

Micropropagation of *Anthurium* through suspension culture using *in vitro* shoots

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Abstract

A simpler and more efficient protocol for mass propagation of *Anthurium* from somatically derived *in vitro* shoots through suspension culture was developed. Shoot proliferation was obtained with agar-solidified MS medium as well as liquid MS medium, at various concentration of BAP. After 45 days of incubation at 80 rpm, both the media gave best results at 8.8 μ M BAP, whereas higher number (29.333 \pm 0.342) of shoots was obtained with liquid medium compared to solid medium (7.667 \pm 0.342). The matured plantlets were harvested and subjected to acclimatization after 30 days of primary and 60 days of secondary hardening and then they were transferred to field. Survival rate of plants (71.3 %) in liquid medium was higher compared to the ones from solid medium (43.0 %).

Key words: *Anthurium*, suspension culture, BAP and acclimatization

Introduction

Anthurium andreaeanum Linden., belonging to family *Araceae*, is an ornamental plant appreciated for its natural beauty and the colour structure of its flowers. It is one of the most important cut flower species normally grown in the tropical and subtropical countries (temperature of 16°C-30°C). A product of great business potential, cultivated mainly for its elegant flowers that come in various sizes and colors, *Anthurium's* global market among all tropical cut flowers is the second biggest, only next to Orchids. It is highly prized not only for its flowers but also for its thickness, long lasting nature, and high durability to post-harvest (Agampodi and Jayawardena, 2007; Gantait and Mandal, 2010, Junqueira and Peetz, 2012).

Conventionally, *Anthurium* is propagated through either seeds or vegetative method. Propagation through seeds is difficult due to their limited availability and poor germination rate (Dufour and Guerin, 2003). Moreover, cross pollination and the resulting off springs being heterozygous (Martin *et al.*, 2003), leads to genetic segregation and uneven production (Bejoy *et al.*, 2008). Vegetative propagation, such as - dividing old plants or taking cuttings and suckers from the rhizome is time-consuming and unprofitable, hence it became necessary to develop a rapid means of propagating *Anthurium* (Martin *et al.*, 2003). For commercial cultivation, large scale propagation through tissue culture in general, and micropropagation in particular has proved to be a better technique (Martin *et al.*, 2003). Even though micropropagation in *Anthurium* has been extensively studied, many reported methods appeared to be unproductive; some require a prolonged time for regeneration and others produce only a restricted number of adventitious shoots (Beyramizade *et al.*, 2008; Gantait and Mandal, 2010; Vargas *et al.*, 2004). *In vitro* propagation via somatic embryogenesis helps overcome the drawbacks mentioned above, such as long end-to-end timeline,

poor germination rate and low viability of seeds associated with conventional methods (Pinto *et al.*, 2011 and Konieczny *et al.*, 2010). Currently, the *Anthurium* species are propagated *in vitro* using somatic embryogenesis from leaves (Fitch *et al.*, 2011)

Tissue culture was first reported in *Anthurium* by Pierik *et al.* (1974), since then many have further refined the protocol, which is now widely applied for commercial production. However, most of the research and established protocols on *Anthurium* have been mostly through solid medium. One of the negative consequences of propagation on solid medium is that the regenerated shoots form clusters, with bases fused together. Separation of individual shoots is not only labour intensive but also time consuming. (Teng, 1997) Researches have shown that, shaken liquid culture on the other hand, can prevent formation of large clusters. (Teng, 1997). Also, under certain conditions, liquid/raft culture gives better results compared to regeneration on solid medium. (Teng, 1997).

This article describes a detailed protocol for micropropagation of *Anthurium* using somatically derived *in vitro* shoots. We have tried to understand whether liquid as a medium is better for multiplication compared to well known solid media. For comparative analysis, *in vitro* shoots from selected *Anthurium* lines (AA1, AA2 and AA3) were cultured on both solid and liquid media. This simplified procedure for rapid shoot multiplication to give qualitative and quantitative plantlets, is a novel method in terms of the hormone and media combinations in *Anthurium*.

Materials and methods

Plant material: Shoots were obtained from *ex vitro* leaves of lines of *A. andreaeanum*, cultured for callus on half-strength modified MS medium (Murashige and Skoog, 1962), supplemented with 6-Benzyladenine (BAP, *Hi-media*) and 2, 4-Dichlorophenoxyacetic acid (2, 4-D, *Hi-media*). Half-strength

MMS medium was prepared by modifying the basal MS medium for lower concentrations of ammonium nitrate (250 mg/L) and 0.1 EDTA Ferric Sodium (Duchefa biochemie). Callus generation and maturation took around 60 days of incubation. For shoot proliferation, the matured callus was incubated for around 30 days in MS medium supplemented with BAP (0.4 and 1.3 μM). The shoots so obtained were considered for further steps in the experiment. There were three replicates for each treatment, and the experiment was repeated twice.

Shoot multiplication in liquid and solid media: In the first set of experiment, liquid MS media was supplemented with different concentrations of BAP (0 - 17.7 μM) along with 3 % (w/v) sucrose. The pH was adjusted to 5.8-6.0 with 1N NaOH before autoclaving at 1.1 kg/cm² pressure (121°C) for 15 min. Then 0.5 g of *in vitro* shoots of the 3 lines (AA-1, AA-2 and AA-3) was transferred to different culture bottles containing 50 mL of media each and were kept in incubator shaker at 40, 60, 80, 100 and 120 rpm separately. Cultures were incubated under cool, white fluorescent lights (16 h photoperiod; 55 $\mu\text{mol m}^{-2}\text{s}^{-1}$, Philips, India) at 25±2.0 °C temperature and 70 % relative humidity (RH).

In the second set of experiment, 0.8 % (w/v) agar (Plant tissue culture tested -PCT0901, Hi-media) was added to the liquid media from the earlier experiment. The cultures were incubated in culture racks (instead of shaker incubator), all the remaining conditions were kept similar to that of the suspension culture. In both the experiments cultures were incubated for 45 days.

Acclimatization: Well-developed plantlets (25 plantlets from each line of both the media) were selected and planted for primary

hardening, after washing with tap water to remove traces of media followed by soaking in bavistin (0.2 %) solution for 2 minutes. The plantlets were transferred to trays containing jiffy plugs in the plant growth chambers.

After 30 days of primary hardening under artificial light, the plantlets were transferred to pots containing cocopeat in the humidity chamber of poly house. After 60 days of secondary hardening, seedlings were transferred to individual pots with potting mixture containing soil, farm yard manure and cocopeat (1:1:1). The observations on number of plants survived were recorded and the data was analysed.

Statistical analysis: The results are presented as mean values ± standard errors. Two factorial CRD was used. 10 sets were maintained under each of the 3 replications. Data were analyzed for significance by analysis of variance (ANOVA) with the mean separation by ($P < 0.05$) by Web Based Agricultural Statistics Software Package (WASP. 2) available at www.icargoa.res.in - Central Coastal Agricultural Research Institute, Goa (Al-Shawaf *et al.*, 2012).

Results

Effect of media on the lines (Table 1) shows that for each line of *Anthurium*, liquid medium gave higher number of shoots and multiplication rate compared to solid medium. Among the three lines, AA2 showed better number of shoots [liquid (11.185±0.114), solid (3.956±0.114)] and growth [liquid (7.129±0.059), solid (3.534±0.059)]. Similar results for AA1 and AA3 also confirmed that liquid as a medium is better compared

Table 1. Effect of media on number of shoots and shoots multiplication in *Anthurium* lines

Media	Number of shoots			Shoot weight (g)		
	AA1	AA2	AA3	AA1	AA2	AA3
Liquid media	8.370	11.185	7.593	3.770	7.129	3.133
Solid media	3.185	3.956	3.704	1.546	3.534	1.045
SE (m)	0.091	0.114	0.105	0.012	0.059	0.014
CD (0.05)	0.230	0.305	0.304	0.034	0.173	0.039

Values are mean of three replicates. Data were scored after 45 d of culture. CD- Critical difference; SE (m) - Standard Error mean.

Table 2. Effect of BAP and media on number of shoots in *Anthurium* lines

Treatments BAP (μM)	AA1		AA2		AA3	
	MS Suspension culture media	MS Solid media	MS Suspension culture media	MS Solid media	MS Suspension culture media	MS Solid media
0.0	1.000g	1.333e	1.000h	1.667g	0.333h	1.333e
2.2	2.000f	2.000de	2.667g	2.333efg	2.667g	1.667de
4.4	5.333e	2.667d	5.667f	3.000e	4.667f	3.000c
6.6	9.333c	4.000c	11.000d	5.333c	7.333d	5.333b
8.8	23.000a	6.333a	29.333a	7.667a	18.667a	7.333a
11.1	11.667b	5.000b	19.333b	6.333b	15.667b	6.667a
13.3	10.000c	3.667c	16.000c	4.333d	8.333c	4.333b
15.5	8.000d	2.333d	9.333e	2.667ef	6.333e	2.667cd
17.7	5.000e	1.333e	6.333f	2.000fg	4.333f	1.000e
SE (m)	0.272	0.272	0.342	0.342	0.314	0.314
CD (0.05)	0.809	0.809	1.144	0.873	0.990	1.044

Values are mean of three replicates. Means followed by the same letter are not significantly different at $P < 0.05$.

Table 3. Effect of BAP and media on shoot multiplication in *Anthurium* lines

Treatments BAP (μ M)	AA-1		AA-2		AA-3	
	MS Suspension culture media	MS Solid media	MS Suspension culture media	MS Solid media	MS Suspension culture media	MS Solid media
0.0	0.003h	0.537f	0.790h	1.130f	0.017f	0.320f
2.2	0.547g	0.717e	4.443f	1.467f	2.823c	0.543e
4.4	2.053e	1.033d	5.900e	2.300e	4.200b	1.073c
6.6	5.223c	1.777c	7.633d	4.300d	4.120b	1.653b
8.8	11.527a	5.040a	16.807a	7.300a	7.550a	3.567a
11.1	7.697b	3.400b	12.890b	6.433b	4.080b	0.820d
13.3	3.390d	0.540f	8.933c	5.340c	2.837c	0.550e
15.5	2.037e	0.397g	4.237f	2.500e	1.533d	0.730d
17.7	1.460f	0.470fg	2.530g	1.033f	1.040e	0.150g
SE (m)	0.036	0.036	0.176	0.176	0.041	0.041
CD (0.05)	0.122	0.088	0.564	0.480	0.123	0.121

Values are mean of three replicates. Means followed by the same letter are not significantly different at $P < 0.05$. Data were scored after 45 d of culture. CD- Critical difference; SE (m) - Standard Error mean.

Table 4. Effects of shaking (rpm) on number of shoots and shoot multiplication in *A. andreum*

RPM	Number. of shoots		Weight of shoots (g)	
	After 15 days	After 45 days	After 15 days	After 45 days
40	1.100 \pm 0.058c	3.000 \pm 0.577d	0.730 \pm 0.010e	1.393 \pm 0.099d
60	1.967 \pm 0.088b	5.333 \pm 0.333c	1.683 \pm 0.044c	3.373 \pm 0.090b
80	3.767 \pm 0.145a	11.000 \pm 0.577a	4.617 \pm 0.060a	8.693 \pm 0.116a
100	1.967 \pm 0.120b	6.667 \pm 0.333b	2.413 \pm 0.070b	3.400 \pm 0.15b
120	0.333 \pm 0.088d	2.667 \pm 0.333c	1.067 \pm 0.088d	2.480 \pm 0.085c
CD (0.05)	0.329	0.191	1.409	0.321

Values are mean of three replicates. Means followed by the same letter are not significantly different at $P < 0.05$. Data were scored after 15 d and 45 d of culture. CD- Critical difference; SE (m) - Standard Error mean



Fig 1. Micropropagation of *Anthurium* lines in suspension culture and solid media (a) and (d) Shoots with well developed roots after 45 d of incubation (b) and (d) weight of plantlets after 45 d of incubation.



Fig 2. Acclimatization of micropropagated *Anthurium* plants. (a) Plants acclimatized under artificial light and room temperature in growth chamber for 30 d - primary hardening (b) Secondary hardening plants in humidity chamber of green house (c) plants after 60 d of secondary hardening (d) plants in potting mixture after 90 days of potting.

to solid medium for shoot multiplication with BAP as the growth hormone.

In the absence of growth hormone (BAP), shoot initiation was negligible in both the media, while solid media showed slight growth (1.130 ± 0.176), liquid did not (0.003 ± 0.036) (Table 3). However, with the addition of BAP, both the media started to show shoot initiation within 15 days of inoculation. Over a period of 45 days of incubation, while both the media responded to their best at $8.8 \mu\text{M}$ BAP. Liquid medium [29.333 ± 0.342] number and [16.807 ± 0.176] multiplication of shoots] gave better results compared to solid medium [7.667 ± 0.342] number and [7.300 ± 0.176] multiplication of shoots] for AA2 line (Table 2 and 3). The interaction effect of BAP with liquid and solid media was explained, whereas liquid media having $8.8 \mu\text{M}$ BAP gives the best result.

Highest number of shoots was obtained at 80 rpm for cultures of both 15d (3.767 ± 0.145) and 45d (11.000 ± 0.577). The same trend was observed for weight of shoots as well (Table 4).

Acclimatization: The *in vitro* plantlets were directly harvested from the media (Figs. 1b and d) and planted into jiffy plugs (Fig. 2a). After 30 days of primary hardening in the plant growth chambers, plantlets from liquid medium showed higher survival rate (75 %) compared to that from solid medium (52 %). The survival rate after 60 days of secondary hardening (Fig 2b) in the polyhouse, was 71.3 % for the liquid medium derived plants and 43 % for the ones from solid medium. Survived plants were successfully transferred to field.

Discussion

Many researchers have reported that the conventional method of *Anthurium* propagation in many *Anthurium* species is very difficult (Hamidah *et al.*, 1997; Pierik and Steegmans, 1976; Pierik *et al.*, 1974). By several studies we came to the conclusion that, micropropagation is an alternative method for vegetative propagation and this is appropriate for selection of spices. Micropropagation helps in the production of pathogen free plants, provides a provision of year round nursery production and preservation of germplasm for use in crop improvement (Etienne and Berthouly, 2002).

On commercial scale production, micropropagation has particular characteristics which may create problems like unpredictable and costly production technology. High production costs generally limit the commercial use of micropropagation to products with a very high unit value, such as ornamentals, foliage plants and selected fruit crops (Sluis and Walker, 1985; Simonton *et al.*, 1991). Generally labour accounts for 40-60 % of production costs especially, cutting and planting represent the most expensive part of micropropagation (Chu, 1995). Along with that tissue handling and maintaining constant aseptic condition is the major and the most technical part of the work (Maene and Debergh, 1985). Variability and production of off-types individuals results in risk in the products emerging from micropropagation (Etienne and Berthouly, 2002). Other major costs results from losses during acclimatization (Reuther, 1985). It has been concluded that commercial application of micropropagation for various species would only take place, if new technologies were available

to automate procedures, and if acclimatization protocols were improved (Kitto, 1997).

To overcome the above disadvantages, liquid media are ideal in micropropagation for reducing plantlet production costs and for automation (Debergh, 1988; Aitken-Christie, 1991). Liquid culture systems can provide much more uniform culturing conditions; the media can easily be renewed without changing the container and sterilization, which is possible by microfiltration and container cleaning after a culture period. Different plant tissues from numerous species have performed better in liquid medium rather than on semi-solid medium. For instance, a larger number of shoots was produced in peach (*Prunus persica* L.) (Hammerschlag, 1982) and more somatic embryos were produced in wheat (*Triticum aestivum*) (Jones and Petolino, 1988) and cotton (*Gossypium hirsutum*) (Gawel and Robacker, 1990).

In *Anthurium*, liquid as a medium for shoot regeneration has not been given much emphasis. While Pierik (1975) for the first time induced callus in liquid medium, Leffring and Soede (1979) showed that shoots can be obtained without intermediate stages of callus formation. In 1997, Teng (1997) did a comparative analysis between solid and liquid / raft media. In alignment with Teng's observation, we see that the media has no impact on the number of days for shoot proliferation. However, due to the difference in the media combination between our study and Teng's (1997), we see an improvement of 2-3 months in the overall timeframe of *Anthurium*'s development life cycle.

With respect to shoot growth, MS basal medium supplemented with $8.8 \mu\text{M}$ BAP produced the maximum number of shoots, as confirmed by Thokchom and Maitra (2017). Teng (1997) observed that the size of the inoculums had an impact on the regeneration rate. While inoculums with $> 1000 \mu\text{m}$ size showed higher growth in raft culture (185-177 shoots/vessel) compared to solid medium, the opposite was observed for inoculums of size 500-1000 μm (20-104 shoots/vessel). However, as confirmed by many workers (Nhut *et al.*, 2006 and Leffring and Soede, 1979) liquid as a medium for shoot proliferation is better than solid medium, which was observed from experiment as well (Table 1).

Bapat and Rao (1988) reported that, higher number of synthetic seeds was produced from sandalwood at 80 rpm. Ruffoni and Massabo (1991) mentioned that, cell separation and isolation were best when the cultures were grown in the dark on a shaker set at 100 rpm in liquid medium containing 2.0 mg/L 2, 4-D. Whereas, Seydel and Dornenburg (2006) and Nhut *et al.* (2006) clearly emphasised that, cell separation and isolation were best on a shaker at 100 rpm in liquid media containing 1.5 mg/L 2, 4-D to get large number of somatic embryos. Leffring and Soede (1979) maintained 120 rpm in the orbital shaker for shoot multiplication. In our study, we standardised rpm by culturing *in vitro* shoots at different rpm 40-120 in the orbital shaker over a period of 15 and 45 days. Higher number (3.767 ± 0.145 for 15d and 11.000 ± 0.577 for 45 d) of shoots was obtained at 80 rpm.

In our present study, jiffy plugs (Bhavana *et al.*, 2018) were used for primary hardening along with cocopeat as a control. Acclimatization of *in vitro* plantlets from liquid media showed significant improvement over those from solid media. At the end of 30 days, the highest percentage (75 %) of the plantlets from

liquid medium were successfully hardened than solid medium derived plants (52 %). After 60 days of secondary hardening, 71.3 % of plants derived from the liquid medium survived and 43 % of plants from solid medium were successfully transferred to field.

In this study, we have established a simple, highly efficient, reproducible and cost effective protocol for multiplication of *Anthurium* lines from *in vitro* shoots, through suspension culture (using liquid MS media supplemented with BAP). The complete plants along with roots were obtained in large quantity within 45 days of suspension culture. Plants obtained from this technique were successfully flowered in the field. In future, Suspension culture technique may be used for large scale propagation of true to type of plants of *Anthurium*. Use of bioreactors as a potential technique, can be explored for further optimization of commercial protocols.

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