Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(5): 1260-1265
Received: 27-07-2017
Accepted: 28-08-2017

Ravindra Kumar Jain
Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India

Arunabh Joshi
Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India

Manvendra Singh
Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India

Yadunandan Sen
Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India

Gunjeet Kaur
Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India

Correspondence
Ravindra Kumar Jain
Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India

Isoenzyme diversity and phylogenetic relationship among soybean (*Glycine max* L.) Genotypes

Ravindra Kumar Jain, Arunabh Joshi, Manvendra Singh, Yadunandan Sen and Gunjeet Kaur

Abstract
Amongst the pulses, soybean (*Glycine max* L.) is an excellent source of high quality protein (42 per cent) and oil (20 per cent), rich in glycine and vitamins A, B and D. In the present investigation, isozymes patterns were studied among 24 genotypes of soybean. Peroxidase (*PER*), Esterase (*EST*), and superoxide dismutase (*SOD*) isozymes were screened by native polyacrylamide gel electrophoresis (PAGE) technique to study the genetic divergence. A total of 15 alleles were generated by the three isozymes. Isozymes of SOD exhibited a maximum of seven activity zones followed by EST and PER that showed six and two activity zones respectively. Isozymes of EST showed 83.33 per cent polymorphism while PER showed 50 per cent polymorphism. Jaccard's Similarity Coefficient values lay between 0.97 to1.00 that divided all the genotypes into two main clusters. PIC values ranged from 0.0 to 0.223 with an average of 0.148. Variation in the isozymes profiles amongst soybean genotypes an indication of genetic variation that could be exploited in breeding programmes for quality improvement.

Keywords: Genetic divergence, Jaccard's similarity coefficient, Polymorphism, Quality improvement.

Introduction
Soybean (*Glycine max* L. Merrill) is also known as Golden bean and Miracle crop of 21st century due to its rich source of protein, amino acid and satisfactory proportion of carbohydrates for predominantly vegetarian population. Soybean belongs to the order *Fabales*, the family *Fabaceae*, the subfamily *Faboidae* and the genus *Glycine*. Soybean is a diploidized allotetraploid (2n = 40), autogamous plant. They account for roughly one fifth of the total area under food grain crops and contribute about one-twelfth of the total food grain production in India.

The use of biochemical marker (allozyme or Isozyme) analysis has been used for over 60 years for various research purposes in biology, viz., to study population genetics, to delineate phylogenetic relationships, to estimate genetic variability and taxonomy, and in developmental biology, towards characterization in plant genetic resources management as well as plant breeding. Isozymes are the biochemical markers of choice for initiating or advancing genetic studies of plants. Isozymes are the multiple molecular forms of an enzyme sharing catalytic activity as derived from tissues of an organism (Markert and Moller, 1959) [14]. Since isozymes are direct gene products, the banding patterns so obtained, called zymograms, can be effectively correlated to the genetic make-up of the particular sample. These zymograms are analogous to "fingerprints". Since the amino acid sequence of protein is determined by nucleotide sequences of structural gene loci, "the analysis of protein structure using electrophoresis has been considered as a first approximation analysis of a gene (Gottlieb, 1977) [11]. The number of polymorphic bands in a zymogram is dependent on the number of loci, the number of alleles per locus, and the quaternary structure of the enzyme (Simpson and Withers, 1986) [19].

In present investigation three isoenzymes PER, EST and SOD were studied. Plant peroxidase (EC 1.11.1.7) are hemoproteins that catalyze the H_2O_2 dependent oxidation of a wide variety of substrates including phenolic compounds. These are ubiquitous in nature and have been found to be involved in broad range of physiological functions in plants (Al-Senaidy and Ismael, 2011) [3]. Esterase is one such enzyme capable of degrading cutin and is produced by several species of *Colletotrichum* (Dickman and Patil, 1986) [7]. Superoxide dismutase (SOD, EC 1.15.1.1) is an essential enzyme for the survival of oxygen utilizing organisms. It protects the cells against the toxic effects of superoxide radicals (Fridovich, 1978) [9].

The present study was undertaken to observe the variations in isozyme banding patterns amongst 24 genotypes from the leaf tissues of seedlings of soybean.

Material and Methods

Plant material: Seeds of twenty four genotypes of soybean were procured from Agriculture Research Station (ARS), Kota, Agriculture University, Kota. Source details of the materials used are given in Table 1.The crop was raised in kharif, 2013 at Agriculture Research Station (ARS), Kota. Laboratory studies were done at the Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, MPUAT, Udaipur. Twenty eight days old young leaves were collected from raised plants for further experiments.

Table 1: Source of the 24 *G. max* L. genotypes

S. No.	Genotype	Source
1.	KDS-726	Sangli (Mh)
2.	PS-1539	Pantnagar
3.	DS-3050	Delhi
4.	SL-983	Ludhiana
5.	DS-2961	Delhi
6.	RKS-109	Kota
7.	SL-955	Ludhiana
8.	DS-3047	Delhi
9.	AMS-1001	Amarawati
10.	JS 20-79	Jabalpur
11.	MACS-1419	Pune
12.	NRC-98	Indore
13.	RVS 2002-4	Sihore
14.	KDS-722	Sangli (Mh)
15.	MAUS-609	Parbani
16.	NRC-107	Indore
17.	MACS-1410	Pune
18.	JS 20-53	Jabalpur
19.	PS-1543	Pantnagar
20.	HIMSO-1685	Palampur
21.	RVS 2002-22	Sihore
22.	RKS111	Kota
23.	BAUS-27	Ranchi
24.	RSC 10-17	Raipur

Biochemical analysis: On the basis of banding pattern of the three enzymes, viz., (POX), (EST) and (SOD), variation in protein profiles for isozymes activity was recorded from the young leaves (28 DAS).

Enzyme extraction: The standardized extraction medium for PER, EST and SOD was prepared which consisted 0.1 M Tris-HCl buffer (pH 7.5) containing 3 per cent (w/v) polyvinylpyrrolidine (PVP), 1mM EDTA and 1mM CaCl2. The crude enzyme was extracted from twenty eight days young leaves by macerating 5 g tissue with 15 ml ice cold extraction medium in a pre-chilled pestle and mortar using acid washed sand as abrasive (Sharma *et al.,* 2008) [17]. The homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged at 10,000 rpm for 10 min. in a refrigerated centrifuge (Sigma, 3K30) at 4 ^{0}C. The supernatant was transferred to microcentrifuge tubes and stored at -20 ^{0}C until use for electrophoresis.

Polyacrylamide gel electrophoresis (PAGE): 400 µl sample (1µg/µl) of each genotype was used in which 15 µl of 5% bromophenol blue and 85 µl of 40 % glycerol was added. The contents were then mixed thoroughly and used for native PAGE as per standard method of Davis (1964) [6].

Isozymes Staining: The side spacers were removed just after electrophoresis. With the help of spatula, the glass plates were then separated. Gels were stained with specific staining solution as described below for each enzyme.

(A) PER: The method described by Guikema and Shermen (1980) was followed with appearance of blue coloured bands that turned brown by 10-15 min.

(B) EST: The method described by Shaw and Prasad (1970) [18] was followed. The gel was incubated at 35 ^{0}C for 20 minutes and washed with distilled water. Brown bands of EST isozyme appeared immediately on a clear background.

(C) SOD: The method of Geburek and Wang (1990) [10] was followed for staining. The gel was exposed to strong fluorescent light for 15 minutes followed by one hour incubation in dark. SOD appeared as light bands (negatively stained) on a dark blue background.

Data analyzing: Gels were visually scored by putting them on a box provided with illumination. All the bands were scored and used to construct the zymograms. Rf (Rm) value of each band was calculated using the following formula (Eeswara and Peiris, 2001) [8].

$$Rm = \frac{\text{Distance travelled by the band}}{\text{Distance travelled by the tracking dye}}$$

Bands were numbered on the basis of increasing Rf value or according to the distance travel by them in the gel.

Statistical analysis for isozyme pattern: In each gel, for each band, the migration rates (Rf) were calculated using the distance migrated by the band divided by that of the running front. The absence and presence of bands were considered as 0 and 1 respectively. The accessions were grouped based on similarity indices (no of common bands divided by the total number of bands) considering the most frequent patterns. The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's (1908) [13] similarity coefficient.

UPGMA dendrogram: Cluster analysis for the genetic distance was then carried out using UPGMA clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationships amongst the genotypes using computer program NTSYSpc version 2.02 (Rohlf, 1933) [16].

Results and Discussion

Peroxidase (PER; E.C.1.11.1.7): Electrophoretic profiles of peroxidase Isozyme (Plate-1) showed two activity zones having Rm value of 0.18 and 0.77. First band was present in all genotypes, the difference was only found in terms of intensity of bands. In genotype RVS-2002-4, the intensity was high at Rm value of 0.18 and less in remaining genotypes. High intensity bands of each genotype lay more towards cathodic side, possibly having a net positive charge and high molecular weight, while rest of the bands were towards anode indicating a net negative charge on them and correspondingly lower molecular weights. The second band having Rm value of 0.77 was present in KDS-726, PS-1539, SL-983, DS-2961, RKS-109, SL-955, DS-3047, JS 20-79, KDS-722, MAUS-609, MACS-1410, JS 20-53, PS-1543, HIMSO-1685, RVS 2002-22 and BAUS-27 genotypes (Figure 1A).Similar findings have been reported by Abdalla (2010) [1]. They found PER isozymes exhibit to 3 polymorphic bands among a total of seven *in Vicia faba* plants.

Esterase (EST; E.C.3.1.1.1): EST isozyme profile in *G. max* L. genotypes has been presented in (Plate 2). Esterase isozymes exhibited a maximum of six bands having Rm value of 0.05, 0.1, 0.38, 0.78, 0.83 and 0.88. Bands having Rm value of 0.05 were present in KDS-726, PS-1539, MACS-1419, NRC-98, RVS 2002-4, NRC-107, RKS111, BAUS-27 and RSC 10-17. Bands of Rm value 0.1 were present in KDS-726, PS-1539, SL-983, DS-2961, DS-3047, AMS-1001, JS 20-79, MACS-1419, NRC-98, KDS-722, MAUS-609, NRC-107, PS-1543, HIMSO-1685, RVS 2002-22, BAUS-27 and RSC 10-17. Bands of Rm value 0.38 was present in all the genotypes. Bands of Rm value 0.78 and 0.83were present in all genotypes except DS-3050 and SL-983. Similarly, bands having Rm value of 0.88 were present in all genotypes except KDS-722 and MAUS-609 (Fig. 1B). Abdalla (2010) [1] also studied EST isozymes that displayed 13 bands, six of them were polymorphic with different genetic responses while the other bands were similar *in Vicia faba* plants.

Superoxide dismutase (SOD; E.C. 1.15.1.1): SOD isozyme profile in *G. max* L. genotypes has been presented in (Plate 3). Corresponding SOD zymogram in all genotypes indicated seven bands having the Rm value 0.25, 0.30, 0.48, 0.55, 0.66, 0.75 and 0.84 respectively. All genotypes were showing similar banding pattern i.e. all bands were present in each genotypes (Fig. 1C). Similar reports also found by Andres and Ortiz (1995) [4]. They studied over twenty five accessions belonging to the genus *Cytisus* and *allies* using iso-enzymes. They found a total of 29 bands for SOD, 18 for PER and 35 for EST. *Genista* showed fewer bands whereas *Cytisus villosus* and *Chameacytisus* shows high intensity of bands in upper zones.

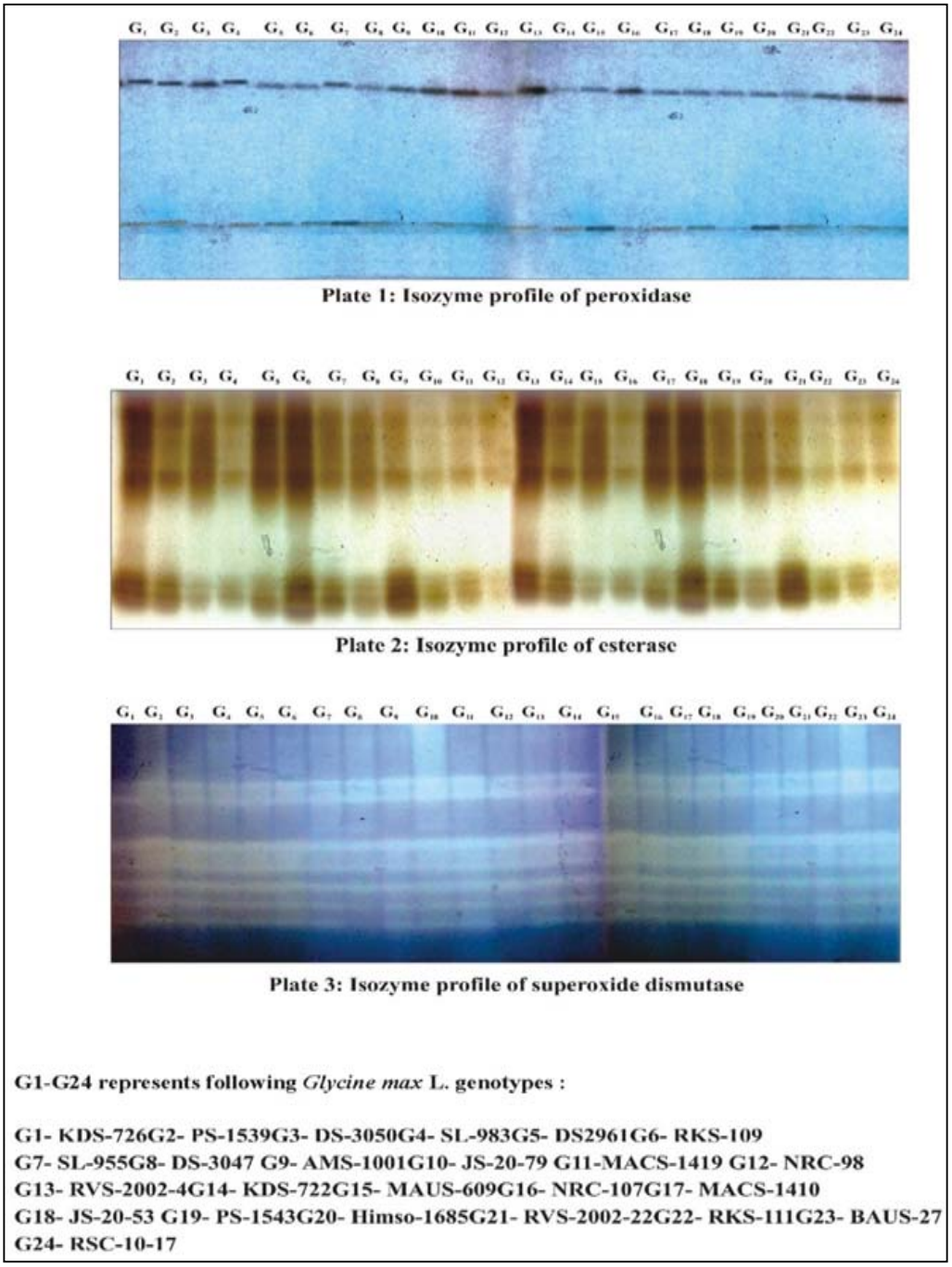

Plate 1: Isozyme profile of peroxidase

Plate 2: Isozyme profile of esterase

Plate 3: Isozyme profile of superoxide dismutase

G1-G24 represents following *Glycine max* L. genotypes :

G1- KDS-726G2- PS-1539G3- DS-3050G4- SL-983G5- DS2961G6- RKS-109
G7- SL-955G8- DS-3047 G9- AMS-1001G10- JS-20-79 G11-MACS-1419 G12- NRC-98
G13- RVS-2002-4G14- KDS-722G15- MAUS-609G16- NRC-107G17- MACS-1410
G18- JS-20-53 G19- PS-1543G20- Himso-1685G21- RVS-2002-22G22- RKS-111G23- BAUS-27
G24- RSC-10-17

Plate 1-3: Banding pattern of Peroxidase, Esterase and Superoxide dismutase isozymes of Soybean

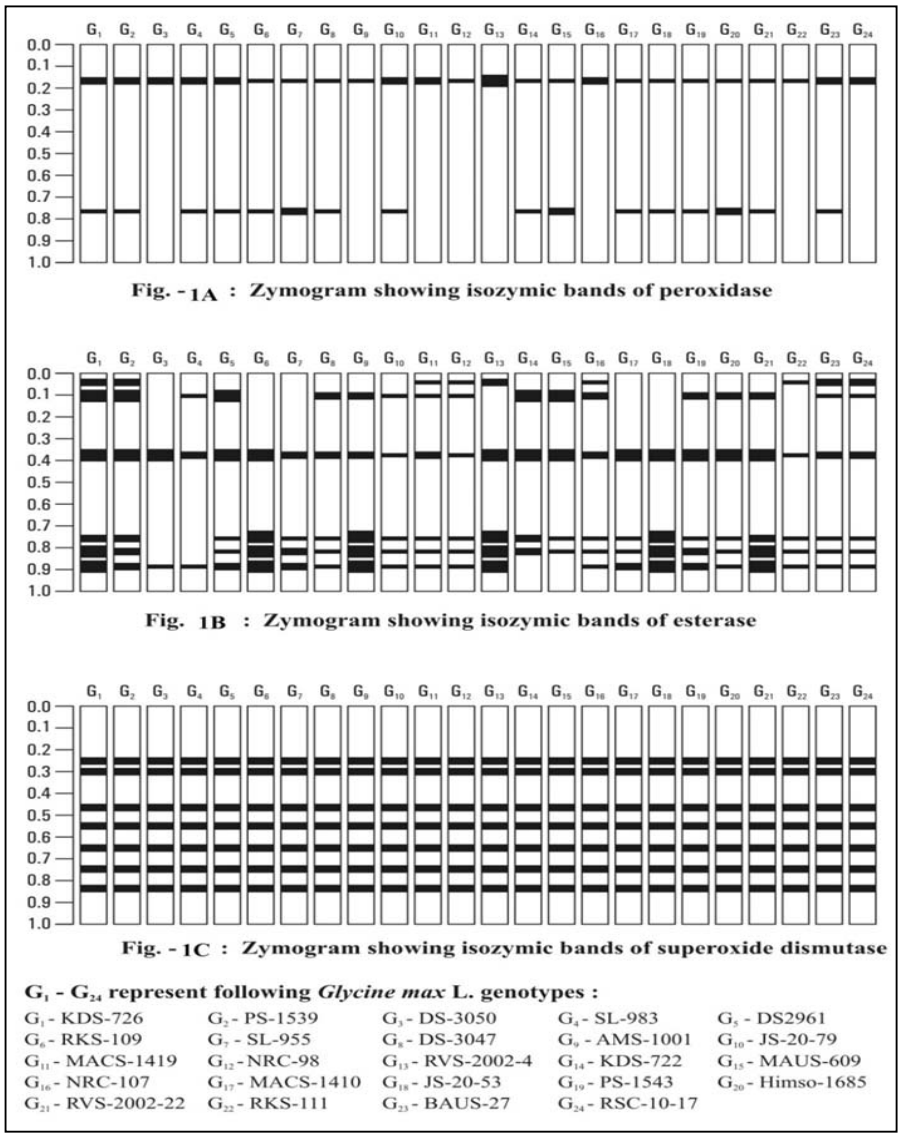

Fig 1(A, B & C): Zymogram of isozymic bands of Peroxidase, Esterase and Superoxide dismutase of Soybean

Genetic relationship and cluster tree analysis: In the present study, observations that lead to discovery of genetic diversity in the 24 genotypes of *G. max* L., comprised a total of 15 alleles that were generated by the three isozymes (Table 2). In scoring the bands obtained, only easily resolved and bright isozyme bands were counted. PER, EST and SOD isozymes showed 50%, 83.33% and 0% polymorphism, respectively. Similarly, Aboel-Atta (2009) [2] studied three isozymes viz., α- and β-esterase and aldehyde oxidase, out of which only the banding patterns of α- and β-esterase revealed slight polymorphism with a percentage of 27.77% between the two species of *Melilotous.*

Table 2: Protein profiling and polymorphism generated in G. *max* L. using 3 isozyme markers

S. No.	Isozyme markers	Total no. of bands	No. of polymorphic bands	% polymorphism	PIC
1.	Peroxidase (POX)	2	1	50	0.222
2.	Esterase (EST)	6	5	83.33	0.223
3.	Superoxide dismutase (SOD)	7	0	0	0
Average		5	2	44.44	0.148

Cluster tree analysis was carried out by UPGMA method based on genetic distance. Similarity coefficient ranged from 0.77 to 1.00 between 24 genotypes of *G. max* L. (Fig. 2). The average similarity across all the genotypes was found out to be 0.88, showing that genotypes were genetically similar. Cluster tree analysis grouped all genotypes into two clusters (Cluster-I & II) at a similarity coefficient of 0.85. Cluster-I included 22 genotypes *i.e.,* KDS-726, PS-1539, BAUS-27, DS-2961, DS-3047, JS-20-79, RVS-2002-22, PS-1543, Himso-1685, RKS-109, SL-955, MACS-1410, JS-20-53, KDS-722, MAUS-609, AMS-1001, MACS-1419, RSC-10-17, NRC-107, NRC-98, RVS-2002-4 and RKS-111 at a similarity coefficient of 0.84. Cluster I was further divided into two sub clusters (Sub cluster-I & II). Sub cluster-I consisted 15 genotypes *i.e.* KDS-726, PS-1539, BAUS-27, DS-2961, DS-3047, JS-20-79, RVS-2002-22, PS-1543, Himso-1685, RKS-109, SL-955, MACS-1410, JS-20-53, KDS-722 and MAUS-609. Sub cluster-I can be again divided into four sub cluster (Sub cluster- A, B, C & D). Sub cluster-A included three genotypes *viz.,* KDS-726, PS-1539 and

BAUS-27 at a similarity coefficient of 1.00. Sub cluster-B included six genotypes *viz.,* DS-2961, DS-3047, JS-20-79, RVS-2002-22, PS-1543 and Himso-1685 at a similarity coefficient of 1.00. Sub cluster-C included four genotypes *viz.,* RKS-109, SL-955, MACS-1410 and JS-20-53 at a similarity coefficient of 1.00. Sub cluster-D included two genotypes *viz.,* KDS-722 and MAUS-609 at a similarity coefficient of 1.00.

Sub cluster-II consisted 7 genotypes *i e.* AMS-1001, MACS-1419, RSC-10-17, NRC-107, NRC-98, RVS-2002-4 and RKS-111. Sub cluster-II can be again divided into two sub cluster (Sub cluster- A& B). Sub cluster-A included four genotypes *viz.,* MACS-1419, RSC-10-17, NRC-107 and NRC-98 at a similarity coefficient of 1.00. Sub cluster-B included two genotypes *viz.,* RVS-2002-4 and RKS-111 at a similarity coefficient of 1.00. Cluster-II included two genotypes *i.e*., DS-3050 and SL-983 at 0.86 similarity coefficient.

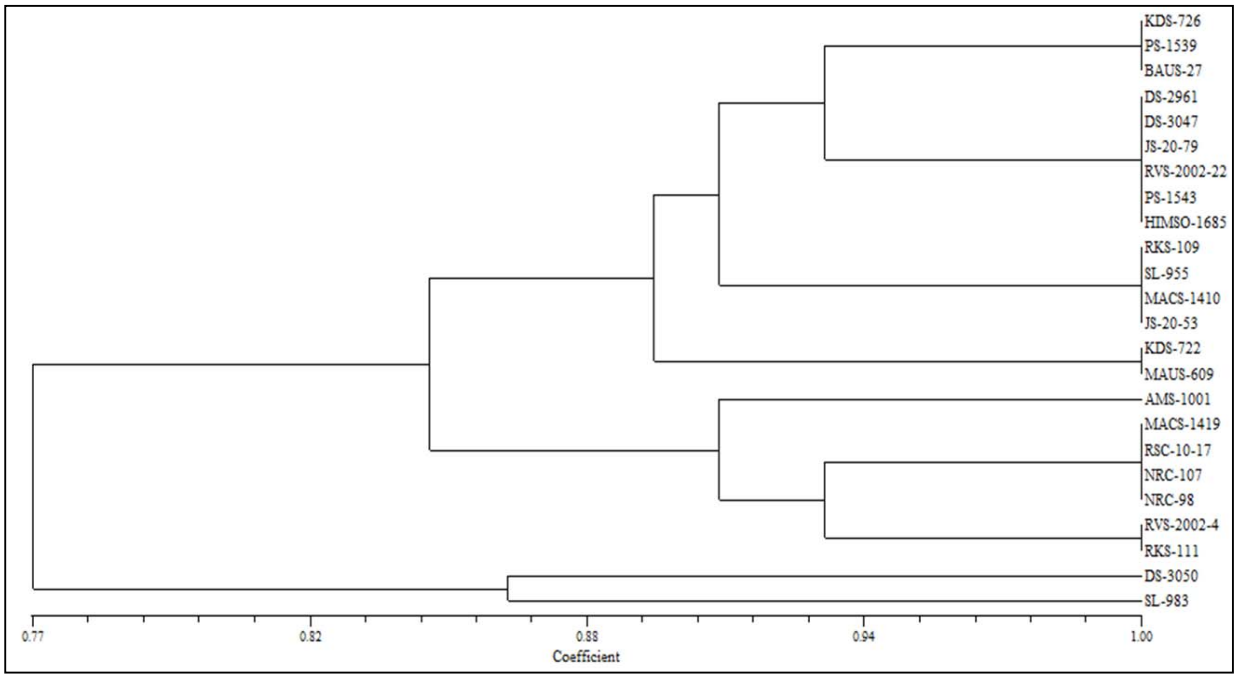

Fig. 2: Dendrogram constructed with UPGMA clustering method among 24 genotypes of *G. max* L. using three isozymes

Genetic diversity and distance derived from isozyme analysis were very low due to small number of polymorphic alleles. This has also been reported by Sonnante *et al.* (1997) [20] *in Vigna* species who had studied 8 isozyme systems.

Due to simplicity, cost effectiveness, speedcity and reproducibility, these biochemical markers are more preferable then some of other marker system. Researchers can use information on genetic similarity to make decisions regarding selection of superior genotypes for improvement or for use as parents for the development of future cultivars through hybridization. Payne and and Koszykowski, 1978 [15] reported that the quantitative differences among cultivars in the activity of Isozyme should serve as aids in identifying soybean. They can be used to evaluate levels of genetic diversity and phenotypic relationships within and between species, and to identify particular races and pathotypes (Brown, 1996) [5]. The results are important because for successful breeding programme and to supply suitable cultivar of crop species to farmers, variations are required.

Acknowledgments

Authors gratefully acknowledge Dr. H. R. Chaudhary and Dr. A. Dashora, ARS Kota for providing seed material and Dr. G. Rajamani, STA, MBBT Dept. RCA for support and encouragement.

References

1. Abdalla MM. Sustainable effects of diatomite on the growth criteria and phytochemical contents of *Vicia faba* plants. Agriculture and Biology Journal of North America. 2010; 1(5):1076-1089.
2. Aboel-Atta AII, Isozymes RAPD, ISSR variation in *Melilotus indica* (L.) All. and *M. siculus* (Turra) B.G. Jacks. (*Leguminosae*). Academic Journal of Plant Sciences. 2009; 2(2):113-118.
3. Al-Senaidy AM, Ismael MA. Purification and characterization of membrane-bound peroxidase from date palm leaves (*Phoenix dactylifera* L.). Saudi Journal of Biological Science. 2011; 18(3):293-298.
4. Andres FG, Ortiz JM. Biochemical Systematics and Ecology. 1995; 23(7-8):813-824.
5. Brown JKM. The choice of molecular marker methods for population genetic studies of plant pathogens. New Phytologist. 1996; 133:183-195.
6. Davis BJ. Disc electrophoresis II. Method and application to human serum proteins. Annals of the New York Academy of Sciences. 1964; 121:404-427.
7. Dickman MB, Patil SS. Cutinase deficient mutants of *Colletotrichum gloeosporioides* are non-pathogenic to papaya fruit. Physiological and Molecular Plant Pathology. 1986; 28:235-242.
8. Eeswara JP, Peiris BCN. Isozyme as marker for identification of mung bean (*Vigna radiata* L.). Seed Science and Technology. 2001; 29:249-254.
9. Fridovich I. The biology of oxygen radicals. Science. 1978; 201:875-880.
10. Geburek T, Wang Q. Inheritance of isoenzyme variants and their linkage relationships in Chinese fir (*Cunninghamia lanceolata* Hook.). Euphytica. 1990; 49:193-201.

11. Gottlieb LD. Evidance for duplication and divergence of the structural gene for phosphoglucoisomerase in diploid species of *Clarkia***.** Genetics. 1977; 86:289-307.
12. Guikema JA, Shermen LA. Electrophoresis profiles of cynobacterial membrane polypeptides showing heme dependent peroxidase activity. Biochimica et Biophysica Acta. 1980; 663:189-201.
13. Jaccard P. Nouvelles researches surla distribution florale. Societe Vaudoise des Science Natulelles Bulletin. 1908; 44:233-270.
14. Markert CL, Moller F. Multiple forms of enzymes**:** tissue, ontogenetic and species specific patterns. Proceedings of the National Academy of Sciences. 1959; 45:753-763.
15. Payne RC, Koszykowski TJ. Esterase isoenzyme differences in seed extracts among soybean cultivars. Crop Science. 1978; 18(4):557-559.
16. Rohlf FJ. NTSYS-PC. Numerical taxonomy and multivariate analysis system. Exeter Software, New York, 1993.
17. Sharma K, Mishra AK, Misra RS. The genetic structure of taro: A comparision of RAPD and isozyme markers. Plant Biotechnology Reports. 2008; 2:191-198.
18. Shaw CR, Prasad R. Starch gel electrophoresis of enzymes. A compilation of recipes. Biochemical Genetics. 1970; 4:297-320.
19. Simpson MJA, Withers LA. Characterization using isozyme electrophoresis: A guide to the literature. International Board for Plant Genetic resources, Rome, 1986.
20. Sonnante G, Spinosa A, Marangi A, Pignone D. Isozyme and RAPD analysis of the genetic diversity within and between *Vigna luteola* and *V. marina*. Annals of Botany. 1997; 80:741-746.