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PHENOTYPIC DIVERSITY OF ALFALFA (*MEDICAGO SATIVA* L.) GERMPLASM

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SUMMARY

The objective of this study was to evaluate phenotypic diversity in the alfalfa germplasm collections using multivariate analysis to examine the extent of genetic diversity and contribution of selected characters to the total diversity and finally to select the most promising clusters/populations for further breeding work. Forty alfalfa populations/cultivars of different geographical origin were evaluated for 12 agro-morphological characters during two consecutive years. The populations/cultivars were grouped into six clusters. In most cases populations/cultivars within clusters were not associated with their geographical origin. Intercluster distances were larger than the intracluster ones. This research revealed a broad phenotypic diversity within and between the alfalfa germplasm collections. The following characters contributed most to the total phenotypic diversity: dry matter yield in the first production year, plant height and length of central leaflet. Based on the mean value of the evaluated characters and determined distances between clusters, the most promising populations/cultivars belong to the clusters IV and V. Selected populations/cultivars could be considered as a valuable genetic material for the yield and quality improvement of alfalfa in our breeding programme.

Key-words: alfalfa, populations, phenotypic diversity, multivariate analysis, breeding

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the most important perennial forage crops on cultivated fields in the world. Agronomical interests for this crop are based on its numerous advantageous characteristics such as (1) high yield potential and suitable feeding value, (2) different methods of utilisation - green mass, hay, silage, greenchop, briquette, pellets, etc., (3) favourable environmental impact – potentiality and no nitrogen fertilizer required, (4) growth over a wide range of soil and climatic conditions and the significance in crop rotation through its positive effects on soil fertility, soil structure and reduced soil erosion (Flajoulot et al., 2005; Tucak et al., 2008; Rashidi et al., 2009).

Cultivated alfalfa is autotetraploid (2n = 4x = 32), open-pollinated species characterized by extreme heterozygosity and severe inbreeding depression. Current commercial alfalfa cultivars are mostly synthetic populations developed by intercrossing a number of superior selected parents to multiplying seeds through several generations of random mating. Therefore, utilizing the genetically diversified selection material is necessary for the improvement yield and quality alfalfa in our breeding programme as well as for producing high-yielding cultivars. Information about level and distribution of genetic diversity, relationship among material within a breeding programme is essential to better utilize available germplasm resources (Musial et al., 2002; Karuri et al., 2010; Tucak et al., 2010).

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The genetic diversity can be determined by several multivariate techniques (clustering methods, analysis by principal components and/or canonical variables), the procedures that are widely used in different crops (Alom et al., 2003; Mohan and Seetharam, 2005; Naghavi and Amirian, 2005; Dias et al., 2009; Thul et al., 2009). The most appropriate methods should be chosen according to the desired precision, ease of analysis and the way the data were collected (Rangel et al., 1991).

The objective of this study was to evaluate phenotypic diversity in the alfalfa germplasm collections using multivariate analysis to examine the extent of genetic diversity and contribution of selected characters to the total diversity and to select the most promising clusters/populations that could contribute to the improvement of yield and quality of alfalfa in our breeding programme.

MATERIAL AND METHODS

The experimental material included alfalfa germplasm (40 populations/cultivars) of different geographical origin (Table 2). Twenty three materials collected at the Agricultural Institute Osijek - Croatia included: twelve breeding populations developed after several cycles of phenotypic recurrent selection for disease resistance and winter survival, eight local populations collected in the Panonian region of Croatia between 1990-2005 and three registered cultivars (Vuka, Slavonka, OS-66). Five Australian cultivars (Genesis, Jindera, Super 7 and 10, Trifecta) were obtained from the South Australian *Medicago* Genetic Resource Centre, SARDI, Waite Campus, Urrbrae, Australia. Three American cultivars Magnum V, Saranac and Alfagraze were provided by Dr. Dan Undersander (University of Wisconsin, Madison, USA). One French local population (Malzeville) and two cultivars (Du Puits, Europe) were provided by Dr. Julier Bernadette (INRA, Lusignan). Serbian cultivars NS Mediana ZMS and Novosađanka H-11 were obtained from the Institute of Field and Vegetable Crops, Novi Sad. Three Argentinian cultivars Barbara, Lujan and Monarca were obtained from Dr. Daniel Basigalup (INTA Estacion Experimental Agropecuaria Manfredi, Argentina). Polish cultivar Radius was obtained from the IHAR National Centre for Plant Genetic Resources at Radzikow.

Seeds of 40 populations/cultivars were sown directly by hand on the experimental field of the Agricultural Institute Osijek (lat 45°32'N, long 18°44'E, altitude 90 m) on 17th of March 2008. The field trial was set up as a randomized block design with four replications. The plots consisted of four rows of 5.5 m length with row spacing of 40 cm and plant spacing of 50 cm. Each population/cultivar was represented with a total of 192 individual plants.

The plants were cut three times in the year of establishment (August 12, September 10, November 03 in 2008) and four times during the second production year (May 12, June 10, July 08, August 13 in 2009). The following agro-morphological characters were collected on individual plants of each population/cultivar at each cutting in the first and second production years: 1. Green mass yield (GMY I and II, g plant^{-1}) was determined by hand cut plants at approximately 5 cm above the ground and weighed on electronic balance directly in the field. The plants were cut at early flowering growth stage (10% flowers). 2. Dry matter yield (DMY I and II, g plant^{-1}) was calculated by dry matter content x GMY/100 formula. For determining dry matter content fresh samples (500 g) of randomly chosen plants were taken from each plot, dried at 105°C for 24 h and weighed. Total GMY and DMY were defined by summing the yield data over cuttings within each year. 3. Plant height (PH, cm) was measured, a prior to cutting, from the ground to the top of the inflorescence of the three longest stems. 4. Regeneration after cutting (RAC, cm) was recorded two weeks after each cutting by individual measurements of the plant height.

Parameters recorded in the second cut of 2009 production year include number of stems (NS), number and length of internodes (NI and LI, mm), stem thickness (ST, mm) width and length of central leaflet (WCL and LCL, mm). NS was recorded directly following cuttings by hands and recounting stems of all individual plants; others were measured at early flower bud stage on the longest stem of 30 randomly chosen individual plants of all populations/cultivars. Observations on the leaf and stem were made on the third leaf, i.e. internodes below inflorescence using digital caliper.

Analysis of variance was performed to evaluate the variation between the tested populations/cultivars for all agro-morphological characters followed by the multivariate analysis. The data were analyzed using Mahalonobis's generalized distance (D^2) to measure the genetic distance between tested populations/cultivars. They were grouped into different clusters using Tocher's method (Mahalanobis, 1936; Rao, 1952). The analysis of canonical variables was used to evaluate the relative contribution of each character to the total diversity. All statistical analyses were performed using PROC IML of SAS 9.1 software (SAS Institute, 2003).

RESULTS AND DISCUSSION

The description of agronomically important and useful characteristics is a crucial prerequisite for effective and efficient utilization of germplasm collections in breeding programs (Duvick, 1984). The analysis of variance on the basis of mean values revealed significant differences among the populations/cultivars for all agro-morphological characters (Table 1).

Table 1. Analysis of variance (significance of F-test) for the evaluated agro-morphological characters

Tablica 1. Analiza varijance (značajnost F-testa) za istraživana agromorfološka svojstva

Source of variation/ *Izvor variranja*	Characters/*Svojstva*						
	DF/ *SS*	GMY I/ *PZM I*	DMY I/ *PST I*	GMY II/ *PZM II*	DMY II/ *PST II*	PH/ *VB*	RAC/ *RBK*
Repetition/*Ponavljanje*	3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Pop.Cultivar/*Pop.Sorta*	39	**	**	**	**	**	**
Source of variation/ *Izvor variranja*	Characters/*Svojstva*						
	DF/ *SS*	NS/ *BS*	NI/ *BI*	LI/ *DI*	ST/ *DS*	WCL/ *ŠSL*	LCL/ *DSL*
Repetition/*Ponavljanje*	3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Pop.Cultivar/*Pop.Sorta*	39	**	*	**	**	*	**

significant at P<0.01/*značajno na P<0,01;* *significant at P<0.05/**značajno na P<0,05;* n.s.-not significant/*n.s.-nije značajno*

DF-Degrees of freedom/*SS-Stupnjevi slobode*, GMY-Green mass yield in the first (I) and second (II) production year/*PZM-Prinos zelene mase u prvoj (I) i drugoj (II) vegetacijskoj godini*, DMY-Dry matter yield in the first (I) and second (II) production year/*PST-Prinos suhe tvari u prvoj (I) i drugoj (II) vegetacijskoj godini*, PH-Plant height/*VB-Visina biljaka*, RAC-Regeneration after cutting/*RBK-Regeneracija biljaka nakon košnje*, NS-Number of stems/*BS-Broj stabljika*, NI-Number of internodes/ *BI-Broj internodija*, LI-Length of internodes/*DI-Dužina internodija*, ST-Stem thickness/*DS-Debljina stabljike*, WCL-Width of central leaflet/*ŠSL-Širina srednjeg listića*, LCL-Length of central leaflet/DSL-*Dužina srednjeg listića*

Phenotypic diversity between the alfalfa populations/ cultivars was estimated using Mahalonobis's D^2 analysis for all 12 characters. Based on degree of divergence among plants, the 40 alfalfa populations/cultivars could be grouped into six clusters (Table 2). The distribution pattern indicates that the largest number of populations/ cultivars was assigned to clusters II (12, 30.00%) and III (10, 25.00%). These two clusters contained the largest number of populations/cultivars of Croatian origin including seven breeding populations - four in cluster II (Rs-06/62, Rs-06/70, Rs-04/63, Rs-04/53) and three in cluster III (LBP-1, LBP-3, LBP-4), six local populations -

Table 2. Origin and distribution of 40 populations/cultivars of alfalfa within six clusters

Tablica 2. Porijeklo i raspodjela 40 populacija/sorti lucerne unutar šest grupa

Cluster number *Broj grupe*	Name/Population/Cultivar/Type *Ime/Populacija/Sorta/Tip*	Origin *Porijeklo*
I	LP-8/local population	Croatia
	LBP-2/breeding population	Croatia
	Novosađanka H-11/cultivar	Serbia
	Europe/cultivar	France
	Super 10/cultivar	Australia
	Monarca/cultivar	Argentina
II	Rs-06/62, Rs-06/70, Rs-04/63, Rs-04/53/breeding population	Croatia
	LP-4, LP-7, LP-2/local population	Croatia
	Slavonka/cultivar	Croatia
	NS Mediana ZMS/cultivar	Serbia
	Du Puits/cultivar	France
	Barbara/cultivar	Argentina
	Super 7/cultivar	Australia
III	LP-1, LP-3, LP-5/local population	Croatia
	LBP-1, LBP-3, LBP-4/breeding population	Croatia
	Trifecta/cultivar	Australia
	Lujan/cultivar	Argentina
	Saranac, Alfagraze/cultivar	USA
IV	LP-6/local population	Croatia
	Rs-04/59, Rs-07/68/breeding population	Croatia
	OS-66/cultivar	Croatia
V	Magnum V/cultivar	USA
	Rs-01/89, PCP/breeding population	Croatia
	Vuka/cultivar	Croatia
	Genesis/cultivar	Australia
VI	Malzeville/local population	France
	Radius/cultivar	Poland
	Jindera/cultivar	Australia

three in cluster II (LP-4, LP-7, LP-2) and three in cluster III (LP-1, LP-3, LP-5), one registered cultivar (Slavonka) in cluster II. The populations/cultivars originated from Australia (cultivars Super 7 in cluster II and Trifecta in cluster III), Argentina (cultivars Barbara in cluster II and Lujan in cluster III), Serbia (cultivar NS Mediana ZMS in cluster II), France (cultivar Du Puits in cluster II) and America (cultivars Saranac and Alfagraze in cluster III) grouped within these clusters as well. Cluster I consisted of six populations/cultivars (15.00%), clusters IV and V of four, respectively, five populations/cultivars (10.00% and 12.50%). Cluster VI included only three population/cultivars (7.50%, local population Malzeville, cultivars Radius and Jindera). Cluster I included local and breeding populations (LP-8, LBP-2) originated from Croatia and four cultivars Monarca (Argentina), Super 10 (Australia), Novosađanka H-11 (Serbia) and Europe (France). Cluster IV was composed only of populations/cultivars originated from Croatia (three local and breeding populations namely LP-6, Rs-04/59 and Rs-07/68 and one registered cultivar OS-66). Cultivars Magnum V (USA), Genesis (Australia) and Vuka (Croatia) and two breeding populations (Rs-01/89, PCP) originated from Croatia were grouped into cluster V.

The clustering pattern of the populations/cultivars under this study revealed that the germplasm collected from the same location can also be grouped into different clusters (Table 2). The populations/cultivars belonging to different geographical origin were grouped in the same clusters. The present results show that geographic diversity is not necessarily directly related to the geographic distribution of these materials. Similar results were reported by other authors who studied genetic diversity in crops by multivariate analysis using morpho-agronomic characters (Naghavi and Jahansouz, 2005; Dias et al., 2007; Sardana et al., 2007; Khan et al., 2008; Tucak et al., 2009).

The results revealed that intercluster distances were larger than the intracluster distances, suggesting broad genetic diversity between populations/cultivars of different groups (Table 3). The intracluster distance ranged from 0.087 (IV) to 3.143 (II). The highest intercluster distance was observed between clusters IV and VI (37.362). Considerable intercluster distances were found between clusters IV and V (29.687) and I and VI (24.990). The lowest intercluster distances were observed between clusters I and II (3.670), followed by II and III (5.055), and I and V (7.009). Higher cluster distances indicate large genetic variability between populations/cultivars among and within clusters, respectively. The lowest cluster distances indicate limited diversity among populations/cultivars of two clusters. Intercrossing populations/cultivars from different clusters may generate large variability that would produce desirable recombination genes for yield and yield-related characters for the development of populations with broad genetic bases. Selection of parents based on the extent of genetic diversity has been successfully done in alfalfa heterosis breeding program (Riday and Brummer, 2002; Maureira et al., 2004; Mackie et al., 2005).

Table 3. Intra (bold values) and intercluster distances (D^2 values) between six clusters of alfalfa populations/cultivars

Tablica 3. Udaljenosti (D^2) između grupa te između populacija/sorti lucerne unutar pojedine grupe (podebljane vrijednosti)

Cluster *Grupe*	I	II	III	IV	V	VI
I	**2.991**	3.670	7.609	14.162	7.009	24.990
II		**3.143**	5.055	16.223	8.829	22.178
III			**2.845**	20.997	13.435	17.769
IV				**0.087**	29.687	37.362
V					**2.346**	8.363
VI						**0.152**

The cluster mean values for 12 agro-morphological characters are presented in Table 4. Cluster V showed the highest mean values for yields of green mass and dry matter in the first and second production year (747.18 and 2513.00 g plant^{-1} for GMY, 161.48 and 519.71 g plant^{-1} for DMY), number of stems (91.41), plant height (72.97 cm), and regeneration after cutting (22.23 cm). Maximum cluster mean for width and length of central leaflet (12.43 and 28.74 mm) were observed in cluster IV. Also, high yields and favourable values of the most of the evaluated characters were found in this cluster. The higher mean values for number of internodes (11.17) were recorded in cluster I. Cluster VI had lowest mean values for yields of green mass and dry matter in the first and second production year (108.04 and 567.83 g plant^{-1} for GMY, 27.32 and 118.22 g plant^{-1} for DMY), number of stems (53.50), plant height (37.00 cm), regeneration after cutting (9.88 cm) and number of internodes (9.22). The highest values for stem thickness (2.90 mm) and length of internodes (52.72 mm) were found in this cluster, while values for width and length of central leaflet (11.36 and 26.18 mm) were similar to the values obtained in other clusters.

Table 4. Cluster mean values for 12 agro-morphological characters of 40 alfalfa populations/cultivars

Tablica 4. Prosječne vrijednosti 12 agromorfoloških svojstava za 40 populacija/sorti lucerne grupiranih unutar šest grupa

Character / *Svojstvo*	I	II	III	IV	V	VI
GMY I/*PZM I* (g plant^{-1}/*g biljka*$^{-1}$)	417.97	481.08	365.00	637.10	747.18	108.04
DMY I/*PST I* (g plant^{-1}/*g biljka*$^{-1}$)	94.80	106.48	81.61	142.25	161.48	27.32
GMY II/*PZM II* (g plant^{-1}/*g biljka*$^{-1}$)	1807.58	1626.32	1384.84	2081.50	2513.00	567.83
DMY II/*PST II* (g plant^{-1}/*g biljka*$^{-1}$)	379.01	340.43	293.91	436.35	519.71	118.22
NS/*BS*	74.12	65.59	57.15	87.69	91.41	53.50
PH/*VB* (cm)	62.31	61.66	57.68	68.53	72.97	37.00
RAC/*RBK* (cm)	17.78	19.46	17.36	20.44	22.23	9.88
NI/*BI*	11.17	10.78	10.65	10.65	9.50	9.22
LI/*DI* (mm)	49.26	51.04	47.11	49.61	48.90	52.72
ST/*DS* (mm)	2.59	2.46	2.60	2.77	2.79	2.90
WCL/*ŠSL* (mm)	10.82	10.71	10.51	12.43	10.80	11.36
LCL/*DSL* (mm)	27.06	26.60	25.75	28.74	27.40	26.18

See description of the investigated characters in Table 1/*Opis istraživanih svojstava pogledati u Tablici 1.*

Relative contribution of the characters to the total diversity within tested alfalfa populations/cultivars is given in Table 5. The analysis of canonical variables revealed that the vectors I and II for dry matter yield in the first production year, plant height and length of central leaflet were positive. The results indicated that these characters had highest contribution to the total phenotypic diversity of the germplasm. More emphasis should be given to these characters in future selection of populations/cultivars in alfalfa breeding programme.

Table 5. Relative contribution of 12 agro-morphological characters to the total diversity within tested alfalfa populations/cultivars

Tablica 5. Relativni doprinos 12 agromorfoloških svojstava u ukupnoj raznolikosti proučavane germplazme lucerne

Character *Svojstvo*	Vector I *Vektor I*	Vector II *Vektor II*
GMY I/*PZM I*	-1.870	-4.296
DMY I/*PST I*	1.465	1.734
GMY II/*PZM II*	-1.374	0.327
DMY II/*PST II*	0.119	-0.082
NS/*BS*	0.152	-0.783
PH/*VB*	0.206	1.875
RAC/*RBK*	-0.368	0.914
NI/*BI*	-0.496	0.632
LI/*DI*	0.048	-0.073
ST/*DS*	0.043	-0.233
WCL/*ŠSL*	-0.284	-0.692
LCL/*DSL*	0.200	0.343

See description of the evaluated characters in Table 1/*Opis istraživanih svojstava pogledati u Tablici 1.*

CONCLUSION

This research revealed broad phenotypic diversity within the tested alfalfa germplasm collections. The cluster analysis grouped populations/cultivars into six clusters. The clustering pattern of the populations/cultivars did not show association between geographical and phenotypic diversity. Dry matter yield in the first production year, plant height and length of central leaflet had **highest** contribution to the total phenotypic diversity of the studied alfalfa germplasm. This indicates that examined properties represent a significant value for further selection of the studied populations/cultivars of alfalfa. Based on the mean value of the evaluated characters and determined distances between clusters of the most promising populations/cultivars belong to the clusters IV and V (cultivars OS-66 and Vuka, five breeding and local populations – Croatia; USA and Australian cultivars Magnum V and Genesis). Evaluated populations/cultivars could be considered as a valuable genetic material for the yield and quality improvement of alfalfa in our breeding programme.

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FENOTIPSKA RAZNOLIKOST GERMPLAZME LUCERNE (*MEDICAGO SATIVA* L.)

SAŽETAK

Cilj ovog istraživanja bio je procijeniti fenotipsku raznolikost u kolekciji germplazme lucerne pomoću multivarijatne analize, utvrditi doprinos izabranih svojstava u ukupnoj raznolikosti i izabrati najzanimljivije grupe/populacije za daljnji oplemenjivački rad. Tijekom dvogodišnjega razdoblja analizirano je 12 agromorfoloških svojstava kod 40 populacija/sorti lucerne različitoga geografskoga porijekla. Proučavane populacije/sorte bile su grupirane unutar šest grupa. U najvećem broju slučajeva grupiranje populacija/sorti nije bilo povezano s njihovim geografskim porijeklom. Udaljenost između grupa bila je veća u odnosu na udaljenost između populacija/sorti unutar grupe. U provedenom istraživanju otkrivena je velika fenotipska raznolikost u proučavanoj kolekciji germplazme lucerne. Najveći doprinos u ukupnoj fenotipskoj raznolikosti imala su svojstva prinos suhe tvari u prvoj vegetacijskoj godini, visina biljaka i dužina srednjeg lističa. Na osnovi ostvarenih prosječnih vrijednosti ispitivanih svojstava te utvrđene udaljenosti između grupa, najperspektivnije populacije/sorte pripadaju grupama IV i V. Izabrane populacije/sorte predstavljaju vrijedan genetski materijal za poboljšanje prinosa i kvalitete lucerne u okviru našega oplemenjivačkoga programa.

Ključne riječi: lucerna, populacije, fenotipska raznolikost, multivarijatna analiza, oplemenjivanje

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