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Isoenzyme Variation in *Medicago sativa* Ecotypes

MERYEM SENGUL†, GULERAY AGAR† and SULEYMAN SENGUL*
Department of Field Crops, Faculty of Agriculture
University of Atatürk, Erzurum, Turkey
E-mail: drsengul@atauni.edu.tr

This study was carried out in order to determine variation of *Medicago sativa* ecotypes, using poliacrilamide slab gel electrophoresis (PAGE) on the 5 enzyme systems (peroxidase, malate dehydrogenase, polyphenol oxidase, superoxide dismutase, glutamate). Differences between ecotypes were analyzed statistically. The highest genetic distance was between Manyas and Refahiye ecotypes, the least was between Manyas and Acipayam ecotypes. The results from isoenzyme analysis correlated with some morphological characteristics. This study of *Medicago sativa* ecotypes suggested that the geographical and ecological distribution of the plants contributes to higher genetic similarity.

Key Words: *Medicago sativa*, Ecotypes, Isoenzymes Variation.

INTRODUCTION

Medicago sativa L. a major forage crop throughout the world is an out crossing seed propagated species whose populations have a genetic structure complicated by the tetrasomic inheritance and by a rate of selfing which varies according to the environmental conditions. In general, alfalfa cultivars are synthetic varieties generated by inter crossing selected plants and by advancing their offspring through limited generations of random mating[1].

A significant percentage of alfalfa cultivated in Turkey is represented by local landraces 'ecotypes'. An ecotype is defined as a population growing in a specific geographical environment, whose individuals share a common gene pool. The geographic, edaphic, climatic and vegetation region of Turkey are extremely diverse. Elevation varies from sea level to more than 3000 m high mountains area. Also much of the country is dominated by a humid, arid and dry climate, the higher elevation is colder and considerable snow accumulates. Annual precipitation varies between 350-800

†Department of Biology, Faculty of Art and Science, University of Atatürk, Erzurum, Turkey.

mm. Thus, the introduction of alfalfa from various sources coupled with the possibility of natural selection as they were transported and used throughout Turkey could have resulted in differentiation of numerous land races or strains of the crop. Concerned about the genetic erosion of this resource due to the introduction of alfalfa seed samples.

Turkish alfalfa ecotypes may cover large geographical regions and the problem arises as to whether they still conform to the definition of ecotype. This situation requires the development of a method for the unambiguous and reproducible identification of alfalfa ecotypes. Numerous studies[2-7] have been conducted to culture *Medicago sativa* L.

The genetic variations are crucial for the success of selection programs. However, these variations could be genetically regulated. Different methods such as DNA isoenzyme and protein electrophoresis should be applied to determine how much the observed differences stem from the gene's structure. Isoenzymes have been used in evaluation studies[8-11]. Protein benefiting, each variety has its own biochemical properties. Separation of proteins by electrophoresis is a common method as a genetic pointer or indicator. Determination of genetic variety in the gene pool is used for characterization of breeding lines which have different biochemical features but of the same morphological properties[12-15]. However, studies on isozymes variations *Medicago sativa* ecotypes have not been carried out.

The aims of the study were: (1) Description of electrophoresis isoenzyme phenotypes and their variation patterns among *Medicago sativa* ecotypes, and (2) Evaluation of possibilities to use isoenzymes as molecular characters to discriminate between *Medicago sativa* ecotypes.

EXPERIMENTAL

Seed of ecotypes used in this research were taken from National Gene Bank material of Aegean Agricultural Research Institute, Izmir, Turkey in 2004 (Table-1). All seeds were sown in shallow boxes of soil (based on 3:1 rate of soil, sands and organic matter), then each of single plant transplanted into plastic pots of soil for use of other agronomic properties. Analyses were carried out on 21-day-old seedling leaves from plants grown under greenhouse conditions[16].

Protein extraction: Individual samples were extracted in 0.05 M sodium phosphate (pH 6.5) buffer for peroxidase (PRX) and polyphenol oxidase (PPO). Malate dehydrogenase (MDH), superoxide dismutase (SOD), glutamate (GDH) were extracted[17] with 0.2 M *Tris*-HCl buffer (pH 7.5) at + 4 ºC for 1 h period. Samples were filtered and centrifuged at 22.075 g for 15 min at 0 ºC. The supernatant was stored at + 4 ºC for electrophoresis.

TABLE-1
SEED SOURCES OF *Medicago sativa* SAMPLES WERE COLLECTED

Acces. no.		Location/Province	Alt. (m)	
TR45243	*M. sativa*	Tutak/Agri	1800	Land races
TR45246	*M. sativa*	Igdir	870	Land races
TR52956	*M. sativa*	Söke Aydin	30	Land races
TR52968	*M. sativa*	Kuyucak/Aydin	140	Land races
TR52961	*M. sativa*	Çine/Aydin	125	Land races
TR53035	*M. sativa*	Manyas/Balikesir	20	Land races
TR53038	*M. sativa*	Gönen/Balikesir	30	Land races
TR44801	*M. sativa*	Tatvan/Bitlis	1580	Land races
TR52989	*M. sativa*	Tavas/Denizli	860	Land races
TR53003	*M. sativa*	Çivril/Denizli	725	Land races
TR52994	*M. sativa*	Acipayam/Denizli	760	Land races
TR44791	*M. sativa*	Diyarbakir	600	Land races (Peruvian)
TR46308	*M. sativa*	Refahiye/Erzincan	1560	Land races
TR52951	*M. sativa*	Bayindir/Izmir	80	Land races
TR52976	*M. sativa*	Tire/Izmir	135	Land races
TR53023	*M. sativa*	Kula/Manisa	350	Land races
TR53024	*M. sativa*	Saruhanli/Manisa	10	Land races
TR44787	*M. sativa*	Gürün/Sivas	1290	Land races (Gürün)
TR46251	*M. sativa*	Hafik/Sivas	1275	Land races
TR45267	*M. sativa*	Akçaabat/Trabzon	340	Land races

Gel electrophoresis: The polyacrylamide gel electrophoresis (PAGE) process was conducted in an OWL dual vertical slab gel apparatus. Resulting gels were 12.5 (h) × 20 (w) × 0.75 (t) cm and contained 7 % acrylamide, while the stacking gels were 2.5 (h) × 20 (w) × 0.75 (t) cm and contained 2.5 % acrylamide. Reservoir buffer[18] contained 0.025 M *Tris* and 0.133 M glycine at a pH 8.3. Protein supernatant was placed in the stacking gel sample wells, followed by 20 μL of reservoir buffer containing bromophenol blue, which served as the tracking dye.

Electrophoresis was done at 2-4 ºC for 0.5 h at a constant voltage of 80 V and followed by 4.5-5.0 h at a voltage of 150 V until the tracking dye was *ca.* 1 cm from the gel bottom[19].

Staining procedure: The staining solution for MDH contained: 100 mL 0.2 M *Tris*-HCl (pH 7.5), 3 mL 1.0 M D-L malate (pH 7.5), l2 mg β-nicotine with adenine dinucleotide (NAD), 15 mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2 mg phenozin metasulfate (PMS). Gels were stained at + 37 ºC in the dark until the bands developed. After the bands became visible, the gel was rinsed with distilled water and stored in 3 % acetic acid solution[17].

The staining solution for PRX enzyme consisted of 20 mL 0, 6 % hydrogen peroxide, 20 mL stock benzidine (2 g benzidine in 18 mL acetic acid was solved by heating slowly. Then 72 mL double distilled water was added), 70.4 mg ascorbic acid and 60 mL distilled water. The gels were stained at room temperature until the bands developed. After the bands become visible the reaction was halted with 10 % acetic acid solution and left in 3 % acetic acid solution[20].

The staining solution for PPO contained 0.3 M dihydroxyphenyl alanine (DOPA) (DOPA resolved 1 % KOH) and 0.1 M sodium phosphate buffer. Gels were stained at 37 ºC in the dark until the bands developed[19].

The staining solution for MDH contained GDH/SOD 75 mL 0.1 M *Tris*-HCI (pH 8), LGlutamic acid 2 g, l2 mg β-nicotine with adenine dinucleotide (NAD), 15 mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2 mg phenozin metasulfate (PMS). Gels were stained at + 37 ºC in the dark until the bands developed. SOD bands are white against the blue background[17].

Statistical analyses: Band frequencies were calculated for each ecotype and for each isoenzyme system. Each isoenzyme system was scored as presence (1) and absence (0) of band for each of the 20 accessions analyzed. Data were used to calculate a Jaccard similarity. Based on the matrix of band frequencies the variation among ecotype was examined by cluster analysis and UPGMA method using program package NTSYS-pc version 2.02 k (Applied Biostatistics Inc. 1986-1998) which was utilized based on Euclidean distances.

RESULTS AND DISCUSSION

Peroxidase enzyme formed bands in cathode and anode regions *i.e.*, 4 bands of different mobility (R_f) values (R_f = 0.5, 0.11, 0.24, 0.37) in all ecotypes. The observation showed that Tatvan, Diyarbakir and Akçabat ecotypes had only one band. Agri and Manyas had the same bands of second and third band, while, Çine, Gönen, Refahiye and Hafik existed similar band of first and third bands. On the other hand Igdir, Acipayam and Bayindir ecotypes had third different bands (Table-2). There was wide variation within and between ecotypes with respect to PRX enzyme.

The result of analysis of electrophoresis showed that PPO enzyme in cathode regions on gels formed a total of 5 bands; R_f values of these bands were calculated as 0.04, 0.18, 0.27, 0.49 and 0.56, respectively. The first band observed only Çine ecotype. Second and four bands existed in Igdir, Çivril, Refahiye, Bayindir and Gürün ecotypes. Agri, Söke and Diyarbakir had the same band of second, third and fifth bands. The other ecotypes revealed either two or three bands. There were differences between ecotypes based on this enzyme.

TABLE-2
BAND PATTERNS FOR PEROXIDASE (PRX), POLYPHENOL OXIDASE (PPO), SUPEROXIDE DISMUTASE (SOD), GLUTAMATE (GDH), MALATE (MDH) ENZYME FORMED RELATIVE MOBILITY VALUES (R_f) (– BAND SEEN ON THE GEL)

Ecotypes	PRX (R_f)				PPO (R_f)					GDH (R_f)				SOD (R_f)					MDH (R_f)				
	.05	.11	.24	.37	.04	.18	.02	.49	.56	.20	.32	.56	.64	.05	.14	.40	.51	.58	.13	.25	.38	.42	.49
Agri		–		–		–	–		–		–		–	–	–			–			–		–
Igdir	–		–	–		–		–		–		–	–		–		–		–			–	–
Söke		–		–		–	–		–		–		–	–		–	–			–		–	
Kuyucak		–	–		–		–		–			–	–		–	–		–	–			–	
Çine	–			–		–					–			–			–			–	–		–
Manyas		–		–		–	–			–		–	–		–		–		–		–		–
Gönen	–			–			–		–	–			–		–	–		–	–			–	
Tatvan		–			–			–			–		–			–	–			–			–
Tavas		–	–		–	–				–		–			–		–		–		–		–
Çivril	–		–			–		–				–			–		–	–		–		–	
Acipayam		–	–	–	–		–			–		–	–		–		–		–				–
Diyarbakir			–			–	–		–		–			–				–		–	–		
Refahiye	–			–		–		–		–		–		–		–			–			–	
Bayindir	–		–	–		–		–			–	–			–		–	–			–		–
Tire		–		–	–			–		–		–		–		–		–		–			–
Kula	–		–			–			–	–			–		–		–		–		–		
Saruhanli		–	–		–		–			–		–		–		–		–		–		–	
Gürün	–		–			–		–			–		–		–		–			–			–
Hafik	–			–			–		–		–	–			–			–			–		
Akçaabat		–			–		–			–			–	–			–		–		–		–

Electrophoresis analysis revealed that a total of 4 bands were formed by GDH enzyme. Their R_f values were 0.2, 0.32, 0.56 and 0.64, respectively. Çine and Diyarbakir ecotypes (0.32) and Çivril ecotype (0.56) had only one band. Similar variation was observed with second and third bands on Agri, Söke, Tatvan and Gürün ecotypes. Also similarities were seen with second and fifth band at Bayindir and Hafik ecotypes. On the other hand Igdir, Manyas and Acipayam ecotypes had similarities with first, third and fourth bands, respectively. As a result of this study, remarkable variations between and within ecotypes, with respect to GDH enzyme were observed (Table-2).

Examining SOD enzyme formed 5 bands at this study. Their R_f values were 0.05, 0.14, 0.40, 0.51 and 0.58, respectively. There was wide variation both within and between ecotypes with respect to SOD enzyme upon evaluation of all ecotypes used in this study. Igdir, Manyas, Tavas, Acipayam, Kula and Gürün ecotypes had similarities with second and four bands. Tire and Saruhanli ecotypes exist with the same band of first, third and fifth bands. On contrary Kuyucak and Gönen ecotypes had the same band of second, third and fifth bands (Table-2).

Result of electrophoresis analysis showed that a total of 5 bands were formed by MDH enzyme. Their R_f values were 0.13, 0.25, 0.38, 0.42 and 0.49, respectively. Hafik ecotype had only one band with R_f value (0.38). Third and fifth bands were observed at Agri and Bayindir ecotypes. On the other hand second and fourth band were examined on Söke, Çivril and Saruhanli ecotypes. Kuyucak, Gönen and Refahiye ecotypes had the same band of first and fourth. Moreover, three bands of first, third and fifth were observed on Manyas, Tavas and Akçabat ecotypes. In summary, there was remarkable variation within and between ecotypes based on MDH enzyme (Table-2).

A dendogram formed from data based on band pattern to determine the degree of relationship of ecotypes is shown in Fig. 1, using UPGMA as the clustering method. The enzymes results showed the presence of 3 clusters among the Medicago sativa ecotypes. Cluster 1 was divided into 2 sub clusters. Manyas, Tavas, Acipayam, Akçaabat and Kula ecotypes formed one sub clusters while Bayindir, Gürün, Çivril and Igdir ecotypes formed the other sub cluster. Cluster 2 was divided into 2 sub clusters and Söke and Tatvan ecotypes formed one sub cluster. Agri, Hafik, Diyarbakir and Çine ecotypes formed the other sub cluster. Cluster 3 was divided into 2 sub clusters and Kuyucak and Gönen formed one sub cluster. Tire, Saruhanli and Refahiye ecotypes formed the other sub cluster. The highest genetic distance was between Manyas and Refahiye ecotypes, the least was between Manyas and Acipayam ecotypes.

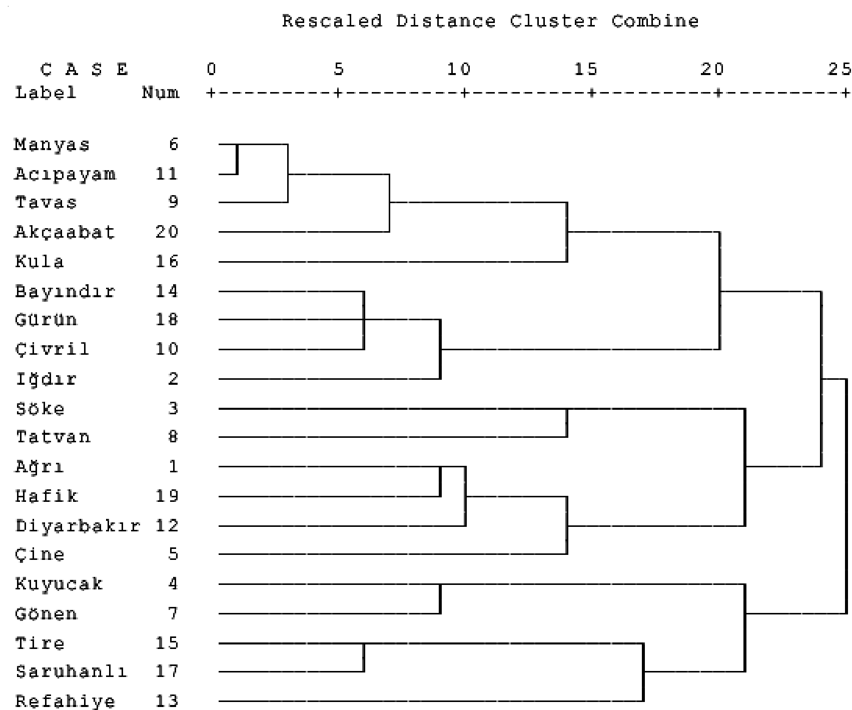

Fig. 1. Dendogram made according to band patterns

Twenty ecotypes were used for this experiment and differences were detected based on the number of bands PRX, PPO, GDH, SOD and MDH enzymes formed and their R_f value. There was also variation within ecotypes based on these five enzymes. The range of this variation was pronounced in some ecotypes based on these enzymes studied in this work. Less variation was observed between Tatvan, Diyarbakir and Akçaabat ecotypes based on PRX enzyme, Çine ecotype based on PPO enzyme, Çine, Çivril and Diyarbakir ecotypes based on GDH, Hafik ecotype based on MDH enzyme. On the other hand the magnitude of higher variation was similar to much of the ecotypes with respect to examined enzymes. The reason for this variation detected within ecotypes may be genetic composition, which is probably due to heterozygosis because of out cross-pollination of alfalfa. Morphological differentiation studied by Small[21] on *Medicago sativa* sl, in relation to ploidy level. Comparative chromosome banding studies on non-dormant alfalfa germplasm were done by Bauchan *et al.*[22]. Population structure of alfalfa was studied by Maureira *et al.*[23]. Genetic control of alfalfa seed yield and its components were examined[6,7,24]. Also genetic variation in alfalfa pointed out by Julier *et al.*[25], forage quality, morphology and seed yield[7].

Rumbaug *et al.*[26] stated that geographic, edaphically and vegetation regions have extremely diverse effect on alfalfa.

Similar and different izoenzyme bands between ecotypes existed based on the five enzymes with R_f value 0.37 band containing PRX enzymes, R_f value 0.18 containing PPO enzyme, two band (0.56; 0.64) containing GDH enzyme, one band (0.14) containing SOD enzyme and one band (0.49) pertaining to MDH enzyme were detected as common bands in the most ecotypes and cultivars.

There were significant differences between ecotypes with respect to morphological, phonological and biological and agronomical properties. Forage yields, stem and leaf properties based on close relationships of ecotypes were investigated and the dendogram analysis was similar to that of the studies mentioned above.

In conclusion, variations exist both within ecotypes and between ecotypes based on isoenzyme polymorphism. Therefore isoenzyme profiling could be an efficient way to explore variation of *Medicago sativa*. Differentiation of hybrid and inbred plants can be easily performed in perennial plants such as alfalfa. The results presented here are a contribution to characterization of *Medicago sativa* ecotypes. The isoenzymes technique is shown to be adequate for discrimination of *Medicago sativa* ecotypes (accessions), although the accuracy of these results should also be confirmed with studies using markers such as RAPD, micro satellite and AFLP. All those applications might contribute considerably to reduce the length of time taken in breeding programs.

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BALACLAVA, TURTLE BAY, MAURITIUS

Contact:
Dr. V. Sivaram,
Organizing Secretary, Department of Botany, Bangalore University,
Bangalore - 560056, INDIA
Phone: 91-(080)-22736305,
Fax: 91-(080) ? 23219295, 22961315
E-mail: icbb2009@gmail.com
http://www.cenfoundindia.org