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An Evaluation of Genetic Diversity in Micropropagated Anthurium Using Starch Gel Electrophoresis

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ABSTRACT. Anthurium andreanum Lind is a cut flower which is in high demand in the local and export cut flower trades in Sri Lanka. For commercial production of export anthurium cut flowers, it is essential that the selected elite qualities of the flowers borne on this original mother stock plants are maintained consistantly through many generations on vegetative multiplication.

This paper reports on starch gel electrophoresis which was adapted successfully on anthuriums to evaluate genetic diversity if any, in plants derived either from seedlings, conventional stem cuttings or micropropagated clonal plants.

Buffered leaf extracts were compared by means of horizontal starch gel electrophoresis to check the variability in cv. 'Crinkled Red'. Isoenzyme banding patterns of malic dehydrogenase (MDH), 6 phosphogluconic dehydrogenase (6 PGDH), phosphogluco isomerase (PGI) and diaphorase (DIAP) were used. Extracts of 105 seedlings, 60 clonal plants produced through rhizome cuttings and 150 in vitro propagated plants were compared to detect possible somaclonal variations in the daughter plants produced.

Results of starch gel electrophoresis showed that extracts of seedling plants contained variations, expressed as percentages showing deviant patterns in all five isoenzyme systems assessed: MDH 14%, 6 PGDH 13%, GOT 12%, PGI 14% and DIAP 16%. None of the clonal plants or in vitro propagated plants tested using this technique showed variations in any of the isoenzyme compared.

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The results of this study showed the genetic diversity in anthurium seedling plants and confirmed that stability was maintained in vegetatively propagated and in vitro propagated plants. Since in vitro propagation produces a large number of uniform and genetically stable plants, in vitro multiplication would appear to be suitable for use in the Sri Lankan floriculture industry.

INTRODUCTION

Micropropagation is the process whereby small plant tissues or organs are cultured on a nutrient medium to induce the production of many new shoots or embryos. Several different routes of differentiation and proliferation of plants are inducible *in vitro*. The most common ones are direct proliferation of existing meristems or the *de novo* production of new meristems from partially differentiated cells and tissues by means of adventitive growth. In some systems, adventitious shoots or somatic embryos originate indirectly from a dedifferentiated mass of cells (callus), in which case, genetic variability may occur at a high frequency in the plantlets. Plants derived from such adventitious routes of regeneration may show increasing levels of phenotypic and or genotypic variability, collectively referred to as somaclonal variation. Such variability occurs particularly when plants are regenerated from callus tissues (Mantell, 1986). Geier (1987) investigated variations in 169 plants of *Anthurium scherzerianum* derived from tissue – cultured explants of a single spadix. Using chromosome counting, he found that in one tetraploid plant and one chimera possessing both diploid and tetraploid chromosome numbers could be identified. There are no reports on attempts made to identifiy variability in micropropagated *Anthurium andreanum*.

Genetic variation among micropropagated plants exists, albeit usually on a limited scale (Davies and Heslop, 1972; Simmonds and Nelson, 1988). However, the presence of any variability above the normal levels expected from natural somatic mutation does nevertheless pose a severe threat to large scale clonal propagation schemes, since a small number of variants would multiply to significant levels in a micropropagation system due to the high multiplication rates achievable through the process. Therefore, awareness of the possible damages of genetic variation in commercial propagation systems is extremely important. Thus, screening of genetic variation should be an essential component

of any large scale micropropagation programme (a sort of quality control). Therefore, relatively quick, reliable and repeatable techniques should be available for screening work. Morphological characteristics (Hashim *et. al.*, 1988; Dewald, 1988; Hussey, 1982; Webb and Watson, 1991) chromosome counting (Hussey, 1982; Geier, 1987; Webb and Watson, 1991) and electrophoresis techniques (Dewald, 1988; Cousineau and Donnelly, 1989) have been employed successfully to detect variations in micropropagated plant populations. However, investigations of morphological characteristics require time and space for the substantial numbers of daughter plants required for evaluation of such characteristics. Clearly the most definitive method available for examination of aberrations at the above level is through the use of Restiction Fragment Length polymorphysm (RFLP) technology (Lee and Phillips, 1988). But this technique is too sophisticated for local conditions in Sri Lanka. However, isoenzyme contents are regulated in plants by genetic factors and their separation using electrophoresis can produce reliable techniques to detect changes in the genetic constitution of plants. Isoenzymes can be detected in rapidly obtained tissue extracts. The banding patterns which can be obtained within a few hours are unique to each genotype (McKee, 1973). Hence electrophoretic techniques were employed in the current study to assess variations among micropropagated anthurium plants. Another advantage of isoenzyme techniques is that they can be carried out when plants are growing under environmentally stable *in vitro* conditions, and only small amounts (100 – 500 mg) of plant tissues are required for assessments. In the current study, starch gel electrophoresis was used which could allow assessments of genetic constitutions of regenerated anthurium plants compared to those of the original mother stock.

MATERIALS AND METHODS

Investigation of genetic stability of micropropagated anthurium plants using starch gel electrophoresis.

Plant material

Three sources of plant materials were used to compare the genetic stability of micropropagated *cv.* `Crinkled Red'. 1) The micropropagated anthurium plants consisted of 150 individual plants of the 8^{th} subculture. These plants were regenerated from meristematic nodular structures

produced on the wounded edges of leaf explants. Multiplication of regenerated plants was carried out using apical shoots with two nodes. 2) Plants propagated vegetatively from suckers (n=60). 3) Seedling plants (n=105). Plants in all three sources were at approximately 6 months of age.

Sample extraction

Anthurium leaf tissues (500 mg) were ground in 1 ml extracting buffer. Standard system: 10 ml buffer (0.1 M Tris malate – pH 7.8) – 80 ml, glycerol – 10 ml, PVP 40 – 10 g), 10 µl 2 mercapthoethanol and 200 µl 25% Triton x 100. Histidine system: 10 ml buffer (0.1 M pottasium phosphate (pH 7.0) – 80 ml, glycerol 10 ml, PVP 40 – 10g), 10 µl 2 mercapthoethanol and 200 µl 25% Triton x 100. Immature leaf pieces were ground in an appropriate extraction solution using a pre cooled (4^0 C) mortar and pestle. The homogenate was centrifuged at 4500 rpm for 20 minutes at $4-5^0$ C. The clear supernatant was frozen (-20^0 C) for subsequant electrophoresis analysis.

Gel preparation

Gels were prepared using two different gel systems *viz.* standard gel system; 20 ml of 0.03 M lithium hydroxide, 0.19 M boric acid (pH 8.1) with 200 ml tris citrate (pH 8.4) and histidine gel system; 55 ml of 0.065 M L – Histidine, 0.007 M citric acid (pH 6.5) and 165 deionized water. Appropriate amounts of gel buffer were mixed with 25 g of hydrolized potato starch. This mixture was heated, then degassed and poured on to a gel mould (160 x 165 x 10 mm^3). The gel was allowed to cool for approximately one hour then covered with a thin polythene film and left overnight at room temperature ($25-30^0$ C).

Gel running conditions

The 5 x 8 mm filter paper wicks (No. 470, Schleicher & Schuell, Inc, USA.), each saturated with leaf extracts, were inserted into the slit which was made vertically, 4 cm from the cathodal end. Each gel contained 15 samples of *cv.* `Crinkled Red' and one sample of *cv.* `Orange' as a control for each electrophoresis run. A wick saturated

with red food colourant was inserted as a dye marker at one end of the slit to determine the movement of sample front. The gel slab was connected with the electrode buffer using two thin, flat, cellulose sponges. The whole unit was then placed in a refrigerator at 2–4° C. The electrodes were connected to a DC supply and 50 mA electric current supplied to each gel. After 15 minutes samples were removed and electrophoresis was continued with the electric supply of 450 volts and 350 volts, respectively for histidine and standard gel systems. This operation continued until the red dye marker reached 7.5 cm, usually for 2.5 – 3 hours.

Gel staining

At the end of the electrophoresis run the gel was trimmed to 150 x 750 mm then sliced into five slices, thickness of each slice was 1.2 mm. Then these slices were stained for different isoenzymes using recipes given below (Peiris, 1986).

Diaphorase (DIAP) – (Histidine gel system)

1M Tris–Hcl (pH 8.5) – 2.5 ml, deionized water – 22.5 ml, NADH – 7.0 mg, 2, 6, dichlorophenol indophenol – trace and 0.2% MTT – 3.5 ml. Add NADH and MTT just prior to staining. Incubated at 37° C for 1 hour.

Glutamate Oxaloacetate Transaminase (GOT) – (Standard gel system)

1M Tris Hcl (pH 8.5) – 2.5 ml, deionized water – 22.5 ml, L–aspartic acid – 50 mg, Ketoglutaric acid – 25 mg, pyrodoxial 5'phosphate – 2mg, fast blue BB salt – 25 mg. Incubated in the dark at 37° C.

Malate Dehydrogenase (MDH) – (Histidine gel system)

1M Tris – Hcl – 2.5 ml, deionized water – 22.5 ml, NAD – 10 mg, 0.2% MTT 1 ml, 0.005% Meldola blue – 1 ml, L – malate 20 mg, incubated in the dark at 37° C.

6 Phosphogluconic Dehydrogenase (6PGDH) – (Histidine gel system)

1M Tris – Malate (pH 7.2) – 2.5 ml, deionized water – 22.5 ml, 6 – phophoguconate – 6 mg, NADP – 4 mg, 0.05% MTT 1 ml, 0.005% meldola blue – 1 ml. Incubated in the dark at 37^0 C.

Phosphoglucoisomerase (PGI) – (Standard gel system)

1M Tris – Hcl (pH 8.0) – 2.5 ml, deionized water – 20 ml, 0.1M magnesium chloride 0.5 ml, glucose 6 phosphate – 5 µl, Fructose 6 phosphate – 5 mg, NADP – 3 mg, 0.2% MTT 1 ml, and 0.005% meldola blue – 1 ml. Incubated in the dark at 37^0 C.

At the end of the staining procedure gels were fixed in 50% (v/v) glycerol, and photographs and mesurements were taken. Banding patterns were compared on the basis of presence or absence of bands and the location (R_f value) and the number of bands present. Band densities were compared only where there were marked differences. In some cases bands were diffused and appeared as columns rather than as well defined bands. These non distinctive bands were also compared according to factors such as length and location. R_f values were calculated according to the equation given below (Kuhns and Frits, 1978).

$$R_f = \frac{\text{distance the origin isoenzyme band migrated}}{\text{distance from the sample application point to the end}}$$

RESULTS AND DISCUSSION

Evaluation of genetic stability

a) Micropropagated and vegetatively propagated plants

The isoenzyme banding patterns of the extracts separated by horizontal starch gel electrophoresis for both batches of micropropagated and vegetatively propagated plants showed no variation between these clones and they were also identical to the banding patterns obtained for the mother plants (Table 1). However, *cv.* `Orange' which was included as a control showed distinctively different banding patterns compared to

Table 1. Banding patterns of extracts obtained from vegetatively and micropropagated plants.

Isoenzyme	R_f	Banding patterns		
		cv 'Crinkled Mother plant	Red' Propagule	cv 'Orange'
DIAP	0.46	= = =	= = =	
	0.56			= = =
	0.64			= = =
GOT	0.20	= = =	= = =	
	0.25			= = =
6 PGDH	0.33	= = =	= = =	
	0.48	= = =	= = =	= = =
	0.56			= = =
	0.60	= = =	= = =	- - -
MDH	0.25	= = =	= = =	= = =
	0.38	= = =	= = =	
PGI	0.13	= = =	= = =	= = =

= = = heavy bands

- - - light bands

those produced by the extracts obtained from the *cv.* 'Crinkled Red' (Table 1).

b) Seedling plants

Banding patterns of the seedlings tested, were not uniform among the 105 seedling extracts compared. In diaphorase, one banding pattern which was different from the mother banding pattern was present in 17 seedlings. Thus variation detected using diaporase in a sample of 105 anthurium seedlings was 16%. Glutamate Oxaloacetate Transaminase had two patterns which were not similar to the mother banding pattern. Two patterns could be found in 13 seedlings of the plants tested. Therefore, variation detected using GOT was 12%. Banding pattern created by 6PGDH were not clear though some bands could be identified. There were three banding patterns which were different from the mother pattern and these different banding patterns appeared on 14 seedlings out of the 105 seedlings tested. Thus the variation with 6PGDH was 13%. Some bands which appeared on MDH isoenzyme were also not clear, but there were three banding patterns which were different from the pattern of the mother plants. These different banding patterns appeared on 15 seedlings of the 105 seedlings tested. The variation detected by MDH was 14%. The pattern of the mother plants of PGI isoenzyme showed only one band in the banding pattern. However, 15 seedlings of the 105 samples tested had an additional band thus 14% variation in these seedlings could be detected (Table 2).

All the isoenzyme samples in 105 seedlings which were compared showed 12 – 16% variability. These results suggested that the isoenzyme systems were probably detecting the same sources of genetic variability in the sample population of 105 seedlings. Furthermore, the fact that no detectable differences in isoenzyme banding pattern were found between extracts obtained from all of the micropropagated and vegetatively propagated plants was a strong indication that these plants were genetically homogeneous.

Comparison of the electrophoretic patterns of isoenzymes extracted from leaves of the anthurium regenerants and the mother stock plants indicated that no genetic changes had resulted from *in vitro* manipulations. In all samples of each zymogram, the banding patterns and the R_f values were identical. These results supported those of

Table 2. Representative zymograms of seedling extracts using starch gel electrophoresis.

Isoenzyme	R_f	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
DIAP	0.46	==	* ==	* ==	* ==	==	==	==	==	* ==	* ==	==	* ==	* ==	* ==	* ==
	0.56	==				==	==	==	==			==				
GOT	0.20			* ==	* ==	* ==	==	* ==	==	* ==	* ==	* ==	* ==			
	0.25	==	==				==		==						==	==
6 PGDH	0.33	* ==	* ==					==								
	0.48	==	==	==	==	==	==	==	==	==	==	==	==	==	==	==
	0.60	==	==	==							==	==	==			
MDH	0.25	==	==			==			==	==				==	==	
	0.38			==	==		==	==			==	==	==			
	0.40				==					==	==	==	==			
PGI	0.13	==	==	==	==	* ==	* ==	==	==	* ==	==	* ==	* ==	==	==	==
	0.33	==	==	==	==			==	==		==			==	==	==

* banding pattern similar to mother plant.

several other workers who have investigated isoenzyme banding pattern variability in micropropagated plants of other species. For example, Swendlund and Vasil (1985) reported that there was no genetic variation in *Pennisetum americanum* L. regenerated through callus cultures. They employed 10% polyacrylamide gels to separate MDH and ADH (Alcohol dehyrogenase) isoenzymes. These two isoenzyme systems showed no variation between mother stock plants and regenerants, as well as between regenerants. In contrast to such situations of uniformity, Heinz and Mee (1971) have reported variations in two clonal populations (H 37 – 1933 and H 50 – 7209) of sugarcane derived through callus cultures. Leaf extracts separated using 7% discontinous gel electrophoresis polyacrylamide showed differences in amylase, PRX and GOT but not in esterase isoenzyme banding patterns. Detected variations in amylase, PRX and GOT were 80.9% in the H 50 – 7209 clonal population and 31.0% in the H 37 – 1933 clonal population.

The causes of somaclonal variation in vegetatively propagated plants are considered to be genetic, epigenetic and/or chimeral in nature (Hartmann *et. al.*, 1990). Genetic variations may be produced through micropropagation because of several factors, such as 1) the high rate of cell division in shoot multiplication, 2) the use of inappropriate techniques *eg.* extended subculturing and/or the application of strong growth regulators like 2, 4 – D (George and Sherrington, 1984), 3) genetic variability, *eg.* polysomatism, which may already exist within the mother plant tissues (Constantin, 1981) and 4) nuclear responses to callus induction and plant regeneration conditions (D'Amato, 1978). Alterations in micropropagated plants may occur either as nuclear rearrangements which have originated in the mother plants themselves or as changes in the normal karyotypes that are a consequence of culture. It can be concluded that *Anthurium andreanum cv.* 'Crinkled Red' plants which have been regenerated through callus and subsequent *in vitro* shoot multiplication for eight subculture passages are genetically stable and uniform.

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