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Anthurium andraeanum (Linden ex André) Culture: *In Vitro* and *Ex Vitro*

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ABSTRACT

In order to optimize the establishment of both *in vitro* and *ex vitro* cultures of *Anthurium andraeanum*, recently expanded leaves from 3- to 4-year-old greenhouse plants were disinfected with 1.43% NaOCl and treated in an antioxidant solution with 150 mg.L^{-1} citric acid, 100 mg.L^{-1} ascorbic acid and 200 mg.L^{-1} cysteine. Callus formation was induced from disinfected leaf explants (± 1 cm^2) on half Murashige and Skoog (MS/2) medium supplemented with 0.08 mg.L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg.L^{-1} 6-benzylaminopurine (BAP), with and without 1 mg.L^{-1} N6-isopentenyl adenine (2iP), while shoots were induced to root on MS medium with BAP (0.0; 0.5 or 1.0 mg.L^{-1}). Somaclonal variation was not observed. Organic soil, or vermiculite, or both (1:1), or both combined with sand, *xaxim*, sphagnum, pine cone, carbonized rice hull, turf, or sawdust (1:1:1), were used to test the efficacy of *ex vitro* acclimatization. This protocol resulted in the production of regenerants in about 120 days from the *in vitro* incubation of foliar explants to the *ex vitro* establishment of plantlets: callus was formed after 56 days of incubation; aerial buds and roots appeared after 28 days on regeneration medium, and the first leaves, after 33 days; after 70 days, 3 to 4 cordiform and dark green leaves were observed. MS/2 medium + 0.08 mg.L^{-1} 2,4-D + 1 mg.L^{-1} BAP + 1 mg.L^{-1} 2iP for callus induction, and MS + BAP 0.5 mg.L^{-1} for regenerating shoots are important protocol steps. Successful *ex vitro* growth was achieved within 7 months on all substrates with the exception of that containing sawdust.

Keywords: acclimatization, Araceae, *Anthurium*, callogenesis, *ex vitro* growth, *in vitro* culture
Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **2iP**, N6-isopentenyl adenine; **BAP**, 6-benzylaminopurine; **IBA**, indole-butyric acid; **Kn**, kinetin; **MS/2**, half strength MS medium; **NAA**, α-naphthalene acetic acid; **PGR**, plant growth regulator

INTRODUCTION

The improvement in the quality of foliage and ornamental plants, especially those which can be mass propagated and have great market value, such as heliconias, bromeliads, and anthuriums, can be achieved through micropropagation. This process can generate pathogen-free clones with homogeneous phenotypes and genotypes in large numbers and within a restricted space.

Anthuriums belong to the Araceae family, which consists of 108 genera and approximately 3750 monocotyledonous species (herbaceous and creepers). Although occurring naturally on all continents (excepting Antarctica), the arums are primarily a tropical family, found in a temperature range from 16ºC to 30ºC, with the exception of *Zantedeschia aethiopica* (calla lily) that will tolerate temperatures below freezing. The genera *Anthurium*, *Dieffenbachia*, *Monstera*, *Philodendron*, *Caladium,* and *Spathiphyllum*, among others, have highly ornamental inflorescences and/or foliage (Grayum 1990). In Brazil, according to Vianna *et al*. (2001), the Araceae are particularly used as ornamentals.

The spadix (inflorescence with small flowers crowded on a thickened, fleshy axis) together with the spathe (showy and solitary bract) form an attractive ensemble, giving the anthurium a renowned and respected status within the economically important ornamentals, and allowing its use in interior and exterior decoration, in addition to its use as a cut-flower (Lopes and Mantovani 1980; Castro *et al.* 1986; Reid and Dodge 2002).

Anthuriums flower all year round, but seed propagation leads to a very heterogeneous progeny. In addition, seeds cannot be conserved, and must be collected immediately after fruit maturation. Moreover, the period from pollination to seed maturity (approximately 6 to 7 months) and the ensuing development of the plants, which takes a further three years, is too lengthy, for instance, for the selection of female traits in a breeding programme. Vegetative propagation through terminal cuttings of the stem, despite producing homogeneous material, is a very slow process, and thus not very advantageous (Pierik *et al*. 1974; Higaki and Rasmussen 1979; Geier 1990).

Parallel to micropropagation, callogenesis (or indirect shoot formation) has been used and is of great interest to genetic transformation studies, *in vitro* selection and the generation of somaclonal variation (Kuehnle and Sugii 1991). Multiplying anthuriums via callogenesis implies a long explant-plant cycle, 12 months long: three months to form calli, two months to multiply them, four months for sprout induction, one month for the formation of chlorophyll and subsequent leaf development, and another two months for rooting (Pierik 1976).

We aimed at optimizing callogenesis (callus induction) in anthuriums (*Anthurium andraeanum* Linden ex André), in order to reduce the time required for the different production stages, focusing not only on *in vitro* culture, but also on acclimatization and *ex vitro* culture.

MATERIALS AND METHODS

Newly-expanded brown leaves from 3 to 4-year-old *Anthurium andraeanum* cv. "Flamingo" plants – cultured in pots and kept at room temperature in the laboratory – were the explant sources. *In*

Received: 28 May, 2006. Accepted: 10 September, 2006.

Original Research Paper

vitro material was kept in culture chambers, at 25 ± 2ºC, in the dark, during callus induction, but under a 16-hour light photoperiod during the regeneration and pre-acclimatization stages. Micropropagated material during the acclimatization and *ex vitro* culture stages were kept indoors.

Leaves were washed in running water for 20 minutes, passed through 70% ethanol, then immersed in commercially-available sodium hypochlorite (1.43% active chlorine) to which two drops of Tween-20 were added. After 20 minutes, they were rinsed three times in sterile distilled water. Leaves were then immersed in an antioxidant solution containing 150 mg.L^{-1} citric acid, 100 mg.L^{-1} ascorbic acid and 200 mg.L^{-1} cysteine for 20 minutes. Explants (± 1 cm^2), were cut on Petri dishes in this antioxidant solution, where they were kept for 10 minutes (M. T. R. Rocha, unpublished data). Subsequently they were placed on the conditioning medium as described by Castro *et al*. (1986).

For callus induction, the basal medium MS (Murashige and Skoog 1962) was used, based on Pierik *et al*. (1974) protocol: half-strength MS medium (i.e. half concentration of macronutients = MS/2) with vitamins, and supplemented with 0.08 mg.L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg.L^{-1} 6-benzyladenine (BAP) and 7.5 mg.L^{-1} agar (Bacteriological Agar, Vetec, Brazil), with or without 1 mg.L^{-1} N6-isopentenyl adenine (2iP). Explants were placed on the medium, adaxial side down. Treatments consisted of 5 explants per flask, with 7 repetitions, in an A x B (A = 2 callus induction media and B = 10 observations) factorial experiment, completely randomized. Material was observed weekly for a period of 70 days in order to detect signs of callogenesis.

The calli were then placed on 30 mL MS, supplemented with 0.0, 0.5 or 1.0 mg.L^{-1} BAP. Each treatment comprised 3 calli per glass jar (with 500 mL capacity), 5 jars, 15 calli total, and were subcultured onto fresh medium every 30 days, in order to remove necrotic sections. This procedure was repeated until the appearance of shoots, then roots. The number of shoots per callus piece was counted weekly up to 70 days. The experiment was conducted in an A x B factorial (A = 3 regeneration media, B = 10 counts), in a completely randomized design, with 5 repetitions.

Table 1 Plant height and number of leaves per plant regenerated from calli induced from *Anthurium andraeanum* leaf mesophyll tissue, cultured *ex vitro* on different substrates for 29 weeks.

Substrate			Variables	
Name	**Composition**	**Ratio**	**Plant height (cm)**	**Number leaves/plant**
S	Organic soil (S)	1	4.26 ab	3.31 ab
V	Vermiculite (V)	1	5.47 ab	3.62 ab
SV	S + V	1:1	7.48 a	4.92 a
SVSan	S + V + sand	1:1:1	6.99 a	5.51 a
SVX	S + V + xaxim	1:1:1	6.96 a	5.62 a
SVSph	S + V + sphagnum	1:1:1	6.86 ab	5.95 a
SVPc	S + V + pine cone	1:1:1	6.67 ab	5.50 a
SVCrh	S + V + carbonized rice husks	1:1:1	5.54 ab	3.39 ab
SVT	S + V + turf	1:1:1	3.96 ab	2.26 ab
SVSaw	S + V + sawdust	1:1:1	2.44 b	1.37 b

Means followed by the same letter within a column remained the same after Duncan's test (α = 0.05).

The jars with 3-4 cm high plantlets, presenting 2 to 4 leaves, were left open in the culture chamber for 3 days (pre-acclimatization period). Then, the plantlets from which agar was removed were placed in pots containing vermiculite moistened with a nutritive solution composed of 1 mL.L^{-1} of each MS stock solution (except for the vitamin solution), where they were kept for 7 days, in the laboratory. After that, they were transplanted into pots, each containing one of the 10 different substrates, simple or mixed (**Table 1**). The vermiculite and the substrates (**Table 1**) were all autoclaved before transplantation. The experiment was conducted in an A x B factorial (A = 10 substrates, B = 29 counts and observations), in randomized blocks with sub-divided parcels, with 4 repetitions. The number of leaves as well as plant height (in cm) was determined on the day of transplantation to the substrates, and then weekly for a total of 29 weeks.

RESULTS AND DISCUSSION

Callogenesis

The importance of using newly-expanded brown leaves instead of green lamina derived explants for calli induction, and the oxidation and necrosis showed by the foliar lamina explants, which die later in culture, was stressed by Martin *et al*. (2003). In 2001, the UPLB protocol stressed that young leaf and petiole explants would oxidize and eventually die after 12 days. As a result, we chose to use brown leaves and immerse them in an antioxidant solution.

The statistical analysis of callus induction with or without 2iP was carried out with data from the 35th day in culture alone, since callogenesis only began after the 40th day in culture. The data in **Table 2** shows that the difference in callus induction, which depends on the culture medium, will only become evident after the 56th day of culture. After this period, it was shown that the 2iP-supplemented medium was significantly superior (α = 0.05) to the 2iP-free medium. At 70 days culture, the shoot number in the 2iP-containing medium is almost double the number of shoots in the 2iP-free medium, when one compares the callus induction cultures 56 and 63 days old.

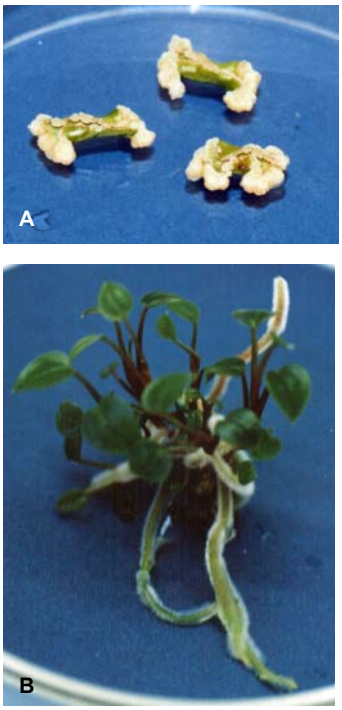

Fig. 1 ***Anthurium andraeanum in vitro*****.** (**A**) creamy colour leaf mesophyll-derived calli, (**B**) plantlets with leaves and vigorous roots regenerated.

Table 2 Average number of calli induced from *Anthurium andraeanum* leaf mesophyll, placed on MS/2 medium supplemented with and without 1 mg.L^{-1} 2iP during 70 days.

MS/2	Days in culture					
medium	**35**	**42**	**49**	**56**	**63**	**70**
with 2iP	0.00 a	0.03 a	0.03 a	0.29 a	0.29 a	0.41 a
without 2iP	0.00 a	0.05 a	0.05 a	0.05 b	0.08 b	0.08 b

Means followed by the same letter within a column remained the same after Duncan's test (α = 0.05).
MS/2 = MS with half the concentration of macronutrients
2iP = N6-isopentenyl adenine.

The number of calli induced from leaf explants from day 1 to 70 days in culture is highly significant (α = 0.01), with a positively-increasing linear regression, and with an 88% coefficient of determination, showing a remarkable impact of the choice of the medium, especially that containing 2iP.

Pierik *et al*. (1974) highlighted that good results could be obtained if MS/2, supplemented with 0.1 or 0.08 mg.L^{-1} 2,4-D, was used. Pierik (1976) also attempted to optimize this shoot induction medium by adding 1.0 mg.L^{-1} BAP. The

importance of the cytokinin BAP for callus induction in anthurium has been reinforced by studies carried out by Kunisaki (1980), Castro *et al*. (1986), and Kuehnle and Sugii (1991), who used 0.2 to 1.0 mg.L^{-1} of this cytokinin in a full-strength MS medium. The importance of applying cytokinins for callus induction in anthurium was demonstrated by the better results obtained by Pierik *et al*. (1979), who added 10 mg.L^{-1} 2iP to the medium.

Soczek and Hempel (1989) confirmed that the induction of calli from hybrid *A. andraeanum* nodal segments improves with an increase in the concentration of the applied cytokinin. They used MS/2 supplemented with 0.125, 0.25, 0.5, 1.0 and 2.0 mg.L^{-1} of each of the following plant growth regulators (PGRs), respectively BAP, kinetin (Kn), zeatin and 2iP. N medium (Nitsch 1969), supple-mented with 1 mg.L^{-1} BAP and 0.1 mg.L^{-1} 2,4-D, was used for callus induction in *A. andraeanum* leaf explants by Malhotra *et al*. (1998) and Puchooa (2005). Puchooa and Sookun (2003) obtained calli after a two-week culture in this medium. Other good results of callus induction in anthuriums were obtained when these two PGRs were used and applied at various concentrations in MS medium: 0.1 mg.L^{-1} 2,4-D and 1.0 mg.L^{-1} BAP in leaf, petiole, internode and root explants (Somaya *et al*. 1998), or 0.3 mg.L^{-1} 2,4-D and 0.5 mg.L^{-1} BAP in leaf blade and basal sprouts explants (Yakandawala *et al*. 2000).

Joseph *et al*. (2003) also concluded that the MS/2 medium supplemented with 0.88 µm BAP, 0.90 µm 2,4-D and 0.46 µm Kn was more effective for callus induction from petioles and leaf blades in three commercial cultivars of *A. andraeanum* ("Lima White", "Tropical White" and "Tropical Red"), having obtained a 60.8, 67.2 and 56.4% callus induction rate in these three cultivars, respectively, during a 50-day period of incubation.

Castro *et al*. (1986) obtained calli formation in 15% of leaf explants. Devinder-Prakash *et al*. (2001) obtained calli from *A. andraeanum* petioles after culture on MS medium with 0.5 or 1.0 mg.L^{-1} 2,4-D. Callogenesis was induced at 50-75% at these concentrations, and higher concentrations proved to be toxic. Vargas *et al*. (2004) obtained calli from plants derived from *in vitro*-germinated seeds and from micro-cuttings after 4 and 8 weeks, respectively, when placed on MS with 4.4 µM BAP and 0.05 µM naphthalene acetic acid (NAA), doubling in size after 6 weeks. Looking for a direct plant regeneration protocol, a good callus induction level was obtained by Martin *et al*. (2003), in 70% of the explants of the cv. "Tinora Red" and "Senator", with the use of MS/2 plus 1,11 µM BAP, 1,14 µM IAA and 0,46 µM Kn, but after shoot initiation. However, these authors did not use those calli because of the possibility of somaclonal variation.

We obtained a good callus induction level (45.7%) when the explants were cultured on MS/2 medium with 0.5 or 1.0 mg.L^{-1} 2,4-D. In addition, when we used this medium supplemented with 2iP, calli appeared within 56 days, a result similar to those reported by Castro *et al.* (1986) and Vargas *et al*. (2004), who observed calli after 60 days, and by Devinder-Prakash *et al*. (2001), who registered them after 42-56 days. All these results are faster than the 120 to 160 days as described by Pierik (1976).

Shoot regeneration

The calli changed from a creamy colour (**Fig. 1A**) to dark green within the first week, initiating growth and differenttiation and increasing somewhat in size when submitted to a 16-hour photoperiod. Primordial shoots appeared after 28 days on regeneration medium, and the first leaves appeared after 5 days. Each shoot produced 3 to 4 leaves throughout the 70 days of observation. These leaves were morphologically normal, dark green in color and cordiform (**Fig. 1A**), typical of *A. andraeanum*. Somaclonal variation was not observed.

The significant differences in growth rate during shoot regeneration were observed only after the 28th day of culture on BAP-supplemented media, or after the 42nd day on BAP-free media. The differences between media containing different concentrations of BAP could only be distinguished after the 63rd day of culture, when medium with 0.5 mg.L^{-1} BAP showed to be superior to that containing 1.0 mg.L^{-1} BAP, as shown in **Table 3**.

Table 3 Average regeneration rate from the shoots of *Anthurium andraeanum* leaf mesophyll-derived calli when on MS medium supplemented with different concentrations of BAP (0.0, 0.5 and 1.0 mg.L^{-1}).

BAP (mg.L^{-1})	Days in culture						
	28	**35**	**42**	**49**	**56**	**63**	**70**
0.0	0.00 a	0.00 a	0.00 b	0.33 b	0.68 b	0.68 c	0.98 c
0.5	0.00 a	0.16 a	0.68 ab	1.47 ab	3.93 a	7.24 a	8.65 a
1.0	0.00 a	0.53 a	1.25 a	1.74 a	2.71 a	3.93 b	4.85 b

Means followed by the same letter within a column remained the same after Duncan's test ($\alpha = 0.05$).
BAP = 6-benzylaminopurine.

The number of shoots formed during the 70-day culture is highly significant ($\alpha = 0.01$), with an increasing positive linear regression, with 91%, 95% and 99% coefficients of determination for 0.0, 0.5 and 1.0 mg.L^{-1} BAP-supplemented MS medium, respectively. These data demonstrate that a medium containing this cytokinin results in constant regeneration and growth of shoots until the 10th week of culture.

Montes *et al*. (1999) observed that *A. cubense* leaf explants produced more than 15 adventitious sprouts in 60 days, when cultured on MS supplemented with low concentrations of BAP (0.001 to 0.004 mg.L^{-1}). However, as Trujillo *et al*. (2000) pointed out, 3 mg.L^{-1} BAP was the best concentration for the regeneration and multiplication of *A. andraeanum* plantlets obtained from leaf-derived callus. Other protocols for *A. andraeanum* show similar results to ours, such as Malhotra *et al*. (1998), Yakandawala *et al*. (2000), and Puchooa (2005), who used MS medium with 0.5 mg.L^{-1} BAP. Puchooa and Sookun (2003) used the same concentration of BAP in N medium. Orlikowska *et al*. (1995) obtained most intensive regeneration of shoots in 18% of root explants, 58% of petioles, and 78% of leaves when incubated in MS medium with 1 mg.L^{-1} BAP and 0.2 mg.L^{-1} 2,4-D. Soczek and Hempel (1989) had already observed better shoot proliferation with 0.5 to 1.0 mg.L^{-1} zeatin on MS medium. Vargas *et al*. (2004) obtained an average of 43.8 plantlets per green callus when the calli were placed on MS medium supplemented with 8.9 µM BAP or 2.7 µM NAA.

All plants rooted spontaneously approximately 40 days after the first shoot's emergence, and no particular medium was needed, a fact that had been observed previously by Pierik *et al*. (1979), Kuehnle *et al*. (1992), Puchooa and Sookun (2003), and Vargas *et al*. (2004). Somaya *et al*. (1998) emphasized that rooting occurs more easily without the addition of PGRs, although their study shows that the addition of 0.25 mg.L^{-1} NAA increases the quality and number of roots produced. Malhotra *et al*. (1998) and Puchooa and Sookun (2003) claimed that the speed and frequency of rooting could be significantly improved with the addition of 0.04% activated charcoal in an N medium supplemented with 1 mg.L^{-1} IBA (indole-butyric acid).

In a unique study, Teixeira da Silva *et al*. (2005) show that well-rooted *A. andraeanum* "Elizabeth" shoots can be obtained within one month on PGR-free medium (rockwool or agar) when cultured under CO_2-enriched MS/2, i.e. photoautotrophic micropropagation.

Our protocol substantially decreases the explant-plant cycle, creating an *in vitro* period of only four months: two for the formation of calli, and two for regeneration and rooting, when compared to almost 12 months required by Pierik (1976), and by Tombolato *et al*. (2002 *apud* Fuzitani and Nomura 2004).

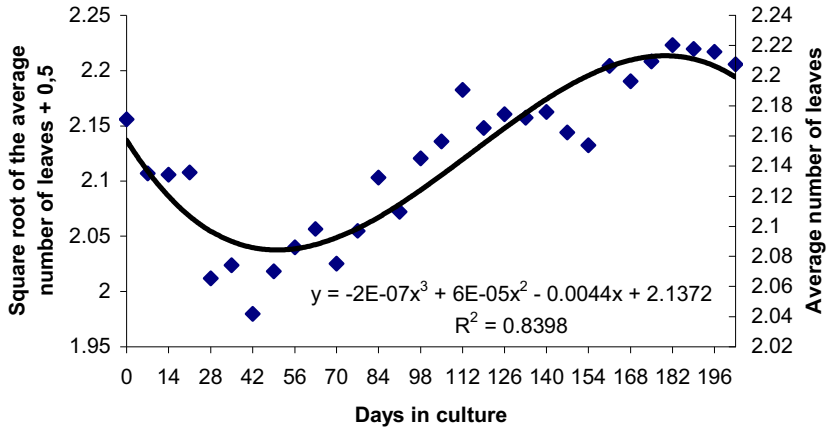

Fig. 2 Number of leaves in regenerants from *Anthurium andraeanum* leaf mesophyll-derived calli during a 29-week culture period *ex vitro* in the 10 different substrates.

Acclimatization and *ex vitro* culture

There were no losses during the pre-acclimatization period, neither in the growth chamber, nor in the vermiculite acclimatization in the laboratory. These results are in agreement with those of Orlikowska *et al*. (1995) who obtained 96% to 100% of shoots – 2-3 cm in length – that were able to acclimatize in the greenhouse. In this same year, Yu and Paek find out that fine bark was effective in shoot and root growth of the regenerants (survival rate over 90% in all treatments) compared with combined medium of peatmoss and perlite. Atta-Alla *et al*. (1998) observed that when *Anthurium parvispathum* micropropagated plants were acclimatized in a mixture of turf, tree bark and soil (1:1:1) there was a loss of just 2% of material during acclimatization and for the following 3-month growth period. According to Puchooa and Sookun (2003), plantlets with well-developed roots maintain well after transplantation to vermiculite, and grow under low light intensity and high humidity, without losses, even when they are transferred to the greenhouse after 2 months. In contrast, plants with either few or without roots showed large losses when transferred to vermiculite. Vermicompost and sand (1:3) maintained a 97% rate of survival when the plantlets were transferred to the greenhouse for a 2-week period of acclimatization. Vargas *et al*. (2004) mantained plantlets in the regeneration medium for 4 months, and another 3 months in PGR-free MS before transplantation into pots with a mixture of organic soil and organic humus (1:1), and had an acclimatization success rate of 80%. Teixeira da Silva *et al*. (2005) demonstrated that, when photoautotrophic micropropagation was used, there was no need for an *ex vitro* acclimatization phase, since plants grown *in vitro* are hardened-off and formed well developed stomata and root complexes, meaning the *in vitro* acclimatization with a subsequent transfer of plantlets directly to the greenhouse resulted in a 100% survival rate.

The plants grown in SV, SVSan and SVX were on average taller, differing statistically only from SVSaw, and the plants that grew on the SVSph, SVX, SVSan, SVPc and SV substrates produced a higher average number of leaves, also differing, in performance, from plants grown on SVSaw (**Table 1**). In general, regenerated anthuriums had a successful *ex vitro* growth on all studied substrates, with the exception of that composed of soil-vermiculite and sawdust (SVSaw).

Growth parameters of the plants grown in the different substrates point towards an effective increase in height up to 29 weeks of growth *ex vitro*, with the greatest number of leaves being evident 182 days after transplant (**Fig. 2**). Similar results were obtained for *A. andraeanum* by Ajithkumar and Nair (1998), and for *A. cubense* by Montes *et al*. (1999), who could achieve an excellent acclimatization and growth *ex vitro* in micropropagated plantlets that were 2.5 to 3.0 cm in height and had 3 to 4 leaves.

Somaclonal variation was never observed in our experiment, results in common with those of Orlikowska *et al*. (1995), Joseph *et al*. (2003) and Vargas *et al*. (2004) who reported that regenerants had the same morphology as the *A. andraeanum* mother-plants which were used as the explant source.

The present study also shows that xaxim – the trunk of a tree fern of the *Dicksonia sellowiana* Hook species (Dicksoniacea) which is becoming extinct and is considered to be of illegal trade in the southern states of Brazil (Rio Grande do Sul, Santa Catarina and São Paulo) – can be effectively substituted by substrates such as sand, or even others that are easily and readily available in those states, such as carbonized rice hulls (residues from rice crops) and pine cones (pine fruit from plants of the *Araucaria* L. Jussie genus, that are abundant in forests and gardens).

CONCLUDING REMARKS

Culture time *in vitro* of *Anthurium andraeanum* (callus induction and plant regeneration from young leaf mesophyll) can be substantially reduced to four months by culturing explants on MS with half the concentration of macronutrients, to which 0.08 $mg.L^{-1}$ 2,4-D, 1 $mg.L^{-1}$ BAP and 1 $mg.L^{-1}$ 2iP have been added to induce calli, and full strength MS with 0.5 $mg.L^{-1}$ BAP to induce shoot regeneration and subsequent rooting.

Anthurium regenerants show an excellent transition and adaptation during both the pre-acclimatization period (in open glass jars, mantained in culture chamber for 3 days) and the acclimatization period (in vermiculite, mantained in the laboratory for 7 days), with subsequent good growth *ex vitro* (for 29 weeks) on all substrates made up of organic soil, or vermiculite, or both (1:1), or both combined with either sand, xaxim, sphagnum, pine cone, carbonized rice hull or turf (1:1:1), with the exception of that containing sawdust.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Beatriz Viégas-Faria for the English revision, Dr. João Baptista da Silva for the statistics assistance, and Nelson Caravaca dos Santos for the laboratory help.

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