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Determination of Variations of Some Enzymes in Orchardgrass (*Dactylis glomerata* L.) Ecotypes

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In the present study, wild orchardgrass (*Dactylis glomerata* L.) collected from four different locations (Oltu, Olur, Uzundere and Ulubağ) of Erzurum (Turkey) were used to investigate various electrophoretic analyses within and between four ecotypes based on peroxidase (PRX), polyphenol oxidase (PPO) and malate dehydrogenase (MDH) enzymes. Four natural populations of *Dactylis glomerata* showed genetic variability within and among ecotypes based on similarities and polymorphism of isoenzyme bands. In addition, a dendogram analysis was made according to band patterns indicating that ecotypes could be grouped showing characteristics in relation to each other, notably Oltu-Ulubağ and Olur-Uzundere. However, the degree of relationship of Oltu-Ulubağ (6 units) was higher than Olur-Uzundere (12 units).

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Introduction

Orchardgrass (*Dactylis glomerata* L.) is one of the earliest and fastest growing grass species in the spring. It grows on a wider range of soil types with slightly lower fertility and is easy to establish. In addition, orchardgrass is shade-tolerant (Miller, 1984).

Numereous studies have been conducted to culture *Dactylis glomerata* L. for Erzurum ecological conditions (high elevation – 1850 m –, cold and dry conditions) and revealed that there were important differences between orchardgrass ecotypes with respect to agricultural properties such as fresh herbage yield, seed yield and crude protein content (Tosun & Sağsöz, 1994; Sağsöz et al., 1996; Tosun et al., 1996, 1997). These 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 evolution studies, species identification, and determination of variation within and between populations. (Seidel, 1989).

Several studies have been performed in order to determine factors producing the genotype not influenced by environmental conditions. Proteins, which are translated directly through gene expression, are used for fingerprints. Proteins benefiting each variety have their own biochemical properties. Separation of proteins by electrophoresis is a common method as a

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genetic pointer or indicator (Ilbi & Eser, 1995; Seyhan et al., 1995). Therefore, especially for identification of variety and biotype control, 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 This could lead to determination of the extent of relationships (Brown, 1978; Du Cros & Wrigley, 1979; Shewry et al., 1983; Köksel et al., 1992; Keskin et al., 1995; Tanyolaç et al., 1995).

The objective of this study was to determine the variation and relationship between four different ecotypes (Oltu, Ulubağ, Uzundere and Olur) of wild orchardgrass (*Dactylis glomerata* L.).

Material and methods

Plant Material

In the present study, four ecotypes (Oltu, Ulubağ, Uzundere and Olur) having agronomic characteristics such as fresh herbage yield, seed yield and crude protein content were selected from wild orchardgrass (*Dactylis glomerata* L.) and the collection made at eight different locations at high elevation (1850 m), under cold and dry conditions (Tosun & Sağsöz, 1994; Sağsöz et al., 1996; Tosun et al., 1996). Plant materials of ecotypes used in this research were previously obtained from natural populations, and planted under field conditions (Table 1). Twenty-four plants from each population and 5 leaves from each plant were sampled and placed in plastic bags separately. The samples were kept at -20°C until examination (Salinas & Benito, 1985; Şenel & Kadıoğlu, 1992).

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) was extracted with 0.2 M Tris–HCL buffer (pH 7.5) (William & Mujeeb-Kazi, 1992). Extractions were made at $+4$°C for 1-hour period. Samples were filtered and centrifuged at $22.075 \times$ g for 15 min at ± 0°C. The supernatant was stored at $+4$°C for electrophoresis.

Table 1. Characteristics of location where samples were collected

Location	Altitude (m)	Characteristics
Ulubağ village	1081	Approximately 5% inclined, few trees
Uzundere town	1100	Meadow, with small creeks
Oltu town	1300	Flat area and samples were collected from water source
Olur town	1340	Flat meadow having small creeks

Gel electrophoresis

The polyacrylamide gel electrophoresis (PAGE) process was conducted in a dual vertical slab gel apparatus. Resulting gels were 12.5 (h) $\times$ 20 (w) $\times$ 0.75 (t) cm and contained 7% acrylamide, while the stacking gels were 2.5 (h) $\times$ 20 (w) $\times$ 0.75 (t) cm and contained 2.5% acrylamide. Reservoir buffer contained 0.025 M Tris and 0.133 M glycine at a pH 8.3 (Ferguson & Grabe, 1986). 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°C for $\frac{1}{2}$ hour at a constant voltage of 80 V and followed by 4.5–5 hours at a voltage of 150 V until the tracking dye was approximately 1 cm from the gel bottom (Ağar, 1996).

Staining procedure

The staining solution for MDH contained: 100 ml 0.2 M Tris–HCI (pH 7.5), 3 ml 1.0 M D-L malate (pH 7.5), 12 mg β-nicotine with adenine dinucleotide, 15 mg 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide, 2 mg phenozin metasulphate. Gels were stained at $+37$°C 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 (William & Mujeeb-Kazi, 1992).

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 dissolved by heating slowly, then 72 ml dd H_2O 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 became visible the reaction was halted with 10% acetic acid solution and left in 3% acetic acid solution (Liu, 1973).

The staining solution for PPO contained 0.3 M dihidroxyphenyl alanine resolved 1% KOH and 0.1 M sodium phosphate buffer. Gels were stained at 37°C in the dark until the bands developed (Şenel & Kadıoğlu, 1992).

Statistical analyses

The isoenzymal band data were evaluated and the description of the phylogeny was from the DROWGRAM program from PHYLIP package (Felsenstein, 1993).

Results

Peroxidase (PRX)

Peroxidase enzyme formed bands in both anode and cathode regions in four ecotypes. PRX enzyme formed 14 bands of different relative mobility (Rf) values (Rf = 0.04, 0.06, 0.08, 0.11, 0.13, 0.16, 0.20, 0.22, 0.24, 0.26, 0.32, 0.35, 0.40, 0.42) in all ecotypes (Fig. 1).

While the first band was observed at Oltu, Olur and Uzundere, the same band did not exist in Ulubağ. Moreover, this band showed variation even within the ecotype, and the most remarkable variation was observed in plants of the Uzundere ecotype. On the other hand, Oltu and Olur ecotypes showed variation in a small number of plants. A second band existed in Olur, Uzundere and Ulubağ ecotype, but not in the Oltu ecotype. Moreover, this band characterized the Ulubağ ecotype in all plants; the other ecotype was observed in the majority of plants. On the other hand, a third band existed in all individuals of the Oltu and Ulubağ ecotypes and the majority of plants of the Olur ecotype but not in Uzundere ecotype. A fourth band was observed in only the Uzundere ecotype. Fifth and tenth bands existed in Olur and Uzundere ecotype, but not in other groups. Three common bands (band nos. 6, 8 and 13) existed in Oltu and Ulubağ in addition to the other two ecotypes. A thirteenth band was observed in all plants of two ecotypes. A seventh band existed in three ecotypes (Oltu, Uzundere and Ulubağ). The common band (ninth) for Oltu and Uzundere did not exist in Olur and Ulubağ ecotypes. While band 11 existed in all ecotypes except for Uzundere, band 12 was observed in Olur, Uzundere and Ulubağ ecotype except for Oltu. There was wide variation both within ecotype and between ecotypes with respect to PPX enzyme upon evaluation of all ecotypes used in this study (Fig. 1).

Polyphenol oxidase (PPO)

The result of analysis of electrophoresis showed that PPO enzyme in anode and cathode regions on gels formed a total of 15 bands; Rf values of these bands were calculated as 0.02, 0.03, 0.05, 0.09, 0.10, 0.11, 0.16, 0.20, 0.28, 0.29, 0.30, 0.35, 0.40, 0.58 and 0.69 respectively. Sixth and twelfth bands were observed in all ecotypes. Additionally, band 1 was observed in two ecotypes (Uzundere and Olur) but not in Ulubağ and Oltu. Similarly, while bands 3 and 4 were common bands for Uzundere and Olur ecotype apart from the other two ecotypes, bands 7 and 8 were not observed as common bands in Oltu and Ulubağ population. Band 13 that existed as a common band in Oltu and Uzundere was not observed in Olur and Ulubağ. While band 14 and 15 occured in Oltu and Ulubağ, they did not occur in other two ecotypes. On the other hand, bands 2, 9, 10 and 11 were found only in Olur. Similarly, band 5 existed in Olur ecotype, but not the others (Fig. 2). In summary, there were differences both within and between ecotypes based on enzyme. Variation within ecotype was remarkable especially in Olur ecotype.

Band Number	Ecotypes			
	Oltu	Olur	Uzundere	Ulubağ
1	—	—	—	
2		—	—	—
3	—	—		—
4			—	
5		—	—	
6	—			—
7	—		—	—
8	—			—
9	—		—	
10		—	—	
11	—	—		—
12		—	—	—
13	—			—
14			—	

Fig. 1. Band patterns for PRX enzyme. (— Band seen on the gel.)

Band Numbero	Ecotypes			
	Oltu	Olur	Uzundere	Ulubağ
1		—	—	
2		—		
3		—	—	
4		—	—	
5		—		
6	—	—	—	—
7	—			—
8	—			—
9		—		
10		—		
11		—		
12	—	—	—	—
13	—		—	
14	—			—
15	—			—

Fig. 2. Band patterns for PPO enzyme. (— Band seen on the gel.)

Malate dehydrogenase (MDH)

Result of electrophoresis analysis showed that a total of 9 bands were formed by MDH enzyme. Their Rf values were 0.03, 0.06, 0.09, 0.11, 0.15, 0.20, 0.23, 0.27 and 0.29, respectively (Fig. 3).

When bands produced by MDH enzyme in four ecotypes of plants used in this experiment were evaluated, band 3 and 8 were observed in all ecotypes. While band 1 existed in all individuals of Olur, Oltu, Ulubağ ecotype, it did not exist in Uzundere ecotype. Again, band 2 was detected in 3 ecotypes (Olur, Uzundere and Ulubağ) but not in Oltu ecotype. Additionally, when band 2 was evaluated in conjuction with variation within ecotypes, it was observed that the number of plants in which this band existed in Olur and Ulubağ ecotypes, was less than that in Uzundere ecotype. On the other hand, band 4 in Ulubağ and Olur ecotypes, bands 5 and 7 in only Ulubag and Uzundere ecotypes, band 6 in Oltu, Olur, and Uzundere ecotypes, and band 9 was observed in Oltu and Olur. As a result of this study, differences both within ecotype and between ecotypes with respect to MDH enzyme were observed (Fig. 3). Variation within ecotypes, especially Ulubağ, is more remarkable than the others.

Dendogram

A dendogram formed from data based on band pattern to determine the degree of relationship of ecotypes is shown in Fig. 4. Dendogram analysis showed that four ecotypes based on degree of relationship were grouped as Oltu-Ulubağ and Olur-Uzundere. However, while there was 6-unit relationship between Oltu-Ulubağ ecotypes, there was 12-unit relationship between Olur-Uzundere ecotypes.

Discussion

In four ecotypes used as material, differences were detected based on the number of band PRX, PPO and MDH enzyme formed and their Rf values. There was also variation within ecotypes based on these three enzymes. The magnitude of this variation was more pronounced in some ecotypes based on three enzymes. For example, less variation was observed

Band Number	Ecotypes			
	Oltu	Olur	Uzundere	Ulubağ
1	—	—		—
2		—	—	—
3	—	—	—	—
4		—		—
5			—	—
6	—	—	—	
7			—	—
8	—	—	—	—
9	—	—		

Fig. 3. Band patterns for MDH enzyme. (— Band seen on the gel.)

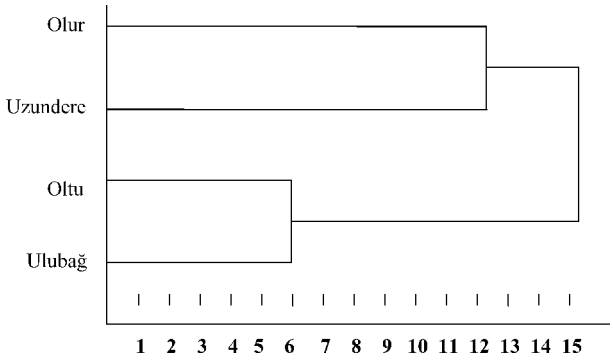

Fig. 4. The dendogram made according to band patterns.

between Olur ecotype based on PRX enzyme, Oltu and Ulubağ ecotypes, based on PPO enzyme, Uzundere ecotypes based on MDH enzyme. On the other hand, the magnitude of variation was high in Uzundere ecotype with respect to PRX enzyme, Olur ecotype with respect to PPO enzyme, and Ulubağ ecotype with respect to MDH enzyme. The reason for this variation detected within ecotypes may be genetic composition, which is probably due to heterozygosis because of cross-pollination of orchardgrass (Sağsöz et al., 1996). Furthermore, the population used in this experiment was from wild plants from natural flora, and no studies were done on selection. For this reason, high genetic variation between plants was not narrowed. This situation may be another reason for variation within plants.

Similar and different izoenzyme bands between ecotypes existed based on the three enzymes. None of the 14 bands, containing PRX enzymes, and two (bands 6 and 12) of 15 bands containing PPO enzyme, and two (band 3 and 8) of 9 bands pertaining to MDH enzyme were detected as common bands in all populations.

As can be understood from above discussion, there were major differences between populations with respect to the three enzymes. The studies which used the same population (Tosun & Sağsöz, 1994; Sağsöz et al., 1996; Tosun et al., 1996) and this study revealed similar findings; for example, there were significant differences between populations with respect to morphological, phenological and biological properties. Fresh herbage 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. Likewise, the close relationship between ecotypes of Oltu and Ulubağ was greater than the others, and the closeness between Olur and Uzundere ecotypes was greater than that of Oltu and Ulubağ ecotypes. In other words, four populations based on the degree of their close relationships, could be grouped as Oltu-Ulubağ and Olur-Uzundere as seen clearly in Fig. 4. Analysis of these results showed that large genetic variations were found between populations with respect to these three enzymes. Parallel to our findings, important variations were observed with respect to morphological, phenological and biological properties between populations in previous studies (Tosun & Sağsöz, 1994; Sağsöz et al., 1996; Tosun et al., 1996). Different ecological conditions from which plants were obtained may have caused the observed variations based on the investigated enzymes. Lumaret (1984), carrying out isoenzyme analysis to determine the variation in orchardgrass plants in different ecological conditions stated that this variation was caused by environmental factors. Pierre et al. (1990) and Zhang et al. (1993), who studied different plants, stated that there were variations between populations with respect to isoenzyme bands and these variations originated from geological and ecological factors. Similarly, different plants which were obtained from different populations were analysed by means of electrophoresis, and variations between plants were demonstrated in previous studies done on this subject (Du Cros & Wrigley, 1979; Gillespie & Langle 1974; Kephart, 1990; Alexander & Nikolay, 1993).

In conclusion, variations exist both within ecotypes and between ecotypes based on isoenzyme polymorphism. Therefore isoenzyme profiling could be an efficient way to explore variations of *Dactylis glomerata*. Differentiation of hybrid and inbred plants can be easily performed in perennial plants such as orchardgrass. This application might contribute considerably to reduce the length of time taken in breeding programs.

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