Callus Induction and Plantlet Regeneration in Tissue Cultures of Hawaiian Anthuriums

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Abstract. Leaf explants of seven cultivars of Hawaiian anthuriums (*Anthurium andraeanum* Linden ex André cv. Kaumana, Kozohara, Marian Seefurth, Mauna Kea, Nitta, Ozaki, and Paradise Pink) produced callus most successfully after 2 to 3 months on a modified Pierik medium containing 0.36 μM 2,4-D and 4.4 μM BA. Petiole explants callused best on Pierik modified Pierik, and Finnies and van Staden media. Long-term cultures of callus from Univ. of Hawaii anthurium selections UH965, UH1060, and UH1003 were maintained for 12 to 13 months and were still capable of plantlet regeneration. Adventitious plantlets were recovered from callus plated on a Kunisaki medium containing 2.2 or 22 μM BA. Regeneration appeared to be organogenic rather than embryogenic and varied among the genotypes tested. Chemical names used: N-(phenylmethyl)-1H-purin-6-amine (BA); (2,4-dichlorophenoxy) acetic acid (2,4-D).

As a clonally propagated, slow-maturing crop (2-3 years from seed to seed) and the single most important cut flower in Hawaii, *Anthurium andraeanum* is a prime candidate for improvement using biotechnology. Reliable proliferation of callus and subsequent plantlet recovery from long-term callus cultures are important for studies on genetic transformation using particle bombardment, in vitro selection, and somaclonal variation.

Establishment of callus from a variety of tissue-cultured explants has previously been described for cultivars of *A. andraeanum* available in Holland (Leffring et al., 1976; Pierik, 1975, 1976; Pierik et al., 1974) and in the Republic of South Africa (Finnie and van Staden, 1986). Differences in callus growth and subsequent plant regeneration in some cases are due to genotype and source of explants. An embryogenic mode of plant regeneration was described only for early stages in spadix-derived callus of *A. scherzerianum* Schott (Geier, 1990). Regenerability of older callus cultures was not examined since these earlier studies focused on rapid micropropagation procedures.

This study compares the effectiveness of several media for 1) callus formation on leaf and petiole explants of Hawaiian anthuriums and 2) plantlet regeneration from long-term callus cultures.

Callus formation from Hawaiian anthurium explants. Mature plants were initially

grown in a saranhouse with 80% shade, overhead irrigation, and an approximate day/night cycle of 29/23C. Plants were moved indoors with pot-level irrigation and grown at a constant 23C with a natural photoperiod of 75 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ for several weeks before initiation of cultures. Leaf and petiole explants from seven common Hawaiian cultivars ('Kaumana', 'Kozohara', 'Marian Seefurth', 'Mauna Kea', 'Nitta', 'Ozaki', and 'Paradise Pink') were tested during 1989-90.

Leaves and petiole segments were disinfested for 5 min in 0.14% Physan 20 followed by 30 min in 0.53% NaOCl + Tween 20 (one drop/100 ml) and 30 min in 0.27% NaOCl + Tween 20, and then rinsed with 500 ml sterile deionized water. Fully expanded, yet tender, leaves were cut into rectangular pieces with sides ranging from 1 to

2 cm, each explant containing at least one major vein. Petiole explants were 1 cm long. Explants were cultured on anthurium media formulated by Finnie and van Staden (1986) (F& vS) and Pierik (1976) (P), including P modified by a substitution of sucrose and Gelrite (Scott Lab., Carson, Calif.) for glucose and Bacto-agar (Difco Labs., Detroit), respectively, (Pmod) (Table 1). Three other media used previously for maintenance of callus cultures but of unknown value for callus initiation (Cmod, D, and Dmod; A.R.K., unpublished data) were included for comparison (Table 1). Media were dispensed in 20-ml aliquots into 100 $\times$ 15-mm plastic petri dishes and sealed with Parafilm M (American National Can, Greenwich, Conn.).

Cultures were maintained in the dark at 23C for 2 to 3 months and then evaluated. The formation of callus visible without the aid of a microscope was scored as a positive response. Data from leaf and petiole explants were analyzed separately. Although the study was originally designed as a two-factor analysis for medium and cultivar, continued incidence of contamination resulted in small sample sizes (less than four explants) or missing data entries for replications testing the media Cmod, Dmod, and D. Therefore, a two-way analysis of variance was done based on the remaining three media to determine whether data from the seven cultivars could be pooled for each medium. Variability among cultivars was nonsignificant ($P = 0.418$ and 0.451 for leaf and petiole explants, respectively), and there was no interaction between cultivar and medium ($P = 0.467$ and 0.810). Consequently, all data entries were pooled within cultivars, and pooled cultivar data were treated as replications for the computation of mean square data for media effects.

Callus was produced along cut edges of both leaf and petiole explants for all cultivars tested. The callus was pale yellow and firm.

Fig. 1. Regenerability of four long-term anthurium callus lines, representing three Univ. of Hawaii selections, 3 months after culture on cytokinin-containing media. Vertical lines represent 1 SE. (A) Percentage of callus pieces that formed shoots on Kunisaki (Hi) and modified Kunisaki (H1mod) media solidified with either Bacto-agar (agar) or Gelrite (gel). (B) Percentage of callus pieces from each selection that formed shoots on regeneration medium. Each callus maintenance medium is indicated after the selection designation.

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Table 1. Media tested for initiating callus from leaf and petiole tissues of Hawaiian anthurium.

Components	Medium and composition ²					
	P ¹	Pmod	F & vS ³	Cmod	Dmod	D
Macronutrients	1/2x MS, except MgSO ₄ , CaCl ₂ as MS	1/2x MS, except MgSO ₄ , CaCl ₂ as MS	1/2x MS	1/2x MS	1/2x MS	1/2x MS
Micronutrients	As MS	As MS	As MS, except H ₃ BO ₃ , MnSO ₄ as 1/2x MS	As MS, except H ₃ BO ₃ , MnSO ₄ as 1/2x MS	As MS, except H ₃ BO ₃ , MnSO ₄ as 1/2x MS	As MS
NaFeEDTA	25 mg·liter ⁻¹	25 mg·liter ⁻¹	43 mg·liter ⁻¹	43 mg·liter ⁻¹	43 mg·liter ⁻¹	25 mg·liter ⁻¹
Vitamins	As MS	As MS	As MS ^w	As MS ^w	As MS ^w	As MS ^w
Sucrose	---	30 g·liter ⁻¹	30 g·liter ⁻¹	30 g·liter ⁻¹	30 g·liter ⁻¹	20 g·liter ⁻¹
Glucose	30 g·liter ⁻¹	---	---	---	---	10 g·liter ⁻¹
Myo-inositol	100 mg·liter ⁻¹	100 mg·liter ⁻¹	---	---	---	100 mg·liter ⁻¹
Auxin	0.36 μM 2,4-D	0.36 μM 2,4-D	0.54 μM NAA	0.36 μM 2,4-D	9.0 μM 2,4-D	9.0 μM 2,4-D
Cytokinin	4.4 μM BA	4.4 μM BA	22 μM BA	4.4 μM BA	---	---

¹From Pierik (1976), with BA substituted N-(phenylmethyl)-9-(tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine (PBA).²From Finnie and van Staden (1986).³Whh 0.4 mg thiamine-HCl/liter.

Table 2. Percent callus formation by leaf and petiole explants of Hawaiian anthuriums 2 to 3 months after initiating cultures on various media.

Explant source	Medium					
	P	Pmod	F & vS	Cmod	Dmod	D
Leaf ^a	24.3 ± 13.1 b ^c	63.2 ± 14.7 a	11.8 ± 3.0 b	19.3 ± 11.6 b	2.7 ± 2.7 b	15.7 ± 15.7 b
Petiole ^a	26.3 ± 12.3 ab	29.2 ± 12.7 ab	49.3 ± 10.8 a	0.0 ± 0.0 b	---	2.5 ± 2.5 b

^aLeaf data are means of six replicates for P, Pmod, F & vS; four replicates for Cmod; and three replicates for Dmod and D; n = 4-21 per replicate.^bData presented are means ±1 SE. Different letters signify mean separation within rows at *P* = 0.05 (repeated t test).^cPetiole data are means of six, five, and seven replicates for P, Pmod, and F & vS, respectively three replicates for Cmod; and two replicates for D; n = 6-41 per replicate. Dmod was excluded from the petiole analysis.

Fig. 2. Comparison of longitudinal sections [shoot (s) and root (r) tissues indicated] through (A) an immature anthurium embryo (seedcoat and endosperm were removed) (scale bar = 100 μm) and (B) a putative embryogenic callus (scale bar = 100 μm) from the Univ. of Hawaii selection UH1060 cultured on Cmod medium.

Analysis of variance showed that the callusing response for leaf explants differed significantly among media (*P* = 0.019), with tissue on Pmod medium being most responsive (Table 2). Although the analysis of variance for petiole explant responses among all media was not highly significant (*P* = 0.084), the cultures grown on P, Pmod, and F & vS

produced much more callus than those on Cmod or D (Table 2). Because Pmod medium gave the best response for leaf tissue and since it costs about \$0.30 and \$0.12 less per liter than either P or F & vS, respectively, our results suggest Pmod to be a suitable medium for establishing callus from the cultivars tested.

Plantlet regeneration from long-term callus cultures. One-year-old callus from three plant selections from the Univ. of Hawaii anthurium breeding program was tested. Callus from four culture lines, derived from either petioles or basal cuttings from plantlets micropropagated via axillary bud culture according to Kunisaki (1980), was subculture monthly during 1988-89. These culture lines included selection UH965 maintained on D medium, selections UH1003 and UH1060 maintained on Dmod medium, and selection UH1060 maintained on Cmod medium (Table 1).

In 1989, 12 to 13 months after the initiation of callus, two replications of 50 callus pieces, each ≈30 to 40 mg in fresh weight, were used for regeneration; four variations of a cytokinin-containing Kunisaki medium (H1) were tested (Kunisaki, 1980). H1 is composed of half-strength MS macronutrients (Murashige and Skoog, 1962), full-strength MS micronutrients, MS vitamins supplemented with 0.4 mg thiamine-HCl/liter, 25 mg NaFeEDTA/liter, 100 mg myo-inositol/liter, 2% sucrose, and 2.2 μM BA. H1 is effective in the light for multiple shoot formation from anthurium nodal cuttings (Kunisaki, 1980). The four media variations were H1 solidified with either 0.7% Bacto-Agar (H1 agar) or 0.18% Gelrite (H1 gel) and H1 with cytokinin content increased from 2.2 to 22 μM BA (H1mod) and solidified with either 0.7% Bacto-Agar (H1mod agar) or 0.18% Gelrite (H1mod gel). H1mod was included since an increase in cytokinin levels increased shoot formation for otherwise recalcitrant genotypes (Leffring and Soede, 1979) and for some Hawaiian callus cultures (F.C. Chen, personal communication).

Following 1 month of culture in the dark at 23C, callus from H1mod was transferred onto H1 and returned to darkness. Two weeks later, all of the cultures were moved to 25 ± 2C with a 16-h photoperiod of 54 μmol·s⁻¹·m⁻² provided by mixed cool-white and Gro-lux Sylvania fluorescent lamps (GTE Corp., Danvers, Mass.). After an additional 2 months without subculture (for a total of 3 months on regeneration medium), the number of callus pieces with at least one

emerging shoot with visible leaves was recorded.

Preliminary histological examinations of long-term callus from UH1060 were made to determine whether the callus was embryogenic or organogenic in organization. The immature embryo of an anthurium seed was also fixed and sectioned to serve as an example of embryonic tissue organization. Callus and embryo samples were fixed in 2 formalin :1 glacial acetic acid :17 ethanol (55.9%) (by volume) (FAA), dehydrated through a tertiary butanol series, and embedded in paraffin according to Berlyn and Miksche (1976). Samples were sectioned (12 μ m thick) on a rotary microtome and stained for 10 min with 0.05% toluidine blue.

All long-term culture lines regenerated plantlets. Two-way analysis of variance showed variability among culture lines to be highly significant ($P = 0.0038$). There was no significant difference among regeneration media ($P = 0.1103$) (Fig. 1A) and there was no culture line $\times$ medium interaction ($P = 0.5394$). These observations are similar to those of Leffring and Soede (1979) in that various genotypes of *A. andraeanum* differed in their regenerability. Furthermore, an increase in cytokinin levels under these conditions did not improve plantlet recovery at $P = 0.05$, yet the BA-rich H1mod medium promoted higher mean regeneration.

Selection UH1003 cultured on Dmod medium yielded the highest percentage of callus pieces producing shoots (32%; Fig. 1B). When compared with a different genotype from the same callus maintenance medium, selection UH1060 cultured on Dmod, the regenerability of UH1003 was significantly higher ($P = 0.0131$). When maintained on Dmod before regeneration, UH1003 callus had an organogenic (shoot-like) organization, while UH1060 produced a more friable, embryogenic callus. Histological examination of similar UH1060 callus cultured on Cmod medium supported a possible embryogenic organization with some resemblance to a seed-derived embryo (Fig. 2). Furthermore, there was a slight difference between regenerability of the same genotype from different media, with UH1060 grown previously on Dmod producing more shoots than UH1060 from Cmod ($P = 0.0644$; Fig. 1B). The callus types also differed in this case, with UH1060 from Cmod being more compact, but still embryogenic, in appearance. These two comparisons suggest that regenerability is strongly affected by the genotype and callus organization before regeneration.

A comparison of UH1060 embryogenic callus with nonembryogenic UH965 and UH1003 callus yielded a lower regenerability for the embryogenic callus ($P = 0.0083$; Fig. 1B). In contrast, when callus was allowed to grow in darkness for 3 months on callus maintenance medium without intervening transfers, callus of UH1060 on Cmod and Dmod showed a higher regeneration than either UH1003 or UH965 (11% and 6% vs. 4% and 0%, respectively).

Since the H1 medium is normally used for

promoting multiple shoots from lateral buds, this medium may be well suited for shoot production from organogenic callus. Adequate development of somatic embryos, however, may require a change in medium composition, such as alteration of the $\text{NH}_4\text{-N} : \text{NO}_3\text{-N}$ ratio as suggested for anthurium (Geier, 1990) and other embryogenic systems (Kohlenbach, 1978). Nevertheless, reliable initiation of callus from tissues of Hawaiian anthurium and progress in the ability to recover plantlets from long-term cultures make it possible to test particle bombardment as a gene transfer method with callus or explants that form callus and to screen variants generated in vitro for use in the breeding of Hawaiian anthuriums.

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