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IMPROVEMENTS IN THE QUALITY OF MICROPROPAGATED *ANTHURIUM ANDREANUM* L. PLANTLETS BY THE USE OF BILAYER CULTURE TECHNIQUES: PRELIMINARY RESULTS

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ABSTRACT

Anthurium andreanum is becoming increasingly important in the Caribbean, playing a part in the agricultural diversification programs of many governments. Planting material is generally mass produced via micropropagation, during which *Anthurium* morphogenic callus is usually transferred to solid multiplication medium for shoot regeneration. The regeneration rate and the shoot quality, however, can sometimes be very poor, necessitating a prolonged culture period to produce plantlets of sufficient size and quality for weaning. The use of liquid media is known to enhance shoot regeneration in some species, but it also requires costly equipment in the form of rotary or oscillating shakers, and can induce shoot hyperhydricity (vitrification). An intermediate media form requiring no additional equipment, namely the bilayer, has many of the advantages of liquid culture with less of a tendency to induce hyperhydricity. The use of bilayer culture media was shown to stimulate and prolong *Anthurium* shoot production. Plantlets produced in this way were larger, with a greater number of leaves. The leaves, also, had a greater surface area and they appeared to be much darker green than those of the control. Further experiments indicate that these plantlets are hardier, surviving the weaning process well and quickly initiating new growth. The simple use of bilayer techniques can, therefore, be used to improve the quality of micropropagated *Anthurium* plantlets, while reducing the production time. This improved micropropagation efficiency better enables planting material produced in the Caribbean to compete with imports.

INTRODUCTION

Governments throughout the Caribbean are increasingly developing agricultural diversification programs to aid in the improvement and expansion of their agricultural export industries. A component of these programs is often ornamental crops, mainly cut flowers, which have a ready local market, including hotels, restaurants and cruise ships, while also having tremendous export potential. Tropical flowers, or 'exotics', form only a small proportion, approximately 3% (Rajkumar, 1991), of the total world trade in cut flowers. The wholesale market for *Anthurium* alone is US$17.8 million in North America and US$25.6 million in Europe (International Trade Centre, 1990) and even a small proportion of this market equates to a fairly large amount of foreign exchange.

Anthurium andreanum is an outbreeding species and can be propagated by seed, but cultivars propagated this way have poor uniformity and it can take four years, after fertilization, before the plants are large enough to be evaluated (Geier, 1990). This wide variation is invaluable to breeders but clearly unsatisfactory for mass propagation. *Anthurium* can also be propagated vegetatively by terminal cuttings, stem sections or suckers, but this method is again unsatisfactory for mass propagation as the multiplication rates are low. Micropropagation techniques have been successfully applied to *Anthurium* and they are now generally mass produced in this way. In the Netherlands alone, over half a million *Anthurium* plants are produced by micropropagation per year (Pierik, 1991).

A number of alternative micropropagation schemes, with varying degrees of success, have been proposed, firstly by Pierik *et al.* (1974) and then Kunisaki (1977, 1980) and Leffring and Soede

(1978, 1979a,b). These have been refined over the intervening years and are summarized by Geier (1990) in Figure 1.

Morphogenic callus tissue is generally transferred to solid multiplication medium for shoot regeneration. These shoots are removed and transferred either to root induction media or to multiplication media for further growth. The remaining clump of callus tissue and shoot initials is returned to fresh multiplication media for further development and shoot production. Shoots cannot be induced to form indefinitely from this clump and the rate of shoot production decreases dramatically after four to five subcultures (Geier, 1990; Personal observation - results not presented).

A series of experiments was performed with three initial aims: to increase the rate of shoot formation from *Anthurium* callus; to increase the number of subcultures from which good quality shoots could be obtained; and to assess the improvement, if any, in the quality of these shoots.

The physical characteristics of the media were manipulated in order to stimulate and prolong shoot production. A number of workers have used liquid culture as part of an *Anthurium* micropropagation scheme, including Leffring and Soede (1979b) and Kunisaki (1980). The use of liquid media is known to enhance shoot regeneration in some species (Chu *et al.*, 1993; Paranjothy, 1984; Short, 1986), but this requires costly equipment, in the form of rotary shakers, and can in some cases induce shoot hyperhydricity (Debergh *et al.*, 1992), whereby the shoots become unusable. An intermediate media form requiring no additional equipment, namely the bilayer, has many of the advantages of liquid culture with less of a tendency to induce hyperhydricity.

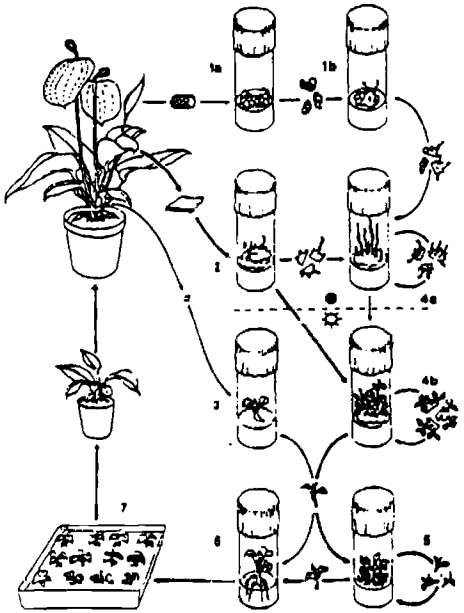

Fig. 1. Micropropagation of *Anthurium*. (1a,b) Establishment of caulogenic callus from spadix sections. (2) Establishment of caulogenic callus from leaf sections. (3) Shoot establishment from axillary bud explants. (4a) Multiplication of caulogenic callus in darkness. (4b) Multiplication of caulogenic callus under illumination. (5) Multiplication of isolated shoots by shoot proliferation. (6) Rooting of isolated shoots. (7) Establishment of plants in soil or potting mix.

MATERIALS AND METHODS

A range of culture media for *in vitro* multiplication of *Anthurium*, mainly based on Murashige and Skoog (1962), are being used at the CARDI Tissue Culture Laboratory, Barbados. There appears to be a varietal response to nitrate levels in the media (data not presented) which has also been noted by other workers (Geier, 1990; Kuehnle and Sugii, 1991), however, one media (lab code - MS2) has shown good results with many of the varieties under test and, therefore, was used for this series of experiments.

The multiplication media, MS2, was as Murashige and Skoog (1962) supplemented with sucrose (20 gl^{-1}) and benzylaminopurine (0.25 mg l^{-1}). Media was placed in Magenta culture vessels (Sigma Chemical Co., USA) either in the form of 50 ml solid media (containing 7gl^{-1} agar), 50 ml liquid media (on a rotating bed) or a bilayer of 25 ml solidified media overlaid with 25 ml liquid media.

Five morphogenic callus clumps each of the varieties Hybrid Red and Hybrid Pink var.2 were used. Each clump was approximately 2 cm in diameter and was divided into three equal pieces which were placed in solid, liquid and bilayer culture conditions. After 4 weeks each clump was hedged of all shoots larger than 0.5 cm. These were transferred to multiplication media for further growth and multiplication. The remaining clump of callus and shoot initials was placed in fresh MS2 media. This cycle was continued until no further usable shoots were obtained. Shoots showing hyperhydricity, fasciation or an abnormal appearance were discarded and not recorded.

In the second experiment four varieties were used, namely Hybrid Pink var.2, Hybrid Red, Common Pink and Hybrid Pink var.1. For each variety, ten large clumps (approximately 4 cm diameter) consisting of morphogenic callus, shoot initials and very small shoots (less than 0.5 cm) were used. These were each equally divided and one half placed on 50ml of solidified MS2 media in a Magenta container. The other half was placed in a Magenta container with a MS2 bilayer (25 ml solid / 25 ml liquid).

The clumps were allowed to grow and multiply for 8 weeks, after which all shoots over 1 cm high were removed. Out of these, 20 shoots per variety per production system were randomly selected and their leaf surface area measured.

RESULTS AND DISCUSSION

The shoots produced by both Hybrid Pink var.2 and Hybrid Red in weeks 4, 8 and 12 generally showed no morphological abnormalities and were used for further micropropagation; however, in weeks 16 and 20 there were many more distorted shoot initials. These were discarded and this is reflected in the low numbers of usable shoots for these weeks (Table 1). The reduced quality of shoots did not appear to correlate with any media type; they were all morphologically abnormal and no hyperhydricity was seen, even in the liquid cultures.

The use of bilayer or liquid media, instead of the more conventional solid media, resulted in a greater number of usable shoots being produced, with the production continuing over a slightly longer time period. In these respects, also, use of the bilayer media form was an improvement over the liquid media.

There appears to be varietal differences in the numbers of usable shoots produced. This may relate to the varying varietal growth responses to different media compositions (Geier, 1990; Kuehnle and Sugii, 1991) and for each variety the media may have to be designed specifically in order to maximize the growth responses.

Table 1. Shoot Production from Callus Clumps of Hybrid Red and Hybrid Pink var.2.

Variety	Media Type	Number of shoots removed at:					
		Week 4	Week 8	Week 12	Week 16	Week 20	TOTAL
Hybrid Red	Solid	mean = 4.4 sd = 0.55	mean = 3.0 sd = 1.22	mean = 1.2 sd = 0.84	mean = 0.4 sd = 0.55	mean = 0.0 sd = 0.00	mean = 9.0[a] sd = 1.87
	Bilayer	mean = 9.4 sd = 0.89	mean = 6.2 sd = 1.30	mean = 4.6 sd = 0.89	mean = 2.0 sd = 0.71	mean = 0.8 sd = 0.84	mean = 23.0[b] sd = 1.22
	Liquid	mean = 7.2 sd = 0.84	mean = 4.4 sd = 1.67	mean = 2.0 sd = 0.71	mean = 1.2 sd = 0.45	mean = 0.2 sd = 0.45	mean = 15.0[c] sd = 2.55
Hybrid Pink var.2	Solid	mean = 2.6 sd = 0.55	mean = 1.8 sd = 0.45	mean = 1.0 sd = 1.0	mean = 0.2 sd = 0.45	mean = 0.0 sd = 0.00	mean = 5.6[d] sd = 2.07
	Bilayer	mean = 6.0 sd = 0.71	mean = 4.0 sd = 1.0	mean = 3.6 sd = 0.89	mean = 1.2 sd = 0.84	mean = 0.2 sd = 0.45	mean = 15.0[e] sd = 2.92
	Liquid	mean = 5.4 sd = 0.55	mean = 3.8 sd = 0.84	mean = 2.8 sd = 1.30	mean = 1.4 sd = 1.14	mean = 0.2 sd = 0.45	mean = 13.6[f] sd = 3.85

Results with differing superscripts are significantly different ($p<0.05$) using ANOVA performed on transformed data.

Shoot production from callus can, therefore, be improved by using a liquid or bilayer system, with the latter eliciting the most improvement. Liquid culture usually necessitates the use of a shaking bed to keep the cultures aerated and this can be an expensive piece of equipment, especially for a small laboratory. It is heartening, therefore, to see that by the simple use of bilayer techniques the advantages of liquid culture can be gained without the extra equipment costs. Consequently the bilayer technique has been introduced into the standard *Anthurium* micropropagation protocol used at the CARDI Tissue Culture Laboratory.

It was noted that the shoots produced in the bilayer system appeared to possess larger, darker green leaves than those in the conventional (solid media) system. Preliminary experiments are underway to quantify those factors which are perceived to indicate improved shoot quality. There was also a need to study the effects of the technique on other varieties and so further work continued with the addition of the varieties Common Pink and Hybrid Pink.

The leaf surface areas of shoots produced in the conventional (solid media) system and in the bilayer system were measured. The preliminary results are shown in Table 2.

Table 2. Leaf surface area of plantlets produced in the conventional and bilayer production systems.

Variety	Leaf surface area - conventional system	Leaf surface area - bilayer system	Percentage increase in leaf area
Common Pink	mean = 0.14cm^2 sd = 0.06cm^2	mean = 0.78cm^2 sd = 0.28cm^2	457%
Hybrid Red	mean = 0.13cm^2 sd = 0.09cm^2	mean = 0.71cm^2 sd = 0.13cm^2	446%
Hybrid Pink var.1	mean = 0.14cm^2 sd = 0.11cm^2	mean = 0.52cm^2 sd = 0.15cm^2	271%
Hybrid Pink var.2	mean = 0.12cm^2 sd = 0.10cm^2	mean = 0.58cm^2 sd = 0.11cm^2	383%

The results from each variety and system have been expressed as means with standard deviation. As a preliminary comparison, for each variety, the leaf surface area using the bilayer system has been expressed as a percentage increase over that of the conventional system. Further results will be gathered before full statistical analysis is performed on the data.

As a preliminary observation, therefore, the use of a bilayer system increases the leaf surface area of shoots in the range of 271 - 457%, depending upon variety. This increased growth

response may be due, in part, to an improved diffusion and uptake of nutrients or growth hormones. The bilayer liquid phase may be more chemically homogeneous, thereby preventing a hormone- or nutrient-deficient 'halo' around the plantlet. The solid phase may also act as a type of slow-release reservoir for hormones or nutrients, thus allowing them to be supplied to the plantlets in a more controlled manner. It must be stressed that these are preliminary deductions and much more work is required before full conclusions can be drawn.

It was noted as a purely subjective observation that the shoots produced in the bilayer system appeared to have leaves that were of a much darker green. Plantlets *in vitro* are generally not fully photosynthetically competent (George and Sherrington, 1984) and an increased chlorophyl content does not necessarily relate to increased photosynthetic activity , however, it does suggests an interesting line of research. It is difficult to easily obtain information about the potential chlorophyll activity and photosynthetic competence of the plantlets but a measurement of the CO_2, O_2, RubisCO or other enzyme activity (Dai *et al.*, 1990; Husemann *et al.*, 1990; Kozai, 1990) would provide an indication as to the photoautotrophic status of these plantlets.

As a further subjective observation it was noted that plants produced in the bilayer system appeared to suffer less stress during the weaning process. There were less losses and the plants appeared to recover more quickly. It is possible that these plants can be weaned without a prior *in vitro* rooting stage, hence reducing the time and expense of the production process. A number of experiments are underway to quantify these observations.

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