SAFEGUARDING *ANTHURIUM* GENETIC RESOURCES: IN VITRO GERMPLASM BANK DEVELOPMENT AND FLOW CYTOMETRY ANALYSIS

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CHAPTER 1

INTRODUCTION

This thesis is a compilation, as chapters, of four individual documents under the scope of safeguarding *Anthurium* genetic resources. The scope of work was to establish in tissue culture a set of *Anthurium* species of horticultural value, develop the tissue culture protocol necessary for doing so, characterize these and additional species for nuclear DNA content, and then make a subset of species available for distribution via the USDA Ornamental Plant Germplasm Center. The chapters are intended for individual publication as follows: 2) In vitro germplasm bank of *Anthurium* species: development and techniques, intended for Plant Cell, Tissue and Organ Culture or similar journal in a Research Notes format; 3) Rapid micropropagation of *Anthurium* species and hybrids by a minimal callus protocol, intended for publication in In Vitro Cellular and Developmental Biology – Plant or similar journal; 4) *Anthurium* germplasm from Hawai'i: collaboration with the Ornamental Plant Germplasm Center, intended as part of an internet newsletter published by the Ornamental Plant Germplasm Center and targeted towards the lay public. It is included as a chapter to illustrate the variety of communications required for successful in vitro germplasm bank development and as a necessary tool for the increased distribution of germplasm resources; and 5) Nuclear DNA content estimations in *Anthurium* (Araceae), intended for Plant Systematics and Evolution or similar journal. The format for each chapter is representative of the requirements for their intended publication outlet.

CHAPTER 2

IN VITRO GERMPLASM BANK OF *ANTHURIUM* SPECIES: DEVELOPMENT AND TECHNIQUES

Abstract

Ovule and lamina culture were employed for the development of an in vitro germplasm bank of nineteen *Anthurium* species. Such a bank would mitigate both the loss of species due to habitat destruction and the difficulty and cost of maintaining live plants in collection. The species include *A. amnicola, A. antioquiense, A. formosum, A. garagaranum, A. kamemotoanum, A. lindenianum, A. nymphaeifolium, A. ravenii, A. roseospadix, A. bicollectivum, A. bakeri, A. gracile, A. watermaliense, A. sanctifidense, A. pallidiflorum, A. folsomianum, A. scandens, A. trinerve,* and *A. jefense.* Recovery of shoots, ready for rooting, after initial establishment through ovule culture was eight to twelve weeks and after minimal callus formation on lamina explants averaged 21 weeks. Plants were transferred to solidified medium specific for medium-term storage or shipping purposes, or to liquid medium for long-term storage purposes. Towards achieving the ultimate goal of sharing materials for research and conservation, eleven species were transferred to the US National Plant Germplasm System for maintenance and further distribution in vitro to qualified organizations, institutions, and stakeholders.

Introduction

Anthurium is the largest and most widely distributed genus within the Araceae. It occurs in the Caribbean Islands and from northern Mexico through Central America to southern Brazil with approximately 990 species and a diverse range of habitats (Croat 1986). The genus is valued for both research and horticultural purposes, yet its genetic base is being eroded (Bown 2000). Live plants are critical to gaining a complete and accurate description of the species, in particular for cytology, fragrance chemistry, and hybridization studies to determine species relationships and conduct horticultural improvement (Croat 1979; Kamemoto and Kuehnle 1996). However, propagation of existing germplasm for distribution of plants to research and botanical institutions can be difficult due to the slow development of *Anthurium* off-shoots in vivo (Pierik et al. 1974; Matsumoto and Kuehnle 1997) and to limited resources. Moreover, with habitat destruction and the difficulty of maintaining live plants in existing aroid collections (A. Meerow; United States Department of Agriculture, Agricultural Research Services, Research Geneticist, plants; personal communication), the need for an in vitro germplasm bank has arisen.

The objective of this study was to establish a variety of *Anthurium* species in vitro, as a partial germplasm bank, using ovule and lamina culture techniques. Cost-saving was implemented through use of table sugar to replace reagent-grade sucrose and gellan gum to replace agar. Ovules and lamina tissues were used for culture initiation whenever available, followed by medium- and long-term storage of resulting plantlets. Medium-term storage is appropriate for cultures destined for distribution purposes. The

plasticware and solidified medium used are less expensive and more suited for shipping than long-term storage materials. Long-term storage is ideally suited to germplasm banking (Watt et al. 2000). It reduces the need for frequent subculture which can lead to increased costs and labor, risk of material loss through contamination and human error, and the loss of genetic integrity through somaclonal variation (Hao et al. 2003). For long-term storage, a liquid system with glass test tubes was used to significantly prolong the time between required sub-culturing.

The following techniques proved successful in the development of the in vitro germplasm bank of *Anthurium* species. To our knowledge this is the first report of such a collection.

Sib and self-pollinations prior to ovule culture

Sib-pollinations within a species were made as flowers became available from January 2005 through January 2006 from screenhouse-grown plants. Berries were also collected from self-pollinated spadices as they became viable for use in ovule culture. Determinations of viability for ovule culture were made on a visual basis. The ideal stage for ovule culture is approximately one to two weeks after the visual emergence of the berries among the tepals. At this stage, 16 to 20 weeks after pollination, storage products

of proteins and starches are present and the embryos are well -suited to germination (Matsumoto et al. 1998). Moreover, removing the berries from the spadix prior to maturation increases the success of the disinfestation process as well as eases the removal of the gelatinous mesocarp encasing the ovule (Matsumoto et al. 1995; Matsumoto et al. 1998).

Ovule culture

Immature berries were manually excised from spadices and disinfested in 10% Physan 20 (Maril Products Inc. Tustin, CA) for 30 min to 24 h and rinsed thoroughly with water. Berries were then soaked in 10% Clorox (Clorox Co. CA; 0.6% NaOCl) for 30 min to 24 h. Soaking for longer periods of time decreases the adherence of the mesocarp to the ovules, but also increases the risk of decreased embryo viability. Under aseptic conditions berries were transferred to a large glass Petri dish containing sterile distilled water. The ovules were extracted from the immature berries by applying pressure to the apical end as described by Croat (1979). Ovules were transferred to a second Petri dish containing sterile distilled water for the removal of the remaining mesocarp to minimize the introduction of contamination in the cultures.

At this stage the ovules were cultured under lights (20 μmol m^{-2} s^{-1}) using liquid or solidified H2 medium (Kuehnle et al. 2001). H2 basal medium contains half-strength MS (Murashige and Skoog 1962) macronutrients and micronutrients and 0.4 mg l^{-1} thiamine (Caisson Cat. No. MSP009, 1 packet for 2 l total), 150 ml l^{-1} coconut water

(PhytoTechnology Laboratories, Shawnee Mission, KS; Cat. No. C195), and 20 g l^{-1} table sugar (used in place of sucrose), pH 5.7 - 5.8.

Liquid medium proved ideal for species intended for long-term storage on site. Ovules were placed directly into five ml of liquid medium dispensed into 16 x 125 mm Pyrex test tubes and cultured without shaking (Fig. 1). The plantlets that developed from these ovules did not need further subculture for at least one year. The subculture interval could be extended beyond one year for some particularly slow-growing species.

Liquid medium was also useful for culture of ovules extracted during earlier or later stages of development, as it facilitated the use of a 250 ml flask on a shaker (Fig. 1) and the optional addition of antimicrobial Plant Preservative Mixture (PPM; Plant Cell Technology). Ovules extracted from berries taken at earlier stages of development benefited from shake-culture at 50 rpm to increase gas exchange around the tissue. Ovules extracted from berries taken at later stages of development were at an increased risk of contamination, which could be mitigated by the addition of 2 ml l^{-1} PPM. Ovules exposed to PPM required subculture to PPM-free media upon germination as tissues exposed to PPM for longer than one month became visibly damaged and/or non-viable.

For the solid medium, there was an addition of 2.5 g l^{-1} gellan gum (Caisson Cat. No. G017) in place of agar (0.7%, Kuehnle and Sugii 1991) to lower cost. The medium was poured into Magenta GA-7 boxes (Sigma-Aldrich Co.) to 25-30% of the total volume (Fig. 1). This medium was ideal for species that were to be shipped to the Ornamental Plant Germplasm Center (Columbus, Ohio) for housing in the US National Plant Germplasm System. Magenta boxes are sturdy and well suited for shipment to

alternative locations. Tissue placed directly into this medium did not need to be sub-cultured for six months to one year depending on the species' individual growth habit.

Lamina culture

For some species in the collection, berries were an impossibility, as the *Anthurium* species were not responsive to pollination efforts. For these limited species, laminar tissue was cultured to establish plantlets in vitro using a minimal callus protocol; bud culture (Kunisaki 1980) was too high risk in terms of loss of a plant specimen of a species. Leaves were removed from mature plants approximately three to five days after unfurling, and cut into sections containing major veins. The sections were then placed in a 1% Physan 20 + Tween 20 (3 drops/100 ml) solution for 1 h, rinsed thoroughly and soaked in a 10% Clorox + Tween 20 (3 drops/100 ml) solution for 30 min. The sections were then transferred to a 5% Clorox + Tween 20 (3 drops/100ml) solution for another 30 min. Following disinfestation the leaves were thoroughly rinsed with sterile distilled water under aseptic conditions. Leaf sections were then cut into rectangular segments (approximately 1x4 cm) and placed on a 100x15 mm plastic Petri dish with solidified H3 medium (Kuehnle and Sugii 1991; Kuehnle et al. 2001) containing MS basal salts with 0.4 mg/L thiamine, 0.4 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg l^{-1} benzyl-9-adenine (BA), 20 g l^{-1} table sugar, 2.5 g l^{-1} gellan gum, and 2ml l^{-1} PPM (to reduce endogenous contamination), pH 5.7 – 5.8.

After culture of one week under darkness at 24 C, leaf segments were removed and transverse cuts were made at 1 cm intervals along the vein. Segments were then

placed on a 100x15 mm plastic Petri dish with 10 ml liquid medium containing MS basal salts with 0.4 mg l^{-1} thiamine, 0.2 mg l^{-1} indole-3-butyric acid (IBA), 0.1 mg l^{-1} thidiazuron (TDZ), and 20 g l^{-1} table sugar. Leaf tissues were stabilized through the strategic placement of sterile cosmetic cotton rounds (Johnson & Johnson, 5.5 cm diameter) in the center of the Petri dish, exposing them to the medium, but keeping them from becoming completely submerged.

Cultures were stored under darkness at 24 C until organogenic callus was visible. Formation of 2x2 mm pale yellow, firm callus averaged 6.74 weeks for the species *A. nymphaeifolium,* and *A. lindeniamum* (Fig. 1). Cultures were then placed near a window under natural light conditions. Once the callus turned green, the entire leaf segment was submerged in a liquid basal medium containing 0.2 mg l^{-1} BA, and 20 g l^{-1} table sugar for culture on a lighted (20 µmol m^{-2} s^{-1}) shaker at 50 rpm. Shoot clusters were transferred directly to solidified medium after an average of 11 weeks with no intermittent subculture. Every effort was made to minimize the time the tissues spent under the influence of external growth regulators. Prolonging the time that the lamina tissue spent in the callusing stages of this protocol would have increased the number of plantlets obtained from the tissue but also the chances for variations. With the use of this protocol it was possible to establish one to twenty plants per leaf segment, depending on genotype, within an average of 21 weeks, or just less than six months. An alternative protocol to test in the future may be that for apparent direct shoot formation from lamina (Martin et al. 2003b), although this has not yet been reported effective with any species.

Outcomes

The objective of this study was met with the successful establishment of nineteen species of *Anthurium* (Table 1), critical to the University of Hawaii collection and breeding program, using ovule and lamina culture techniques. Ovule culture produced rooted shoots within two to three months. This is the recommended method since non-adventitious tissue is preferred for genetic conservation purposes (Pence et al. 2002) and ovules are relatively easy to culture. Lamina explant culture was required for only two of the nineteen species; this produced shoots ready for rooting within an average of 21 weeks. All plantlets appear true to type, based on vegetative morphology. Species of particular breeding interest to the University of Hawaii were placed into long-term storage. Species suited for distribution were placed into medium-term culture to facilitate shipping during 2005 and 2006 and are expected to be available at the US National Plant Germplasm System via the Ornamental Plant Germplasm Center (OPGC May 2006 Newsletter, http://opgc.osu.edu/). This first example of an in vitro gene bank for *Anthurium* can now be expanded to include additional species using the protocols described here.

Confirmation of the genetic stability of species established in vitro would be a desirable aspect of the development and validation of a long-term in vitro *Anthurium* germplasm bank. One suitable method may be amplified fragment length polymorphism (AFLP; Karp et al. 1997) analysis that proved useful to confirm the genetic stability of long-term, slow-growth cultures of apple (Hao 2003) and has previously been used for

aroid analysis (Chen et al. 2004). It would also be important to determine what level of variation in a gene bank is acceptable, if any at all.

Table 1. *Anthurium* species established in vitro and their University of Hawai'i identification numbers for reference.

***Anthurium* Species**	**UH Accession Number**
A. amnicola Dressler	A417
A. antioquiense Engler	A490**
A. formosum Schott	A291
A. garagaranum Standl.	A263
A. kamemotoanum Croat	A800
A. lindenianum K. Koch & Augustin	A220**
A. nymphaeifolium K. Koch & Bouché	A213*
A. ravenii Croat and & Baker	A228
A. roseospadix Croat	A293
A. bicollectivum Croat	A237
A. bakeri Hook. f.	A116
A. gracile (Rudge) Lindl.	A345
A. watermaliense Hort ex L.H. Bailey	A754
A. sanctifidense Croat	A503
A. pallidiflorum Engl.	A449
A. folsomianum Croat	A276
A. scandens (Aubl.) Engl.	A773
A. trinerve Miquel	A774
A. jefense Croat	A324

* Established in vitro via lamina culture
** Established in vitro via lamina and ovule culture

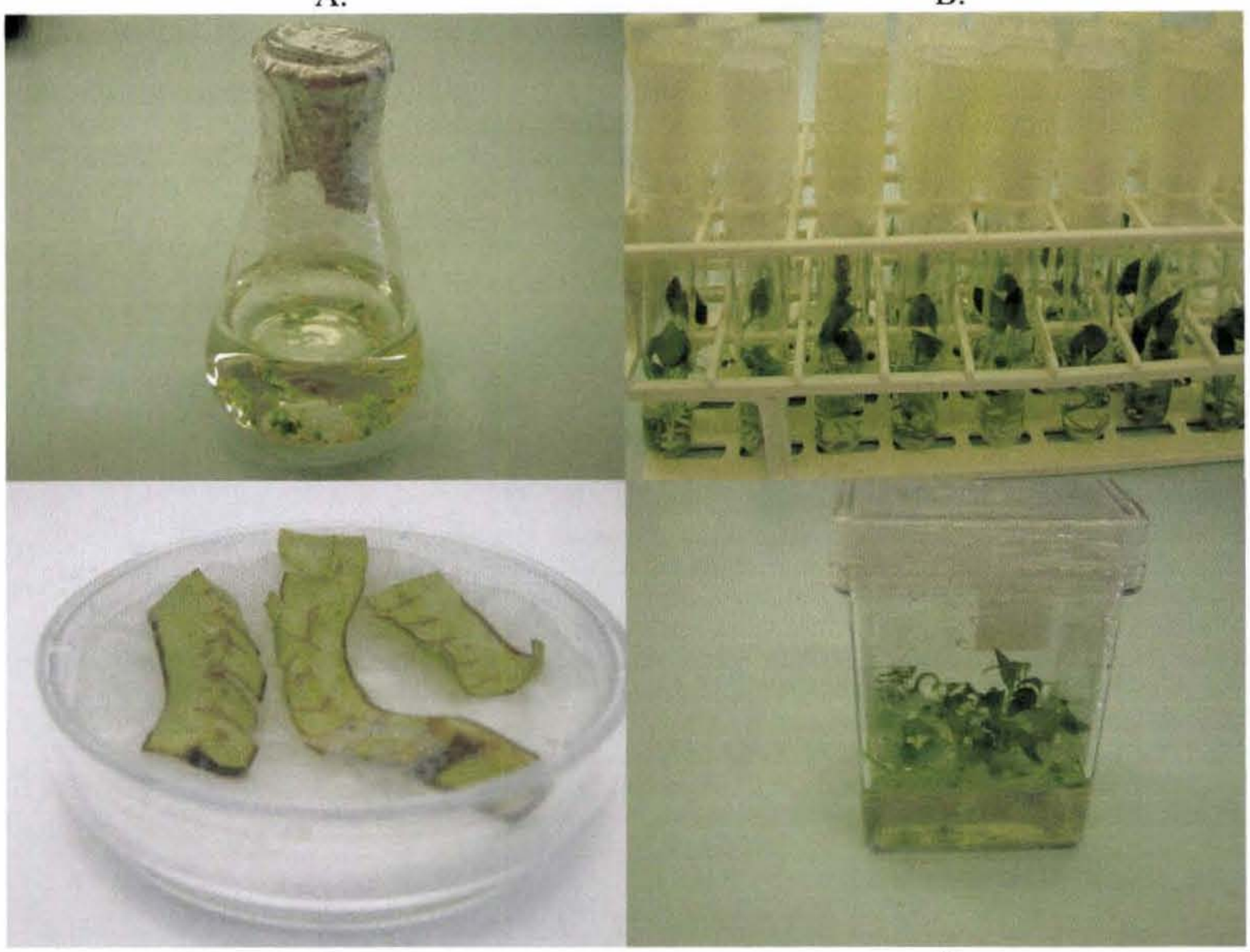


Figure 1. (A) Developing ovules of *A. folsomianum* 5 weeks after initial placement in liquid medium with shaking. (B) *A. amnicola* in stationary liquid medium for long - term storage. (C) *A. nymphaeifolium* in the second stage of lamina culture (D) *A. garagaranum* in solidified medium, ready for distribution.

CHAPTER 3

RAPID MICROPROPAGATION OF *ANTHURIUM* SPECIES AND HYBRIDS BY A MINIMAL CALLUS PROTOCOL

Abstract

Creating an in vitro collection of rare *Anthurium* species is critical due to specimen loss in nature and in botanic gardens (Kuehnle and Sugii 1991). Tissue culture of commercial hybrid *Anthurium* can be difficult, but is required by industry for mass production. This research examined alternative in vitro media combinations to more effectively establish callus and initiate plantlets from leaves of three species and three hybrid cultivars. Protocols were examined to determine the effectiveness of different auxin – cytokinin combinations and sequences in establishing callus namely, 0.2 mg l^{-1} indole-3-butyric acid (IBA)/ 0.1 mg l^{-1} thidiazuron (TDZ) and 0.4 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D)/ 0.2 mg l^{-1} benzyl-9-adenine (BA). After callus formation, the explants were placed in either liquid or solidified medium containing 0.2 mg l^{-1} BA. Comparisons of the different media combinations were made based on percent callus response across genotypes, mean weeks to callus formation, and percent shooting response to either the liquid or solid BA media. The most effective treatment for

establishment of *Anthurium* in vitro was the placement of the lamina explant on IBA/TDZ solid medium for one week followed by placement in 2,4-D/BA liquid medium until callus development approximately five to seven weeks later. The results of this study will help to more rapidly and reliably establish an in vitro collection of different genotypes of *Anthurium*, with significant impacts for conservation efforts as well as the Anthurium industry.

Introduction

Anthurium is the single most valuable cut flower commodity in Hawai'i, and is consistently traded in the top fifteen of the Dutch cut flower auctions. Programs at the University of Hawai'i and around the world rely on breeding with species to bring new colors and shapes of this flower into the marketplace, as well as disease resistant plants into the growers' fields. Breeding programs are threatened by plant specimen loss in nature and in botanic gardens due to habitat encroachment, bacterial blight and nematode damage (Higaki et al. 1995; Meerow, personal communication). With these losses the ability to infuse new genetic characteristics into the stately, long-lasting cut flower diminishes. It is therefore critical to create and maintain an *in vitro* collection of rare *Anthurium* species through micropropagation to ensure a future of new and exciting varieties for the marketplace. *Anthurium* of different genetic backgrounds can take upwards of four months to establish callus *in vitro* from lamina explants (Pierik et al. 1974; Kunisaki 1980; Matsumoto and Kuehnle 1997) or seven months for direct shoot regeneration (Orlikowska et al. 1995; Martin et al. 2003) and are not always responsive to

current protocols. Thus, there is a need for alternative and reliable tissue culture media suitable for micropropagation (Matsumoto and Kuehnle 1997).

This study compares the effectiveness of several media combinations for a minimal callus formation stage using leaf explants, followed by plantlet regeneration. Two auxin and cytokinin combinations (Gaspar et al., 1996), namely 2,4-dichlorophenoxyacetic acid (2,4-D) / benzyl-9-adenine (BA) and indole-3-butyric acid (IBA) / thidiazuron (TDZ; Yang et al. 2003), were compared to establish callus across genotypes in two months or less. Callus forming explants were then placed in either liquid or solidified medium containing 0.2 mg l^{-1} BA for shoot development. The objective of this study was to develop a rapid tissue culture protocol for the in vitro establishment of critical *Anthurium* species as well as to efficiently meet clonal production requirements for Hawaii's growers.

Materials and Methods

Leaf selection and removal

Leaves were taken from the Magoon greenhouse facilities at the University of Hawai'i at Manoa. Leaves of three species, *A. nymphaeifolium*, *A. antioquiense*, *A. lindenianum*, and three hybrids, 'Midori' (Higaki et al. 1994), 'ARCS' (Kamemoto et al. 1998) and breeding selection UH1992, were selected from mature plants three to five days after unfurling. Leaves were removed in the morning and directly transferred to the

lab for culture initiation. 80 segments from each genotype, 480 segments total, were placed into culture to obtain 10 final repetitions for each genotype in each media combination.

Leaf sterilization

Leaf blades were cut into 2x5 cm strips containing central or lateral major veins. The sections were placed into flasks and submerged in distilled water, 1% Physan 20, and Tween 20 (polyoxyethylene (20) sorbitan; 3 drops/100 ml). The solution was replaced after 1 h with distilled water, 10% Clorox (0.06% NaOCl), and Tween 20 (3 drops/100 ml). The solution was replaced after 30 min with distilled water, 5% Clorox, and Tween 20 (3 drops/100 ml) and soaked for 30 min. Leaf strips were then thoroughly rinsed with sterile de-ionized and distilled water in preparation for sectioning.

Sectioning and initial placement on solidified media for detection of contamination

Initial sectioning to remove the bleach damaged tissues reduced the leaves into 1x4 cm pieces with the vein running parallel to the long edges. Three sections each were placed adaxial side up on one of two solidified media in 100x15 mm plastic Petri dishes. Medium one, H3, contained Murashige & Skoog (1962) basal salts (MS) with 0.4 mg l^{-1} thiamine, 0.4 mg l^{-1} 2,4-D, 0.2 mg l^{-1} BA, 2% sucrose, and 0.25% gellan gum, at pH 5.7-5.8. Medium one is the control based on previously published work by

Kuehnle and Sugii (1991) and Kuehnle et al (2001). Medium 2, Hit, contained MS basal salts with 0.4mg l^{-1} thiamine, 0.2 mg l^{-1} IBA, 0.1 mg l^{-1} TDZ, 2% sucrose, and 0.25% gellan gum.

Transfer to secondary media.

After one week in the dark at 24°C and daily observation for bacterial or fungal growth, the non-contaminated sections were transferred to 100x15 mm Petri dishes containing H3 or HIT solidified or liquid media with a cosmetic cotton round (Johnson & Johnson) used for support. Transverse cuts were made along the vein of the leaf sections at approximately 1 cm intervals. The media sequence and composition are summarized in Fig. 2.

Callus initiation and observations.

Petri dishes containing leaf explants were stored in the dark at 24°C and observed weekly for two months. The date of formation of 2x2 mm pale yellow, firm callus was recorded. The mean response of number of explants forming callus per total number of viable explants was compared using the Duncan's multiple range test (at the 5% level, with uneven repetition to determine the mean square error) to determine significantly different responses to the treatments. Explants that showed browning were not accounted for in the final responsive tissue results and were treated similarly to contaminated explants in that they were not considered viable for plantlet formation.

Transfer to light for organogenesis.

The viable callusing explants were moved under lights (20 μMol m-2 s-1, 24 h light cycle) for greening, then segmented in half and placed either in 75 ml of liquid in a flask or on solidified medium in a Magenta box containing MS basal salts with 0.4 mg l^{-1} thiamine, 0.2 mg l^{-1} BA, and 2% sucrose, solidified with 0.25% gellan gum at pH 5.7-5.8. This medium, H1, in the solidified form was the control based on the previous work of Kuehnle et al. (2001). Explants were observed weekly for the presence of shoots visible to the naked eye. The explants were then placed on a solidified medium proven effective for root initiation in the past, H2 (Kuehnle et al. 2001). The H2 medium contained MS basal salts with 0.4 mg l^{-1} thiamine, 15% coconut water, and 2% sucrose, solidified with 0.25% gellan gum at pH 5.7-5.8.

Results and Discussion

The *Anthurium* genotypes chosen for this experiment had different phenotypic characteristics and genetic backgrounds that might elicit a possible range of responses to the media combinations. The three species, *A. nymphaeifolium*, *A. lindenianum*, and *A. antioquiense,* display distinctively different floral characteristics as well as growth habits (Kamemoto and Kuehnle 1996). *A. antioquiense* is a small pot-type plant with purple spadices while both *A. nymphaeifolium* and *A. lindenianum* are large plants with white spathes and spadices (*A. lindenianum* can also present light pink spathes). These two

species were difficult to establish in vitro through lamina explants with solidified H3 as described by Kuehnle and Sugii (1991). The three hybrids, 'Midori', 'ARCS', and UH1992, were chosen based on their different flower-types (Kamemoto and Kuehnle 1996). 'ARCS' has a purple tulip-shaped flower and both 'Midori' and UH1992 have a heart-shaped flower, in colors of green and orange, respectively. UH1992 also proved difficult to establish in tissue culture, using solidified H3 (Kuehnle and Sugii 1991). The explants from the different genotypes showed variable callus formation on the different media. To best reach the objectives set out by this experiment, developing an alternative rapid protocol for the establishment of *Anthurium* plants in vitro, the critical data points examined were the positive or negative callus response across genotypes, and the mean number of weeks to callus development for each treatment.

Tissues that turned brown, presumably due to phenolic exudates and did not lead to plantlet formation were treated as contaminated or non-viable tissues. The browning response was present in all media combinations throughout the experiment, and did not appear to be stage or genotype dependent. In accordance with George (1996) the secondary solidified medium had a higher incidence of browning as the toxic substances were not as easily dispersed away from the explants. Some future considerations for decreasing the number of explants exhibiting browning would be the addition of polyvinyl pyrrolidone (PVP) or antioxidants such as ascorbic acid to the media (George 1996).

Callus was produced along the cut edges of all genotypes in all the media tested. Results indicated that culture on the solidified HIT medium followed by transfer to the H3 liquid combination was the most effective for callus establishment, with a positive

callusing response across genotypes of 69.2% (Table 2). This growth regulator combination had the fastest time to callus with a mean of 5.1 weeks to viable callus development (Table 2). Somaclonal variations in *Anthurium* plantlets established from callus were shown to occur at a low rate of about 4% in plants micropropagated via long-term callus culture (Kuehnle and Sugii 1992). This variation could possibly be further reduced by decreasing the time spent in callus culture (Martin et al. 2003). All of the media combinations examined in this experiment had a range of 5.1 to 7.8 mean weeks to 2x2 mm callus development (Table 2).

As a result of this study, a new plant growth regulator combination and sequence may be incorporated into a minimal callus lamina explant micropropagation protocol. However, all treatments were effective to some extent in initiating callus (Table 2). Therefore, if a particular genotype is unresponsive on one media combination it may prove beneficial to try the other combinations examined in this study.

The shooting response of the callus using the media examined in this study, H1 solid or liquid, did not give ideal results in all genotypes for mass production. These media were examined based on previous studies which showed suitability, in the solidified form, for shoot generation from callus (Matsumoto and Kuehnle 1997; Kuehnle et al. 2001). For 'Midori,' the most responsive genotype, 14 out of 52 (26.9%) callusing explants developed shoots in the liquid BA medium (Fig. 2) and 6 out of 52 (11.5%) explants developed shoots on the solidified BA medium (Table 3). Although the species genotypes were less responsive, with an average response of 7.1% to both the solidified and liquid medium, they still showed some shooting. One explant of a species established

in tissue culture is enough to develop a germplasm base for further in vitro replication. With this protocol we were able to establish one to 20 shoots ready for rooting per leaf segment within an average of 21 weeks or just less than six months.

These findings open up the possibility for the micropropagation of *Anthurium* genotypes that were previously very difficult or impossible to establish in vitro. They also show a rapid alternative media combination for commercial production and species replication. Further research is needed to develop a suitable and effective shooting medium for mass production from lamina explant callus.

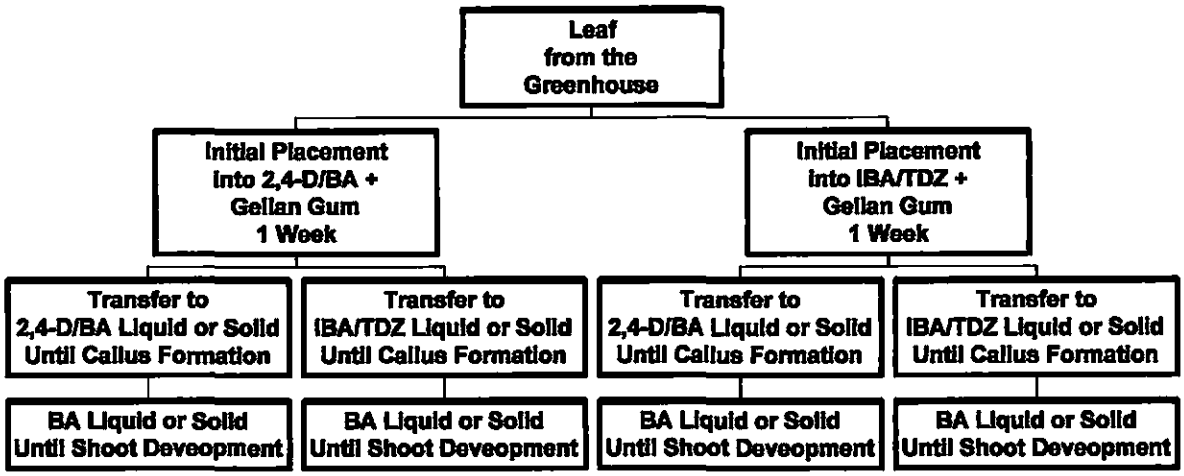


Figure 2. Summary of media sequences and composition tested for rapid micropropagation of *Anthurium*.

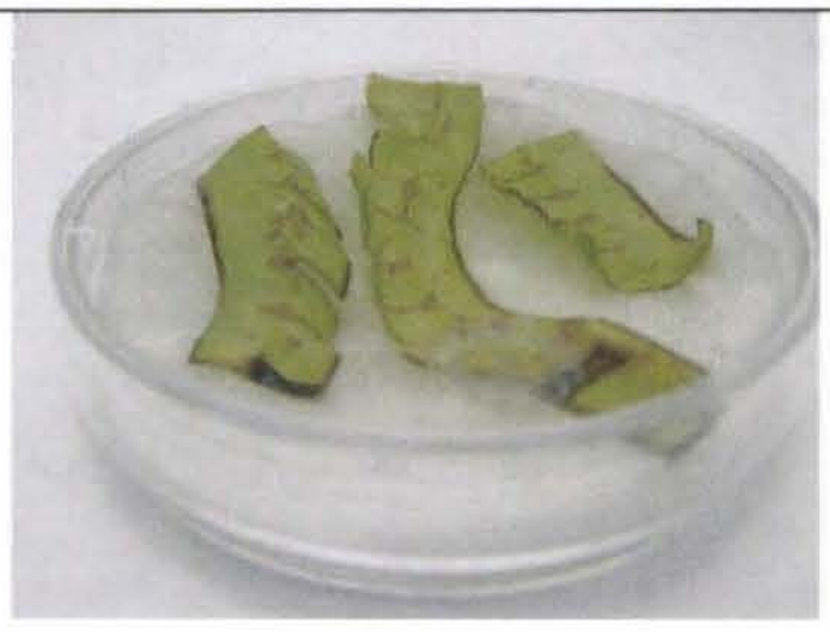
Callus forming along the edges of *A. nymphaeifolium* in IBA/TDZ liquid medium after 6 weeks in tissue culture.	Plantlets forming from callus on leaf explants in liquid BA medium after 9 weeks.
	

Figure 3. Visual results of micropropagation protocol evaluation.

Table 2. Results of media combinations showing the mean percentage of positive callus response across genotypes and mean weeks to callus formation..

Growth Regulator/Media Combinations	**% Callus Response Across Genotypes +/- SE (# with 2x2 mm callus/ total viable explants) ***	**Mean Weeks to Callus Formation +/- SE**
2,4-D/BA solid->2,4D/BA liquid	53.3% **C** +/- 0.2 (8/15)	6.0 +/- 0.35
IBA/TDZ solid->IBA/TDZ liquid	36.4% **A** +/- 0.5 (8/22)	7.8 +/- 0.68
2,4-D/BA solid->2,4-D/BA solid	47.6% **B** +/- 0.35 (10/21)	7.1 +/- 0.46
IBA/TDZ solid->IBA/TDZ solid	44.4% **B** +/- 0.45 (4/9)	7.0 +/- 0.12
IBA/TDZ solid->2,4-D/BA liquid	69.2% **F** +/- 0.25 (9/13)	5.1 +/- 0.18
2,4-D/BA solid->IBA/TDZ liquid	64.7% **E** +/- 0.15 (11/17)	6.7 +/- 0.66
IBA/TDZ solid->2,4-D/BA solid	60.0% **D** +/- 0.25 (6/10)	7.3 +/- 0.22
2,4-D/BA solid-> IBA/TDZ solid	65.0% **E** +/- 0.36 (13/20)	6.7 +/- 0.13

* Mean percentages of positive callus response followed by the same letters are not significantly different at the 5% level using Duncan's multiple range test.

Table 3. Results of % shoot production response for each genotype examined, and the mean weeks to shoot formation.

Genotype Examined	**% of Explants Producing Shoots on Solidified Medium**	**% of Explants Producing Shoots in Liquid Medium**	**Mean Weeks to Shoot Formation**
A. nymphaeifolium	9.1% (1/11)*	0% (0/12)	12.0
A. lindenianum	0% (0/12)	8.3% (1/12)	10.0
A. antioquiense	0% (0/4)	25% (1/4)	11.0
'Midori'	11.5% (6/52)	26.9% (14/52)	10.3
'ARCS'	22.9% (8/35)	11.4% (4/35)	9.2
UH1992	16.7% (2/12)	8.3% (1/12)	9.0

* The total number of callusing explants producing shoots/total # of callusing explants for each genotype.

CHAPTER 4

ANTHURIUM GERMPLASM FROM HAWAI'I, COLLABORATION WITH THE ORNAMENTAL PLANT GERMPLASM CENTER

A. bakeri Hook f.

A. folsomianum Croat

A. garagaranum Standl.

A. gracile (Rudge) Schott

A. jefense Croat

A. pallidiflorum Engl.

A. sanctifidense Croat

A. supianum Engl.

A. trinerve Miq.

Figure 4. *Anthurium* species established in tissue culture at the University of Hawai'i and deposited in the National Plant Germplasm System. (Photographs courtesy of Dr. Haruyuki Kamemoto, University of Hawai'i)

In 1889, the first *Anthurium* species was imported to Hawai'i. Since then, Anthurium has grown to be the most important cut flower crop in Hawai'i. The grace and durability of the plant and the exotic long-lasting flower make it a rich and beautiful part of our history as well as our future.

Researchers at the University of Hawai'i have been studying and collecting this dynamic genus for more than fifty years. An important part of the University of Hawai'i's mission is to develop new disease-resistant, fragrant and other novel varieties for the growers in our state. Part of this mission over the years has been the development of a species collection for infusion of good genetics (disease-resistance, fragrance, color, flower-shape, etc.) back into plants suitable for commercial production. Over 300 species have been collected through early explorations of Panama and donations from colleagues and hobbyists.

Unfortunately this collection is under constant threat. Hawai'i is definitely a tropical paradise, but with the good must come the bad, and our locale sits in the path of hurricanes, floods, pests and disease. To avoid the loss of this precious material, the University of Hawai'i has undertaken the establishment of a tissue culture germplasm bank of *Anthurium*.

Over the last few years pollinations were made and berries were collected from the species. After sterilization of the berries, the seeds from those berries were placed in liquid or solid media containing sugar, coconut water, and vitamins. Once the seeds germinated and developed at least two leaves they were transferred to a storage vessel appropriate for long-term growth.

In addition to the collection of berries, alternative methods were explored for the establishment of these important species in tissue culture. This is necessary because not all species will make berries under the conditions provided at the University of Hawai'i greenhouse facilities. The explorations focused on the tissue culture of leaves. Through the manipulation of hormones it was possible to initiate callus (cells that can become different plant organs, such as shoots and roots.) Once callus was initiated different hormones were added to the media mix to develop shoots and eventually plantlets.

It is the University of Hawai'i's good fortune to be able to share the material established in tissue culture with the National Plant Germplasm System.

CHAPTER 5

NUCLEAR DNA CONTENT ESTIMATIONS IN *ANTHURIUM* (ARACEAE)

Abstract

Nuclear DNA contents of 21 *Anthurium* species from eight different taxonomic sections and three *Anthurium* hybrid cultivars were determined using laser flow cytometry. The buffer used to extract intact nuclei was comprised of 45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 3-[N-morpholino] propanesulphonic acid (MOPS), 1% Triton X100, 5% polyvinyl pyrrolidone, 10 mM dithiothreitol, and 10 mM ascorbate, pH 7.0. DNA content values ranged from 2.40 pg to 8.38 pg among species, and from 5.71 pg to 6.03 pg among the hybrids examined. Use of recently unfurled leaf tissues maximized the population of cells in the G1 cycle, with the general absence of a distinctive G2 peak and minimal endopolyploidy.

Introduction

With a current approximation of 990 species (T. Croat, Missouri Botanic Gardens; unpublished), and habitats ranging from sea level to 3000 m, *Anthurium* is the largest and most diverse genus in the Araceae (Croat 1986). *Anthurium* is of particular interest for establishing DNA content due to its range of epiphytic and terrestrial habitats, variety of growth habits, and its value as floricultural and foliage crops. DNA content

estimations have utility for applications in *Anthurium* molecular biology, ecology, phytogeography, and systematics, among other disciplines (Bennett and Leitch 2005). Currently, DNA content values are only available for two *Anthurium* species, *A. tetragonum*: 15.28 pg/2C and *A. grande* 27.04 pg/2C (Gosh et al. 2001), and a limited number of monocot species (Bharathan et al.1994; Bennett and Leitch 2004). Previous determinations of *Anthurium* DNA content were made using Feulgen microdensitometry, a technique considered to be less accurate than laser flow cytometry (Galbraith et al. 1983; Bennett and Leitch 2004). Laser flow cytometry is also rapid, permitting replicated and numerous samples to be analyzed for accurate nuclear DNA content determination (Galbraith et al.1983; Dolezel 1997).

The objective of this study was to determine nuclear DNA content for 21 species of *Anthurium* from eight different sections of the genus, and for three commercial *Anthurium* hybrids using flow cytometry. DNA content within and among sections was evaluated. Chromosome count comparisons with DNA content were made.

Materials and Methods

Leaf tissue was taken from mature plants (Table 1) grown at the University of Hawaii Magoon greenhouses in Honolulu and at the Waiakea research station in Hilo in screenhouses with 80% shade cloth, overhead irrigation, and an average day/night temperature cycle of 29/23°C, respectively. Plants were fertilized using Osmocote 14-14-14 (N-P-K) ninety day formulation, and were treated with an integrated pest management program appropriate to the environmental conditions. Most leaf samples were analyzed

within 24 hours of removal from the plant, as recommended in Bharathan et al. (1994). Three to six replicates of each sample were analyzed. If possible, the tissues were taken from recently unfurled leaves, approximately three to five days after opening. According to Galbraith et al. (1983), the immaturity of leaves will decrease the population of cells in the G2 cycle, therefore maximizing the population of cells in the G1 cycle (G1=2C=2N, for *Anthurium*; Greilhuber et al. 2005). This is advantageous for this particular experiment due to the primary goal of attaining DNA content values, as increasing the population of cells in the G1 cycle decreases the standard error of the mean between samples.

All materials were kept on ice throughout the nuclei extraction process (Galbraith et al. 1983). For each sample, 0.8 grams of leaf tissue, excluding the midrib, were finely chopped using a scalpel with a Feather No. 10 blade (Electron Microscopy Sciences, Hatfield, PA) while submerged in 2 ml of buffer used to extract intact nuclei. The buffer used was: 45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 3-[N-morpholino] propanesulphonic acid (MOPS), and 1% Triton X100 (Galbraith et al. 1983) modified with the addition of 5% polyvinyl pyrrolidone, 10 mM dithiothreitol, and 10 mM ascorbate (Bharathan et al. 1994) adjusted to pH 7.0 with 1 M sodium hydroxide. Buffer was made just prior to use.

The suspension of nuclei resulting from leaves macerated for 10 min in buffer was passed through two 30 μm pore size nylon mesh filters seared onto the cut end of a small syringe to remove debris. Approximately fifteen minutes prior to beginning the flow cytometer analysis, 0.1mg ml^{-1} propidium iodide (Invitrogen) for staining and 0.01

mg ml^{-1} DNAase-free ribonuclease (Qiagen) to eliminate doublets were added to 1 ml of nuclei suspension (Bharathan et al. 1994).

Nuclei, ranging in number from 5000 to 15000 per sample, were analyzed with a Beckman-Coulter Altra flow cytometer (www.soest.hawaii.edu/sfcf) using the 488 nm line of a Coherent I90C argon ion laser set at 200 mW. The linear, log and peak fluorescence signals of the propidium iodide-stained nuclei were collected (610 BP filter), along with forward and side scatter signals. Propidium-iodide stained chicken erythrocyte nuclei (CEN; Biosure Inc.) were used as an internal or external standard. In instances where CEN was used as an external standard, it was run through the flow cytometer before and immediately after the plant tissue sample under the same instrument settings as the plant nuclei (Arumuganathan et al. 1991). Plots of peak versus linear propidium iodide fluorescence were used to eliminate doublets. Histograms of linear DNA fluorescence were analyzed using the FlowJo computer program (v. 6.3.4, Treestar Inc., www.flowjo.com) to determine mean peak positions for use in comparative analysis. Standard errors were calculated based on three DNA content values per species.

DNA content values were calculated in accordance with Arumuganathan and Earle (1991). The mean channel of the 2C peak of the sample was divided by the mean channel of the 2C peak of the CEN (arbitrary units). That value was then multiplied by the 2C DNA content of the CEN (2.33 pg/2C nucleus; Galbraith et al. 1983).

Dried specimens of all taxa, except *A. andraeanum*, have been deposited in the herbarium at Lyon Arboretum, University of Hawai'i. Live plant material is also maintained at the University of Hawai'i. Many of the species examined in this study are preserved in tissue culture as plantlets both at the University of Hawai'i and at the Ornamental Plant Germplasm Center as part of a germplasm conservation effort.

Results and Discussion

The peaks of the *Anthurium* samples fell slightly above those of the CEN, making CEN a useful reference standard (Fig. 5). The first major peak of fluorescence represented those nuclei in the G1 (2C) phase of the cell cycle, and the second major peak represented nuclei in the G2 (4C) phase (Fig. 5). A G2 peak, when present, appears at the expected channel equal to exactly two times that of the G1 peak (Galbraith et al. 1983). Most samples observed showed a distinctive G1 peak with very few nuclei at the expected range for a G2 peak or beyond. This lack of representative endopolyploidy was also shown in leaf DNA content analysis of various monocots by Bharathan et al. (1994) but differed from the high endopolyploidy reported for the monocot *Dendrobium* (Jones and Kuehnle 1998). Endopolyploidy is not indicative of DNA content and is most likely under developmental control (Arumuganathan et al. 1991). Based on the G1 peaks, the mean DNA content values across at least eight botanical sections of *Anthurium* ranged from 2.40 pg DNA for *A. pallidiflorum* to 8.38 pg DNA for *A. aripoense* (Table 4). These values differed greatly from those reported previously by Gosh et al of 15.28 pg and 27.04 pg.

The taxa examined ranged in 2C chromosome number from 24 to 90 (Table 4). The larger chromosome counts corresponded to larger DNA contents, as in *A. aripoense*, which has a chromosome count of 90 and an estimated DNA content of 8.38 pg. However, there were also examples of cryptopolyploidy seen as an increase in DNA content without an increase in chromosome number (Costich et al. 1993). A large DNA content unexplained by chromosome number may represent a primitive species (Dressler 1981). The theory that evolutionary adaptation in a group of related species results in a decreased genome size was put forth by Price (1988). While this study is not broad enough to draw strong phylogenetic conclusions, preliminary analysis supports such a theory for the section *Calomystrium* Schott, with *A. antioquiense*, A. *nymphaeifolium*, and *A. roseospadix* possibly representing ancestral species with 2C chromosome numbers of 30, and DNA contents of 7.14 pg, 7.25 pg, and 7.43 pg, respectively, compared to the seven other species of 2N=2C=30 examined in this section with 4.87 to 5.9 pg DNA/2C nuclei. These species are generally cross-compatible, producing interspecific hybrid offspring, despite the roughly 40% difference in DNA content (Kamemoto et al. 1996).

The hybrid *A.* 'Andraecola-1' (Kamemoto et al. 1995), a cross between *A. andraeanum* (5.72 pg) and *A. amnicola* (5.90 pg), had a 2C value of 5.71 pg, indicating a DNA content value closer to the female parent, *A. andraeanum*. The hybrid cultivars, 'Paradise Pink' (Kamemoto et al. 1981) and 'Tropic Flame' (Kamemoto et al. 1990), were very close in 2C values of 5.87 pg and 6.03 pg, respectively, despite their contrasting appearances and distinct genetic backgrounds. 'Paradise Pink' has a heart-shaped spathe typical of *A. andraeanum* (Kamemoto and Kuehnle 1996) and 'Tropic Flame' has a tulip-type spathe resulting from a cross with 'Calypso' and 'Trinidad'

(Kamemoto and Kuehnle 1996). These hybrids were chosen for this experiment due to the need for baseline DNA content to determine DNA loading quantities for gel electrophoresis to assess transgene copy number in genetically engineered plants (Kuehnle et al. 2004).

This efficient and effective protocol for measuring DNA content of *Anthurium* nuclei through flow cytometry techniques should be applicable to the advancement of this important genus. With environmental threats to the natural habitats of these species and the difficulty presented with keeping them alive in a botanical collection, these studies may be useful in determining the risk of extinction for a particular species based on DNA content (Vinogradov 2003; Fay et al. 2005). Vinogradov states that extinction risk increases with DNA content. Flow cytometry of *Anthurium* also opens up the possibility to study chromosomal differentiation due to organizational changes within the chromosome, and DNA content changes (Costich 1993). One useful application will be as a quality control tool for comparative assessment of plants resulting from clonal propagation by tissue culture. Continued surveying of *Anthurium* DNA content will provide researchers and breeders an additional trait for future systematic studies and molecular manipulations.

Table 4. Mean DNA content, in pg/2C and Mbp (mega base pairs)/1C, and chromosome number of *Anthurium* species and hybrids by taxonomic section.

Section	Species	Chromosome Number (2C)	Authority	Mean 2C DNA content (pg) +/- SE	Mbp/1C nuclei
Belolonchium Schott	*A. brownii* Masters	30	Sheffer et al. (1983)	6.23 +/- 0.20	3008
	A. aripoense N.E. Br.	90	Sheffer et al. (1976)	8.38 +/- 0.25	4043
Calomystrium Schott	*A. amnicola* Dressler	30	Marutani et al. (1988)	5.9 +/- 0.13	2847
	A. andraeanum Andre	30	Sheffer et al. (1976)	5.72 +/- 0.09	2760
	A. antioquiense Engler	30	Sheffer et al.t (1983)	7.14 +/- 0.15	3445
	A. formosum Schott	30	Marutani et al. (1993)	5.0 +/- 0.13	2413
	A. kamemotoanum Croat	30	Sheffer et al. (1976)	4.87 +/- 0.07	2350
	A. lindenianum K . Koch & Augustin	30	Marutani et al. (1993)	5.17 +/- 0.34	2496
	A. nymphaeifolium K . Koch & Bouché	30	Marutani et al. (1993)	7.25 +/- 0.47	3498
	A. ravenii Croat and & Baker	30	Marutani et al. (1993)	5.18 +/- 0.32	2499
	A. roseospadix Croat	30	Marutani et al. (1993)	7.43 +/- 0.59	3585
	A. sanctifidense Croat	30	Sheffer et al. (1983)	5.68 +/- 0.06	2741
Cardiolonchium	*A. falsomianum* Croat	30	Sheffer et al. (1976)	2.90 +/- 0.15	1399
Leptanthurium Schott	*A. gracile* (Rudge) Lindl.	40, 60	Sheffer et al. (1983)	4.94 +/- 0.12	2384
Pachyneurium Schott	*A. standleyi Croat & Baker*	60	Sheffer et al. (1983)	7.92 +/- 0.15	3821
	A. watermalense Hort ex L.H. Bailey	30	Sheffer et al. (1983)	5.26 +/- 0.09	2538
Porphyrochitonium Schott	*A. wendlingeii* G.M. Barroso	30	Sheffer et al. (1983)	5.07 +/- 0.18	2446
Semaeophyllium Schott	*A. gargavanum* Standl.	30	Marutani et al. (1993)	5.31 +/- 0.05	2562
Tetraspermium Schott	*A. trinervi* Miquel	24, 30	Sheffer et al. (1976)	3.94 +/- 0.11	1901
Undetermined	*A. hookeri* Kunth	30, 60	Sheffer et al. (1976)	6.31 +/- 0.13	3045
	A. pallidiflorum Engl.	Undetermined	Not applicable	2.40 +/- 0.06	1159
Hybrids	'Andraecola' -1	Undetermined	Kamemoto et al. (1994)	5.71 +/- 0.28	2755
	'Paradise Pink'	Undetermined	Kamemoto et al. (1981)	5.87 +/- 0.18	2832
	'Tropic Flame'	Undetermined	Kamemoto et al. (1990)	6.03 +/- 0.04	2909

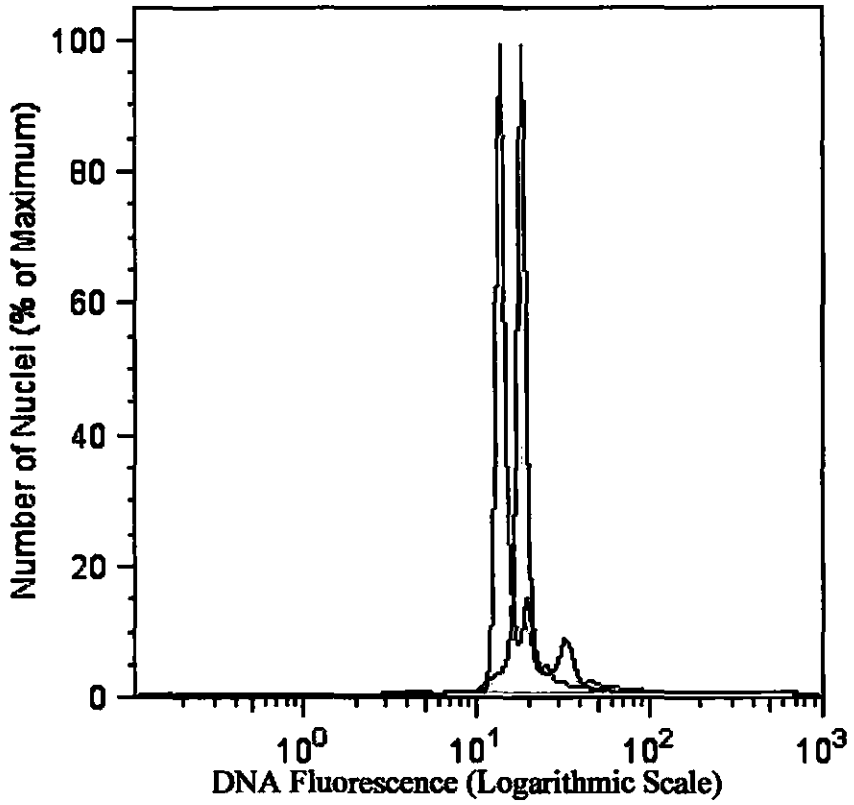


Figure 5. Ungated logarithmic histogram of *A. folsomianum* (formerly *A. folsomii*; shaded peak) and chicken erythrocyte nuclei (unshaded peak) showing distinctive G1(100% of maximum) and G2 peaks. Calculations were based on linear fluorescence channels of the sample in comparison to the reference standard. Counts of 15,325 nuclei of *A. folsomianum* and 6,124 nuclei of the reference standard were run through the flow cytometer in this sample.

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