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Isozyme analysis based genetic fidelity assessment of micropropagated banana plants

Sugandh Suman*, Ranju Kumari, V. K. Sharma and Harsh Kumar

Department of Agricultural Biotechnology and Molecular Biology, Faculty of Basic Sciences and Humanities, Rajendra Agricultural University, Pusa, Samastipur-848125 (Bihar), INDIA
*Corresponding author. E-mail: sugandhsuman@gmail.com

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Abstract: Isozyme studies of micropropagated and mother plants of banana cvs. Matti, Ney Poovan, Kechulepa, Dwarf Cavendish, Malbhog, Champa, B.B. Battisa and FHIA-1 were done to test their genetic fidelity. The banding patterns as revealed by electrophoretic variations were evaluated with respect to isozymes of acid phosphatase, catalase, esterase and peroxidase as markers. The genetic fidelity of micropropagated plants and the relationship of the different cultivars were determined by dendrogram using numerical taxonomy and multivariate analysis system (NTSYS). A clustered dendrogram was prepared by unweighted pair group method using averages (UPGAMA) method. At 87% similarity, the micropropagated and mother plants were clustered in four groups reflecting their genomic constitution. Cvs. Matti (AA) and Dwarf Cavendish (AAA) with similar 'A' genome were categorized in Cluster I. Cluster II comprised of cvs. Ney Poovan (AB), B.B. Battisa (ABB) and FHIA-1(AAAB) with genomic constitution of both 'A' and 'B' type. Cvs. Champa (AAB) and Malbhog (AAB) with similar genome were grouped in Cluster III. Cluster IV contained the cv. Kechulepa (BB) having only 'B' genome. However, there was no somaclonal variation among the micropropagated plants and they showed 100% genetic similarity. Thus, the isozyme studies could be a reliable marker for testing the genetic fidelity of micropropagated plants and for evaluating the diversity among the banana germplasm.

Keywords: Banana, Genetic fidelity, Isozyme, Micropropagation

INTRODUCTION

Modern banana and plantain have originated from inter and intra specific hybridization of two wild diploid (2n = 2x =22) species, *Musa acuminata* and *Musa balbisiana* that possess the A and B genomes respectively (Simmonds, 1962). Edible bananas have 22, 33 or 44 chromosomes representing diploid, triploid and tetraploid cultivars (Stover and Simmonds, 1987). Karamura *et al*. (1998) reported that the diploids AA gave rise to AAA triploids by meiotic chromosome restitution, while inter-specific hybridization between AA and AAA types and *M. balbisiana* (BB) resulted into various AAB and ABB types. Other combinations such as ABBB, AAAB and AABB are also thought to have developed in South East Asia (Richardson *et al*., 1965).

In vitro multiplication of banana is an excellent alternative to conventional propagation with the plantlets having many advantages over field-grown material. The advantages of *in vitro* propagation include a high multiplication rate, physiological uniformity, availability of disease free material all the year round and rapid dissemination of new plant materials throughout the world. Though micropropagated plants are mostly true to the parent genotype however, some tissue cultured plants show somaclonal variation (Larkin and Scowcroft, 1981).

To test the genetic fidelity and to rule out the variant plants, the micropropagated plants should be evaluated through morphological, biochemical and molecular markers. Standard morphological markers are inadequate for identification of cultivars and for scoring genetic uniformity of regenerated plants, because they are affected by environmental conditions. Electrophoretic pattern of isozyme provides a good biochemical marker independent of environmental conditions. Differences in isozyme in growth, development and differentiation of plants, are reflections of changes in metabolic activities (Rao *et al.*, 1972). Important biochemical enzyme markers, namely, acid phosphatase, catalase, esterase and peroxidase have significant roles in plant cells. During photosynthesis, photorespiration, respiration, flowering and other cellular reactions, reactive oxygen species such as O_2 and H_2O_2 are produced. 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). 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. Acid phosphatase catalytically breaks down a wide variety of phosphate esters (Vincent *et al.*, 1992). In plant roots, acid phosphatase is involved in the solubilization of macromolecular organic phosphates in soils which can then be utilized (Panara *et al.*, 1990). They also regulate the efficiency of phosphorus and thus play an important role in many physiological processes. In the present study, isozyme was used for cultivar identification, evaluation of genetic fidelity of micropropagated and *in vivo* plants and for the phylogenetic assessment of eight cultivars of banana.

MATERIALS AND METHODS

Suckers of eight cultivars namely Matti, Ney Poovan, Kechulepa, Dwarf Cavendish, Malbhog, Champa, B.B. Battisa and FHIA- 1 were collected from the local farms. The roots and the outer leaves with leaf sheaths were removed. A cube of tissue of about 2 cm^3 containing the apical meristem was excised from the base of the sucker. The rhizome shoot tips were prepared, washed, pretreated and surface sterilized following the method of Suman *et al*. (2013 a). The outer two to three layers of the rhizome shoot tips were carefully removed using a scalpel and a cube of tissue of about 1 cm^3 containing the apical meristem was excised. The individual explants were inoculated and cultured on Murashige and Skoog (1962) medium supplemented with different concentrations and combinations of IAA and BAP in culture tubes and bottles. The cultured tubes and bottles were incubated in the thermal insulated tissue culture room with temperature around 25 ^{0}C and relative humidity 50 - 80 %. A continuous light of 2 kilo lux intensity was provided through fluorescent tubes. The regenerated plantlets having healthy roots were selected for pot transfer. The rooted plantlets along with the agar medium were carefully taken out. The agar medium was removed without damaging the root. Plantlets were now transferred to pots having sterilized sand and farm yard manure in 1:1 ratio and progressively acclimatized to reduced humidity for their hardening and acclimatization (Suman *et al*., 2013 b). The plants were finally transferred to the field leading to the establishment of micropropagation technique in selected banana cultivars.

Isozyme study: Isozymes study of acid phosphatase (ACP, E.C.3.1.3.2), catalase (CAT, 1.1.1.48), esterase (EST, E.C.3.1.1.1) and peroxidase (PRX, E.C.1.11.1.7) in the leaf tissues of banana were used to test the genetic fidelity of micropropagated and mother plants. Protocols outlined by Shield *et al.* (1983) with minor modifications were used for extraction and electrophoretic separation of isozymes. 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 anodal bands were designated with prefix 'A' and cathode bands with prefix 'C'. A number was assigned to each band; the closet to the origin (site of application) was numbered as 1. The Rm values of all the concerned bands of the different enzymes were calculated. A band having the same relative mobility in the different cultivars is designated with the same number and was treated as the same isozyme. The resultant bands of

Table 1. Intensity of anodal and cathodal Acid Phosphatase bands of mother and tissue cultured genotypes of banana.

Bands	R_m value	G_1	G_2	G_2T	G_3	G_3T	G_4	G_4T_1	G_4T_2	G_5	G_6	G_6T_1	G_6T_2	G_7	G_7T	G_8
A_1	0.56	+	+	+	+++	++	++	+	+		+	+	+	+	+	+
A_2	0.77	+	+	+			++	+	+	+	+	+	+	+	+	++
A_3	0.91		+	+	++	++								++	+	+

Table 2. Intensity of anodal Catalase bands of mother and tissue cultured genotypes of banana.

Bands	R_m value	G_1	G_2	G_2T	G_3	G_3T	G_4	G_4T_1	G_4T_2	G_5	G_6	G_6T_1	G_6T_2	G_7	G_7T	G_8
A_1	0.28	+	+	++	+	++	+	+	+++	+	+	+	+++	+	+++	+
A_2	0.49	++	+	++	+	++	++	++	+++	++	++	+	++	++	+++	++

Table 3. Intensity of cathodal Esterase bands of mother and tissue cultured genotypes of banana.

Bands	R_m value	G_1	G_2	G_2T	G_3	G_3T	G_4	G_4T_1	G_4T_2	G_5	G_6	G_6T_1	G_6T_2	G_7	G_7T	G_8
A_1	0.51	+	+	+	+	+	+	++	+					++	+	+++
A_2	0.65	+	+	+			+	+	+	+	+	+	+	+	+	
A_3	0.75	++					+++	+	+					+	+	++

Table 4. Intensity of anodal and cathodal Peroxidise bands of mother and tissue cultured genotypes of banana.

Bands	R_m value	G_1	G_2	G_2T	G_3	G_3T	G_4	G_4T_1	G_4T_2	G_5	G_6	G_6T_1	G_6T_2	G_7	G_7T	G_8
A_1	0.51	+	++	+	+++	++	+++	++	++	++	+	++	++	+	+	+++
A_2	0.77	+++	++	++	++	++	+++	++	++	++	++	++	++	++	++	++
C_1	0.28	+++	+	++	++	+	+++	+	+	+	+	++	++			+++
C_2	0.43	+					+	++	++							

Table 5. Estimates of four isozyme based similarity coefficient among micropropagated and *in vivo* plants of banana cultivars.

	G_1	G_2	G_2T	G_3	G_3T	G_4	G_4T_1	G_4T_2	G_5	G_6	G_6T_1	G_6T_2	G_7	G_7T	G_8
G_1	0.00														
G_2	0.90														
G_2T	0.90	1.00													
G_3	0.78	0.89	0.89												
G_3T	0.78	0.89	0.89	1.00											
G_4	0.95	0.86	0.86	0.74	0.74										
G_4T_1	0.95	0.86	0.86	0.74	0.74	1.00									
G_4T_2	0.95	0.86	0.86	0.74	0.74	1.00	1.00								
G_5	0.78	0.89	0.89	0.75	0.75	0.74	0.76	0.76							
G_6	0.89	0.89	0.89	0.75	0.75	0.84	0.84	0.84	0.88						
G_6T_1	0.89	0.89	0.89	0.75	0.75	0.84	0.84	0.84	0.88	1.00					
G_6T_2	0.89	0.89	0.89	0.75	0.75	0.84	0.84	0.84	0.88	1.00	1.00				
G_7	0.90	0.90	0.90	0.78	0.78	0.86	0.86	0.86	0.78	0.78	0.78	0.78			
G_7T	0.90	0.90	0.90	0.78	0.78	0.86	0.86	0.86	0.78	0.78	0.78	0.78	1.00		
G_8	0.90	0.90	0.90	0.89	0.89	0.86	0.86	0.86	0.78	0.78	0.78	0.78	0.90	0.90	0.00

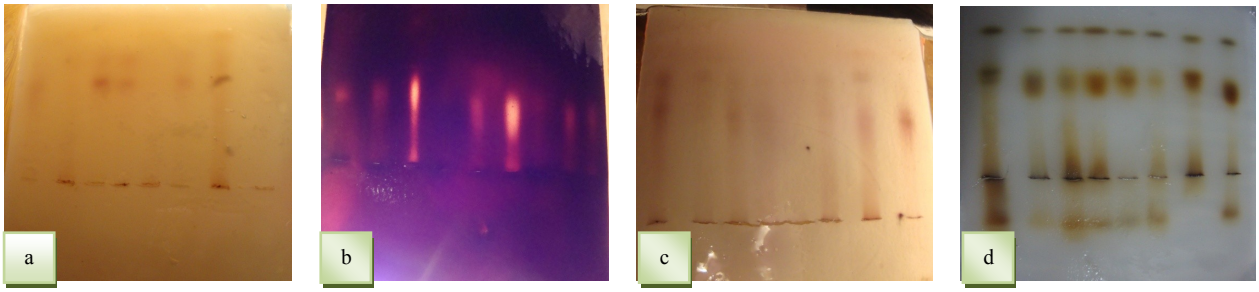

Fig. 1. *Banding patterns of different isozyme in leaf tissues of field grown and tissue cultured plants of different cultivars of banana. (a) Acid Phosphatase (b) Catalase (c) Esterase (d) Peroxidase.*

isozymes were scored.

RESULTS AND DISCUSSION

Leaf tissues from only the mother plants were used in cvs. Matti (G_1), Champa (G_5) and FHIA-1(G_8) while in cvs. Ney Poovan (G_2 and G_2T), Kechulepa (G_3 and G_3T), Dwarf Cavendish (G_4, G_4T_1 and G_4T_2), Malbhog (G_6, G_6T_1 and G_6T_2) and B.B. Battisa (G_7 and G_7T) leaf tissue from both field grown and *in vitro* developed plants were used for the study of isozymes of acid phosphatase, catalase, esterase and peroxidase (Fig. 1). The response of micropropagated and mother plants for different isozyme studies were as follows:

Acid Phosphatase (ACP): The electrophoresis assay for acid phosphates activity revealed three anodal bands, namely A_1 (Rm = 0.56), A_2 (Rm = 0.77) and A_3 (Rm = 0.91) with reddish brown bands in the selected cultivars of banana. The anodal band A_1 was present in all cultivars and tissue cultured plants except cv. Champa. The band intensity varied from light to dark. Acid phosphatase band A_2 was also present in all the cultivars in the field grown plants and their tissue cultured plantlets with light to moderate intensity, except cv. Kechulepa. The band A_3 was present in cvs. Ney Poovan, Kechulepa, B.B. Battisa; their tissue cultured plants and was also present in cvs. Champa and FHIA-1with light to moderate intensity (Table 1, Fig. 1a).

Catalase (CAT): Catalase isozyme pattern in leaf tissue of banana were visualized as yellow fluorescent bands in blue background on the anodal side of the gel. Two anodal catalase bands namely, A_1 (Rm = 0.28) and A_2 (Rm = 0.49) were present in the field grown and tissue cultured plants of all the cultivars viz. Matti, Ney Poovan, Kechulepa, Dwarf Cavendish, Malbhog Champa, B.B. Battisa and FHIA-1 (Table 2, Fig. 1b). The intensity varied form light to dark in the different cultivars of banana.

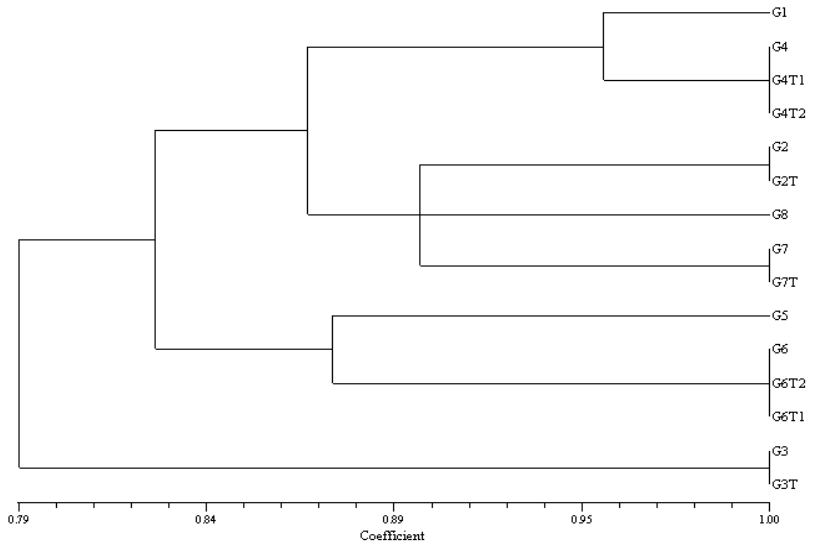

Fig. 2. *Dendrogram displaying the genetic distance among banana cultivars obtained from UPGAMA analysis based on Dice (1945) similarity coefficient.*

Esterase (EST): The esterase bands were observed as dark brown bands on a colourless background only on anodal side of the gel. Three esterase bands, viz., A_1 (Rm = 0.50), A_2 (Rm = 0.65) and A_3 (Rm = 0.75) were found in the plants of different cultivars of banana. The band A_1 was present in the selected field grown and tissue culture plants of the cvs. Matti, Ney Poovan, Kechulepa, Dwarf Cavendish, B.B. Battisa and FHIA -1 and the intensity varied from light to dark. The band A_2 was observed in the field grown and tissue cultured plants of six cultivars namely Matti, Ney Poovan, Dwarf Cavendish, Champa, Malbhog and B.B. Battisa with light intensity. Esterase band A_3 showed its expression of cvs. Matti and FHIA-1 field grown and tissue culture plants of cvs. Dwarf Cavendish and B.B. Battisa (Table 3, Fig. 1c).

Peroxidase (PRX): Four different peroxidase brown bands were observed on both cathodal and anodal sides of the gel. The anodal and cathodal bands were A_1 (Rm = 0.51), A_2 (Rm = 0.77), C_1 (Rm = 0.28) and C_2 (Rm = 0.43) respectively. The anodal bands A_1 (0.51) and A_2 (0.77) was present in the field grown and tissue cultured plants of all the cultivars of banana with moderate to dark intensity. The cathodal band C_1 (Rm = 0.28) was observed in the field grown and tissue cultured plants of seven cultivars Matti, Ney Poovan, Kechulepa, Dwarf Cavendish, Champa, Malbhog and FHIA-1 with varying intensities from light to dark. The cathodal band C_2 (Rm = 0.43) was present only in field grown cv. and tissue cultured plants of cv. Dwarf Cavendish with light and moderate intensity (Table 4, Fig. 1d).

Genetic similarity analysis and clustering of micropropagated and in mother plants of selected cultivars of banana: The genetic similarity of the micropropagated and mother plants were estimated on the basis of Dice (1945) similarity coefficient considering the four isozyme bands as markers. A clustered dendrogram was prepared by unweighted pair group method using averages (UPGAMA) method. At 87% similarity coefficient, the micropropagated and mother plants were clustered in four groups (I, II, III and IV) on the basis of their genomic constitution. Cvs. Matti (AA) and Dwarf Cavendish (AAA) with common 'A' genome were categorized in Cluster I. Cluster II comprised of cvs. Ney Poovan (AB), B.B. Battisa (ABB) and FHIA-1 (AAAB) with genomic constitution of both 'A' and 'B'. Cvs. Champa (AAB) and Malbhog (AAB) with same genome were grouped in Cluster III. Cluster IV contained the cv. Kechulepa (BB) having only 'B' genome. Based on estimated genetic similarity matrix, cent percent genetic similarity was observed between mother and micropropagated plants of cv. Ney Poovan (G_2 and G_2T), Kechulepa (G_3 and G_3T), Dwarf Cavendish (G_4, G_4T_1 and G_4T_2), Champa (G_6, G_6T_1 and G_6T_2) and B.B.Battisa (G_7 and G_7T, Table 5). Thus, there was no somaclonal variation among the micropropagated plants and thus they showed 100% genetic similarity with those of the mother plants.

Tissue culture processes and plant regeneration protocol may result in genetic changes. These genetic changes are the causes of variation in tissue cultured plants known as somaclonal variation. Plants regenerated in banana cultivars were through either shoot tip culture or subculture of differentiated shoots. These cultures preserved genetic stability much better than callus and suspension cultures. The number of subculture cycles increases the rate of such variations. Thus, the genetic similarity based on isozyme studies could be a reliable marker for analyzing the diversity among the germplasm.

Molecular markers have been used for the detection of levels of somaclonal variation in *Musa* (Pancholi *et al.*, 1996). Isozyme technique has been used to score the variability among banana cultivars as well as to score somaclonal variation in micropropagated plants (Sahijram *et al.*, 2003; James *et al.*, 2007). The isozyme based markers can be used for the identification of cultivars, species and subspecies (Jarret and Litz, 1986). Several workers have used isozyme polymorphism for clonal identification in banana (Horry and Jay, 1988; Bhat *et al.*, 1997). In the present study, different isozyme namely acid phosphatase, catalase, esterase and peroxidase have been used to test the genetic fidelity of micropropagated plants and to score somaclonal variation to differentiate the banana genotypes differing in their doses of 'A' and 'B' genomes. Similarly, Espino and Pascua (1992) reported the utilization of three isozyme for differentiating A and B genomes as well as AAB and ABB cultivars from BB/BBB cultivars. Lebot *et al*. (1993) studied isozyme variation among 563 accessions of Musa including diploid AA, BB, triploid AAA, AAB, ABB and some tetraploid ABBB clones to determine genetic relationship. They focused on elucidation of genetic relationship between pacific plantains (AAB subgroup) and found that pacific plantains were similar to *acuminata/banksii* complex of Papua New Guinea. Liyanage *et al*. (1998) differentiated A and B genome of banana and plantain by esterase isozyme variations detected by polyacrylamide gel electrophoresis. The accessions having AA, AAA, AAAA, AAB, ABB and BB genomic groups showed a high degree of polymorphism for isozymes allozymes of esterase. Mendioro *et al*. (2007) studies the isozymes of malate dehydrogenase (MDH), 6-phosphoglucodehydrogenase (PGI), phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM) for genome identification of 20 table-type and 6 cooking type of banana (*Musa* spp.). A dendrogram was created using NTSYS to determine the genetic relationships of the different table types of cultivars of banana. Based on UPGAMA analysis, the different table type cultivars differentiated into 6 clusters at similarity coefficient of 0.85. The cultivars with similar genome do not get differentiated into same cluster. El-Dougdoug *et al*. (2007) analyzed somaclonal variation in tissue cultured derived banana plants using isozyme and RAPD as markers and found that the frequency of somaclonal variations depended on the number of subcultures. The genetic variations were only detected in 7 months old cultures. Venkatachalam *et al.* (2008) used genetic markers for detecting gene polymorphism, genotypes identification and phylogenetic relationship among banana cultivars through similar analysis of genetic similarity.Izquierdo *et al*. (2014) evaluated the genetic stability of micropropagated plants of banana cv. 'FHIA-18' (AAAB) with non-traditional growth regulators using isozyme systems. The results revealed no genetic variability among the clones.

Conclusion

It was concluded that there was no somaclonal variation among the micropropagated plants and they were true to type to parents. On the basis of isozyme studies, the cvs. having 'A' genome were grouped separately from the group having only 'B' genome. The cvs. having both 'A' and 'B' genome formed an intermediate group. Thus, the genetic similarity based on isozyme studies could be a reliable marker for analyzing the diversity among the banana germplasm.

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