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Isozyme analysis based genetic purity assessment of single cross hybrid and its parental line in maize (*Zea mays* L.)

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Abstract
Seed is the basic and most critical input for sustainable agriculture. The quality seed is determined by its genetic purity in which the main source of genetic contamination is the self-pollination of the female parent due to incomplete removal of the tassel. Traditionally, the approach adopted for the genetic purity certification in maize is not accurate and fast method. Thus, the present investigation was carried out to test the genetic purity of single cross by using isozyme as a marker. The horizontal starch gel electrophoresis was used to study the isozyme patterns in eight inbred lines namely, CML 142, CML 144, CML 150, CML 178, CML 186, CM 300, CM 400, CM 600 and some selected fifteen single cross hybrids having high heterosis to distinguish single cross hybrids from their parental lines. The parental lines were obtained from AICRP on Maize, Dholi Centre and hybrids were generated by crossing these inbreds in all possible combinations excluding reciprocal. These single cross hybrids and their parental lines were evaluated by isozymatic pattern of peroxidase, catalase, amylase and esterase. Amongest these isozyme systems, banding pattern produced by peroxidase, catalase, and esterase isozyme systems made it possible to distinguish single crossed hybrids from their parental lines. Morever, this technique was fast, precise and without environmental effects.

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

Introduction
The seeds plays a vital role in agriculture and acts as a carrier of the genetic potential of varieties. It is estimated that the contribution of quality seed alone to the total production is about 15–20%. Quality of seeds can be highlighted as the combination of genetic, physiological and sanitary attributes. Maintenance of isolation distance of seed production fields to minimize natural crossing, the removal of female parent tassel, using of clean threshing floor and processing unit are some of criteria adopted to maintain the genetic purity of maize hybrid seeds. The main source of genetic contamination in hybrid seed production in case of maize is the self-pollination of female parent due to incomplete removal of its tassel. This contamination increases the end ogamic levels, reducing the genetical and physiological quality of seeds that consequently decrease the crop productivity.
Traditionally, the approach adopted for the genetic purity certification in maize has been utilization of easily recognizable morphological traits as descriptors. Recently, the use of the isozyme markers has been proposed as an additional method to test and certify the genetic purity of seeds. Isozyme based markers have been successfully used as a reliable genetic markers to certify genetic seed purity. Such markers exhibit relatively more consistency in their expression than morphological features irrespective of environmental factors. Cooke (1995) [3] provided the evidence to suggest that isozymatic profile can be effectively used in the genetic purity evaluation of seeds from a great number of plant species, using even a single seed from each material. The International Union for protection of New varieties of plants (UPOV) has harmonized and adopted test guidelines and procedures for the use of isozyme polymorphism as characteristic features for establishing uniqueness of cultivars (UPOV, 1997) [13] of crop plants including maize. Evaluation of genetic purity of seed lots of maize using electrophoresis technique has also been reported earlier

(Orman *et al*., 1991; Smith and Register 1998) [7, 11]. Commercial seed companies are increasingly using isozymes to assess pollination control (Hattemer, 1982) [5], to detect possible mixtures of seed lots and to eliminate a large proportion of the grow outs in winter nurseries. Salgado *et al* (2006) [9] studied genetic purity in hybrid maize seed using isozyme as a markers and conferred that enzymatic systems that were able to distinguish the hybrids from their parental line were catalase, isocitrate dehydrogenase and esterase. Hamill and Brewbaker (2008) [4] identified twenty four peroxidase isozyme by starch gel electrophoresis of 250 varieties of maize. Maize tissues varied greatly in isozyme pattern, many tissues having a characteristic and distinguishing complement. Important biochemical enzyme markers, namely, amylase, catalase, esterase and peroxidase have significant roles in plant cells. During photosynthesis, photorespiration, respiration, flowering and other cellular reactions, reactive oxygen species such as O2 and H2O2 are produced. The plant protects themselves from reactive oxygen species by antioxidants like catalase and peroxidase. Catalase acts as a primary H2O2 scavenger in the peroxisomes and the mitochondria (Anderson *et al*., 1995) [1]. Peroxidase is also involved in various physiological and biochemical processes and changes with growth and development of plant. 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. In the present study, isozyme was used for evaluation of genetic purity of single cross hybrids generated from their parental inbreds.

Materials and Methods

The experiment was conducted at the research farm of Tirhut College of Agriculture, Dholi, under Rajendra Agricultural University, Bihar, and Pusa. The experimental materials for the present investigation were generated from eight inbred lines, including five quality protein maize (QPM) inbreds and three non-QPM inbreds obtained from AICRP, Dholi Centre. These inbreds were crossed in all possible combinations excluding reciprocals and 28 single crosses were generated. Out of 28 single crosses, 15 crosses were selected on the basis of high *per se* performance and high heterosis.

Isozyme studies : Four isozymes viz., peroxidise (PRX, E.C.1.11.1.7), esterase (EST, E.C.3.1.1.1), catalase (CAT, 1.1.1.48) and amylase (AMY, E.C. 3.2.1.1.) patters were studied in germinating coleoptile tissue of eight inbred lines and some selected fifteen single crosses to distinguish single cross hybrids from their parental lines. The horizontal starch gel electrophoresis was used to study the isozyme patterns using discontinuous buffer system as described by Poulik (1957) [8]. Protocols outlined by Shield *et al* (1983) [10] 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) [15], catalase (Thorup, 1961) [12], amylase (Vellejos, 1983) and esterase. 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 (+++).

Results and Discussion

In the present study, peroxidase isozyme pattern were visualized as green bands on both anodal and cathodal sides of the gel with varying intensity (Fig. 1 and Table1). A total of five anodal PRX-1, PRX-2, PRX-3, PRX-4 and PRX-5 (Rm - 0.20, 0.27, 0.56, 0.64 and 0.70) and five cathodal PRX-1C, PRX-2C, PRX-3C, PRX-4C and PRX-5C (Rm- 0.18, 0.28, 0.35, 0.55 and 0.65) of peroxidase were observed in both parental lines and their respective single cross hybrids. Four anodal bands, namely, PRX-1, PRX-2, PRX-3 and PRX-4 were visualized in all the eight inbreds. Difference was observed for band PRX-5 which was absent in three inbreds, namely, CM 300, CM 400 and CM 600.. It was appeared that single crosses exhibited similarity in number and mobility of band to the parents which was involved in making single crosses (Fig 1). Differences was observed in respect of intensity of the bands. It was observed that the cross combination CM 300 x CML 142 exhibited all the five anodal bands, but only four cathodal bands, namely, PRX-1C, PRX-3C, PRX-4C and PRX-5C. While inbred CM 300, one of the parental lines of this cross, had only four anodal bands. The anodal band PRX-5 was not present in CM 300. Banding pattern of cross CM 300 x CML 142 was similar to the banding pattern of parental line CM 300 and CML 142. All bands were present in both parental line and single cross except cathodal band PRX-2C (Rm- 0.25) which was absent in parental lines as well as single cross, proved the genuiness of the cross. Similarly, the inbred CML 142, the other parental line of this cross, had five anodal bands and four cathodal bands. The cathodal band PRX-1C was absent in CML 142. In cross CM 300 x CML 144, all bands were observed except cathodal bands PRX-2C (Rm-0.28) and PRX-5C (Rm-0.65) which was also absent in parental line CM 300 and CML 144. A similar trend was observed in the case of other hybrid combinations under consideration in this study. The results, therefore, revealed that presence of a band in either of the inbreds was the essential requirement for showed its presence in their cross combination. Differences were observed in respect of intensity of some of the bands. No hybrids bands were observed in the present study in accordance with the earlier report that all of the peroxidase isozymes appear to be monomers, as no hybrid bands are observed (Brewbaker and Hasegawa, 1975) [2]. Thus, it is evident from the results of the present investigation that single cross hybrids generated and evaluated during the present study were genetically pure. Furthermore, the isozyme pattern of peroxidase was able to distinguish the hybrids from their parental lines.

Amylase isozyme variation

Amylase isozyme visualized as achromatic band in white colour gel. Only anodal bands were observed in case of amylase. The scoring of amylase bands was somewhat difficult due to diffused, achromatic broad band.in respect of

other isozymes. Banding pattern of amylase isozyme in some inbreds and single cross hybrids generated from cross combinations between these inbreds was presented in Fig. 1. The hybrid (CM 300 x CML 176) generated from the cross between these two inbreds exhibited the same relative mobility values as obtained in the case of parental inbreds, thereby indicating the genetic purity of the cross.

It was found that amylase isozyme patterns in four crosses, namely, CM 300 x CML 142, CM 300 x CML 150, CM 400 x CML 186 and CM 400 x CML 150 did not show a close correspondence with the banding patterns obtained in their parental lines. Difference in banding pattern was observed in respect of relative mobility and intensity of the bands. The results, therefore, indicated that amylase isozyme patterns may not be effective in providing precise information regarding genetic purity of hybrids. However, further investigation in this direction is warranted to explore the possibility of utilization of amylase isozyme pattern for testing the genetic purity of maize hybrids.

Catalase isozyme variation

Catalase isozyme was visualized as achromatic band (Fig 1) in the bluish back ground of the gel in anodal side. Catalase bands were visualized as a broad, fused band with varying thickness. Thus, scoring of the band was very difficult. The highest and the lowest point of the band was considered as relative mobility (Rm value) of the band. Rm value with varying intensity was the basis to distinguish the eight inbreds and their single crosses as presented in Table 3. In germinating coleoptile altogether six catalase isozyme bands namely, Cat-1, Cat-2, Cat-3, Cat-4, Cat-5 and Cat-6 with Rm value 0.27, 0.30, 0.33, 0.37, 0.40 and 0.43 were observed. The purity of the single cross was identified as comparing the thickness of the band of single cross with the inbreds involved in crosses (Fig.1 and Table 3). The banding pattern of all the single crosses were same as that of their respective parents involved in development of that single cross. This showed that the profile of catalase isozyme in coleoptile allowed to distinguish the fifteen single cross hybrids from their parental lines. These results are in accordance with the earlier report (Salgado *et al.*, 2006) [9]. However, these findings are different from the ones reported by earlier researcher (Heidrich-Sobrinho, 1982) [6] that catalase isozyme pattern did not make it possible to distinguish nine inbreds lines of maize. The divergences in the results was probably due to differences in the genetic materials. However, this enzymatic system has been applied to the cultivar differentiation.

Esterase bands only two esterase bands namely EST-1 (Rm-0.66) and EST-2 (Rm-0.72), (Fig. 1 (a b and Table 3) were observed in all the eight inbreds of maize and single crosses with medium and dark intensity. No genotypic variation was observed among the inbreds for the number of the bands, variation was observed only in respect of intensity of the bands. r Esterase showed monomorphic electrophoretic profile among all the fifteen selected single crosses in maize and its parental lines. Similar monomorphic banding patterns were observed by Salgado *et al* (2006) [9] using coleoptiles for purity assessment of single cross from its parents in case of maize. This results was also in agreement with Yen (1987) that found polymorphism between the parental lines in arice single-cross hybrid, reinforcing the applicability of this system in the evaluation of genetic purity in seeds from different crops.

From the present study it was concluded that out of four isozyme systems of peroxidase, catalase, esterase and amylase which were used to characterize the eight parental lines and fifteen single cross hybrids, isozyme patterns produced by peroxidise, esterase and catalase isozyme systems made it possible to distinguish hybrids from their parental lines.

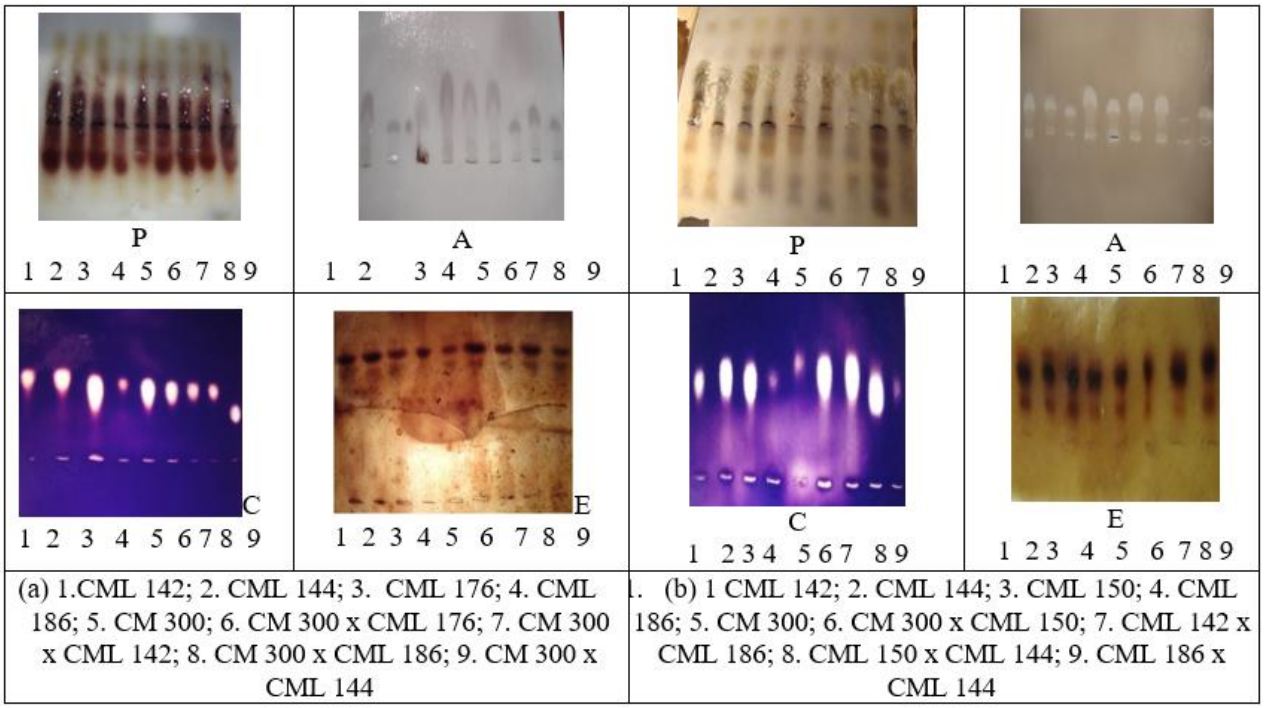

Fig.1 Banding pattern of peroxidase (PRX), amylase (AMY), catalase (CAT) and (4) esterase (EST) isozymes in some parental lines and their single crosses combinations in maize

Table 1: Peroxidase isozyme banding patterns in parents and single cross hybrids

Parents & Single crosses	Anodal						Cathodal			
	Band No.	PRX-1	PRX-2	PRX-3	PRX-4	PRX-5	PRX-1C	PRX-2C	PRX-3C	PRX-4C	PRX-5C
	Rm Value	0.20	0.27	0.56	0.64	0.7 0	0.18	0.28	0.35	0.55	0.65
CML 142		+++	+++	++	+++	++	+++	_	+++	+++	+
CML 144		+++	+++	+++	+++	++	+++	_	++ +	+++	_
CML 150		+++	+++	+++	+++	++	+++	_	+++	++	_
CML 176		+++	+++	++	+++	++	+++	_	+++	++	+
CML 186		+++	+++	++	+++	+	+++	_	+++	+++	_
CM 300		+++	+++	+++	++	_	+++	_	_	_	_
CM 400		+++	+++	++	++	_	+++	_	+	++	_
CM 600		+++	+++	+++	+++	_	+++	+++	+	++	_
CM 300 × CML 142		+++	+++	++	++	+	+++	_	+++	+++	+
CM 300 × CML 144		+++	+++	++	++	+	+++	_	+++	+++	_
CM 300 × CML176		+++	+++	++	++	+	+++	_	+++	+++	+
CM 300 × CML 186		+++	+++	++	++	+	+++	_	+++	+++	_
CM 400 × CML 144		+++	+++	+	+	++	++ +	_	+	+++	_
CM 400 × CML 186		+++	+++	+++	+ ++	++	+	_	+	+++	_
CM 400 × CML 176		+++	+++	+	+	++	+	_	+	+++	+
CM 400 × CML 142		+++	+++	++	+++	+	+	_	+	+++	+
CM 300 × CML 150		+++	+++	++	++	+ +	++	_	++	+++	_
CML 142 × CM 186		+++	+++	++	++	+	+++	_	++	++	+
CML 150 × CML 144		++ +	+++	+++	++	++	+++	_	++	+++	_
CML 186 × CML 144		+++	+++	+++	++	+	+	_	+	++	_
CM 600 × CML 176		+++	+++	++	++	+	+++	+++	+	++	+
CM 600 × CM 400		+++	+++	++	++	_	+++	+++	+	+++	_
CM 400 × CML 150		+++	+++	++	++	_	+++	_	+	+++	_

Table 2: Catalase isozyme banding pattern in parents and single cross hybrids of maize

Genotypes	Band No.	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6
	Rm Value	0.27	0.30	0.33	0.37	0.40	0.43
CML 142		_	+++	_	++	_	_
CML 144		_	_	++	_	++	_
CML 150		_	_	++	+	_	_
CML 176		_	+++	_	_	++	_
CML 186		_	_	_	++	+	_
CM 300		_	_	_	_	_	+
CM 400		_	_	++	_	_	+
CM 600		+++	_	_	_	++	_
CM 300 × CML 176		_	+++	_	_	_	+
CM 300 × CML 142		_	+++	_	_	_	++
CM 300 × CML 186		_	++	_	_	_	++
CM 300 × CML 144		_	+++	_	_	_	++
CM 300 × CML 150		_	+++	_	_	_	++
CML 142 × CML 186		_	+++	_	++	_	_
CML 150 × CML 144		_	_	++	_	+	_
CML 186 × CML 144		_	_	+++	_	++	_
CM 400 × CML144		_	_	+++	_	_	++
CM 400 × CML 186		_	_	+++	_	_	+++
CM 400 × CML 176		_	_	+++	_	_	+++
CM 400 × CML 142		_	_	++	_	_	++
CM 600 × CML 176	+++	_	_	_	_	+++	_
CM 600 × CM 400	+++	_	_	_	_	_	++
CM 400 × CML 150		_	_	_	++	_	++

Table 3: Esterase isozyme banding pattern in parents and single cross hybrids

Genotype	Anodal		
	Band No.	EST – 1	EST – 2
	Rm Value	0.66	0.72
CML 142		+ +	+ +
CML 144		+ + +	+ +
CML 150		+ ++	+ + +
CML 176		+ +	+ + +
CML 186		+ + +	+ +
CM 300		+ +	+ + +
CM 400		+ + +	+ ++
CM 600		+ +	+ + +
CM 300 × CML 176		+	+++

CM 300 × CML 142		++	+++
CM 300 × CML 186		++	+++
CM 300 × CML 144		+	+++
CM 300 × CML 150		++	+++
CML 142 × CML 186		++	+++
CML 150 × CML 144		++	+++
CML 186 × CML 144		++	+++
CM 400 × CML144		++	+++
CM 400 × CML 186		++	+++
CM 400 × CML 176		++	+++
CM 400 × CML 142		+++	+++
CM 600 × CML 176		++	+++
CM 600 × CM 400		++	+++
CM 400 × CML 150		++	+++

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