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Foliar Regeneration in *Anthurium andraeanum* Hort. cv. Agnihothri

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Abstract: A tissue culture protocol for mass propagation of the cut-flower crop, *Anthurium andraeanum* cv. Agnihothri has been worked out. Callus development was observed along the cut margins and midrib regions of the leaf lamina on half-strength MS medium supplemented with BAP and 2,4-D. Relatively older explants from pale green leaves exhibited better responses and the calli were creamy, compact and slow growing. The best dedifferentiation response of 53 percent was recorded in BAP (1 mg L^{-1}) and 2, 4-D (0. 5 mg L^{-1}) in the dark. The highest shoot regeneration potential was observed when the calli were sub cultured on BAP enriched medium. The influence of PGR regime in the induction medium on shoot production was similar to that in subsequent multiplication passage. The *in vitro* plantlets were hardened in potting mixture containing 3:1 coarse river sand and charcoal under greenhouse conditions with 89 percent survival. The technique is a suitable tool for meeting the floricultural needs of growers through mass propagation of this cut-flower crop.

Key words: *Anthurium*, Araceae, callus culture, floriculture, micropropagation

INTRODUCTION

The monocotyledonous genus *Anthurium* belongs to the family Araceae comprising of highly prized perennial herbs or climbers. Among the 500 species, only two (*A. andraeanum* and *A. scherzerianum*) are popular for their attractive long lasting flowers. The inflorescence, popularly known as the flower, is a spadix usually surrounded by a highly colourful and petaloid spathe. Anthuriums are one of the floricultural resources in the forefront of cut-flower production in India, especially in Kerala. As a high value crop with high productivity per unit area and amenable to cultivation both in metros and rural areas, anthuriums and orchids are the first among the priority floricultural crops for Kerala (Seeni and Bejoy, 1997; Singh, 1987). Because of the suitability of this crop for low cost plantation and production in the prevailing agro climatic conditions in the State, many small and medium scale entrepreneurs are engaged in this agri-business. However, availability of quality planting material is essential for commercial cultivation of such market conscious flower crops. *Anthurium* has been traditionally propagated through stem cutting or division and seeds. As a cross-pollinating species, the offsprings show poor uniformity. Besides, poor germination and short viability span make seed propagation difficult. Vegetative propagation is slow and time consuming to achieve large-scale production of propagules. Tissue culture offers an alternative tool for rapid multiplication of selected clones in a short period. In spite of the importance of this cut-flower crop, only a few tissue culture studies have been reported (George, 1996; Pierik, 1976; Joseph *et al*., 2003; Kuehnule and Sugii, 1991). In aroids, different explants such as leaf segments from *A. andraeanum* (Pierik, 1976; Joseph *et al*., 2003) and *A. scherzerianum* (Hamidah *et al*., 1997); stem apices from *Xanthosoma sagittifolium* (Gomez *et al*., 1992); tuber segments from *Zantedeschia* (Duquenne *et al*., 2006); anther filament from *Spathiphyllum* (Werhrouck *et al*., 2000) etc. have been utilised for *in vitro* regeneration studies. *A. andraeanum* cv. Agnihothri is a promising cultivar for cut-flower cultivation in the State. Therefore, the present study is carried out with an objective to develop a non-conventional mass propagation system in this plant.

MATERIALS AND METHODS

Healthy and disease free *A. andraeanum* cv. Agnihothri plants maintained in the greenhouse of the Commercial Tissue Culture Unit, TBGRI were used as mother plants for the study. Young leaf lamina collected from mature and healthy plants, 5-10 days after unfolding at the pale brown or pale green stage were used as explant source. They were surface sterilised in 15% commercial bleach for 20 min and in 0.1% $HgCl_2$ for 7 min followed by thorough washing with sterile distilled water. The lamina segments (1-1.5 cm^2) were inoculated onto half-strength

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Table 1: Dedifferentiation responses of foliar tissues in *Anthurium andraeanum* cv. agnihothri

Treatment (mg L^{-1})*			Responsiveness of explants (%)**		Callus development**	
BAP	KN	2,4-D	Pale brown	Pale green	Pale brown	Pale green
-	-	-	-	-	-	-
0.5	-	-	-	4.0	-	+
1.0	-	-	29.4	35.3	++	++
1.5	-	-	29.4	32.4	+	++
-	0.5	-	-	-	-	-
	1.0	-	8.8	14.7	+	+
	1.5	-	16.4	8.8	+	+
1.0	-	0.5	41.2	52.9	++	+++
1.0	-	1.0	35.3	44.1	+	++

*: Basal medium: ½ MS + 30 g L^{-1} sucrose + 6 g L^{-1} agar and pH 5.5; **: All data from 20 replicates after 45 days of culture. +: Poor; ++: Medium; +++: Good

Table 2: Shoot regeneration from callus cultures of *Anthurium andraeanum* cv. Agnihothri

Treatment (mg L^{-1})*		Shoot production (±SE)**	
BAP	KN	Initial regeneration	First multiplication
0.1	-	3.3±0.68^{c}	3.9±0.69^{b}
0.3	-	6.7±1.20^{d}	9.7±1.28^{e}
0.5	-	6.6±1.05^{d}	9.4±1.31^{e}
1.0	-	2.2±0.57^{ab}	2.3±0.50^{ab}
-	0.1	0.0±0.00	0.0±0.00
-	0.3	0.8±0.25^{ab}	2.3±0.87^{ab}
-	0.5	0.8±0.24^{ab}	1.3±0.37^{a}

*: Basal medium: ½ MS + 30 g L^{-1} sucrose + 6 g L^{-1} agar and pH 5.5; **: All data from 18 replicates after 60 days of culture and shoots above 5 mm. Values with same letters within the column are not significantly different ($p<0.05$)

MS basal medium (Murashige and Skoog, 1962) enriched with various combinations and concentrations of plant growth regulators (PGR), viz. 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and kinetin (KN) (Table 1). The calli obtained were subjected to various PGR treatments for inducing caulogenesis (Table 2). *In vitro* rooting of micro-shoots was investigated by using basal medium supplemented with different concentrations (0.1-1.0 mg L^{-1}) of α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) individually.

All media were supplemented with 3.0% sucrose and gelled with 0.6% (w/v) bacteriological grade agar. The pH of the media was adjusted to 5.5, prior to autoclaving at 121°C and 1.05 kg cm^{-2} for 20 min. All cultures were incubated at 25±2°C and in 16/8 h light and dark photoperiod, unless otherwise specified. At least 15-20 replicates comprised each experiment and the results were confirmed twice. The rooted shoots with 3 or more leaves were treated with 3% commercial fungicide (Dithane M 45) for 5 min and planted in earthen community pots containing coarse river sand and charcoal in the ratio 3:1. The hardened plants were re-potted in small plastic cups for further establishment.

RESULTS AND DISCUSSION

Morphogenetic response of explants cultured on induction medium was first observed along the cut ends of the lamina especially at the midrib and vein region in 4-5 weeks of culture (Fig. 1A). Foliar explants exhibited more potential for callus induction when they contained midrib or vein which was consistent with results reported earlier (Kumar *et al*., 1992; Kato, 1974). Callus development is often reported along the cut edges of explants as in *Rosa damascena* (Ishioka and Tanimoto, 1990) and *Prunus persica* (Declerck and Korban, 1996). On the other hand, callus formation was observed on the entire surface of leaf explants from adult male kiwifruit (Prado *et al*., 2007). Callus could be established at various frequencies in most of the PGR regime within 5 weeks in the dark. The explants kept in the light did not develop callus and a few of them turned brown in 2-3 weeks, while those remaining green became yellow and died in 5-6 weeks. Similar culture conditions for *in vitro* differentiation of plant tissues have been reported for other cultivars of *Anthurium* (Pierik, 1976; Kuehnule and Sugii, 1991). Whereas in *Rhododendron,* both callus production and callus survival rates were better when incubated under continuous light for six weeks than in continuous darkness (Economou and Reed, 1988). Quantitative differences in morphogenic responses observed between treatments were significant as shown in Table 1. Individual treatment of cytokinins induced callus to a maximum of 35.3% when pale green unfolded foliar explants were incubated in 1.0 mg L^{-1} BAP. KN seems to be less effective for the initiation of callus from both types of explants, while PGR-free control medium failed to induce morphogenic responses. The results are in agreement with those reported in *A. patulum*, in which BAP was found superior to other cytokinins for establishing callus cultures (Eapen and Rao, 1985). *In vitro* morphogenic responses of explants are expressed in accordance with favourable culture conditions, usually achieved by regulating PGR balance in the culture medium. For the PGRs tested, BAP along with 2, 4-D were found better in respect to rate of responsiveness and the extent of callus development. In the present study, the best dedifferentiation response of 53% was recorded in BAP (1 mg L^{-1}) and 2, 4-D (0.5 mg L^{-1}) in 6 weeks.

Studies on the suitability of different stages of leaf maturity revealed that segments from pale green leaves were preferred for callus initiation to relatively younger pale brown leaves. The explants from pale brown leaves grew in culture and attained 2-3 fold increase in size. The size of the explant is also one of the critical factors for the induction of *in vitro* response. The difference in size of

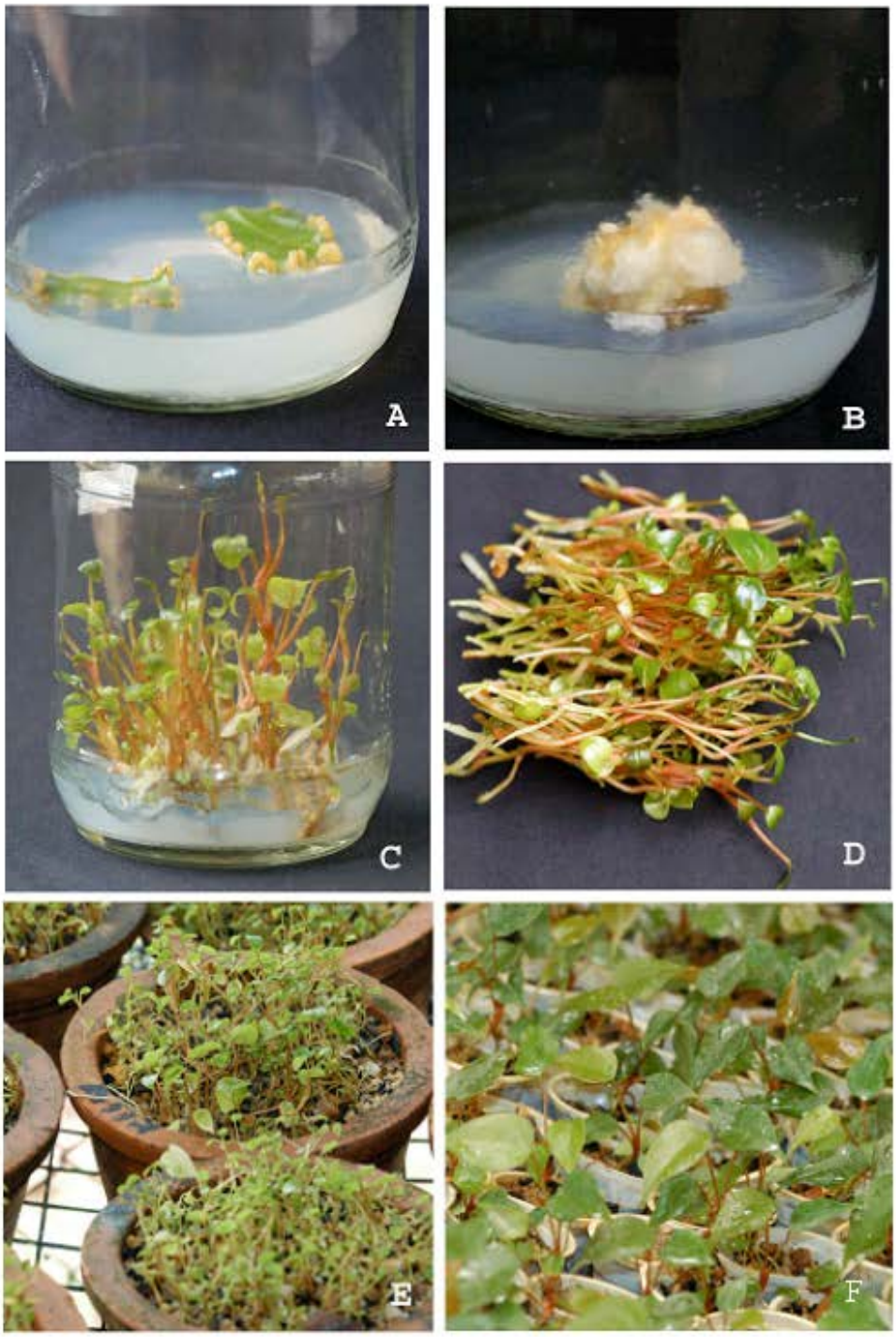

Fig. 1: *In vitro* regeneration of *A. andraeanum* cv. Agnihothri using foliar explants; (A) Callus initiation from pale green leaf segments in ½ MS + BAP 1 mg L^{-1} + 0.5 mg L^{-1} 2,4-D after 6 weeks, (B) Proliferating callus after 8 weeks of subculture, (C) Shoot regeneration from callus tissue in 10 weeks of incubation, (D) Rooted plantlets harvested for greenhouse transfer, (E) Plantlets establishing in the greenhouse after 6 weeks of transfer, (F) Fourteen week-old hardened Agnihothri plantlets in plastic wine cups

the explants has been proved as an influencing factor in early callus development in carrot roots and artichoke tubers, where optimum size consists of twenty to twenty five thousand cells (Yeoman, 1970). The growth of the explants in culture that changed the critical size is possibly the reason for low morphogenic responses from younger leaves. The rate of response of explants from pale green leaves was better and showed good callus development in the initial medium (Table 1). Superior *in vitro* morphogenic response from relatively older explants to younger ones in the present study is contrary to the report in another hybrid, wherein explants from folded brown leaves exhibited better response (Joseph *et al*., 2003).

Calli obtained initially were subcultured for further proliferation in the same medium after 6-8 weeks of incubation and they were creamy, compact and slow growing (Fig. 1B). About two-fold increase in volume of calli units has been achieved during proliferation stage. They were then subcultured onto auxin-free medium for shoot organogenesis. In the present study, the meristemoids originated as protuberances on the callus surface within 2-3 weeks of incubation in cytokinin-fortified medium were grown into small shoots. Effective regeneration and healthy shoot development were observed when the cultures were shifted from dark to 16/8 h light condition. The effect of cytokinin on morphogenic response of callus cultures of Agnihothri was significant. Basal medium containing BAP induced organogenic responses and development of shoots from foliar callus tissues (Table 2). Initially, buds appeared as pink or light brownish protuberances in 3-4 weeks when the cultures were shifted to 16/8 h light condition. Bud induction was observed more or less in all the concentrations of BAP tested. Though buds were developed on 0.1 mg L^{-1}, they showed slow growth. The best initial response of average 6.7 shoots per unit callus was observed in basal medium supplemented with 0.3 mg L^{-1} BAP. Further increase in BAP concentration did not improve the rate of shoot production while concentrations above 0.5 mg L^{-1} supported callus growth. This indicates that there is a requisite for auxin free condition to accomplish the organogenic competence and a lower concentration of BAP and light enhances shoot development and its proliferation. Similarly, callus formation has been achieved in higher concentrations of PGR while their reduction promoted shoot regeneration from callus cultures of *Scabiosa columbaria* (Romeijn and Lammeren, 1999), whereas in *Potentilla potaninni* increase in BAP concentration from 0.5-5 mg L^{-1} significantly improved the rate of multiplication (He *et al*., 2006). In KN substituted medium, 1-3 shoot buds appeared occasionally but they failed to develop in to healthy shoots. Similar reports on the efficacy of BAP on *de novo* shoot induction has been reported in many taxa such as *Hieracium auantiacum* (Bicknell, 1994), Hawaiian anthuriums (Kuehnule and Sugii, 1991) etc. Even though a few adventitious buds were initiated in the dark on 0.3 mg L^{-1} BAP, they failed to attain normal shoots. Callus grown in complete darkness developed etiolated, slender and pale pink shoots. However, they turned to normal shoots upon transfer to the light. Previous studies on micropropagation of *A. andraeanum* cultivars have also reported that the etiolated shoots produced in the dark were transformed into green shoots when reared under light (Pierik, 1976).

Further shoot multiplication has been achieved by subculturing the regenerating callus tissues after removing shoots above 1 cm, but with 2-4 young shoots below 5 mm. It was observed that the multiplication was further enhanced in the next multiplication stage (Table 2). In the second multiplication stage, the rate of shoot production was improved on basal medium containing 0.3 mg L^{-1} BAP under 16/8 h photoperiod, developed an average of 9.7 shoots/explant in 60 days. Some workers have reported initial shoot differentiation in different *Anthurium* taxa (Kuehnule and Sugii, 1991; Pierik, 1976; Eapen and Rao, 1985), but results of subsequent multiplications were never reported. Alike the influence of PGR regime in the initial regeneration media, the callus tissues followed similar shoot production response in the next passage (Table 2). In both cases BAP 0.3 mg L^{-1} was found optimal for maximum shoot production and KN seems to be less effective. The presence of BAP at lower concentrations stimulated shoot differentiation in subsequent regeneration cycles too. The basal calli were routinely subcultured after harvesting elongated shoots in 7-9 weeks of interval and achieved multiplication (Fig. 1C). In *Houttuynia cordata*, gradual declining of shoot multiplication from average 91.9-36 shoots has been found at the end of third subculture (Chakraborti *et al*., 2006). In contrast, about 2-3 fold increase in shoot production has been achieved during subsequent multiplication cycles in this cut-flower crop.

Roots developed simultaneously from about 40% of the newly formed shoots made the multiplication easier and cost effective. NAA supplemented half-MS medium was found suitable for rooting and continued shoot growth of isolated shoots. About 98% of the shoots rooted in 0.5 mg L^{-1} NAA in 6 weeks. Other auxins like IBA and IAA seems to be less effective for developing roots from *in vitro* shoots and the rhizogenic response of IBA and IAA were 75 and 65%, respectively. After the formation of good roots, the plantlets were washed thoroughly in running water (Fig. 1D) and transferred in to community pots containing coarse river sand and charcoal (3:1) mixture. The microplants regained growth within 4-5 weeks and developed new leaves in

greenhouse conditions (50% shade and 80-90% RH) and recorded 89% survival (Fig. 1E). After 2-3 months of establishment in community pots, the young plants were re-potted in small plastic cups and they reached 5-8 cm size in 3-4 months (Fig. 1F). Vegetative characters of the young tissue culture plants were identical to clonal saplings. Around 14000 plantlets could be produced in 17 months from a single leaf using this protocol.

The results revealed that propagation of phenotypically similar plantlets is possible with leaf explants of *A. andraeanum* cv. Agnihothri using tissue culture techniques. The techniques reported herein, therefore, will help to cater the needs of floriculturists through quality production of a dependable number of plants within a short span of time.

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