

## CLONAL PROPAGATION AND HISTOLOGICAL STUDIES OF *ANTHURIUM ANDRENUM* LIND. THROUGH SOMATIC EMBRYOS

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### ABSTRACT

*Anthurium* industry plays a significant role in the global floriculture trade. To overcome the demerits of conventional vegetative propagation, micropropagation of *Anthurium*, through tissue culture is the most alternative techniques to increase the production. Somatic embryogenesis is the maximum expression of cell totipotency in plant cells. Somatic embryos initiation has been studied by using different explants, growth regulators and basal salt media in *Anthurium andraenum* Lind.. By using mid, proximal and distal end of young leaves on full, half and  $\frac{3}{4}$  strength of MS media by reducing only the macro nutrients. The media were supplemented with benzylaminopurine (BAP) and 2, 4-Dichlorophenoxy acetic acid (2, 4-D) - in combinations.

The embryoids were harvested at various stages and examined macro- and microscopically. Histological analysis of the somatic embryo initiation and development from embryogenic mass derived from leaf explants of *Anthurium andraenum* were conducted. Embryogenic mass along with somatic embryos at different developmental stages were fixed for histological examination, stained with hematoxylin-eosin and observed under a microscope. Embryos showed a sequence of developmental patterns from globular to heart and finally cotyledon stages. Only BAP 1mg/L and 2, 4-D 0.1 mg/L combination with  $\frac{3}{4}$  strength MS was found to be the best for somatic embryogenesis using mid and distal region of the leaf. Shoot multiplication was observed best on MS medium supplemented with BAP 1mg/L and Kinetin 1mg/L. Optimum number of roots were obtained on simple MS medium without plant growth regulator. The regenerated plants were shifted to green house for acclimatization. Various potting mixtures were also studied, only coconut husk and charcoal in 1:1 ratio gave the best result for acclimatization of *Anthurium*.

**Key words:** *Anthurium*, Somatic embryos, Micropropagation, Leaves, Hematoxylin & Eosin, Histology

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### INTRODUCTION

*Anthurium* is one of the most popular and largest genus with 500 species of Araceae family. *Anthurium* is known for its attractive long lasting inflorescence with beautiful colors and shapes. These characters make the *Anthurium* an ever demanding plant as it is being used continuously as indoor decors (Dufour and Guerin, 2003). Among tropical cut flowers, its trade value stands second to Orchids. One of the vegetative propagation methods for *Anthurium* is through seed propagation which requires approximately three years for the plant to be completely developed in a breeding program (Hamidah *et al.*, 1997). Moreover, slow germination rate and low seed viability are limiting factors for the large scale production of *Anthurium* (Martin *et al.*, 2003). Plants obtained through seeds have usually poor uniformity and heterozygosity due to cross pollination (Bejoy *et al.*, 2008). On the other hand plant tissue culture technology has emerged as an alternative method for large scale production of true-to-type *Anthurium* at faster rate than conventional methods (Chen *et al.*, 1996).

Various explants like seedling, leaf, petiole, spathe, lateral bud, or shoot tips are used for *Anthurium* micro-propagation (Atta-Alla *et al.*, 1998; Joseph *et al.*, 2003; Atak and Celik, 2009). The factors affecting regeneration of *Anthurium* are age, genotype and choice of explant for the initiation. Although, regeneration from embryogenic calli is more reliable for rapid mass propagation direct somatic embryogenesis results in high multiplication rate with high genetic integrity. The development of somatic embryos can also be maintained for long periods of time by repeated cycles of secondary somatic embryogenesis (Te-chato *et al.*, 2007) and by producing artificial seeds (Hamidah *et al.*, 1997). The handling of enormous number of embryos at the same time could be managed in bioreactors (Merkle *et al.*, 1991). The regeneration of *Anthurium* from somatic embryos has been reported (Gier 1982; Kuehnle *et al.*, 1992; Hamidah *et al.*, 1997; Farsi *et al.*, 2012) by using different induction medium with reduced multiplication time span (Gantait and Mandal, 2010). Somatic embryogenesis was confirmed through histological analysis. Histological knowledge concerning the ontogeny of embryoids can provide important information for improving the somatic embryogenesis process for this species. Studies on the origin and development of somatic embryos in *Anthurium* are still inadequate. It is important to observe the key events in somatic embryogenesis from different explants sources and follow the plant regeneration efficiencies.

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On the other hand, Silva *et al.*, 2005 reported the involvement of various physical and biological factors including media during *in vitro* propagation of *Anthurium*. In this regard, some modifications of different hormonal and macronutrient concentrations in culture media such as reduced amount of macronutrients for somatic embryos induction may lead to ideal regeneration system for massive plant propagation of *Anthurium*. This improvement could have economic importance by contributing towards enhanced production of *Anthurium* plants along with limited consumption of nutrients and lead to its commercial application on a large scale development of *Anthurium* by tissue culture technology.

The aim of study was to optimize protocol for the maximum production of somatic embryos in short period of time by using  $\frac{3}{4}$  strength of MS salts concentrations especially Ammonium Nitrate and to investigate the events for somatic embryogenesis of *Anthurium* through histological analysis.

## MATERIAL AND METHODS

### Plant material

Young green leaves of *Anthurium andraenum* were collected from mature plants grown in the green houses of the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. Initially different explant parts such as spadix, petal, and leaves were sterilized with different sterilants such as NaOCl (bleach) (20%) and mercuric chloride (0.1%) for different time intervals (Fig.1). Young leaf explants were sterilized with different concentrations of NaOCl (bleach) and mercuric chloride (Fig. 2). After 20 minutes of gentle agitation during sterilization, the leaves were rinsed thrice with sterile distilled water. Sterilized leaves were cut into three parts, proximal, midrib and distal end of appropriate sizes ( $1\text{cm}^3$ ) in the laminar flow cabinet under sterile conditions.

### Embryo initiation of *Anthurium andraenum*

All different types of explants were inoculated on modified MS (Murashige & Skoog, 1962) medium. MS basal salt mixtures were used with macronutrients at full,  $\frac{1}{2}$  and  $\frac{3}{4}$  strengths. The micronutrients used were full strength in each case with 100 mg/L myo-inositol and MS vitamins. Table sugar instead of sucrose was added to the media as a source of carbon at the concentration of 30g/L. Plant hormones like 6-Benzylaminopurine (BAP), 2, 4 – Dichlorophenoxyacetic acid (2, 4-D) alone and in combinations were supplemented with MS  $\frac{3}{4}$  +BAP (0, 0.5, 1, 1.5 and 2) mg/L with 2, 4-D (0, 0.1 and 0.2) mg/L for somatic embryogenesis whereas for multiplication MS + BAP (0, 0.5, 1, 1.5) mg/L + Kinetin (0, 0.5, 1) mg/L + Sugar 40g/L was used. Rooting was done on simple MS without plant growth regulator. pH was adjusted to 5.7-5.8 with 1M NaOH prior to adding the agar. Media were autoclaved for 15 minutes at  $121^{\circ}\text{C}$  and dispensed as 25 ml aliquots into 125 ml glass jars. All cultures were placed under dark condition at  $25\pm 2^{\circ}\text{C}$ . Somatic embryo formation was started in *Anthurium andraenum* after three weeks at  $\frac{3}{4}$  MS in combination with 1 mg/L BAP and 0.1 mg/L 2, 4-D and then followed by embryoid differentiation. The embryos were white to pale yellow in color. After 2 months embryos turned green in color. The induced shoots were transferred to full MS supplemented with various concentrations of BAP (0-1.5mg/L) and Kinetin (0-1mg/L) to enhance shoot multiplication. All cultures were placed at  $25\pm 2^{\circ}\text{C}$  with a 16:8h photoperiod at a light intensity of  $25\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ . The newly formed shoots were excised and used for the induction of *in-vitro* rooting. MS media without any plant growth regulator with 30 g/L sugar was used for rooting. Twenty explants were selected for each treatment and all the best treatments were repeated twice.

### Histological Observations

For histological studies, somatic embryos were fixed in Formalin Acetic Acid (FAA) solution containing 90ml 70% ethyl alcohol, 5ml glacial acetic acid and 5ml formalin solution. After three days of fixation, somatic embryos were dehydrated through 70%, 90% and 100% isopropyl alcohol for over night incubation. These dehydrated embryos were embedded in paraffin and then microtome was used to make  $6\mu\text{m}$  to  $10\mu\text{m}$  sections. The sections thus obtained were put in water bath where temperature was maintained at  $47^{\circ}\text{C}$  to expand the sections. These sections were taken on to slides and placed on the slide warming plate for overnight incubation for Hematoxylin and Eosin staining, after deparaffinization and rehydration, slides were stained with aqueous hematoxylin for 4 minutes, followed by washing the slide with distilled water. After washing, the slides were stained with aqueous eosin for 3 minutes and again followed by washing it thoroughly with distilled water. Then slide were dehydrated with graded alcohol, placed in xylene and were mounted with mounting media DEPEX. A Nikon TE2000E microscope was used to visualize the different developmental stages of somatic embryos as shown in the Fig. 6 (j, k, l).

### Acclimatization

Plantlets with fully developed roots were removed from jars, washed in lukewarm water to ensure that all media/gel were removed from the roots of plantlet and plants were transferred to pots containing different types of

potting mix like charcoal (100%), coconut husk (100%), mixture of coconut husk + charcoal (50%:50%), soil (100%), marble peats (100%) and soil + manure (50%:50%) in the green net.

## RESULTS AND DISCUSSION

Various explants from different sources exhibited different responses on treatment with various sterilants (Fig. 1). After screening explants on the basis of low contamination rate, only leaf explants were processed for various concentrations of NaOCl (20 min.) and mercuric chloride (10 min.). 20% NaOCl for 20 min. showed maximum survival rate and considered as the best for sterilization (Fig. 2). Leaf segments were chosen as the best explant for further experiments on somatic embryo induction. Leaf explants from micropropagated plants were found to be more responsive than other plant tissues for the induction of somatic embryos in *Anthurium andraenum* (Hamidah *et al.*, 1997).

In a preliminary experiment, three portions of the leaf explants (proximal end, mid and distal region) were used for initiation on modified Murashige and Skoog (1962) medium of full,  $\frac{1}{2}$  and  $\frac{3}{4}$  strength of macronutrients along with various concentrations of BAP and 2, 4-D. Full and  $\frac{1}{2}$  strength MS media showed slow development of somatic embryos on all leaf portions whereas  $\frac{3}{4}$  strength MS medium proved to be the best for somatic embryo induction. After 8 weeks (Fig. 3, Table 1) only M8 (BAP 1 mg/L and 2, 4-D 0.1 mg/L), M9 media (BAP 1 mg/L and 2, 4-D 0.2 mg/L), M10 (BAP 1.5 mg/L and 2, 4-D 0 mg/L) and M11 (BAP 1.5 mg/L and 2, 4-D 0.1 mg/L) showed their effect on all parts of leaf but maximum somatic embryo percentage was observed from mid and distal regions of leaf on M8 media (Table 1). From this preliminary result, mid and distal regions of the leaf were chosen as the best part for further experiments to get somatic embryos (Table 2). It is believed that proximal end of the leaf had more shoot regeneration potential as compared to the distal end which may be due to the difference in maturity between the regions (Welander 1988; Martin *et al.*, 2003).

Typically, when embryogenic tissue is exposed to a medium lacking auxin (or to one containing only a low auxin concentration) numerous somatic embryos are formed superficially. Together with embryos which may have been present in the tissue prior to the transfer, these sometimes develop into plantlets, but usually a second transfer onto an auxin-free medium may be necessary. Somatic embryos formed in the presence of low concentration of auxin give rise to clumps of cells if they are again exposed to high auxin concentrations. Assuming that the level within cultured tissues is in equilibrium with that in the medium, there are several ways in which the concentration of exogenously supplied auxin may be reduced to promote embryo formation (George, 1996).

Naturally, plants reproduce through the zygotic embryo development. The large number of plantlets regenerated from a group of somatic cells as compared from the natural plantlets, although they have the same genetic makeup.

Somatic embryos were originated in two ways directly from a single cell or from the proembryonic cell complex. This could indicate a relationship between the isolated somatic embryos and the tightly clustered somatic embryos among the same explants and the fact that mutually, these systems arose from the mesophyll (Geier, 1982; Kuehnle *et al.*, 1992).

Histologically, it was observed that young leaf of *Anthurium andraenum* was the most receptive for the stimulation and escalation of embryogenic masses and somatic embryos. Histological sections of the embryogenic culture of *Anthurium andraenum* surrounded with the somatic embryos of different developmental stages are shown in Figures 5 & 6. In histological sections of the intact embryogenic masses, the somatic embryos appeared similar in size but different in shape. Discernible differences between somatic embryos were observed in all the proliferating masses. Globular, heart and torpedo shape somatic embryos were markedly visible as shown in Fig. 5 (g, h, i).

From the histological analysis it was concluded that the somatic embryos derived from the young green leaf of *Anthurium andraenum* have the ability to originate indirectly from the dominant callus phase or embryogenic culture as shown in Fig. 4(b). Thus the small cells have the potential for the induction and development of somatic embryos of different shape and size during continued cell divisions in embryogenic culture of the *Anthurium andraenum* as shown in Fig. 5 (g).

After 3 weeks, somatic embryo induction started in dark along cut edges of the explants. Only M8 media (BAP 1mg/L and 2, 4-D 0.1mg/L) induced the best somatic embryos. It was also observed at lower and higher concentrations of above mentioned plant growth hormones but with lower frequencies (Table 2). The lowest concentration of 2, 4-D indicated that there is requirement for auxin free environment for the induction of somatic embryos. The continuous incubation in darkness was found to enhance the process of somatic embryogenesis. The calli were pale yellow in color, but after 2 months when it was transferred to light it turned green in color and induced shoots Fig. 4 (a, b, c), by the variation in the endogenous levels of these growth hormones in the leaf tissues. The division and sub culturing of somatic embryos were done after every 8 weeks. For the study of the

different stages of the development of somatic embryos, the embryoids were harvested at various time intervals, fixed, sectioned, stained and examined microscopically. These showed globular, heart and torpedo shaped stages and their respective histological structures are shown in Fig. 5 & 6 (g, h, i, j, k, l).

Table 1. Percentage of somatic embryo induction on different leaf portions of *anthurium andreanum*.

Media code	Plant growth regulators		Somatic embryos percentage of leaf mid portion explant	Somatic embryos percentage of leaf proximal end explant	Somatic embryos percentage of leaf distal end explant
	Cytokinins BAP	Auxins 2, 4- D			
M1	0	0	0±0	0	0
M2		0.1	29.8±0.62	0	34.1±0.9
M3		0.2	15.07±0.21	0	10.66±0.76
M4	0.5	0	10.33±0.34	0	10.73±0.66
M5		0.1	29.9±0.34	0	40.5±0.62
M6		0.2	20.0±0.20	0	24.53±0.50
M7	1.0	0	49.96±0.35	0	55.06±0.20
M8		0.1	89.76±0.68	20.03±0.45	94.9±0.75
M9		0.2	80.9±1.08	10.73±0.66	82.9±0.78
M10	1.5	0	19.96±0.65	8.67±0.58	14.96±0.65
M11		0.1	50.3±0.3	5.09±0.17	50.06±0.20
M12		0.2	29.33±0.58	0	40.6±0.65
M13	2.0	0	5.1±0.18	0	10.16±0.15
M14		0.1	25.39±0.45	0	30.3±0.32
M15		0.2	30.6±0.79	0	35.44±0.42

Means ± standard errors

Table 2. Somatic embryos induction at  $\frac{3}{4}$  MS media with different plant growth regulators and their combinations

Media code	Plant growth regulators		% of explants produced somatic embryos	Response of Explant
	Cytokinins (BAP)	Auxins 2, 4- D		
M1	0	0	0±0	-
M2		0.1	34.6±0.36	+
M3		0.2	15.10±0.26	+
M4	0.5	0	19.65±0.34	+
M5		0.1	44.80±0.53	+
M6		0.2	40.03±0.15	+
M7	1.0	0	60.93±0.81	++
M8		0.1	89.76±0.58	++++
M9		0.2	74.8±0.43	+++
M10	1.5	0	22.6±0.79	+
M11		0.1	54.5±0.78	++
M12		0.2	45.73±0.66	+
M13	2.0	0	10.12±0.12	-
M14		0.1	34.63±0.35	+
M15		0.2	40.26±0.92	+

Key: +, ++, +++, ++++ represents, 25%, 50%, 75% and 100% respectively Means ± SE

Table 3. Effect of plant growth regulators on length and no. of shoots of *Anthurium andreanum* regenerated from somatic embryos.

Media code	Cytokinins		No. of regenerated shoots	Length of shoot (cm)
	BAP	Kinetin		
R1	0	0	0±0	0±0
R2		0.5	10±0.1	1.64±0.55
R3		1	14.8±0.32	1.44±0.05
R4	0.5	0	18.06±0.12	2.09±0.17
R5		0.5	37.43±0.51	3.38±0.15
R6		1	41.66±0.57	4.89±0.06
R7	1.0	0	46±0.1	4.98±0.01
R8		0.5	45±1	4.37±0.14
R9		1	50.43±0.51	6.57±0.20
R10	1.5	0	34.26±0.10	5.03±0.06
R11		0.5	32.1±0.1	6.04±0.05
R12		1	40.43±0.51	5.03±0.07

Means ± standard errors

Table 4. Relative growth pattern of *Anthurium andreanum* under different acclimatization supporting mediums.

Medium for acclimatization	Code	Ratio	% Growth	Pattern	Remarks
Charcoal	AA1	100	70	Healthy Leaves and flowers	Verygood
Soil	AA2	100	40	Small curling leaves, no flowering	Not good
Sand	AA3	100	45	Small curling leaves, no flowering	Not good
Farm Yard Manure	AA4	100	40	Small curling leaves, no flowering	Not good
Coconut Husk	AA5	100	60	Healthy leaves and flowers	Better
Coconut Husk + Charcoal	AA6	50:50	95	Healthy with full size Leaves and flowers	Best
Soil + Farmyard Manure	AA7	50:50	45	Small curling leaves, no flowering	Not good
Soil + Sand	AA8	50:50	50	Small curling leaves and flowers	Not good

Transferring the somatic embryos from the dark to light and to full MS supplemented with various concentrations of BAP and kinetin gave shoot induction and multiplication (Table 3). Effective regeneration and healthy shoot development was observed on R8 media (BAP 1mg/L and Kinetin 0.5 mg/L) on MS full strength which showed maximum shoot formation in number and length of the shoots (Table 3; Fig. 4 d). Further increase in BAP concentration did not improve the rate of shoot production whereas lower amount of BAP and light enhances shoot development and proliferation. These shoots were both adventitious and axillary in nature. The shoots were sub-cultured after 60 days intervals on the same media. Subsequent cultures exhibited faster multiplication of shoots at the rate of 50 shoots within 60 days of culture. Indirect plant regeneration in the presence of BAP has been reported in different cultivars of *Anthurium* (Matsumoto and Kuehnle 1997; Joseph *et al.*, 2003).

Allowing the regenerated shoots to stand for two months on MS basal medium supplemented with BAP (1mg/L) and Kinetin (0.5mg/L) caused spontaneous rooting to occur. However, transferring the shoots to full strength MS medium without any plant growth regulators improved rooting by developing 7-9 roots on each shoot which was good for acclimatization Fig. (4e).

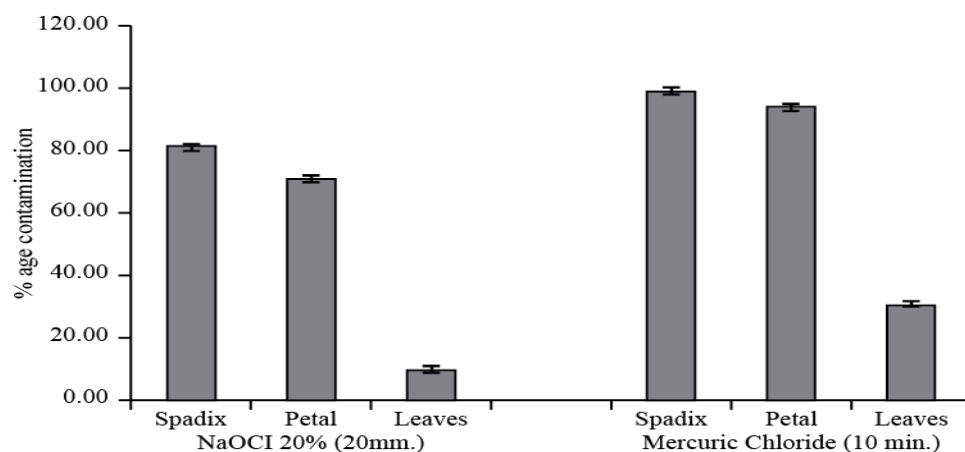


Fig. 1. Response of *Anthurium andreanum* explants to different sterilants.

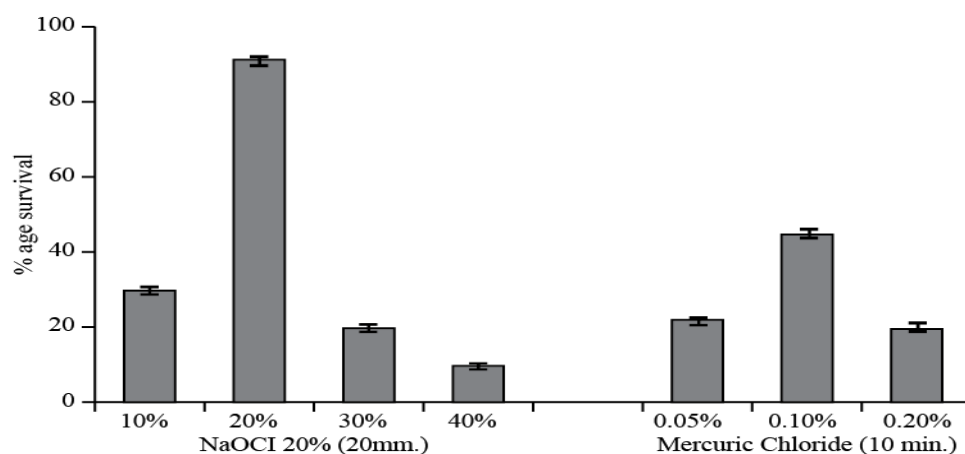


Fig. 2. Percent survival of leaf explants of *Anthurium andreanum* for various sterilant concentrations.

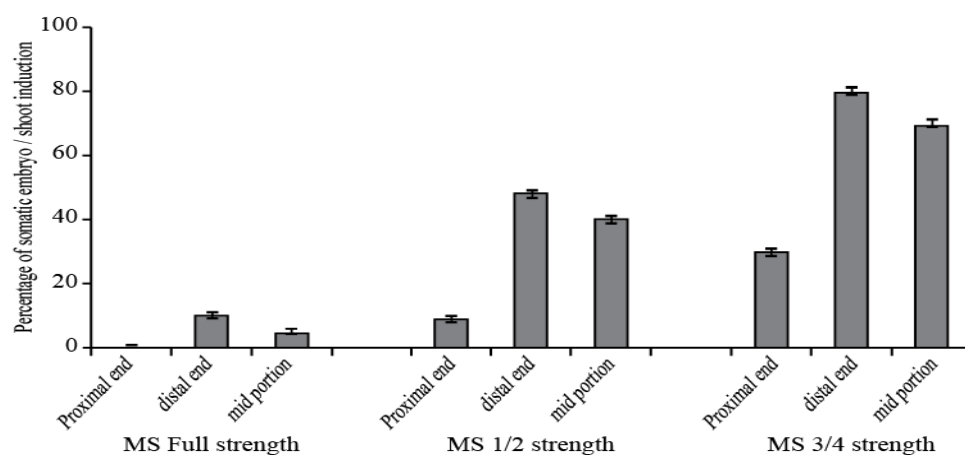
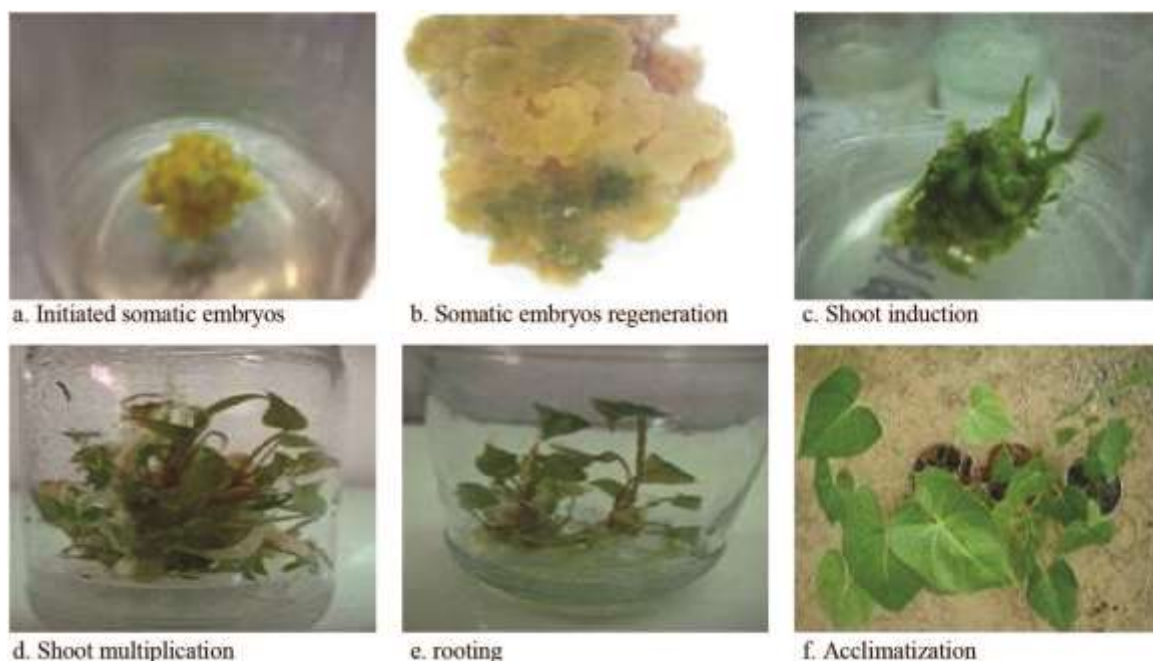
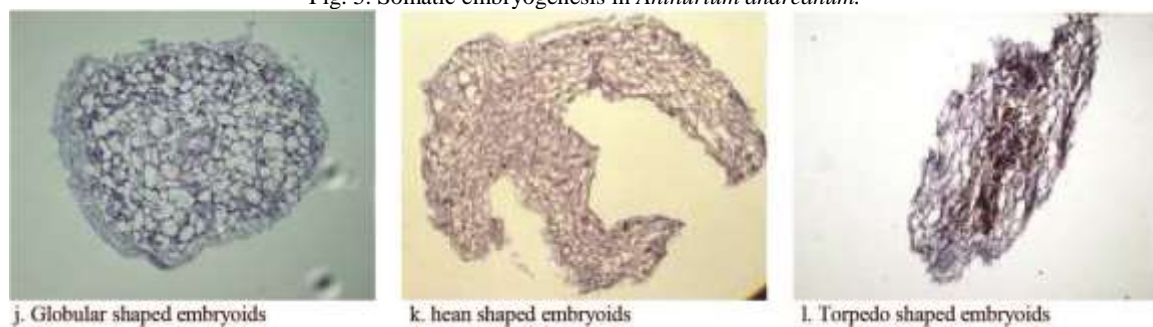


Fig. 3. Shoot induction percentage in different leaf parts of *Anthurium andreanum* under various strengths of MS salts.

After four weeks of development on the rooting medium, plantlets were transferred to pots with different potting mixtures (Table 4) which were kept in the green house for acclimatization. Under these conditions, coconut husk and charcoal (1:1 ratio) gave the best growth during acclimatization, started new growth within 15 days and continuously grew quite well Fig. 4(f). After 2-3 months of acclimatization, 95% of the plantlets survived in the green houses Fig. 7 (m, n). Finally, survived plantlets were shifted to the field.



Fig. 4. Micropropagation of *Anthurium andreaum*.Fig. 5. Somatic embryogenesis in *Anthurium andreaum*.Fig. 6. Histological structure of somatic embryos in *Anthurium andreaum*.Fig. 7. Mass scale propagation of *Anthurium andreaum* in green-house.

## Conclusion

It is concluded from the present study that the mid and distal end of the leaf explants were more responsive than the spadix and petioles. The use of less inorganic nutrient resources (low amount of ammonium nitrate) in  $\frac{3}{4}$  strength MS medium (media 8) was optimal for somatic embryogenesis in *Anthurium andreanum* which is a very rapid and direct method of propagation. Rapid clonal propagation of *Anthurium* through somatic embryos is an important technique in the field of tissue culture technology which can fulfill the demand of growers in very short time period.

## Acknowledgments

One of the authors (SN) is thankful to the HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi for providing all facilities for this research.

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(Accepted for publication July 2013)