type	D	a lot of symptoms	Lw La Ls B	neg. neg. pos. neg.	neg. neg. pos. neg.	neg. (0.027) susp. (0.069) pos. (1.072) neg. (0.048)	neg. pos. pos. pos.	neg. (38) pos. (21) pos. (15) pos. (24)
plum rootstock	M	no symptoms	Lw B	neg. neg.	neg. neg.	neg. (0.048) neg. (0.046)	pos. pos.	pos. (28) pos. (22)
plum 'Jojo'		no symptoms	Lw B	neg. neg.	neg. neg.	neg. (0.034) neg. (0.026)	neg. neg.	neg. neg.
plum, 'Katinka'		no symptoms	Lw B	neg. neg.	neg. neg.	neg. (0.029) neg. (0.037)	neg. neg.	neg. neg.
			negative control			0.026 - 0.036		

pos. = PPV detected = potrjena okužba s PPV
neg. = PPV not detected = okužba s PPV ni potrjena
susp. = OD suspiciously elevated, but below the threshold

Using Immunochromato test infection could not be detected in asymptomatic parts of leaves with symptoms (Tables 2 and 3). AgriStrip and DAS-ELISA testing proved to more successful in May, but failed in August. In one sample tested in May PPV was not detected using even more sensitive molecular techniques. The observation that there may be infected and virus-free zones even in the same leaf (Nemeth, 1986) seams to hold also after using much more sensitive molecular techniques. Adams (1978) found that PPV was frequently undetected by ELISA test in asymptomatic parts of symptomatic plum leaves. The same was found for leaves of apricot 'Tyrinthos' by Myrta *et al.* (2003).

The use of leaf stalks is recommended by the producer of Immunochromato tests. Stalks were tested in May (Table 2). In contrast to leave blades without symptoms stalks taken from the same leaves occasionally gave positive results also with field test and DAS-ELISA. PPV could be confirmed in only some of the stalk samples from leaves with symptoms using field test and DAS-ELISA. Testing leaf stalks using molecular techniques in May proved to be more reliable, since infection was always confirmed in stalks of leaves with symptoms and in stalks of leaves without symptoms. The concentration estimated from Ct values of real time RT-PCR was always significantly lower in stalks from symptomatic leaves when compared with symptomatic parts of the leaves of the same sample. In contrast, the estimated concentration of PPV in stalks of the leaves without symptoms was always higher in comparison with the asymptomatic leave blades.

Our results show that buds are suitable for testing graft-wood in summer if molecular techniques are used. In Slovenia, grafting of *Prunus* is mostly

done in August therefore reliable detection in buds of graft-wood material is very important. Buds were tested only on one location. Infection was not confirmed by field test or by DAS-ELISA (Table 3). Some of the samples gave suspiciously elevated OD values using DAS-ELISA, but the infection needed to be confirmed with molecular techniques. Both tested molecular techniques confirmed the infection in all samples taken from non-resistant plants.

4 CONCLUSIONS

Sensitivity of different detection methods (field tests, DAS-ELISA, two-step RT-PCR and real-time RT-PCR) was tested in different tissues of apricot, plum, damson plum and peach trees infected with isolates of *Plum pox virus* PPV-D, PPV-M or PPV-Rec. Flowers of apricots and plums in full bloom proved to be a very good source for detection of PPV, since infection could be detected with all tested techniques. Detection in mature leaves depended on the presence of symptoms. PPV could be detected with all tested techniques in symptomatic parts of the leaves in May and with one exception even in the beginning of August. PPV was not detected in asymptomatic leaves and even in asymptomatic parts of the symptomatic leaves using field tests, DAS-ELISA and partly also molecular techniques. These results show that PPV is not always present in asymptomatic leaves or the amount of virus is very low; therefore, detection in latently infected trees is not always reliable. Additionally, the observation that there may be infected and virus-free zones even in the same leaf seams to hold also after using much more sensitive molecular techniques. Stalks were tested only in May and proved to be a good tissue source for detection with molecular techniques, since the presence of PPV was always confirmed in stalks from symptomatic as well as asymptomatic leaves. Reliable detection in buds is very important for testing of graft-wood. Unfortunately, infection could not be confirmed in buds in August using field tests or DAS-ELISA, therefore molecular techniques must be used for detection of PPV in graft-wood taken in summer.

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Kakovost jabolk sort 'Gala Brookfield' in 'Fuji Kiku 8' pod in izven protitočne mreže

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IZVLEČEK

Protitočna mreža je pri pridelavi jabolk postala običajen ukrep pri napravi novega ali posodobitvi starega nasada. Med letoma 2007 in 2008 smo v Sadjarskem centru Maribor – Gačnik proučevali razvoj parametrov kakovosti in zrelosti jabolk sort 'Gala Brookfield' in 'Fuji Kiku 8' sajene na podlagi M9 kot posledico vpliva črne protitočne mreže. Rezultati so pokazali, da plodovi pod črno protitočno mrežo pri obeh sortah dosegajo večjo povprečno maso, manjšo vsebnost suhe snovi (od 0,6 do 1 °Brix), manjši škrobni indeks, medtem ko vpliva črne protitočne mreže na trdoto mesa plodov in večji pridelek nismo zaznali.

Ključne besede: jablana, protitočna mreža, kakovost

ABSTRACT

QUALITY OF CV. 'GALA BROOKFIELD' AND 'FUJI KIKU 8' APPLES GROWN UNDER AND OUTSIDE HAIL NET

Anti-hail nets for the production of apples has become a normally action for a new or update an old apple orchard. In 2007 and 2008, the development of quality and maturity parameters of cv. 'Gala Brookfield' and 'Fuji Kiku 8' apples grafted on M9 were studied in the Fruit growing centre Maribor-Gačnik as a factor of being grown under a blackhail net. The results showed that when grown under the black hail net fruit of both cultivars reach a higher mean mass, lower soluble solids content (0.6 to 1 ° Brix), and lower starch index, while no effect of the black hail net was detected on fruit flesh firmness and yield.

Key words: apple tree, anti−hail net, quality

1 UVOD

V pridelavi jabolk je cilj doseči velike, redne in kakovostne pridelke in pridelati jabolka, ki bodo lepa na videz, dobrega okusa ter bodo imela dobro skladiščno sposobnost. Postavitev protitočne mreže in namakalnega sistema omogoča hitrejše vračilo vloženih sredstev v napravo nasada, zgodnejše in redne pridelke velike kakovosti, in tako večjo gospodarnost pridelave jabolk.

Svetloba ima odločilen pomen pri razvoju in zorenju plodov, s tem pa posledično vpliva na kakovost plodov in diferenciacijo cvetnih brstov ter druge razvojne fiziološke procese sadnih dreves (Holzwarth, 2008). Različni tipi protitočnih mrež različno močno ovirajo prehod svetlobe skozi mrežo in imajo različno dolgo življenjsko dobo. Bele mreže prepuščajo nekaj več svetlobe (86 %), vendar je ob enaki nabavni ceni življenjska doba več kot pol manjša (Dobaja, 2005). Uporaba kristalnih mrež dovoljuje maksimalno osvetljenost. Ta tip mreže zmanjša osvetljenost pod mrežo za 14 %. Uporaba sivih in črnih protitočnih mrež (najbolj pogosta tipa protitočnih mrež) osvetljenost zmanjšata za 16 % oz. 20 %. Zaradi umazanije, ki

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se nabira na mrežah tekom let, se razlika v prepustnosti svetlobe med različnimi tipi mrež z leti zmanjšuje (Dobaja, 2005). Slabost kristalne mreže je, da ne dosega zmeraj »zahtevanih« 15 let življenjske dobe, medtem ko zaradi uporabljenih materialov pri črni mreži, to v praksi ne predstavlja težave (Holzwarth, 2008).

Raziskave iz Sadjarskega centra Maribor – Gačnik (v nadaljevanju: SC Maribor – Gačnik) in tujine kažejo, da se zaradi zmanjšane osvetlitve pod črno mrežo posledično zmanjša neto fotosinteza. Ena od prednosti protitočne mreže se kaže tudi v tem, da so plodovi pod mrežo v manjši meri izpostavljeni sončnim ožigom, kljub nespremenjeni maksimalni temperaturi listja na drevesih. Po podatkih iz SC Maribor – Gačnik je tudi trajanje vlažnosti listja enako in ni mogoče pričakovati povečane nevarnosti pojava bolezni na drevesih pod mrežo (Zadravec, 1998).

Svetloba je odločilna tudi pri barvi, okusu in trpežnosti plodov (Jazbec in sod., 1995). Prav tako ima svetloba odločilen vpliv na proces tvorbe cvetnih brstov ter začetek cvetenja (Štampar in sod., 2005). Čim tanjša je mreža in tem večje so zanke, vse večja so nihanja v prepustnosti za svetlobo. Blanke (2007) navaja, da se pri kristalnih mrežah fotosintetska aktivna radiacija zmanjša v povprečju za 7 %, pri rdeče-belih za 11 %, pri zeleno-belih za 12 %, pri svetlo zelenih za 13 %, 15 % pri zeleno-črnih, pri rdeče-črnih za 16 % in črnih za 18 %, izmerjeno 50 cm izpod mreže. Količina UV-svetlobe se pri prosojni mreži zmanjša za 20 % in pri črni za 29 %. Zmanjšana temperatura plodov in temperatura listov imata za posledico manjše poškodbe sončnih ožigov. Črna mreža zmanjšuje temperaturo plodov za 4 °C, bela za 2,5 °C. V oblačnih dneh z zmanjšanim sončnim sevanjem so razlike med obravnavanji manjše. Prav tako so razlike med temperaturo zraka in plodov manjše kot v sončnih dneh (Iglesias in Alegre, 2006). V poskusih v tujini in v SC Maribor – Gačnik so bile razlike v relativni zračni vlagi minimalne ter občasno pod mrežo celo nižje kot izven nje, zaradi tega naj ne bi bilo večjih možnosti za razvoj bolezni (Zadravec, 1998). V kasnejših poskusih Zadravec (2009) navaja, da je relativna zračna vlaga pod mrežo nekoliko povečana.

Na vidno dojemanje barve ne vplivajo samo absolutne koncentracije posameznih barvil v kožici plodov, ampak tudi dimenzije vakuol ter razporeditev in velikost celic v kožici jabolk. Končna zaznava barve je posledica vizualnega mešanja vseh naštetih dejavnikov. Antociani se v kožici jabolk kopičijo dvakrat, najprej se kopičijo med prvo fazo razvoja plodov, to je, ko se celice delijo. Prvi fazi intenzivnega nastajanja antocianov sledi obdobje, v katerem se koncentracija antocianov zmanjša, včasih celo popolnoma izginejo. Dolžino tega obdobja določa prisotnost rastnih hormonov, giberelinov, ki regulirajo razgradnjo klorofila, zorenje in nastanek etilena ter abscizinske kisline. Večja kot je vsebnost giberelinov, dlje časa bo trajalo vmesno obdobje in toliko večja bo nevarnost slabega obarvanja plodov. Formiranje antocianov ob koncu rastne dobe (pred zorenjem) je sortno pogojeno. V tem primeru se koncentracija antocianov uporablja kot pokazatelj zrelosti (Curry, 1997).

1.1 Trdota mesa plodov

Trdota mesa plodov je eden izmed najpomembnejših kriterijev kakovosti jabolk in se uporablja kot merilo zrelosti jabolk. Dejavniki pred in po obiranju, ki vplivajo na trdoto mesa jabolk, so: genetski in rastni dejavniki, oskrba z minerali, zrelost ob obiranju in način skladiščenja. Čvrstost plodov se z zorenjem zmanjšuje, zmanjševanje pa je odvisno od sestave celičnih sten ter količine pektinov, celuloze in hemiceluloze, pa tudi od količine sladkorjev s petimi ali šestimi atomi ogljika. Med dozorevanjem in staranjem plodov se spreminja trdota mesa in odpornost tkiva, ki je neposredno odvisna od čvrstosti celičnih membran in od kemične oblike pektina. Med dozorevanjem jabolk se del netopnega pektina spremeni v topnega, kar povzroča mehčanje tkiva in vizualna znamenja staranja. Na čvrstost vplivajo velikost, oblika in turgor celic (Gvozdenović, 1989). Med zorenjem prihaja do povečanih encimskih aktivnosti predvsem poligalakturonaz, celulaz in pektin-metil-esteraze. Ti encimi razgrajujejo polimere v snovi z manjšo molekulsko maso, ki so bolj topne. Polikturonaza povzroča razgradnjo protopektina v topni pektin in tako prispeva k mehčanju ploda.

2 MATERIAL IN METODE

Poskus je potekal v starosti od četrte do šeste rastne dobe jablan. Postavljen je bil po sistemu naključnih skupin. V poskus je bilo iz vsake sorte vključenih 80 dreves, ki so bila po rasti in rodnosti izenačena. Polovico dreves je prekrivala protitočna mreža, polovica je bila izven protitočne mreže.

V letu 2007 smo na izbranih drevesih pri vsaki sorti izbrali po skupno 160 plodov (po 2 na izbrano drevo), polovico pod in polovico izven mreže. Izbrani so bili plodovi, ki so bili po en plod iz socvetja. Izbrani plodovi so bili primerljivega položaja v krošnji glede osvetlitve in iz primerljive starosti rodnega lesa, brez mehanskih poškodb ali poškodb zaradi bolezni in insektov. Pred pričetkom izvajanja meritev je bila na peclje teh plodov pripeta oznaka s šifro, sestavljena iz oznake zaporedne številke plodu in obravnavanja. Plodove smo označili z zaporedno številko in znakom L. Znak L je predstavljal mesto na plodu, kjer so bile izvajane vse meritve barve (Unuk, 2006 in Germšek, 2008).

Slika 1: Jabolka, ki so primerno označena z znakom L in potek meritve.

Figure 1: Apples appropriately marked with a symbol L, and the measurement procedure.

Ovesek ima pomembno vlogo v povezavi s parametri kakovosti plodov (Unuk, 2008), zato smo ga z regulacijo pridelka na želeno obremenitev dreves s pridelkom odstranili kot dejavnik, ki bi lahko motil rezultate. V letu 2007 in 2008 so bila po rasti (presek debla, $sd < 10$ %) in rodnosti (število socvetij, $sd < 10$ %) izenačena drevesa odbrana za testiranje glede njihove primernosti vključitve v poskus. Po formuli za izračun gostote pridelka CD (crop density) smo izračunali obremenitev drevesa s pridelkom (Westwood, 1993).

$$CD = \text{število plodov/cm}^2 \text{ preseka debla}$$

Pri doseženih 15. mm premera smo plodiče ročno po celi krošnji enakomerno poredčili na želeno obremenitev, ki je v poskusnih letih 2007 in 2008 pri sorti 'Gala Brookfield' znašala 6 plodov/cm^2 in pri sorti 'Fuji Kiku 8' 5 plodov/cm^2. V obravnavanem letu 2007 smo jabolka sorte 'Gala Brookfield' obirali 21. avgusta, sorto 'Fuji Kiku 8' pa 10. oktobra. V obravnavanem letu 2008 smo pri sorti 'Gala Brookfield' z obiranjem začeli dne 1. septembra, pri sorti 'Fuji Kiku 8' pa 13. oktobra.

2.1 Mikroklima v nasadu v letih 2007 in 2008

SC Maribor − Gačnik je pod vplivom kontinentalne in alpske klime in leži na 270−310 m nadmorske višine. Na območju Maribora je bilo v tridesetletnem obdobju (1961−1990) 1122,5 mm padavin, v rastni dobi (april−september) pa 681,5 mm padavin. Povprečna letna temperatura zraka je v tridesetletnem obdobju (1961−1990) na območju Maribora znašala 10,5 °C. V tridesetletnem obdobju ni bilo zaznati pomanjkanja padavin. Na območju SC Maribor – Gačnik je bilo v letu 2007 983,6 mm padavin, v rastni dobi pa 618,4 mm padavin. V tem letu je bila povprečna letna temperatura zraka enaka kot leta 2008, tj. 11,1 °C. Povprečna temperatura zraka med rastno dobo (april−september) pa je znašala 17,4 °C. V letu 2008 je bilo 852,4 mm padavin, v rastni dobi pa

597,6 mm padavin. Povprečna temperatura zraka med rastno dobo je bila 17,0 °C (KGZS-Zavod Maribor).

Srednja dnevna temperatura zraka je bila v letih 2007 in 2008 pod mrežo za 0,17 °C nižja kot izven mreže, povprečna mesečna maksimalna temperatura zraka pod mrežo je bila s 27,4 °C višja od temperature izven nje, ki je znašala 26,5 °C. Višja je bila tudi povprečna minimalna temperatura zraka pod mrežo s 13,8 °C v primerjavi s 13,1 °C izven protitočne mreže. Prav tako je večja tudi povprečna relativna zračna vlaga (77,9 %) pod mrežo od povprečne relativne zračne vlage (76,7 %) izven mreže. Globalno obsevanje, ki so ga merili s piranometrom, je pod protitočno mrežo na dnevnem nivoju doseglo le 52,3 % tistega globalnega obsevanja, ki smo ga izmerili izven protitočne mreže. Relativna zračna vlaga je v povprečju 33 ur za 1,1 % večja pod protitočno mrežo v primerjavi z relativno zračno vlago izmerjeno izven protitočne mreže. Dolžino omočenosti listja smo merili s senzorjem omočenosti listja, ki je bil nameščen neposredno v krošnji drevesa, drevo pa je bilo posajeno v obravnavanem nasadu. Izmerjena dolžina omočenosti listja je po nočnih padavinah pod protitočno mrežo za 1 uro in 35 minut daljša kot izven protitočne mreže.

2.2 Spravilo in vrednotenje pridelka

Med tehnološko zrelostjo je bil obran in stehtan pridelek ter prešteto število plodov po drevesu. Plodovi so bili razvrščeni v kakovostne razrede, ki so definirani glede na velikost in obarvanost plodov. Pridelek smo tehtali posebej po velikostnem razredu, nato pa skupno količino pridelka za posamezno drevo. V 1. kakovostni razred smo razvrstili plodove, ki so bili večji od 70 mm in obarvani nad 50 %. V drugi kakovostni razred smo razvrstili plodove velikosti od 60 do 70 mm in obarvane najmanj 30 %.

Plodovi za vrednotenje parametrov zrelosti so bili obrani v optimalni tehnološki zrelosti. Parametri zrelosti so bili v letih 2007 in 2008 določeni z uporabo stroja Pimprenelle (Setop giraud technologie, 248 Route du Moulin De Losque, 84300 Cavaillon, Francija), ki da ob testiranju plodov naslednje podatke: povprečna masa plodov, vsebnost topne suhe snovi, trdota mesa plodov, vsebnost skupnih kislin in pH soka. Škrob smo določali po standardni metodi. Prerezani plodovi so bili potopljeni v 0,02 M raztopino J_2 v kalijevem jodidu (KJ). Intenzivnost in delež obarvanega dela sta bila ocenjena s pomočjo primerjalne škrobne lestvice EVROFRU z vrednostmi 1−10 (Ctifl 1993). Kot vrednost, ki združuje posamezne parametre zrelosti, je bil izračunan Streifov indeks zrelosti (Streif, 1996).

3 REZULTATI IN RAZPRAVA

3.1 Vpliv protitočne mreže na količino in maso pridelka

Vzorčno polje pod in izven protitočne mreže je bilo po pedoloških lastnostih enako, tako da same razlike lokacije obeh delov nasada niso prispevale k različnosti rezultatov. Vse zunanje in notranje lastnosti plodov so močno odvisne od obloženosti plodov; rezultate povprečnega pridelka izraženega v kilogramih na drevo in povprečno število plodov na drevo so predstavljeni v preglednici 1.

Preglednica 1: Razvrstitev plodov glede na dosežen pridelek in število plodov pri sortah 'Gala Brookfield' in 'Fuji Kiku 8' v letu 2007 in 2008 pod in izven protitočne mreže

Table 1: Classification of cv. 'Gala Brookfield' and 'Fuji Kiku 8' apple fruit according to the achieved yield and number of fruit for fruit grown under and outside hail net in 2007 and 2008.

Leto Year	Sorta Variety	Obravnavanje Treatment	Povp. pridelek (kg/drevo) Avg. yield (kg/tree)	Povp. št. plodov/drevo Avg. fruits/tree
2007	'Gala Brookfield'	Pod mrežo Under the hail net	5,21	26,84
		Izven mreže Outside the hail net	5,74	33,22
	'Fuji Kiku 8'	Pod mrežo Under the hail net	6,32	26,15
		Izven mreže Outside the hail net	4,27	26,51
2008	'Gala Brookfield'	Pod mrežo Under the hail net	7,1	28,43
		Izven mreže Outside the hail net	6,4	20,75
	'Fuji Kiku 8'	Pod mrežo Under the hail net	5,89	25,64
		Izven mreže Outside the hail net	4,23	19,31

Povprečni pridelek pri sorti 'Gala Brookfield' je v obravnavanem letu 2007 pod protitočno mrežo znašal 5,21 kg pridelka na drevo, kar je za 10,17 % manj kot pri povprečnem pridelku izmerjenem izven mreže. Pri sorti 'Fuji Kiku 8' je v enakem obravnavanem letu (2007) povprečni pridelek izmerjen pod protitočno mrežo znašal 6,32 kg na drevo, kar je za 32,43 % več kot pri povprečnem pridelku izven protitočne mreže. V obravnavanem letu 2007 je na območju poskusnega nasada (julij 2007) padala huda toča, kar se je pokazalo v boljši diferenciaciji cvetnih brstov na drevesih obravnavanjih pod protitočno mrežo v letu 2008. Sklepamo, da so zaradi tega povprečni pridelki v letu 2008 pod protitočno mrežo večji. Pri sorti 'Gala Brookfield' smo v tem letu (2008) pod protitočno mrežo zabeležili za 9,8 % večji pridelek, prav tako smo zabeležili večji pridelek pri sorti 'Fuji Kiku 8' v obravnavanju pod protitočno mrežo za 28,18 %. Povprečno število plodov na drevo je bilo pri sorti 'Gala Brookfield' v letu 2007 za 23,77 % manjše kot pri obravnavanju izven mreže. V tem letu (2007) med variantama pod in izven protitočne mreže pri sorti 'Fuji Kiku 8' v povprečnem številu plodov na drevo nismo zaznali razlik. Vpliv toče v letu 2007 se pri število plodov na drevo kaže v letu 2008, saj smo zabeležili večje število plodov pri varianti pod protitočno mrežo za obe obravnavani sorti. Pri sorti 'Gala Brookfield' je bilo v obravnavanju pod protitočno mrežo na drevo v povprečju za 37,01 % več plodov kot v varianti izven protitočne mreže. Pri sorti 'Fuji Kiku 8' je bil ta odstotek nekoliko manjši in je v varianti pod protitočno mrežo znašal 24,68 % plodov več kot v varianti izven protitočne.

3.2 Vpliv protitočne mreže na delež obarvanosti plodov sort 'Gala Brookfield' in 'Fuji Kiku 8'

Iz preglednice 2 je razvidno, da so bili pri obeh sortah deleži obarvanosti plodov zelo podobni. Pri obeh preskušanih sortah gre za dobro obarvane mutante sort 'Gala Brookfield' in 'Fuji Kiku 8'. Pod mrežo je doseglo nad 50 % obarvanosti pri sorti 'Gala Brookfield' 93,6 % plodov in pri sorti 'Fuji Kiku 8' 92,55 %, kar je pri sorti 'Gala Brookfield' slab odstotek manj, pri sorti 'Fuji Kiku 8' pa 2,5 % manj od obarvanosti plodov izven mreže. Čeprav je v rezultatih zaznana tendenca zelo blagega zmanjšanja deleža krovne barve pri vseh

obravnavanjih pod mrežo, so razlike tako majhne, da ne obstajajo statistično značilne razlike med obravnavanji in lahko trdimo, da črna protitočna mreža v letu 2008 ni negativno vplivala na obarvanost plodov.

Preglednica 2: Razvrstitev plodov (%) glede na dosežen delež krovne barve pri sortah 'Gala Brookfield' in 'Fuji Kiku 8' v letu 2008 pod in izven protitočne mreže

Table 2: Classification of fruit (%) according to the proportion of the blush colour in cv. 'Gala Brookfield' and 'Fuji Kiku 8' grown under or outside of the hail net in 2008

% obarvanosti Colouring in %	31−50 %		Nad 50 % Over the 50 %	
Sorta Variety	Pod mrežo Under the hail net	Izven mreže Outside the hail net	Pod mrežo Under the hail net	Izven mreže Outside the hail net
'Gala Brookfield'	6,4	5,6	93,6	94,4
'Fuji Kiku 8'	7,5	5,0	92,5	95,0

3.3 Vpliv protitočne mreže na parametre zrelosti

Povprečna masa ploda pri sorti 'Gala Brookfield' izven protitočne mreže je v letu 2007 znašala 174,11 g, kar je za 12,6 % manj kot v obravnavanju pod mrežo, kjer je povprečna masa plodov dosegla 196,09 g. Razlika v povprečni masi ploda je bila statistično značilna (preglednica 3). Povprečne vrednosti suhe snovi so bile pri sorti 'Gala Brookfield' v obravnavanju pod mrežo za 6,5 % manjše (statistično značilna razlika) in so dosegle vrednost 12,27 °Brix. Povprečna vrednost trdote pri obravnavanju pod mrežo je bila za 2,3 % večja od povprečnih vrednosti pri obravnavanju izven mreže, kjer znaša povprečna trdota mesa 8,15 kg/cm^2, vendar razlika ni statistično značilna. Povprečne škrobne vrednosti pri sorti 'Gala Brookfield' so pri obravnavanju pod mrežo dosegle vrednosti 6,45, pri obravnavanju izven mreže pa 7,76, kar je 16,9 % več, razlika pa je bila statistično značilna. Streifov indeks zrelosti potrjuje značilen vpliv mreže na stopnjo zrelosti plodov, ki je pod mrežo manjša. Pri sorti 'Fuji Kiku 8' je povprečna masa plodov pri obravnavanju pod mrežo dosegla 277,4 g, kar je za 12,1 % več kot pri obravnavanju izven mreže, kjer so povprečne vrednosti mase plodov dosegle 247,38 g. Povprečne vrednosti trdote mesa plodov so pri obravnavanju izven mreže dosegle za cca 3,2 % večjo vrednost kot pri obravnavanju pod mrežo, kjer je bila povprečna vrednost 6,95 kg/cm^2. Tudi pri sorti 'Fuji Kiku 8' razlike v trdoti mesa niso bile statistično značilne. Povprečne vrednosti škroba so bile v plodovih izven mreže večje za 4,7 %. Streifov indeks zrelosti tudi pri sorti 'Fuji Kiku 8' nakazuje trend manjše stopnje zrelosti plodov pod mrežo.

Obema preskušanima sortama je v letu 2007 skupno značilno zmanjšanje vsebnosti suhe snovi pri plodovih pod mrežo (od 0,8 do 0,9 °Brix), kar je tudi glavni razlog manjše vrednosti Streifovega indeksa zrelosti pri plodovih izven mreže. V trdoti mesa plodov so razlike med obravnavanji manjše in niso značilne.

Ob izenačenem ovesku, 6 plodov/cm^2 debla pri sorti 'Gala Brookfield' in 5 plodov/cm^2 debla pri sorti 'Fuji Kiku 8', je tudi v letu 2008 viden trend večje povprečne mase plodov pri drevesih pod mrežo, vendar v tem letu razlike niso značilne. Pri sorti 'Gala Brookfield' povprečna masa plodov doseže 225,8 g pod mrežo, kar je 6,7 % več kot pri plodovih izven mreže. Pri sorti 'Fuji Kiku 8' plodovi pod mrežo dosežejo povprečno maso 236,9 g, kar je v povprečju 5 % več od plodov izven mreže. Plodovi sorte 'Gala Brookfield' so izven mreže dosegli vsebnost topne suhe snovi 11,3 °Brix, kar je cca 0,13 °Brix več kot plodovi pod mrežo. Plodovi sorte 'Fuji Kiku 8' pa izven mreže dosežejo 15,4 °Brix, kar je cca 6,2 % več (signifikantno) kot plodovi pod mrežo. Pri obeh sortah je v bila v letu 2008 trdota mesa plodov izven mreže statistično značilno višja kot pri

plodovih pod mrežo. Pri sorti 'Gala Brookfield' je trdota mesa plodov izven mreže dosegla vrednost 8,6 kg/cm^2, pri sorti 'Fuji Kiku 8' pa 7,9 kg/cm^2. Pri škrobnem indeksu pri sorti 'Gala Brookfield' v tem letu ni bilo dokazanih razlik, medtem ko je bil škrobni indeks pri sorti 'Fuji Kiku 8' presenetljivo signifikantno višji v plodovih pod mrežo in je dosegel vrednost 8,1, kar je 6,8 % več kot pri plodovih izven mreže. Vrednosti Streifovih indeksov zrelosti so bile v tem letu pri obeh sortah zelo podobne in razlike v vrednostih niso signifikantne. Stopnja zrelosti plodov je bila pri obeh sortah ne glede na obravnavanje primerljiva.

Preglednica 3: Povprečne vrednosti parametrov zrelosti plodov jablan sorte 'Gala Brookfield' in 'Fuji Kiku 8' in standardni odklon (sd) ob obiranja v letu 2007 in 2008)

Table 3: Mean values with standard deviation (sd) of the maturity parameters for cv. 'Gala Brookfield' and 'Fuji Kiku 8' apples at harvest in 2007 and 2008

Leto Year	Sorta Variety	Obravnavanje Treatment	Obravnavanje Treatment	Masa (g)* The mass (g)	Sladkor (°Brix) Sugar (°Brix)	Trdota (kg/cm^2) Hardness (kg/cm^2)	Škrob (1–10) Starch (1–10)	Streifov Indeks Streif Index
2007	'Gala Brookfield'	Pod mrežo Under the hail net	Povp. Avg.	196,09 a	12,27 b	8,34 ns	6,45 b	0,105a
			sd	19,74	0,61	1,28	1,74	0,06
		Izven mreže Outside the hail net	Povp. Avg.	174,11 b	13,12 a	8,15 ns	7,76 a	0,080b
			sd	15,09	0,58	0,90	1,46	0,02
	'Fuji Kiku 8'	Pod mrežo Under the hail net	Povp. Avg.	277,41 a	13,63 b	6,95 ns	7,90 ns	0,065ns
			sd	40,52	0,93	0,50	0,87	0,01
		Izven mreže Outside the hail net	Povp. Avg.	247,38 b	14,25 a	7,18 ns	7,53 ns	0,067ns
			sd	38,28	0,87	0,93	1,56	0,01
2008	'Gala Brookfield'	Pod mrežo Onder the hail net	Povp. Avg.	225,81 ns	11,17 ns	8,13 b	5,93ns	0,15 ns
			sd	28,06	0,59	0,68	1,80	0,09
		Izven mreže Outside the hail net	Povp. Avg.	211,50 ns	11,29 ns	8,57 a	5,38ns	0,17 ns
			sd	34,70	0,80	0,83	1,94	0,10
	'Fuji Kiku 8'	Pod mrežo Under the hail net	Povp. Avg.	236,91 ns	14,49 b	7,17 b	8,07 a	0,06 ns
			sd	41,27	0,66	0,95	0,84	0,01
		Izven mreže Outside the hail net	Povp. Avg.	225,04 ns	15,42 a	7,93 a	7,52 b	0,07 ns
			sd	36,57	0,69	0,79	1,24	0,02

*Vrednosti posameznega parametra, označene z enako črko, se med seboj statistično značilno ne razlikujejo (t-test)

4 SKLEPI

Glede na rezultate poskusa smo prišli do naslednjih sklepov:

- vpliv protitočne mreže se pri sortah 'Gala Brookfield' in 'Fuji Kiku 8' med tehnološko zrelostjo odrazi v zmanjšani vsebnosti suhe snovi (od 0,6 do 0,9 %) in večji vsebnosti škroba, kar se odrazi v večji vrednosti Streifovega indeksa zrelosti oz. manjši zrelosti,

– protitočna mreža nima konsistentnega vpliva na pridelek in trdoto mesa plodov.

Kot navajajo avtorji (Štampar, Hudina, Usenik, Sturm Zadravec 2001; Jakopič, Veberič, Štampar 2007 in Cassandro 2011) tudi naši rezultati raziskave potrdijo upravičenost uporabe protitočnih mrež v nasadu jablan s stališča varnosti pridelave in izničujejo strah pred morebitnimi negativnimi posledicami protitočne mreže.

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CONTENT ANALYSIS OF THE PAPERS IN THE ACTA AGRICULTURAE SLOVENICA

VSEBINSKA OBDELAVA PRISPEVKOV V ACTA AGRICULTURAE SLOVENICA let. 103 št. 1

Tomaž BARTOL[a], Karmen STOPAR[b],

SUBJECT INDEX BY AGROVOC DESCRIPTORS
PREDMETNO KAZALO PO DESKRIPTORJIH AGROVOC

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VSEBINSKO KAZALO PO SKUPINAH ZNANJA (PREDMETNIH KATEGORIJAH)

In memoriam

JOŽE KOROŠEC (1930-2013)

Pričujoči zapis je spomin na akademsko in strokovno pot prof. dr. Jožeta Korošca, priznanega strokovnjaka za travništvo in pridelovanje krme, ki je preminil 26. decembra 2013.

Prof. Jože Korošec se je rodil 24. februarja 1930 v Kobilju v občini Lendava kot sin kmečkih staršev, ki so kmetovali na pet hektarjev veliki kmetiji. Tu je obiskoval tudi osnovno šolo. Prve tri razrede nižje gimnazije je med okupacijo obiskoval v Murski Soboti, četrti razred z malo maturo pa je končal leta 1946 v Lendavi. Na Srednji kmetijski šoli Maribor je maturiral junija 1949. Nato je bil kot kmetijski tehnik dobro leto zaposlen na takratni Okrajni kmetijski zadružni zvezi v Grosupljem in na Poverjeništvu za kmetijstvo pri Oblastnem ljudskem odboru v Ljubljani. Jeseni 1950 se je vpisal na Fakulteto za agronomijo, gozdarstvo in veterinarstvo v Ljubljani in septembra 1955 diplomiral. Nato je odšel na služenje vojaškega roka, kjer je končal enoletno šolo za rezervne oficirje.

Poklicna pot prof. Jožeta Korošca se je začela v času uveljavljanja novega družbenega sistema v takratni Jugoslaviji, ki je bil še močno obremenjen z drugo svetovno vojno. To je vsekakor v precejšnji meri opredelilo tako njegovo znanstveno kot tudi poznejše pedagoško delovanje. Seveda je tudi vplivalo na njegov osebni pogled na slovensko oziroma jugoslovansko kmetijstvo in družbo nasploh. Iz te izkušnje je izhajala njegova velika zavzetost za ohranjanje kmetijskih zemljišč in njihovo smotrno rabo.

Po končanem šolanju se je zaposlil na Kmetijskem inštitutu Slovenije, kjer je v okviru Zavoda za poljedelstvo, travništvo in vrtnarstvo delal na referatu za travništvo in večletne krmne rastline. Leta 1957 je uspešno opravil državni strokovni izpit in bil na podlagi tega in drugih del leto pozneje izvoljen v naziv asistenta za področje travništva in pašništva.

Na Kmetijskem inštitutu Slovenije je prva tri leta delal pod strokovnim vodstvom agronomov Valentina Petkovška in Gvida Fajdige. Takrat, na začetku velikih tehnoloških sprememb, je bil priča uvajanju vprežnih in traktorskih kosilnikov v travništvo in prvim poskusom siliranja travniške krme – dvema prelomnima dogodkoma v razvoju slovenskega kmetijstva. Kljub njegovi šali, da je služil za prenašanje torb svojih mentorjev, je bil to pravi začetek za njegovo poznejšo samostojno strokovno pot. S prof. dr. Gvidom Fajdigom sta bila sodelavca tudi na Biotehniški fakulteti, kjer se je prof. Korošec posvečal pridelavi krme na travinju in njivah, prof. Gvido Fajdiga pa pašništvu.

S samostojnim raziskovalnim delom je prof. Jože Korošec začel leta 1959. Pri tem se je ukvarjal s temeljnimi težavami pridelovanja travniške krme. Stroka je namreč v tistem času potrebovala znanje o tem, kako povečati količino in kakovost pridelkov na travnikih in pašnikih, da bi zadostili potrebam tudi hitro razvijajoče živinoreje oziroma po hrani nasploh, tako v Sloveniji kot v drugih republikah Jugoslavije. Konkretno je bilo njegovo raziskovanje omejeno na vprašanja gnojenja in košnje travne ruše – torej tehnoloških dejavnikov, za katera bi lahko rekli, da pretežno odločata o tem, kakšen bo pridelek krme.

Vzporedno se je začel ukvarjati tudi z žlahtnjenjem trav in metuljnic in pri tem je bil zelo uspešen, saj še danes velja za žlahtnitelja, ki je vzgojil največ sort trav in metuljnic v Sloveniji. Med rastlinskimi vrstami, s katerimi se je ukvarjal, prvo mesto pripada njemu posebno ljubi črni detelji. Iz populacij kranjske črne detelje je vzgojil sorti 'Poljanka' in 'Živa'. Črna detelja in druge krmne metuljnice so ga povezovale z njegovo drugo ljubeznijo – čebelarstvom, ki ga je vseskozi dojemal v širšem kmetijskem pomenu in ne le zgolj kot pridelavo medu. Črni detelji so pri žlahtnjenju sledile pomembne trave in druge krmne metuljnice. Med njimi velja izpostaviti trpežno in mnogocvetno ljuljko, pasjo travo in mačji rep, pri katerih so nastale sorte 'Ilirka', 'Draga', 'Kopa' in 'Krim'. Vse te sorte so skupaj s sortama črne detelje v desetletjih, ki so sledila njihovemu vpisu v sortno listo, odločilno

prispevale k izboljšanju pridelave koševin in travniške krme na sejanih travnikih. Ponosen je bil, da mu je v okviru strokovnega dela uspelo popularizirati pridelovanje koševin, med njimi še posebno pomembne mešanice črne detelje in mnogocvetne ljuljke.

V času službovanja na Kmetijskem inštitutu Slovenije je opravil na različnih ustanovah v nekdanji skupni državi in drugih evropskih državah različne specializacije s področij travništva, pridelovanja travno-deteljnih mešanic, žlahtnjenja in preizkušanja sort metuljnic in trav. Izpostaviti je treba profesorjeva gostovanja na večini jugoslovanskih strokovno-znanstvenih institucijah ter pod mentorstvom priznanih strokovnjakov na raziskovalnih postajah v italijanskih Lodiju, Firencah in Padovi, v švicarskih Zürichu in Reckenholzu, v avstrijskem Gumpensteinu, v nemškem Münchebergu, madžarskih Martonvasaru, Budimpešti in Keszthelyju.

Leta 1975 je bil na Univerzi v Ljubljani izvoljen v naziv višjega znanstvenega sodelavca za travništvo in večletne krmne rastline in v letu 1977 v naziv izrednega profesorja za področje travništva in pridelovanja krme. Tako se je v začetku leta 1978 zaposlil na Biotehniški fakulteti, tedanjem VTOZD-u za agronomijo, kjer je bil leta 1982 izvoljen v naziv rednega profesorja za področje travništva in pridelovanja krme. Na tem delovnem mestu je s ponovno izvolitvijo v isti naziv leta 1988 ostal vse do upokojitve leta 1997.

Po prihodu na Biotehniško fakulteto se je skupaj s sodelavci ponovno bolj posvetil preučevanju agro-ekoloških in tehnoloških vprašanj pri pridelovanju voluminozne krme na travinju in njivah. Narejena je bila cela vrsta raziskav s travno-deteljnimi mešanicami z namenom iskanja njihove optimalne sestave in izrabe metuljnic za preskrbo sestojev z biotsko vezanim dušikom iz zraka. V teh raziskavah je bilo potrjeno, da tudi Slovenija spada med tiste evropske države, kjer večvrstne mešanice trav in metuljnic bolje uspevajo in so bolj trpežne od monokultur in enostavnih mešanic, kar je pomembno – kot bi danes rekli – za bolj trajnostno pridelavo krme. Prispevek k trajnostni pridelavi krme je tudi ugotovitev, da metuljnice v mešanicah s travami omogočajo precejšnjo mero samooskrbe sestojev z dušikom. Takšni sestoji so tudi po tehnološki zahtevnosti in prehranski vrednosti boljši od samih metuljnic.

Mednarodno znanstveno sodelovanje prof. Jožeta Korošca je bilo najbolj intenzivno v raziskovalni skupini za gorski travnati svet pri Organizaciji za prehrano in kmetijstvo v obdobju od 1968 do 1987. V tej skupini je bil vključen skupaj s kolegoma – prof. dr. Mirkom Leskoškom in prof. dr. Francetom Šuštarjem – v evropske raziskave izboljševanja degradiranega travinja, vplivov rabe in gnojenja na pridelovalno sposobnost različnih travišč in njihovo floristično sestavo ter vpliva nadmorske višine na fenološki razvoj indikatorskih travniških rastlin. Ena od njihovih pomembnih ugotovitev je bila, da je redko in zapleveljeno travno rušo, ki ima majhno pridelovalno in prehransko vrednost, možno obnoviti z novo setvijo brez oranja, z uporabo herbicidov in minimalno obdelavo. Ta ugotovitev je še posebno pomembna za Slovenijo, kjer absolutnih travnatih zemljišč – takšna pa je velika večina – ni mogoče obnoviti s klasično pripravo tal za setev.

Prof. Jože Korošec je sodeloval pri izobraževanju številnih generacij študentov na različnih študijskih stopnjah na Biotehniški fakulteti v Ljubljani in Višji agronomski šoli Maribor. S predavanji je pokrival vsebine klasičnega travništva in pridelovanja krme na njivah. Plodovitost tega dela se najbolj nazorno kaže v številu mentorstev. Teh je bilo triintrideset na dodiplomskem študiju, sedem na magistrskem in šest na doktorskem študiju. Večina njegovih diplomantov, magistrov in doktorjev znanosti dela na kmetijskem področju in po najboljših močeh nadaljuje delo in poslanstvo prof. Jožeta Korošca.

Izsledke raziskav njegove raziskovalne skupine je redno objavljal tako v znanstveni kot strokovni literaturi. Napisal je tudi številna monografska strokovna dela, ki so še sedaj pomemben vir znanja tako za študente kmetijstva kot pridelovalce krme v praksi. Slednje je zagotovo pomembno prispevalo k napredku kmetijske stroke.

Prof. Jože Korošec je poleg obsežnega pedagoškega in bogatega znanstveno-raziskovalnega ter strokovnega dela opravljal

tudi številne vodstvene naloge. Tako je bil v obdobju od 1983 do 1987 namestnik predstojnika VTOZD-a za agronomijo in v obdobju od 1983 do 1990 predstojnik Katedre za poljedelstvo, pridelovanje krme in vrtnarstvo. Do leta 1985 je bil tudi predsednik skupščine Posebne raziskovalne skupnosti za kmetijstvo, živilstvo in veterinarstvo Slovenije. Pri Razvojni skupnosti za kmetijstvo, živilstvo in prehrano SRS je bil predsednik strokovnega sveta za rastlinsko poljedelsko proizvodnjo, do leta 1986 pa je opravljal funkcijo predsednika podkomisije za priznavanje novih sort krmnih rastlin pri Zveznem komiteju za kmetijstvo.

Prof. dr. Jože Korošec je prejel številna državna in druga priznanja za svoje znanstveno in drugo udejstvovanje. Med drugim je bil dvakratni dobitnik nagrade Sklada Borisa Kidriča za žlahtniteljsko delo, dobitnik Jesenkovega priznanja Biotehniške fakultete za znanstvene in pedagoške dosežke, predsednik republike pa ga je leta 1964 nagradil z redom zaslug za narod.

Jure Čop in Stanislav Trdan, Biotehniška fakulteta, Univerza v Ljubljani

Slika 1: Profesor dr. Jože Korošec na poskusnem polju v Gumpenstenu – Avstrija.

NAVODILA AVTORJEM

(letniki z liho številko - rastlinska proizvodnja)

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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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