Indian J. Genet., 68(2): 195-200 (2008)

Isozyme variability in *Trifolium alexandrinum* accessions

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(Received: June 2004; Revised: February 2008; Accepted: April 2008)

Abstract

The present investigation was carried out on 65 accessions of berseem (*Trifolium alexandrinum*) to estimate the genetic variability based on five enzymes – peroxidase, acid phosphatase, esterase, super oxide dismutase and glutamate oxalo acetate transaminase using starch gel electrophoresis. A similarity matrix showed 95-100% similarity among 64 accessions. Accession EC 329299 belonging to 'Saidi' ecotype showed 79% similarity with other accessions. The accessions grouped in three major clusters based on the cutting points in the dendrogram at root level. However, phenetic line drawn at 98% identified 25 groups comprising of 1 to 10 accessions. Of these, 13 groups with more than one germplasm lines showed 100% similarity. Morphologically distinct accessions (i.e. multifoliates and red flowered) formed one cluster. Monomorphic bands (13 out of 26) were in high number. Exotic accessions also showed close similarity to many indigenous accessions which indicated less genetic variability among the germplasm lines introduced from other countries. Polymorphism was recorded for 13 bands (2 for SOD, 3 for GOT, 1 for ACP and 7 for Peroxidase).

Key words: Isozyme, *Trifolium alexandrinum,* Egyptian clover

Introduction

T. alexandrinum (Egyptian clover or berseem) is the most important winter season fodder legume in the tropical and subtropical regions and occupies 2 m ha in India. Of the two ecotypes of the crop 'Mescavi' and 'Fahli', introduced in India during 1903, the former became highly adaptable. Considering its high production potential and wide adaptation in the Indian sub-continent, the crop has attracted attention for its further genetic improvement. However, the crop suffers from narrow genetic base, which has been a major impediment in genetic improvement programmes. At IGFRI, Jhansi, efforts were made to create genetic variability in the crop through introduction, selection, mutations and polyploidization. This has resulted in the isolation of lines showing distinct morphological traits such as pentafoliate leaves, red flower and induced tetraploids.

Isozyme analysis has been used widely to estimate genetic variability, genetic structure, evolutionary and ecological studies and have also been used for the identification of cultivars. Although there are reports on isozymic studies in different *Trifolium* species such as *T. subterraneum, T. ambiguum, T. hirtum*, *T. hybridum, T. uniflorum, T. occidentale, T. pratense, T. repens* etc. [1-6], little has so far been reported on isozymic diversity in *T. alexandrinum.* Hence, present investigation was carried out to estimate the genetic variability based on different isozyme electrophoretic banding pattern in 65 accessions of this species including some exotic ones.

Material and methods

Sixty five germplasm lines comprising of 52 indigenous and 13 exotic germplasm lines of *T. alexandrinum* were used in the present study (Table 1). Sixty three accessions belonged to 'Mescavi' group and one each to 'Saidi' and 'Fahli'. The accessions grouped in exotic old introductions were introduced in Jhansi 8-10 years back and were regularly rejuvenated in local conditions whereas the lines grouped under new introductions were raised for the first season in these conditions. The healthy seeds of different accessions were sown in 3m rows at 50 cm distance. Plant to plant distance was maintained at 15 cm.

The accessions were compared for five enzymes i.e. peroxidase (E.C.1.11.1.7), acid phosphatase (ACP) (E.C.3.1.3.2), esterase (Est) (E.C.3.1.1.2), super oxide dismutase (SOD) (E.C.1.15.1.1) and glutamate oxalo acetate transaminase (GOT) (E.C.2.6.1.1). Horizontal starch gel electrophoresis technique with the discontinuous buffer system was used for studying the enzyme systems. The crude extract of young, green leaves was prepared by homogenizing 1g of sample

Table 1. Details of *T. alexandrinum* accessions used in present study

Ecotype	Ploidy	Exotic/ indigenous	Flower colour	Leaflet	Accession No. number
Mescavi	2x	Exotic	White	Trifoliate	EC 401710[1], EC 400733[6], EC 401709[1], EC 400977[2], EC 402161, EC 400976[2], EC 401711[1], EC 318951[4], EC 318951[4]-1, EC 318954-Mes[3], EC 318952[4], EC 318953[3]
Fahli	2x	Exotic	White	Trifoliate	EC 318954[3]
Saidi	2x	Exotic	White	Trifoliate	EC 329299[5]
Mescavi	2x	Indigenous (Jhansi)	White	Trifoliate	JHB 94-18/11, JHB 34/22, Wardan, JHB 94-31, JHB 94-25, JHB 57P3, JHB 94-56, JHB 15-27, JHB P17-1, JHB 36/5-54, JHB CT2 6/35, JHB 6/54, JHB 16/2, Wardan S-1, Wardan S-2, Wardan S-3, Wardan S-4, JHB 5-13/12, JHB 146
Mescavi	2x (Jhansi)	Indigenous	Red	Trifoliate	JHB 94-R-16, JHB 94-R-35, JHB 94-R-13, JHB 94-R-25
Mescavi	2x	Indigenous (Jhansi)	White	Pentafoliate	JHB 91 P-20, JHB 94 P-22, JHB P-23/35, JHB 94 P/T-34, JHB 94-P-60, JHB 6/54 p/t
Mescavi	4x	Indigenous (Jhansi)	White	Trifoliate	JHTB 1-90-P3, JHTB 1-90-A1, JHTB 5-90-2, JHTB 9-90 N1, JHTB 3-90-H, JHTB 13-90-B, JHTB 5-90-1
Mescavi	2x	Indigenous (Rajasthan)	White	Trifoliate	Raj-Bundi-O, Raj 7/53-54-O, Raj 7/53-54-2, Raj 7/13-14-O, Raj 7/13-25, Raj 7/49-50, Raj 7/13-14, Raj 7/53-54
Mescavi	2x	Indigenous (Punjab)	White	Trifoliate	BL 144, BL 142, BL 131, BL 122
Mescavi	2x	Indigenous (Jabalpur)	White	Trifoliate	JB92-1
Mescavi	2x	Indigenous (Hisar)	White	Trifoliate	HFB 155, HFB 155-1

Source: [1] = Bulgaria, [2] = Ethiopia, [3] = Germany, [4] = Italy, [5] = Egypt, [6] = UK and [7] = USA

with 0.3 ml of chilled tris buffer (pH 8.65). Hydrolyzed starch (14%) was used for analysis for good elasticity and translucence. Electrophoresis was carried out at a constant current of 24 mA for first 30 minutes followed with 34 mA in 'Genei' horizontal migration chamber. The gels were stained following the method of Veech [7] for peroxidase and Wendal and Weeden [8] for esterase, aspartate amino transferase (AAT or GOT), acid phosphatase and super oxide dismutase. The data on banding pattern was recorded as per their relative mobility (RM values). One control was put in all the plates and movement of bands was equalized based on movement of the major band of this control across the plates. The binary data matrix was generated using presence or absence of bands The dendrogram was prepared following genetic similarity and SAHN clustering and UPGMA analysis using NTSYS computer software. Major clusters and sub clusters were identified from the cutting points in the dendrogram considering the dedrogram topologies i.e. groups joining the other major group coinciding with the cutting points at root level. However, groups within sub cluster were identified by drawing a phenetic line at 98%.

Results and discussion

A tolal of 5 esterase bands (at 0.28, 0.55, 0.63, 0.77 and 0.90 RM values), 3 ACP bands (at 045, 0.69 and 0.78 RM values), 5 GOT bands (at 0.17, 0.31, 0.39, 0.44 and 0.47 RM values), 4 SOD bands (at 0.52, 0.73, 0.81 and 0.87 RM values), 3 peroxidase anodal bands (at 0.38, 0.48 and 0.92 RM values) and 6 peroxidase cathodal bands (at 0.20, 042, 0.49, 0.63, 0.79 and 0.92 RM values) were recorded. In all, 26 bands were generated through five enzyme system. Except esterase, wherein five bands were invariably present in all the accessions (Table 2), the rest four enzymes exhibited polymorphic bands.

SOD isozyme banding pattern, revealed presence of four bands, of which band number 3 and 4 showed polymorphism resulting grouping of the accessions in three groups. Band 1, 2 and 4 were seen in 52 lines out

Table 2. Zymogram pattern for different enzymes among accessions of *T. alexandrinum*

Enzyme	Zymogram	No. accessions pattern
Esterase	1, 2, 3, 4, 5	65
SOD	1, 2, 4	52
	1, 2, 3, 4	2
	1, 2	11
GOT	1, 2, 3, 4	21
	1, 2, 3, 4, 5	4
	1, 3, 4, 5	13
	3, 4, 5	4
	1, 3, 4	23
ACP	1, 2, 3	34
	1, 3	31
Peroxidase	2, 3/1, 2, 4, 6 anodal/cathodal	18
	2, 3/1, 2, 4	25
	2, 3/1, 2, 4, 5	17
	2, 3/1, 2, 5	1
	2, 3/1, 2, 4, 5, 6	2
	2, 3/1, 2, 5, 6	1
	1, 3/2, 3	1

of 65. This group of 52 lines included exotic lines and lines from Indian states Rajasthan, Punjab, Uttar Pradesh and Haryana. The presence of only two bands i.e 1 and 2 was observed in 11 lines representing red flowered and pentafoliates and its derived progenies. SOD activity as represented by number of bands revealed lesser activity among red flowered and pentafoliate accessions. EC 329299 and EC 318954 showed maximum activity wherein all four SOD bands were present and, of these two accessions, the first one is 'Saidi' type and the latter has a 'Fahli' background. 'Saidi' and 'Fahli' both have poor regeneration capacity as compared to the 'Mescavi' types. Hence, presence of additional band 3 could be associated with regeneration capability of the genotype.

GOT banding pattern revealed five types of zymograms i.e. (i) a group of 21 lines comprising of mainly exotic lines, a few tetraploids and lines from Rajasthan with 1, 2, 3 and 4 bands (ii) two trifoliate and two pentafoliate lines with 1, 2, 3, 4 and 5 bands, (iii) 4 lines with 3, 4 and 5 bands, (iv) 23 lines with 1, 3 and 4 bands and (v) 13 lines with 1, 3, 4 and 5 bands (Table 2). All the five groups showed presence of band Nos. 3 and 4 whereas band number 1 was noticed in 4 groups.

ACP enzyme study revealed that band numbers 1 and 3 were invariably present in all the lines. Three bands i.e. 1, 2 and 3 were present in 34 lines whereas the remaining 31 lines showed the presence of only two bands i.e. 1 and 3. Most of the exotic lines possessed three bands. There was no distinction observed for Saidi and Mescavi type of lines. Tetraploid lines showed both types of zymograms having two and three bands. The majority of pentafoliate and red flowered lines were marked for the presence of band number 1 and 3 only.

For the peroxidase enzyme, both anodal and cathodal bands were considered. Two anodal bands i.e. band 2 and 3 were present in 64 lines whereas in EC 329299, band 1 was present in place of band 2. Towards the cathodal end, a maximum number of 5 bands (i.e 1, 2, 4, 5 and 6) were present in two accessions i.e. Raj 7/53-54-2 and EC 400977 whereas most of the lines possessed four bands. Band number 2 was invariably present in all the accessions. The second highest frequency was that of band 1 present in 64 accessions. Band 4 was also found in 62 accessions. Bands 5 and 6 were represented each in 21 lines in different zymogram pattern. Band number 3 was present only in EC 329299 (Saidi type). Cathodal zymogram pattern 1, 2 and 4 was observed among 25 accessions. Among 18 accessions 4 bands *viz*., 1, 2, 4 and 6 were present whereas band 1, 2, 4 and 5 were observed in 17 accessions. Presence of anodal band 1, in place of band 2 in the only 'Saidi' accession EC 329299 was marked. Similarly absence of cathodal band 1 and presence of cathodal band 3 in this accession gives sufficient insight that these anodal and cathodal bands are specific to 'Saidi' type and has some correlation with regeneration and flowering behaviour of the ecotype.

Clustering based on five enzyme system revealed 3 major clusters which were further sub-grouped in five subclusters (Table 3, Fig. 1). However, phenetic line drawn at 98% identified 25 groups comprising of one to maximum 10 accessions in the 4th group wherein 10 genotypes showed 100% similarity. This group contained mostly exotic accessions with three indigenous and two tetraploid indigenous accessions. Sixteenth group had eight accessions mostly belonging to the breeding lines along with some indigenous lines from Ludhiana and Hisar showing that accessions from other places within country are also having high degree of similarity. Thirteen groups at 98% phenetic lines were

Table 3. Clustering of *T. alexandrinum* accessions based on isozyme banding

Cluster	Sub-cluster	Group	Accession grouped as phenetic line at 98%
1	1	1	EC 329299
2	2A	2	EC 318954
		3	EC 401711, JHTB 9-90 NI, EC 318953, JHTB 3-90-H, JHTB-1-90-P3, Raj 7/53-54-O
		4	EC 400733, EC 401710, EC 401709, Wardan, EC 402161, EC 400976, JHB 5-13/12, JHTB 5-90-2, JHTB 13-90-B, Raj 7/53-54
		5	BL 131, JHB CT2 6/35, JHB 36/5-54
		6	EC 400977, Raj 7/53-54-2
		7	JB 92-1
		8	Raj-Bundi-O, Wardan S-4, Wardan S-1, Wardan S-2, Wardan S-3
		9	JHB 94-31, EC 318951
2	2B	10	EC 318951-1
		11	Raj 7/13-25
		12	BL 122, Raj 7/49-50
		13	JHB 146
		14	Raj 7/13-14-O, JHTB-1-90-A1
		15	JHTB 5-90-1, JHB 94-P-60, EC 318952, BL 144, JHB 94-18/11
		16	HFB 155, BL 142, HFB 155-1, JHB 34/22, JHB 16/2, JHB 94-56, JHB 94-25, JHB 6/54
3	3A	17	JHB 94 P-22
		18	JHB 91 P-20
		19	EC 318954-Mes
		20	JHB-P-23/35
3	3B	21	JHB 94-R-16, JHB 94-R-25, JHB 57P3
		22	JHB P17-1
		23	JHB 94-R-35, JHB 15-27, JHB6/54 p/t
		24	JHB 94-R-13
		25	JHB 94 P/T-34, Raj 7/13-14

having more than one germplasm lines showed 100% similarity. EC 329299, a 'Saidi' type accession was distinct and placed in cluster 1 showing 79% similarity with other accessions. Cluster 2A comprised of 30 accessions and most of the old exotic accessions and all new exotic accessions were present in this cluster. Many accessions in this cluster showed 100% similarity. EC 318954 showed 97% similarity with other six accessions. This cluster also included tetraploids and lines from different parts of India. Cluster 2B comprised of 20 accessions and was represented by two exotic and 18 indigenous accessions from northwest and central India. Red and pink flowered plants grouped in cluster 3B. The pentafoliate accessions also grouped in major cluster 3.

The study established distinctness of EC 329299 which showed only 79% similarity with other accessions whereas most of accessions showed more than 95% similarity and many of them exhibited identical zymogram pattern for all the enzymes. *T. alexandrinum* ('Miskawi') has been placed in a separate group on the basis of seed protein profile whereas *T. alexandrinum* ('Fahli') showed more similarity with *T. lappaceum, T. pratense, T. medium* and *T. hirtum* [9]. Further study on these species and ecotypes of Egyptian clover is likely to reveal the role of these species in evolution of ecotypes of *T. alexandrinum.* EC 318954 of 'Fahli' background also showed 97% similarity with other accession probably because after acclimatization for nearly a decade in India many plants are behaving like

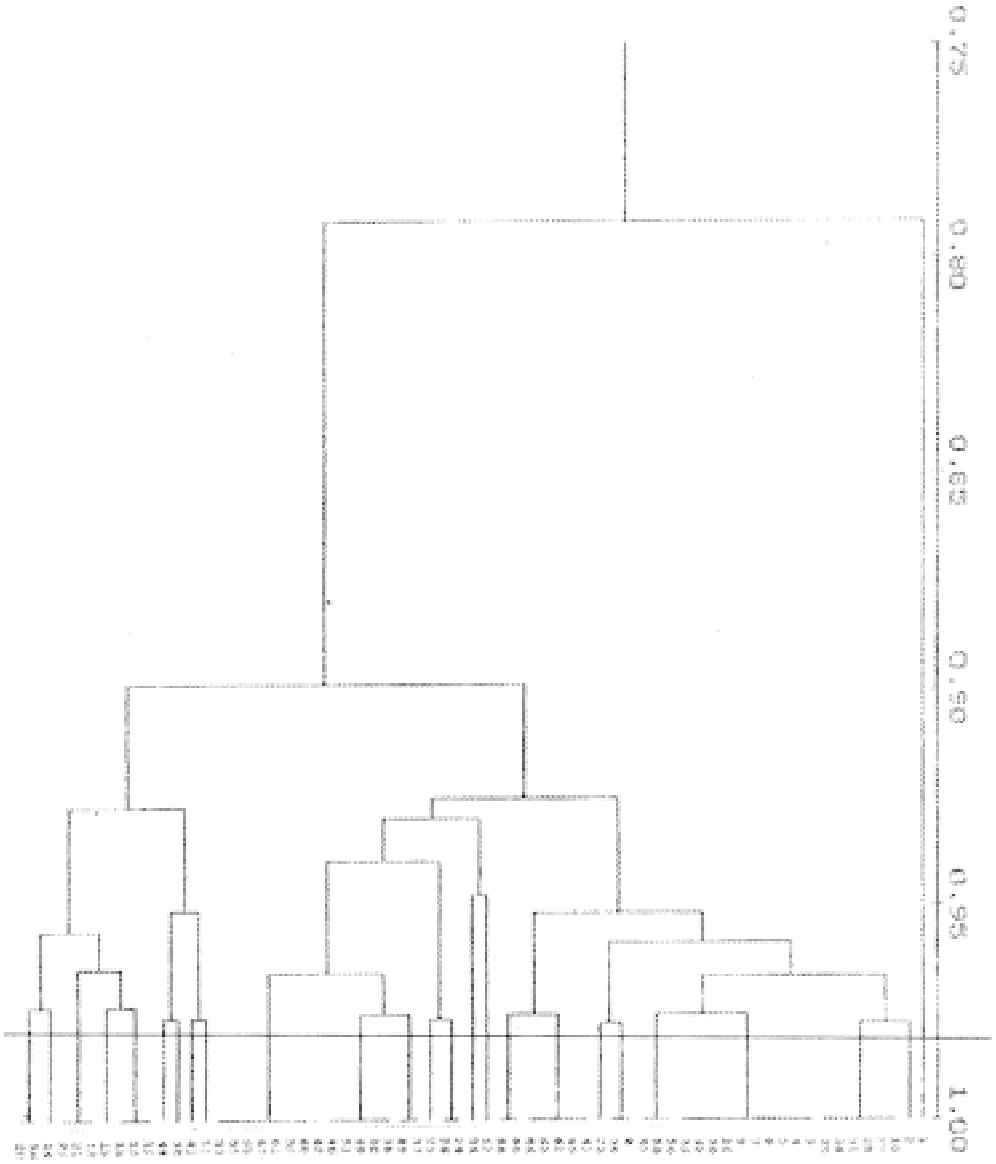

Fig. 1. Dendogram showing relatedness among different accessions of *Trifolium alexandrinum* (Phenetic line drawn at 98%): 1. EC 329299, 2. EC 318954, 3. EC 400733, 4. EC 401710, 5. EC 401709, 6. Wardan, 7. EC 402161, 8. EC 400977, 9. EC 400976, 10. EC 401711, 11. JHB 94 P-22, 12. JHB 94-R-16, 13. JHB94-R-35, 14. JHB 94-R-13, 15. JHB 94-R-25, 16. JHB 94 P/T-34, 17. EC 318951-1, 18. JHB 57P3, 19. JHB P17-1, 20. Raj 7/13-14, 21. JHB 15-27, 22. JHB 6/54 p/t, 23. JB92-1, 24. BL 122, 25. JHB 146, 26. Raj 7/49-50, 27. JHTB 9-90 NI, 28. JHB 5-13/12, 29. EC 318953, 30. JHTB 5-90-2, 31. JHTB 3-90-H, 32. JHTB 13-90-B, 33. Raj 7/53-54, 34. JHTB-1-90-P3, 35. Raj 7/53-54-O, 36. Raj 7/53-54-2, 37. Raj 7/13-14-O, 38. JHTB 5-90-1, 39. EC 318954-Mes, 40. JHB 94-18/11, 41. JHB 91 P-20, 42. JHB 34/22, 43. Raj-Bundi-O, 44. JHB-P-23/35, 45. JHTB-1-90-A1, 46. JHB 94-31, 47. JHB 94-25, 48. EC 318951, 49. EC 318952, 50. JHB 94-P-60, 51. BL 144, 52. JHB94-56, 53. BL 142, 54. Raj 7/13-25, 55. HFB 155, 56. BL 131, 57. JHB 36/5-54, 58. JHB CT2 6/35, 59. JHB 6/54, 60. JHB 16/2, 61. HFB 155, 62. Wardan S-1, 63. Wardan S-2, 64. Wardan S-3 and 65. Wardan S-4

'Mescavi' type [10]. Presence of red and pink flowered accessions and pentafoliate accessions in cluster 3 confirmed their distinctiveness. The genotypes exhibited no banding pattern specific either to indigenous or exotic collection.

Out of total 26 bands observed, seven peroxidase bands, two SOD bands, one ACP band and three GOT bands showed polymorphism. It is generally considered that with the ongoing process of domestication of some species, some bands get fixed while others get

eliminated [11]. The study revealed that the accessions have reached a stable place in the course of evolution and most of the bands have got fixed.

It has been reported that peroxidase and esterase enzymes are good and reliable genetic markers for identification and discrimination between good yielder and inferior yielder in Egyptian clover [12]. This study also provides an isozyme data base for 65 accessions of the crop which can further be utilized in identification of accessions as well as linking with agronomic traits.

Many of the accessions collected from Jhansi, Ludhiana, Hisar and Jabalpur got intermixed in different cluster and showing more than 95% similarity indicate that germplasm being used for breeding programme across the country has narrow genetic base. Further, clustering of morphologically distinct accessions such as tetraploids with diploids also revealed that autotetraploids developed from the accessions of narrow genetic base don't reveal the variation. Exotic accessions also showed close similarity to many indigenous accessions, which indicated less genetic variability among the germplasm lines introduced from other countries.

Thus, this study confirms that the crop suffers from narrow genetic base and needs efforts for broadening its base to achieve the goals in breeding high yielding varieties. One of the reasons for narrow genetic base of *Trifolium* species is incompatibility barrier. In a recent study on isozyme variation in wild and cultivated species of genus *Trifolium*, it has been reported that different accessions of eight *Trifolium* species grouped together, thus, indicating hybridity has not played major role in evolution of the species [13].

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