Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; SP1: 390-393

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Isozyme analysis based genetic diversity studies in maize inbreds (*Zea mays* L.)

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Abstract
Electrophoretic separation of isozymes has provided a reasonably precise and quantitative approach for the analysis of genetic diversity. Thus, in the present study isozymes markers was used for assessing the genetic diversity in eight inbred lines of maize. namely CML 142, CML 144, CML 150, CML 176, CML 186 CM 300, CM 400 and CM 600. The horizontal starch gel electrophoresis was used to study the isozyme patterns. These parental lines were obtained from AICRP on Maize, Dholi Centre under RAU, Pusa, and Bihar. Six isozyme systems, namely peroxidase, esterase, acid phosphatase, catalase, amylase and alcohol dehydrogenase were used for characterization and diversity studies of inbreds. Polymorphism of isozyme was visualized in respect of number and mobility of bands. An analysis of divergence based on isozyme patterns was performed by calculating genetic distances among the eight inbred lines. Based on the presence or absence of bands, the genetic distance was computed by using the Nie and Li coefficients. The dendrogram was obtained following unweighted pair group (UPGMA) method using arithmetic mean. Inbreds were classified into five groups A, B, C, D, and E. Analysis revealed that isozyme patterns in the inbred CM 600 was least similar to the isozyme patterns obtained in other inbreds. Similarity coefficients between CM 600 and CML 186 was least (0.4615) whereas, the highest (0.8511) existed between CML 142 and CML 144. Similarity coefficients ranged from 0.46 to 0.85, indicating moderate levels of diversity present among the inbreds under evaluation in the present study. Besides, this electrophoresis technique was fast, precise and without environmental effects. Thus, isozyme pattern based diverse group might be used in hybridization programme for obtaining heterotic hybrids.

Keywords: Maize, *Zea mays* L., inbreds, isozyme marker, starch gel electrophoresis

Introduction
Maize is one of the most important crops occupying prominent position in the agricultural economy. Maize breeders face a continuing challenge to identify germplasm sources that will provide inbreds with superior heterosis in single cross combinations. The general View is that genetically diverse parents are likely to produce high heterotic effects in cross combinations. Assessment of germplasm diversity based on standard morphological markers in maize has proved to be inadequate because of the existence of wide spectrum of phenotytpic variation, interaction of morphometric and morphologic characters with environment, epistatic interaction and the unknown genetic control of the traits (Mannetji, 1984; Smith and Smith, 1989). Several studies have been conducted in maize with an effort to evaluate the associations of alleles at isozyme loci with agronomically useful traits and to test the possible use of isozymes for more efficiently improving certain agronomic traits such as grain yield. Isozymes have been extensively used to examine the genetic variability in breeding materials since the 1978s (Tanksley and Orton, 1983). It is an useful tool to detect genetic variability/ diversity analysis among cultivars/parental lines variation (Mauria *et al*., 2000; Sanchez *et al*. 2000), and is particularly suitable for developing countries because it is relatively simple rapid and inexpensive. Isozyme based genetic diversity studies between different populations and species, evolution of maize and evaluation of genetic purity of seed lots of maize has also been reported (Doebley, 1990; Lu *et al*. 2002; Salgado *et al*. 2006; Hamill and Brewbaker 2008). Maize tissues varied greatly in isozyme pattern, many tissues having a characteristic and distinguishing complement. Important biochemical enzyme markers, namely, amylase, catalase, esterase, peroxidase, acid phosphatase and alcohol dehydrogenase have significant roles in plant cells. The plant protects themselves from reactive oxygen species by antioxidants like catalase and peroxidase. Catalase acts as a primary H_2O_2 scavenger in the peroxisomes and the mitochondria (Anderson *et al*., 1995). The activity of peroxidase increases in stressed conditions. They also play a role in biosynthesis of lignin. Esterase is a hydrolyzing enzyme which catalyzes the addition or removal of water in biological reactions.

It has been extensively explored as biochemical markers in plants due to its high allele polymorphism (Resende *et al*. 2004). Use of isozymes to assess genetic diversity among parental lines of maize and predict hybrid performance among pairs of lines has only been carried out to a limited extent. Keeping in view of heterosis breeding in the present study, isozymes were used for assess the isozyme polymorphism in inbreds lines of maize and to make an assessment of the extent of genetic diversity among these inbreds.

Materials and Methods

The experimental materials for the present investigation were obtained from AICRP, Dholi Centre under Rajendra Agricultural University, Bihar, Pusa. Materials used were eight inbreds including five quality protein maize (QPM) and three non-quality protein maize (Non-QPM): CML 142, CML 144, CML 150, CML 176, CML 186 and CM 300, CM 400, CM 600 respectively were evaluated for isozyme pattern to study genetic divergence amongst them. Six isozymes viz., peroxidase (PRX, E.C.1.11.1.7), esterase (EST, E.C.3.1.1.2), catalase (CAT, 1.11.1.6), amylase (AMY, E.C. 3.2.1.1.) acid phosphatase (ACP, E.C 3.1.3.2) and alcohol dehydrogenase (ADH, E.C. 1.1.1.1.) patterns were studied in germinating coleoptile tissue of eight inbred lines. The horizontal starch gel electrophoresis technique of smithies (1955) was used to study the isozyme patterns using discontinuous buffer system as described by Poulik (1957). This system has better resolving power (Brewer and Singh, 1970) which results from gradation of molecular sieving effect of starch gel matrix besides electrophoretic separation. Besides this, isozyme of more than one enzyme can be studied at a time because two slices can be stained separately for different enzymes. Protocols outlined by Shield *et al* (1983) with some minor modifications were used for extraction and electrophoretic separation of isozyme. Various steps involved in the starch gel electrophoresis were hydrolysis of starch, preparation of buffer system comprising gel buffer, electrode buffer, sodium acetate buffer, phosphate buffer, sodium thiosulphate buffer, preparation of gel, extraction and application of the sample, running of gel, slicing the gel and enzymatic staining. The gel were stained following the procedure prescribed for peroxidase (Veech, 1969), catalase (Thorup, 1961), amylase (Vellejos, 1983), esterase, Acid phosphatise and alcohol dehydrogenase (Tanksley, 1979). The anodal bands were designated with prefix 'A' and cathode bands with prefix 'C'. Symbol 'A' and 'C' are entirely operational as net change of a molecule that varies with the pH. A number was also assigned to each band, the closest to the origin is number 1 with more rapidly moving bands being assigned progressively higher numbers. A band having same mobility, in different tissues at a particular stage of development in different genotypes, carries the same number and has been treated as the same isozyme. Relative mobility (Rm) was calculated as the ratio of the distance of the band from the origin to the distance of the dye front. Depending on staining intensity, isozyme band (electromorphs) were characterized into light intensity (+), moderate intensity (++) and dark intensity (+++).

Analysis of isozyme divergence: Presence or absence of each band on the gel was scored as '1' or '0' respectively. Similarly between every pair of entries included in the present study was ascertained on the basis of isozyme pattern. The similarity coefficient was estimated as Nei and Li's coefficient (Nei and Li, 1979). The method used for tree building in the analysis involved sequential agglomerative hierarchical non-overlapping clustering based on similarity coefficients. The dendrograms based on similarity indices were obtained by unweighted pair group method (UPGMA) using arithmetic mean. The level of diversity for isozyme of peroxidase, esterase, acid phosphatase, catalase, amylase and alcohol dehydrogenase by identifying the clusters at appropriate phenon levels.

Nei and Li's coefficient = 2 a /(2a + b + c)

Where,

a. = No. of shared bands between j^{th} & k^{th} genotypes
b. = No. of bands present in j^{th} genotypes but absent in k^{th} genotypes
c. = No. of bands absent in j^{th} genotypes but present in k^{th} genotypes

Results and Discussions

Six isozymes viz., peroxidase, esterase, catalase, amylase, acid phosphatise and alcohol dehydrogenase patterns were studied and used for evaluating the genetic divergence among the inbreds. Total number of bands obtained and band number of each inbred for different isozymes patterns were presented in table 1. Presence or absence of isozyme bands was also used as marker for characterizing the inbreds by earlier workers (Mauria *et al*., 2000; Baishya *et al*., 2003). A total of five anodal bands, PRX-1 (Rm- 0.20), PRX-2 (Rm-0.27), PRX-3 (Rm-0.46), PRX-4 (Rm-0.53) and PRX-5 (Rm- 0.67) and five cathodal bands, PRX -1C (Rm-0.18), PRX-2C (Rm-0.28), PRX-3C (Rm-0.35), PRX-4C (Rm-0.55) and PRX-5C (Rm- 0.65) of peroxidase isozyme were observed. The mobility values (Rm) indicated a wide range of variability in molecular weights for peroxidase bands (Table 1). Thus, it appeared that peroxidase can be considered as useful biochemical marker for the characterization of maize inbreds. The results of the present study was in agreement with the earlier reports (Yang *et al*., 1995; Hamilla and Brewbaker, 2008). Only two esterase bands EST-1 (Rm- 0.67) and EST-2 (Rm-0.73) were present in all the eight inbreds. Observable variation was recorded in respect of mobility indicating a wide range of variation in molecular weight of esterase bands. Thus, it is evident that isozyme analysis provided information regarding the existence of genetic variability among the inbreds under consideration in this study. Similar information was also provided earlier by several researchers (Yang *et al*., 1995; Lu *et al*., 2002; Salgado *et al*., 2006 and Pereira *et al*., 2008).

Altogether six acid phosphatase bands viz., ACP-1, ACP-2, ACP-3, ACP-4, ACP-5 and ACP-6 (Rm-0.37, 0.40, 0.45, 0.53, 0.60 and 0.63) were visualized on gel In case of alcohol dehydrogenase seven anodal, ADH-1, ADH-2, ADH-3, ADH-4, ADH-5, ADH-6 and ADH-7 (Rm- 0.09, 0.13, 0.16, 0.23, 0.53, 0.67 and 0.73) and five cathodal bands viz., ADH-1C, ADH-2C, ADH-3C, ADH-4C and ADH-5C (Rm- 0.17, 0.20, 0.25, 0.38 and 0.47) were observed.. In addition to differences in the number of bands, observable variations were also recorded in respect of mobility of bands in both enzymes (Table 1). Differences in mobility values indicated a wide range of variability in molecular weight of acid phosphatase and alcohol dehydrogenase isozyme and can be considered as the basis of characterization of maize inbreds (Table 1). Similar observation was also reported earlier (Haider *et al*., 2000; Mauria *et al*., 2000; Zlokolica and Milosevic, 2001; Lu *et al*., 2002; Salgado *et al*. 2006; Cortes *et al*., 2007)

Five amylase bands Amy-1, Amy-2, Amy-3, Amy-4 and Amy-5 (Rm – 0.01, 0.04, 0.1, 0.16 and 0.26) were observed in eight inbreds. In addition to differences in the number of

bands, observable variations were also recorded in respect of mobility. Six catalase isozyme bands, Cat-1, Cat-2, Cat-3, Cat-4, Cat-5 and Cat-6 with Rm value 0.22, 0.24, 0.28, 0.30, 0.32 and 0.34 were observed (Table 1). The differential mobility exhibited by amylase and catalase bands gave an indication of their different molecular weight. Variation in mobility and number of bands may be considered as the biochemical marker for characterization of maize inbred lines. This observation is in agreement with the earlier report documented from the studies conducted by several researchers (Yang *et al*., 1995; Lu *et al*., 2002; Salgado *et al*., 2006). Presence of a particular isozyme in one inbred and its absence in other may be due to the fact that structural gene responsible for production of particular isozyme, has mutated resulting in complete loss of its activity in some inbreds. The altered electrophoretic mobility, as revealed by the position of bands, reflects a change in net charge of the protein molecule. This is possible when the substituted amino acid carries a charge that is different from one it replaces. This change may be caused by mutation in structural gene. Another possibility of its presence and absence of bands be due to change in regulatory gene which did not allow the structural gene for the isozyme to be expressed in some inbreds.

Analysis of genetic divergence

Combined analysis of six isozyme systems was done for assessment of divergence in present studies. Based on the presence or absence of bands, the genetic distance was computed by using the similarity coefficients. Inbreds were classified into five groups, A, B, C, D and E by drawing the Phenon level at forty similarity units and allowing only inbreds with increasing similar pattern of isozymes to be clustered together. Cluster A accommodated three inbreds, namely, CML 142, CML 144 and CML 176 while the cluster B accommodated two inbreds, namely, CML 150 and CM 300. Cluster C and D was monogenotypic contained only one inbred in each. Inbred CM 400 was accommodated in cluster D while cluster E accommodated inbred CM 600. Isozyme patterns of inbreds CML 142, CML 144 and CML 176 of cluster A were most similar followed by isozyme patterns of inbreds CML 150 and CM 300 of cluster B (Table 4.29). Analysis (Fig. 1 and Table 2) revealed that isozyme patterns in the inbred CM 600 were least similar to the isozyme patterns obtained in other inbreds. Similarity coefficient between CM 600 and CML 186 was the least (0.4615) whereas; the highest similarity coefficient (0.8511) existed between CML 142 and CML 144 (Table 3). Similarity coefficients ranged from 0.46 to 0.85, indicating moderate levels of diversity present among the inbreds under evaluation in the present study (Table 3). Isozyme patterns based genetic diversity studied among landraces/populations and evolution of the maize have also been reported ealtier (Mauria *et al*. 2000; Lu *et al*.2002; Revilla *et al*., 2003).

Table 1: Isozyme banding patterns in maize inbreds

Inbreds	Band number present in inbreds					
	PRX (10)	EST (2)	ACP(6)	ADH (12)	CAT (6)	AMY (5)
CML 142	1, 2, 3, 4, 5, 1c, 3c, 4c	1, 2	1, 4	3, 4, 5, 6, 1C, 4C	6	2, 3, 4, 5
CML 144	1, 2, 3, 4, 5, 1c, 3c, 4c	1, 2	2, 4	5, 6, 1C, 4C	5	2, 3, 4, 5
CML 150	1, 2, 3, 4, 5, 1c, 3c, 4c	1, 2	3, 5	5, 6, 1C, 4C	4	2, 3, 4
CML 176	1, 2, 3, 4, 5, 1c, 3c, 4c	1, 2	2, 5	4, 5, 7, 2C,	3	2, 3, 4, 5
CML 186	1, 2, 3, 4, 5.3c	1, 2	2, 5	2, 5, 6, 7, 1C, 5C	2, 3	4, 5
CM300	1, 2, 3, 4, 1C	1, 2	3, 5	1, 5, 6, 7, 1C, 4C	2	1, 3, 4, 5
CM400	1, 2, 3, 4, 1C, 4C, 5C	1, 2	3, 5	2, 5, 7, 3C	1	2, 3, 4, 5
CM600	1, 2, 3, 4, 2C, 3C, 4C	1, 2	3, 6	3, 5, 7, 1C, 4C	1	1, 3

Parenthesis (-) indicate total number of bands present in maize inbreds ; C – Cathodal bands

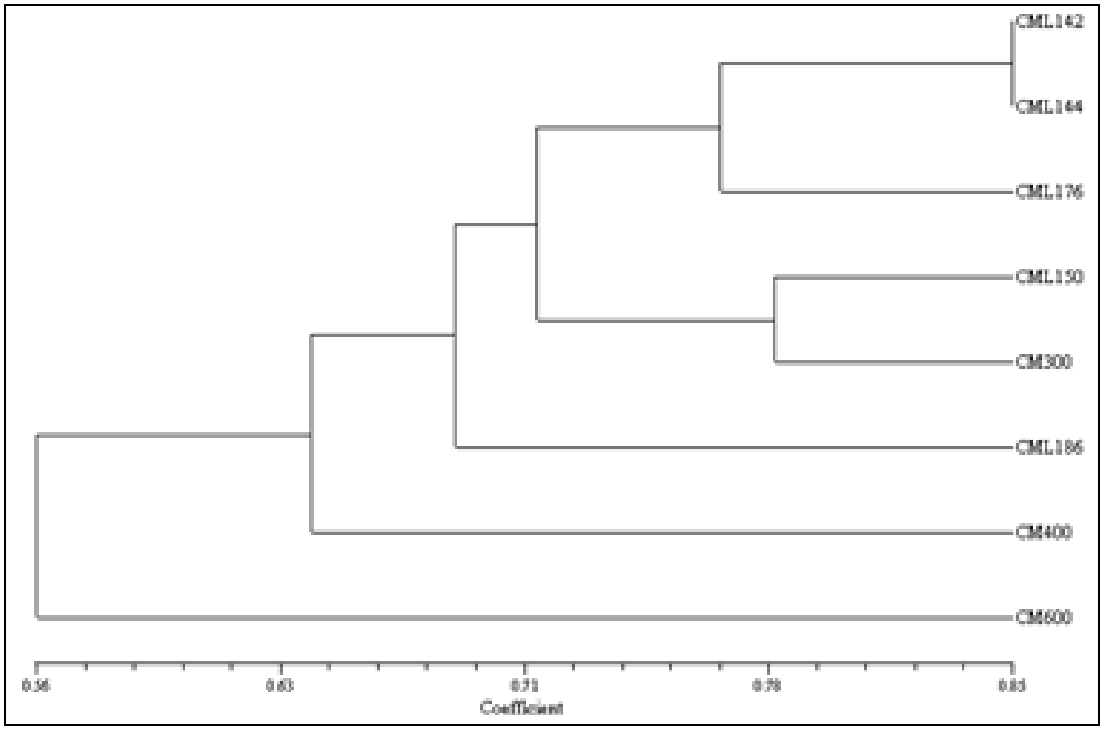

Fig 1: Dendrogram based on Nei and Li (1979) similarity coefficient for six isozyme system of eight inbreds of maize

Table 2. Composition of clusters based on six isozyme patterns in maize

Cluster	No. of entries	Composition of cluster
A	3	CML 142, CML 144, CML 176
B	2	CML 150, CM 300
C	1	CML 186
D	1	CM 400
E	1	CM 600

Table 3. Nei and Li (1979) similarity coefficient in eight inbreds of maize for six isozyme systems

	CML 142	CML 144	CML 150	CML 176	CML 186	CM 300	CM 400	CM 600
CML 142	1.0000							
CML 144	0.8511	1.0000						
CML 150	0.7556	0.7727	1.0000					
CML 176	0.7556	0.7727	0.7619	1.0000				
CML 186	0.6512	0.7619	0.7000	0.7000	1.0000			
CM 300	0.6818	0.6512	0.7805	0.6341	0.6154	1.0000		
CM 400	0.6818	0.7442	0.6341	0.6341	0.5128	0.6500	1.0000	
CM 600	0.6364	0.5581	0.6341	0.4878	0.4615	0.6000	0.5500	1.0000

Acknowledgement
Present investigation is a part of Ph. D. Research. The senior author is thankful to the All India Coordinated Project on Maize, Dholi centre, RAU, Pusa, Bihar for providing the materials and labourers for carrying out the research works of the present studies.

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