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Morphological, biochemical and molecular characterization of Egyptian clover (*Trifolium alexandrinum* L.) varieties

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Abstract

Seeds of five Egyptian clover varieties, Helaly, Serw 1, Gemmiza 1, Giza 6 and Sakha 4 were planted during 2007 at Giza Agricultural Research Farm, Egypt. The highest total dry matter yield of 15.86 t ha^{-1} was recorded for Gemmiza 1 variety. Polyacrylamide gel electrophoresis (PAGE) was performed for native and SDS protein and isozyme variations. RAPD was conducted using 8 arbitrary 10-mer primers. Combined analysis based on four isozymes, PAGE protein electrophoresis and RAPD analyses revealed highest similarity of 0.85 between the two varieties Sakha 4 and Gemmiza 1, while the lowest similarity (0.53) was observed between Giza 6 and Helaly.

Key words : Egyptian clover, Field traits, Isozymes, PAGE, RAPD

Introduction

The genus *Trifolium* contains approximately 240 species. Of these, Egyptian clover (*Trifolium alexandrinum* L.) commonly known as 'Berseem' is an important winter season annual forage legume cultivated in Egypt, the Mediterranean basin and Indian subcontinent. The merit of the crop lies in its multicut nature (four to six cuts), long duration of green forage availability, high green forage yield (100 t ha^{-1}), good forage quality (20% crude protein), digestibility (up to 65%) high palatability (Malaviya *et al*., 2004). Being a leguminous crop, it has an additional advantage of enriching the soil for sustainable cultivation in Egypt. Study of genetic variation within a species is important for breeding of new varieties. For an effective improvement programme, it is imperative to have an adequate knowledge of the breeding system (Roy *et al*., 2005). Characterizing the genetic diversity and the degree of association between Egyptian clover varieties is the first step toward developing and improving varieties.

Random amplified polymorphic DNA (RAPD) technology is a reliable method for characterizing variation within and among species and populations (Huff *et al*., 1993; Gustine and Huff, 1999). Additionally isozymes and protein studies have also been used for characterizing the germplasm and varieties. Hence, objective of the present study was to evaluate Egyptian clover varieties for yield and study genetic variations using isozyme, protein and molecular markers.

Materials and Methods

A field experiment was conducted at Giza Agricultural Research Farm. Seeds of five Egyptian clover varieties, *i.e.* Helaly, Serw 1, Gemmiza 1, Giza 6 and Sakha 4 were planted on 5th November, 2007 in a randomized complete block design (RCBD), replicated four times in 2 m^2 plots. Recommended agronomical practices were followed. Four consecutive cuts were obtained at 77, 118, 139 and 165 days after sowing. Green and dry forage yield (t ha^{-1}) were recorded for each cut and for the whole season. Representative sample of five plants were taken to estimate: plant height (Ph; cm), number of leaves per main stem (L/MS), main stem diameter (MSD; mm) and leaf to stem ratio (on dry basis; L/S) in each cut separately. Analysis of variance (ANOVA) was done to find out the least significant differences among the varieties following Gomez and Gomez (1984).

Peroxidase (Px), Polyphenol Oxidase (PPO), Esterase (Est) and Alcohol dehydrogenase (Adh) isozymes were studied through polyacrylamide gel electrophoresis (PAGE) to identify isozyme variations among cultivars according to Stegemann *et al.,* (1985), Soltis and Soltis (1989, 1990), Brown (1978). Fresh seedling samples were used for isozymes extraction. Extraction of seedling total proteins SDS-polyacrylamide gel electrophoresis was performed following Laemmli (1970) and modified by Studier (1973). Young and fresh seedling samples were used for DNA extraction for RAPD analysis. Electrophoresis was done

following (Sambrook *et al.* 1989; Sambrook and Russell, 2001). Randomly amplified polymorphic DNA-PCR (RAPD-PCR) procedure PCR reactions were conducted using 8 arbitrary 10-mer primers (Table 1). Genetic similarity was estimated by simple matching co-efficient (Sokal and Michener, 1958).

Results and Discussion

Field trial

Fresh and dry forage yield for the studied clover varieties are presented in table 2, in each cut and the cumulative yield. Gemmiza 1 had the best total fresh (136.91 t ha^{-1}) as well as dry yield (15.86 t ha^{-1}) over the studied varieties. Total dry yield of Serw 1 (15.31 t ha^{-1}) insignificantly differed from that of Gemmiza 1 whereas Helaly and/or Sakha 4 had the least insignificant, fresh as well as dry yield.

Plant height (Ph) and leaves to stem ratio (L/S) are shown in table 3. Gemmiza 1 had the tallest plants in the 1st, 2nd, 4th and the mean over cuts. Helaly had the best L/S ratio in the 1st cut only while Gemmiza 1 had the best values of L/S ratio at the 2nd, 3rd, 4th and mean over cuts. Sakha 4 had least L/S values at 3rd, 4th and mean over cuts. Number of leaves per main stem and main stem diameter of clover varieties are presented in table 4. At the 4th cut only, new upper branches emerged, which increased total number of leaves compared to the three earlier cuts. Gemmiza 1 had the maximum leaves at the 1st cut and also in mean of all cuts. Main stem diameter slightly differed from cut to cut and for all tested varieties.

Table 1 : List of the primer names and their nucleotide sequences used in the study

No	Name	Sequence
1	OP-A19	5´ CAA TCG CCG T 3‘
2	OP-B05	5´ TGC TCT GCC C 3‘
3	OP-B07	5´ AGG TGA CCG T 3‘
4	OP-A05	5´ AGGGGTCTTG 3‘
5	OP-C15	5‘ GACGGATCAG 3‘
6	OP-D01	5‘ ACCGCGAAGC 3‘
7	OP-F06	5‘ GGG CGG TAC T 3‘
8	OP-D07	5‘ TTGGCACGGG 3‘

The highest total dry matter yield of 15.86 t ha^{-1} (Table 2) was recorded for Gemmiza 1 variety. It appears that the plant height (79.8 cm) and the maximum mean number of leaves (10.73) of the cultivar has contributed in maximum biomass. Thus, Gemmiza 1 variety was best in forage production.

Isozyme study

A total of 55 bands were generated from the four isozymes peroxidase (PRX), polyphenyl oxidase (PPO), esterase (Est) and alcohol dehydrogenase (ADH) studied among cultivars. Only 14.6 % bands *i.e.* 8 of 55 bands showed polymorphism among the genotypes. The genotype Gemmiza 1 showed polymorphism for one band of Est isozyme (band no. 2) and two bands of Prx isozyme. Genotype Helaly showed low polymorphic percentage. ADH gave monomorphic bands in all genotypes. Peroxidase band number 3 was polymorphic, being absent in Serw1 but present in rest of cultivars. Genotypes Sakha 4 and Giza 6 had one peroxidase band (number 5) present. Isozymes are useful when infra-specific taxa are compared, since the assumption of homology can be more accurate (Klass, 1998). Molina-Freaner and Jain (1992) used isozymes to study population variation of colonizing species using Californian and Turkish cultivars. The isozyme polymorphism was also used for cultivar identification of white clover (*Trifolium repens*) and red clover (*T. pratense*) by Sawada and Yamouchi (1994) and Kongkiatongam *et al.* (1995). Similarity indices among the five Egyptian clover genotypes showed the lowest similarity between Sakha 4 and Gemmiza 1 (75%) and the highest between Sakha 4 and Giza 6 (96%). Similarity values of variety Helaly with Sakha 4, Serw1, Giza 6 and Gemmiza 1 was 87, 84.2, 81.8 and 76.2 % respectively. Serw 1 had a value 85.7, 90 and 81.8 % with Giza 6, Gemmiza 1 and Sakha 4 respectively. The varieties Giza 6 and Gemmiza 1 showed similarity of 78.3%.

Protein profile

The results of SDS-PAGE for the water soluble proteins in the five genotypes (Plate 1) revealed a total number of 9 bands with molecular weights (MW) ranging from about 14 to 100 KDa. The genotypes Gemmiza 1 and Serw1 revealed a total number of 3 polymorphic bands. In the Serw 1 genotype three bands were found at 28.5, 26 and14 KDa. Sakha 4 gave two bands at 24 and 14 KDa. Helaly gave one positive band at 28.5 KDa. Gemmiza 1 and Sakha 4 genotypes possessed specific bands.

Similarity index among the five Egyptian clover genotypes based on protein analysis, was carried out using UPGMA program. The highest similarity index (92.3%) was recorded between two Helaly and Giza 6. Lowest similarity index (50%) was observed between Serw1 and Gemmiza1. Similarity values among the five ranged from 6 to 50 %. The variety Giza 6 possessed 80, 85.7 and 66.7% similarity with Serw 1, Sakha 4 and Gemmiza 1 respectively. The variety Sakha 4 and Gemmiza 1 showed 54.5 % similarity. Helaly had 87.5, 80 and 60% similarity with Serw 1, Sakha 4 and Gemmiza 1 respectively. The similarity between Serw1 and Sakha 4 was 82.4%.

Table 2: Fresh and dry forage yield of clover varieties at each cut (C) and the total yield

Clover varieties	Fresh forage yield (t ha^{-1})					Dry forage yield (t ha^{-1})				
	1st C	2nd C	3rd C	4th C	Total	1st C	2nd C	3rd C	4th C	Total
Helaly	33.10	23.33	34.05	29.76	120.29	3.55	2.41	3.52	3.81	13.26
Serw 1	38.10	30.00	36.91	24.76	129.76	4.31	3.14	4.05	3.81	15.31
Gemmiza 1	36.67	31.43	40.24	28.33	136.91	3.79	3.31	4.33	4.43	15.86
Giza 6	29.67	31.67	35.95	27.38	124.76	3.45	3.36	3.76	3.33	13.91
Sakha 4	32.14	30.00	29.29	25.00	116.43	3.60	3.07	3.02	3.76	13.45
$LSD_{0.05}$	1.26	3.67	3.67	3.29	5.98	0.20	0.43	0.47	0.62	0.74

Table 3 : Plant height and leaf-stem ratio (L/S) of the clover varieties

Clover varieties	Plant height (Ph; cm)					Leaf -Stem ratio (L/S)				
	1st C	2nd C	3rd C	4th C	Mean	1st C	2nd C	3rd C	4th C	Mean
Helaly	81.8	79.1	79.5	73.2	78.4	0.64	0.55	0.57	0.59	0.59
Serw 1	82.8	79.9	75.7	79.9	79.6	0.57	0.50	0.63	0.56	0.57
Gemmiza 1	83.5	80.5	75.3	80.0	79.8	0.53	0.68	0.72	0.63	0.64
Giza 6	80.0	75.9	72.6	75.1	75.9	0.57	0.55	0.66	0.55	0.58
Sakha 4	78.8	78.0	80.2	77.9	78.7	0.60	0.57	0.47	0.54	0.55
$LSD_{0.05}$	3.6	NS	3.9	2.8	1.6	0.05	0.05	0.05	0.05	0.05

Table 4 : Number of leaves per main stem and main stem diameter of the clover varieties

Clover varieties	Number of leaves (L/MS)					Main stem diameter (mm)				
	1st C	2nd C	3rd C	4th C	Mean	1st C	2nd C	3rd C	4th C	Mean
Helaly	8.7	9.2	9.1	13.6	10.3	4.4	4.3	4.2	4.0	4.3
Serw 1	9.0	10.0	9.0	13.4	10.4	3.7	4.6	4.1	4.2	4.0
Gemmiza 1	11.0	9.4	7.8	14.7	10.7	4.8	4.1	4.0	4.3	4.3
Giza 6	8.3	9.0	8.4	12.2	9.5	3.8	3.7	4.1	3.9	3.9
Sakha 4	8.6	9.3	9.2	14.9	10.5	3.2	4.2	4.5	3.7	3.9
$LSD_{0.05}$	1.04	NS	0.57	1.10	0.41	0.52	NS	0.31	0.23	0.22

Table 5 : Total number of band RAPD-PCR products by OP-primers in five Egyptian clover cultivars

Primers	Total no. of band	Helaly	Serw 1	Gemmiza 1	Giza 6	Sakha 4
OP-A19	13	1,2,3,4,5, 6,7,8,13	1,3,4,5,6,7, 8,9,10,11,12	1,3,4,5,8, 9,10,11,12	1, 3,5,8,9, 10,11,12,13	1,3,4,5, 8,9,11,12
OP-BO7	10	4,5	2,4,5,6,7,9	2,4,6,7,8,9	2,4,6,7, 8, 9, 10	2,4,6,7, 8,9,10
OP-B05	12	8,9,10,11,12	1,2,3,4,5,6,7, 8,9,10,11, 12	1,2,3,4,5,6,7, 8, 10, 11,12	4,5,6,7,8,9, 10, 11,12	1,2,3,4,5, 6,7,8,10
OPD01	15	1,4,6,8,9,10	1,2,3,4,5,6,7, 8,9,11,12,14,15	1,2,3,4,5,6,7,8, 9,10,12,13,15	1,2,3,4,5,6,7,8, 9,10,12,13,15	1,2,3,4,5,6,8, 11,12,14,15
OP-F06	13	0	1,4,10	1,2,3,4,5,6,7, 8,9,10,11,12	1,3,4,5,6,7,8, 9,10,11,12,13	1,3,4,5,6,7, 8,9,10,11
OP-C15	12	2,5,9,10	2,4,5,6,7, 8,9,10,12	2,5,8,9,10, 11, 12	2,3,4,5,6,7,8, 9,10,11,12	1,2,3,4,5, 6,8,12
OP-AO 5	11	4,5,6, 7,8,10	4,5,6,7, 8,9,10	1,3,4,5,6,7, 8,9,10,11	2,4,5,6, 7,8,9,10	3,5,6,7, 8,9,10
OP-DO7	14	3,8,13	1,2,3,4,5,6,8,9, 10,11,12,13,14	1,2,3,4,5,8, 9,10,11,13	1,2,3,5,7,8, 9,10,11,13	1,2,4,6,7,8,9
Total no. of band	100	35	75	79	80	68