Middle East Journal of Agriculture Research, 2(2): 36-43, 2013
ISSN 2077-4605

Conservation Plantlets of *Anthurium andraeanum* (Lindeu ex Andre) By using *in Vitro* Technique

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ABSTRACT

To determine the appropriate method to conserve *Anthurium andraeanum* Lind, *in vitro* at Tissue Culture and Germplasm Conservation, Laboratory, of Horticultural Researches Institut,. This investigation was achieved during December, 2011 to July, 2012,by using micro nodes produced from adventitious buds induced by culturing of single explant of *Anthurium andraeanum* Linden ex André. The excised micro nodes were precultured on solidified Murashige and Skoog (1962)medium, containing 25 g/l sucrose and 0.5, 1.0 and 1.5 g/l sorbitol for 8 months. After storage period, the longevity of explants was studied by culturing the survived explants on MS medium containing 1.5 mg/l BAP for 4 weeks. The rate of survival, shoot number, shoot length, leaves number and rooting rate were approximately 100%, 2.83 shootlets/explants, 1.53 cm and 7.77 leaves/shootlet, respectively for the explants treated with sorbitol at 0.5 g/l. while sorbitol at1.0 g/l gave 66.6 % rooting. On liquid nitrogen study, the micro nodes treated with PVS2 at 10 and 5 % at 25˚C for 10 min or cultured in MS medium containing 25 %DMSO at 25˚C for 7 days prior to a plunge into liquid nitrogen and after rapid thawing in a water bath at 40˚C. After 4 weeks in MS medium the rate of survival, shoot number, length and leaves number were significantly increased for explants cultured in MS medium containing PVS2 for 7 days. RAPD analysis showed genetic stability for DNA whereas liquid nitrogen treatment (VIII) has the lowest polymorphism (16.6 %) compared with other treatments. Moreover, isoenzymes PPO and POD showed the highest density in control compared with the lowest density of sorbitol at 0.5 g/l. It could be recommended to storage *Anthurium andraeanum* in *vitro* precultured on MS medium containing sorbitol at 0.5 g/l for 8 months or culturing in MS medium containing PVS2 for 7 days.

Key words: Anthurium; *In vitro*; Vitrification; Liquid nitrogen; Sorbitol; RAPD and Isoenzymes POD and PPO.

Introduction

Anthurium is possibly one of the most complex genera in the Araceae family and is reported to encompass approximately 1000 species (Croat, 1992; Matsumoto and Kuehnle, 1997). Numerous Anthurium species are produced and traded internationally as cut-flowers, flowering potted plants and landscape plants. Most of the cut-flower anthuriums are believed to be hybrids of *Anthurium andraeanum* Linden ex André with several closely related species in the section Calomystrium (Croat and Sheffer, 1983) and have been referred to as *Anthurium andraeanum* Hort. (Kamemoto and Kuehnle,1996).

Anthurium is conventionally propagated by seeds and, therefore, cultivation is hindered by problems due to the inherent heterozygosity. Although traditional techniques of vegetative propagation such as the use of stem cuttings and suckers exist, they are tedious and not practical when carried out on a large scale. Tissue culture greatly increases the normal multiplication rate of plants and can provide a source of clean material which has become increasingly important due to outbreak of bacterial and other diseases such as anthracnose, blight, leaf spot, root knot and bacterial wilt caused by *Xanthomonas campestris* pv. diffenbachiae. According to *in situ* preservation, to conserve plant species only in field is also risky, as valuable germplasm can be lost (genetic erosion) because of pests, diseases and adverse weather conditions. Moreover, the maintenance of clonally orchids is labour-intensive and expensive. The maintenance of *in vitro* collections (established for some vegetatively propagated species) is also labour-intensive and there is the risk of losing accessions due to contamination, human errors or somaclonal variations (i.e., mutations that occur spontaneously in tissue culture, with a frequency that increases with repeated subculture). According to Bajaj (1983) *in vitro* storage offers several advantages over field collections because of minimal spatial and maintenance requirements. There are two methods for *in vitro* storage of plant cultures by slowing down or suspending their growth. Slow growth is achieved for short and mid-term storage by modifying the culturre medium or reducing temperature requirements (Withers, 1991). A further application, to use *in vitro* techniques to store specific genotypes was recognized by several scientists as a way of conserving the genetic resources of such problem crops (Kartha *et al*., 1981, Fletcher 1994 and Bekheet 2000).Application of *in vitro* techniques for germplasm conservation will mainly relyon the system's ability to regenerate a whole plant that will survive in the field and exhibit genetic

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stability over time (Withers, 1984). Among the different methods of *in vitro* preservation is the short- and medium-term storage to increase the interval period between subcultures by reducing growth. This might be achieved by the use of modified environmental conditions and modified culture medium (Withers, 1987 and 1991). This paper describes a method for preservation of globe artichoke cultures for short and mid-term period through *in vitro* storage of shoot buds and callus cultures by slow growth method.

Engelmann, (1997) *in vitro* conservation is used as complementary methods to field maintenance and involves the sustainment of explants in a pathogen-free environment and distribution of clonal material. It is widely used for the conservation of species which produce recalcitrant or no seed, and for vegetatively propagated plant material. Liu (2009) found that the optimized transformation conditions of leaves were preculturing for 4 d, incubating for 20 min and co-culturing for 3 d *with Agrobacterium tumefaciens*, with 30 mg/l. kanamycin as selection pressure. Meanwhile the optimized conditions for transformation of roots were preculturing for 5 d, incubating for 15 min and co-cultured for 5 d. Zhang SuFeng(2010) reported that methods to control vitrification :Under the conditions of increasing the concentration of carbon dioxide or 60～80μmol·m-2·s-, can effectively prevent Dendrobium occurrence of vitrification and get the divert normal plants. Wang Geng-liang et al.,(2010) reported that the suitable technology process of vitrification cryopreservation of *A.andraeanum* embryonic suspension cells is as follow: embryonic suspension cell mass(diameter of 2 mm) subcultured for 3-5 d is pre-cultured in 1/2MS liquid medium containing 0.5 mol·l-1 sorbitol for 2 d, then treated for 24 h in 4 ℃,and pre-treated with 25% PVS2 for 15 min in room temperature, dehydrated with 100% PVS2 for 10 min in 0 ℃,at last ,rapidly put the cell mass into liquid nitrogen for cryopreservation. Put the cryopreservation cells in 40 ℃ water bath to thaw for 3 min, then wash with 1/2MS liquid medium containing 1.2 mol·l-1 sucrose for three times(each time for 10 min),afterward carry on renewal culture. Aslam *et al.* (2011) evaluated the effect of freezing and non-freezing temperature on somatic embryogenesis in *Catharanthus roseus* (L.), their results showed that somatic embryo development (production, maturation and germination) was sensitive to temperature variations. Yousify Shaza (2011) storage *Anthurium andraeanum* in liquid nitrogen, of embryos were warmed for *in vitro* culture and the results showed that ES40 gave the highest survival rate of the embryos following vitrification-thawing procedure among the others cryoprotectant solutions used in this experiment.

Materials And Methods

Plant source:

The present study was carried out at the Tissue Culture and Germplasm Conservation Laboratory, Horticultural Researches Institute, Agricultural Researches Center, Giza during December, 2011 to July, 2012. *Anthurium andraeanum* Linden ex André was collected from the Ornamental Department of Hort. Res. Inst. The small shoots of young plants were used as explants to give the shoot multiplication in MS medium supplemented with BAP at 1.5 mg/l. These explants were used as a source of conservation treatments.

Plant disinfection and culture:

The shoot segments were collected and washed thoroughly under a running tap water and dead tissues were carefully removed with the help of a sharp stainless steel knife (Plate, 1). The shoot segment was chopped into small pieces (2 – 3 cm) and sterilized by 70% ethanol for 60 seconds then washed 3 times with double distilled water. Afterwards, it was sterilized with 20 % commercial bleach solution (Colorox 5.5 %) for 15 minutes and washed several times then inoculated into glass jars containing 25 ml MS medium with BAP at 1.5 mg/l. The culture medium, glassware's and instruments were sterilized by autoclave at 15-psi pressure and 121°C for 20 minutes. Cultures were maintained in an growth room at 25°C temperature at 16 hours light period by the illumination from white florescent tube light (Phillips).

Conservation treatments:

When the shoots grew about 3-4 cm in length with 4-5 well developed leaves were rescued aseptically from the conservation culture treatments and were separated from each other and again cultured on freshly prepared MS medium containing different concentrations of sorbitol (0.5, 1.0 and 1.5 g/l) for chemical conservation for 8 months inoculation.

For liquid nitrogen treatment, the explants were treated with PVS2 (protectant vitrification solution 1) as shown in the next following:

1- Soaking in liquid nitrogen directly (control)
2- Soaking in PEG 5 % + mannitol ($C_6H_8(OH)_6$). 5 % + Dimethyl sulfoxide (DMSO) $(CH_3)_2$ SO at 5 % for 10 min before culture (V.I).

3- Soaking in PEG 10 % + mannitol 10 % + DMSO 10 % for 10 min before culture (V. II)
4- Culture in MS + DMSO 10 % + Polyethylene glycol (PEG) "H-(O-CH_2 –CH_2)$_n$ - OH at 10% + mannitol 10% for 7 days (V.III)
5- Culture in MS + DMSO 25 % for 7 days (V. IV)

After pretreatment of the explants with protection solutions, they were immersed in a liquid nitrogen for 12 days. The effect of different conservation treatments on survivability (%), percent of shoot induction, shoots number per explant, shoot length (cm), leaves number and percentage of root development, after conservation period (12 days and 8 months) and culturing on growth medium was studied and it was collected and statistically analyzed. The experiment was conducted in growth room and arranged in completely randomized design (CRD) (Mead *et al.,* 1993) each treatment included three replicates as each contained five jars with four explants for each .Data were subjected to analysis of variance by MSTAT-C (1990) a computer statistical analysis program by least significant difference (LSD) test at the 5% level of significance (P=0.05) was computed to differentiate between means.

PCR (RAPD analysis):

Reagents used – Target, DNA (10-100ng), oligonucleotide primers (10-mers Primers OP A-10, OP A-12, OP CO-9, OPB 20, OPC 05, OP D-01 and OP D07), sterile de-ionized distilled water, Taq polymerase, dNTP mix (dATP, dCTP, dGTP, dTTP), light mineral oil, agarose (Sigma, Molecular biological grade), TBE buffer (X0.5), Molecular marker VI, Gel-loading buffer (ULB – 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water; stored at 4˚ C), ethidium bromide (10 mg mL–1).The reaction mix was prepared on ice for the PCR analysis. The reaction mix was dispensed into the reaction tubes. The Taq Polymerase was added last. One drop of mineral oil was added to each tube to prevent evaporation during reaction. The tubes were placed in the thermal cycler and the PCR program for RAPD was then run - reaction initiation at 94˚C for 2 min followed by cycles at: 94˚C for 1 minute, 35˚C for 1 min and 72˚C for 1 min. forty such cycles were done (Williams *et al.* 1990).

Isozymes electrophoresis:

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among studied cultivars using three isozyme systems according to Stegemann *et al.* (1985). Fresh and young leaf samples for each treatment was used separately for isozymes extraction. The utilized isozymes are peroxidase (Px) and polyphenyl oxidase (PPO).

Results And Discussions

Effect of sorbitol on vegetative growth of Anthurium andraeanum:

Survival rate:

Data shown in Table (1) and illustrated in Fig. (1) and Photo(1) indicate that using MS medium supplemented with sorbitol at 0.5, 1.0 and 1.5 g/l of the study of sorbitol conserved explants. The increase of explants survival recorded for some treatments over the control was significant. Among these treatments, MS+ sorbitol at 0.5 g/l gave the highest rate of survival (100%) followed by sorbitol at 1.0 g/l which gave 88.9 with non significant differences between them.

Shoot number:

Data in the same Table (1) clearly indicate that using MS medium with sorbitol at 0.5 g/l successeded to produce shoots for cultured explants. The maximum number of shoots that developed was for the treatment of 0.5 g/l sorbitol (2.83) followed by treatment of sorbitol 1.0 g/l (2.0) with significant difference. in between

Shoot length:

Data presented in Table (1) and illustrated in Fig. (1 and 2) indicate that using MS medium supplemented with sorbitol at 0.5, 1.0 and 1.5 g/l of the study of sorbitol conserved explants. The increase of explants shoot length recorded some treatments than the control was significant. Among these treatments, MS+ sorbitol at 0.5 g/l gave the highest rate of shoot length (1.53 cm) followed by sorbitol at 1.0 g/l which gave 1.49 cm with non significant difference between them.

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Table 1: Effect of sorbitol at different concentrations on some growth characteristics after conservation periods (8 months) of *in vitro* conservation

Treatment	Survival	Shoot No.	Shoot length(cm)	Leaves No.	Rooting (%)
Control	19.44	1.367	0.50	4.833	00.00
Sorbitol at 0.5 g/l	100.0	2.833	1.53	7.77	22.10
Sorbitol at 1.0 g/l	88.9	2.00	1.49	6.63	66.63
Sorbitol at 1.5 g/l	55.53	1.80	0.93	6.43	00.00
L.S.D. at 5%	40.7	0.2895	0.433	1.603	33.31

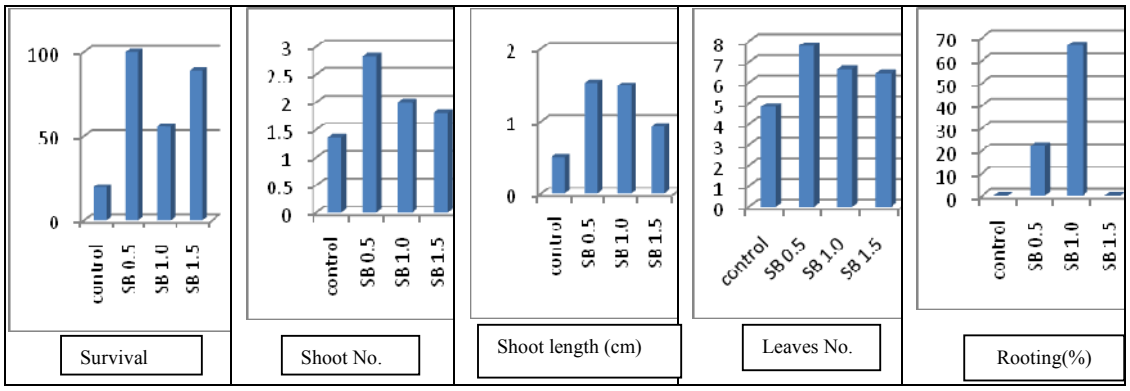


Fig. 1: Effect of sorbitol at different concentrations on some growth characteristics after conservation period (8 months) of *in vitro* conservation

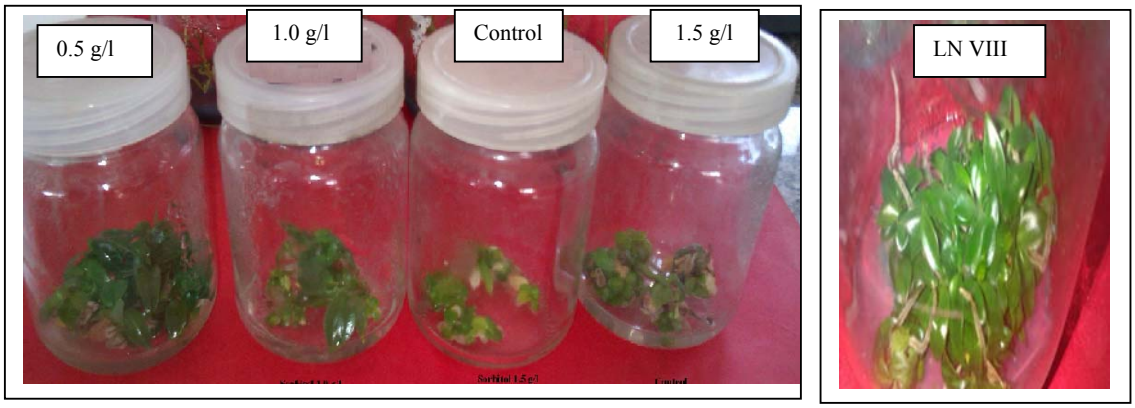


Photo 2: Effect of different concentrations of sorbitol (0.5, 1.0 and 1.5 g/l) and liquid nitrogen (LN VIII) treatments *Anthurium andraeanum***.**

Leaves number:

Data in Table (1) clearly indicate that using MS medium with sorbitol at 0.5 g/l successeded to produce leaves for cultured explants. The maximum number of leaves that developed was for the treatment of 0.5 g/l sorbitol (7.77) followed by treatment of sorbitol at1.0 g/l (6.63) with no significant differences in between.

Rooting percentage:

On the other hand, Data registered in Table (1) and illustrated in Fig. (1) Indicate that using MS medium supplemented with sorbitol at 0.5, 1.0 and 1.5 g/l formed roots on explants. The increase of explants which was rooting recorded for some treatments over the control was significant. Among these treatments, MS+ sorbitol 1.0 g/l gave the highest rate of rooting (66.6%) followed by sorbitol at 0.5 g/l which gave 22.1% compared with control (0.00%). Engelmann (1991) reported that growth reduction could be achieved by modifying the environmental conditions and/or the culture medium. He added that, the most widely applied technique is temperature reduction, which can be combined with a decrease in light intensity or culture in dark. The present results are also in accordance with those reported by Bekheet (2000), in his study on *Asparagus officinalis*. He

mentioned that tissue cultures remained viable after eighteen months of storage at 5ºC. In addition, 80% of pear and 85% of apple shoot bud cultures grown *in vitro* remained alive after eighteen months storage at 4ºC and 8ºC, respectively Fletcher (1994) mentioned that, *in vitro* shoot cultures of asparagus survived for20 months when stored on medium containing 3% sucrose and 4% sorbitol.

Effect of liquid nitrogen on vegetative growth of Anthurium andraeanum:

Data recorded in Table (2) explained the death effect of liquid nitrogen in all treatments except with that cultured on MS medium containing PVS2 10% for 7 days and then soaking in liquid nitrogen directly, only explants treated with this treatments are successfully survived (44.4%), gave shoot number (1.17 shootlets/ explants), shootlet length (0.77 cm) with leaves number of 3.11 leaves/shootlets and finally rise rooting rate to 14.8 % compared with all treatments and agreed with those reported by Zhang SuFeng (2010) on Dendrobium,Wang Geng-liang *et al*.,(2010) on *Anthurium andraeanum* and Yousify Shaza (2011) that storage of *Anthurium andraeanum* in liquid nitrogen of embryos which were warmed for *in vitro* culture the results showed that ES40 gave the highest survival rate of the embryos following vitrification-thawing procedure among the other cryoprotectant solutions used in this experiment.

Table 2: Effect of cryopreservation-vitrification on some growth characteristics after conservation period (12 days) in liquid nitrogen of *in vitro* conservation

Treatment	Survival (%)	Shoot No.	Shoot Length(cm)	Leaves No.	Rooting (%)
Control	0.0	0.0	0.0	0.0	0.0
V. I	0.0	0.0	0.0	0.0	0.0
V. II	0.0	0.0	0.0	0.0	0.0
V. III	44.4	1.17	0.77	3.11	14.8
V. IV	0.0	0.0	0.0	0.0	0.0
LSD at 5%	16.19	0.1331	0.0595	0.1575	3.792

Genetic stability study:

RAPD analysis:

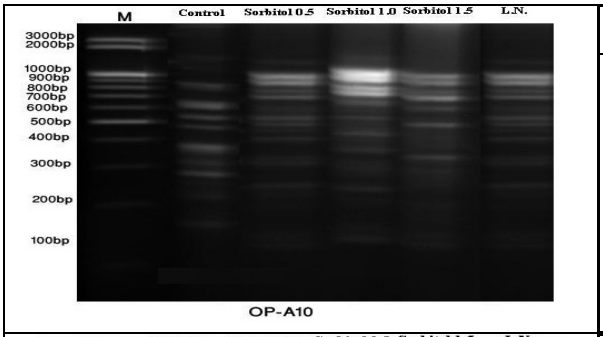
Data presented in Table (3) and illustrated in Fig. (3) showed that the best results of total polymorphism bands in DNA PCR for the explants storage in liquid nitrogen was 16.6% compared with sorbitol treatments.

Isoenzyme pattern:

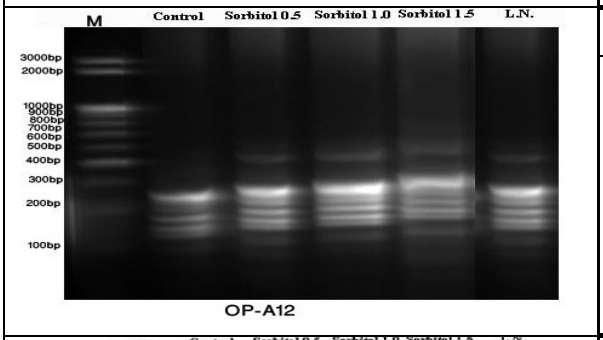
In isoenzyme pattern as shown in Fig. (4) the pattern shows that control treatments are more effective for storage period whereas gave highest density bands compared with other treatments. However, the sorbitol treatment at 0.5 g/l gave the lowest pattern density. On the other hand, poly phenol oxidaze pattern showed traces density of bands in all treatments.

Table 3: Number of amplified bands, monomorphic bands, polymorphic bands and percentage of polymorphic bands of preserved shootlets based on RAPD-PCR analysis using five primers.

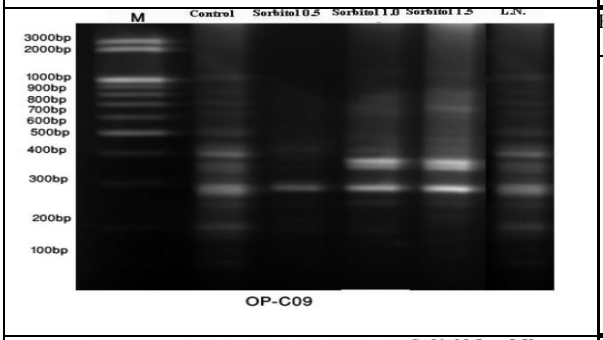
Primer name	Absent bands/marker					Present bands/marker					Polymorphism bands/ control					Monomorphism bands/control					Total bands amp.	Poly morphism (%)
	Cont	Sorb 0.5	Sorb 1.0	Sorb 1.5	LN VIII	Cont	Sorb 0.5	Sorb 1.0	Sorb 1.5	LN VII I	Cont	Sorb 0.5	Sorb 1.0	Sorb 1.5	LN VII I	Cont	Sorb 0.5	Sorb 1.0	Sorb 1.5	LN VII I		
OP – A10	4	4	3	3	3	8	8	9	9	9	-	-	1	1	1	-	8	8	8	8	12	6.9 %
OP - A12	9	7	7	6	7	3	5	5	6	5	-	2	2	3	2	-	-	-	-	-	12	37.5 %
OP – C09	3	9	3	4	2	9	3	9	8	10	-	6	-	1	1	-	3	9	8	9	12	20.5 %
OP – D01	2	1	-	3	1	10	11	12	9	11	-	1	2	1	1	-	10	10	10	10	12	9.4 %
OP – D07	5	4	2	3	4	7	8	10	9	8	-	1	3	2	1	-	7	7	7	7	12	16.6 %
Total	23					37	35	45	41	43	-	10	8	8	6	-					60	
Polymo %												28.5	17.7	19.5	13.6							



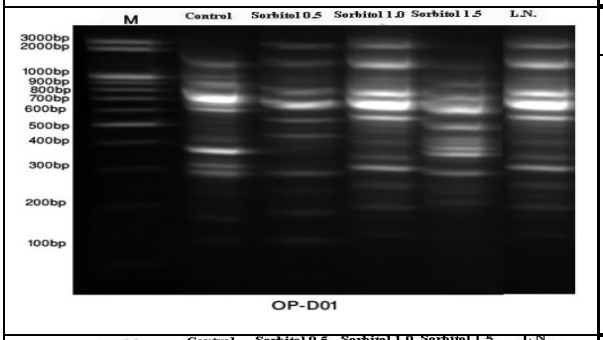
Band No	Mw bp	M	Cont	Sor 0.5 g/l	Sor 1.0 g/l	Sor 1.5 g/l	VI L.N
1	3000	1	0	0	0	0	0
2	2000	1	0	0	0	0	0
3	1000	1	0	0	0	0	1
4	900	1	1	1	1	1	1
5	800	1	1	1	1	1	1
6	700	1	1	1	1	1	1
7	600	1	1	1	1	1	1
8	500	1	1	1	1	1	1
9	400	1	1	1	1	1	1
10	300	0	1	1	1	1	1
11	200	0	1	1	1	1	1
12	100	0	0	0	1	1	0



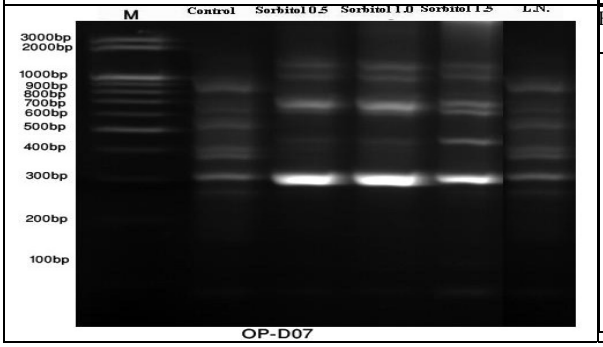
Band No	Mw bp	M	Cont rol	Sor 0.5 g/l	Sor 1.0 g/l	Sor 1.5 g/l	VI L.N
1	3000	1	0	0	0	0	0
2	2000	1	0	0	0	0	0
3	1000	1	0	0	0	0	0
4	900	1	0	0	0	0	0
5	800	1	0	0	0	0	0
6	700	1	0	0	0	0	0
7	600	1	0	0	0	1	0
8	500	1	0	1	1	1	1
9	400	1	0	1	1	1	1
10	300	1	1	1	1	1	1
11	200	1	1	1	1	1	1
12	100	0	1	1	1	1	1



Band No	Mw bp	M	Cont rol	Sor 0.5 g/l	Sor 1.0 g/l	Sor 1.5 g/l	VI L.N
1	3000	1	0	0	0	0	0
2	2000	1	0	0	0	0	1
3	1000	1	1	0	1	1	1
4	900	1	1	0	1	1	1
5	800	1	1	0	1	1	1
6	700	1	1	0	1	1	1
7	600	1	1	0	1	1	1
8	500	1	1	0	1	1	1
9	400	1	1	1	1	1	1
10	300	1	1	1	1	1	1
11	200	0	1	1	1	0	1
12	100	0	0	0	0	0	0



Band No	Mw bp	M	Cont rol	Sor 0.5 g/l	Sor 1.0 g/l	Sor 1.5 g/l	VI L.N
1	3000	1	0	1	1	0	1
2	2000	1	1	1	1	0	1
3	1000	1	1	0	1	1	1
4	900	1	1	1	1	1	1
5	800	1	1	1	1	1	1
6	700	1	1	1	1	1	1
7	600	1	1	1	1	1	1
8	500	1	1	1	1	1	1
9	400	1	1	1	1	1	1
10	300	1	1	1	1	1	1
11	200	1	1	1	1	1	1
12	100	0	0	1	1	0	0



Band No	Mw bp	M	Cont rol	Sor 0.5 g/l	Sor 1.0 g/l	Sor 1.5 g/l	VI L.N
1	3000	1	0	0	1	0	0
2	2000	1	0	0	1	1	0
3	1000	1	1	1	1	1	1
4	900	1	1	1	1	1	1
5	800	1	1	1	1	1	1
6	700	1	1	1	1	1	1
7	600	1	1	1	1	1	1
8	500	1	1	1	1	1	1
9	400	1	1	1	1	1	1
10	300	0	0	1	1	1	1
11	200	0	0	0	0	0	0
12	100	0	0	0	0	0	0

Fig. 3: Number of amplified bands, monomorphic bands, polymorphic bands and percentage of polymorphic bands of preserved shootlets based on RAPD-PCR analysis using five primers.

In conclusion, RAPD markers have successfully been used to differentiate and assess the extent of genetic variation among conservation methods. Cultivars were found to be very closely related to each other. Knowledge on genetic diversity will help in the efficient management of anthurium germplasm conservation.

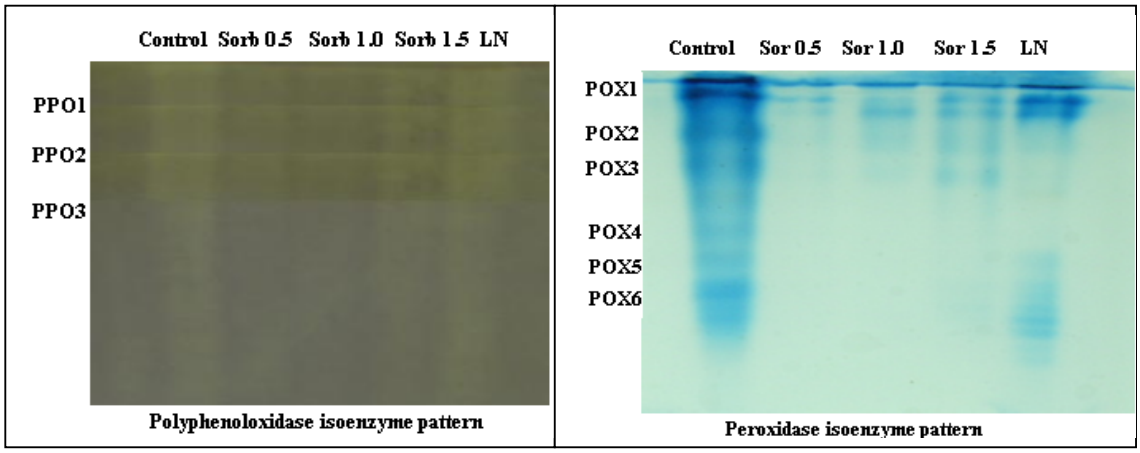


Fig. 4: Peroxidase and poly phenol oxidase isoenzymes pattern for the viable conserved explants.

The present results are in line with those of Saker *et al.* (2000), who mentioned that non significant variations were observed in tissue cultures derived for date palm plantlets. RAPD analysis showed genetic variation in only 4% of analyzed plants (70 regenerants), which were incubated for 6 - 12 months under 25°C.

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