RAPID IN VITRO MULTIPLICATION OF ANTHURIUM USING TEMPORARY IMMERSION SYSTEM (RITA®)

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ABSTRACT

Anthuriums have been considered as the most important cut flower in Hawaii and have been consistently among the top floriculture products. Despite efforts to improve in vitro propagation, problems such as inadequate generation of microplants and slow microplant to field turnover are still encountered. Hence, commercial propagation and cultivar release of anthurium is hindered. To address current problems encountered in anthurium micropropagation, this thesis explored the use of RITA® bioreactors to accelerate anthurium shoot initiation and multiplication. The in vitro shoot production capacity of *Anthurium andraeanum* Hort. 'New Pahoa Red' under the RITA® bioreactor system and the conventional flask system were compared. The RITA® bioreactor system produced higher numbers of initiated shoots (3-4 fold) and increased proliferation rates by 1.6-2.6 fold compared to the conventional flask system. Culture conditions, namely immersion time, media volume and resting interval, for the RITA® bioreactor system were also optimized. A 5-minute immersion time, media volume of 20 mL/explant with a resting interval of 2 hours increased in vitro secondary shoot production and axillary bud mass volume. A comparative analysis of ten anthurium genotypes was done to assess variability of in vitro growth responses under the RITA® bioreactor system. Shannon-Weaver diversity indices revealed low to moderate diversity/variability for the in vitro responses. Through cluster analysis, five clusters were classified based on quantitative and qualitative parameters. Cross-referencing clusters with existing pedigrees revealed similarities within the lineages of the genotypes and that genotype exerts a greater influence over secondary shoot production compared to growth habit. The inclusion of RITA® bioreactors in micropropagation systems and assessment of genotype dependency will enhance microplant production and accelerate cultivar release of anthuriums. Along with pedigree records and historic data,

comparative analysis of in vitro growth responses could provide a benchmark for protocol optimization. It could also help with the identification of genotypes that perform well under in vitro conditions which can be used to introgress in vitro culture suitability in cultivar development.

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CHAPTER 1: INTRODUCTION

Anthuriums are one of the most important cut flowers of Hawaii. In 2018, it ranked second in floriculture and nursery products with a sales value of 2.7 M USD and had the highest volume sales (2.8 M stems) among the cut flowers produced (NASS, 2019). It has consistently been the top cut flower produced in the state for 28 years (NASS, 1997; NASS, 2001; NASS, 2006; NASS, 2011; NASS 2016; NASS, 2019).

Commercial production of anthuriums in the field has significantly evolved from the use of seeds and top cuttings to micropropagation. Seeds were initially used as the main method for disseminating anthuriums in the late 1930s and 1940s (Kamemoto and Kuehnle, 1996). Its flowers are protogynous and favor cross pollination. Seeds that develop from these flowers are highly heterogenous particularly in terms of flower quality, color, yield, and time to first flowering (Geier,1990; Kamemoto and Kuehnle, 1996; Matsumoto and Kuehnle, 1997). To maintain uniformity in production, growers resorted to the use of vegetative propagation methods. Field propagation is mainly done through top cuttings. Rooted top cuttings are slow to develop. Mature flowers will develop in approximately 6 months depending on the cultivar (Matsumoto and Kuehnle, 1997). In addition, bacterial blight caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* can be spread through mechanical injury during cutting (Nishijima and Fujiyama, 1985). The majority of anthurium nurseries in Hawaii rely on micropropagation for the supply of true to type and clean planting materials.

The University of Hawaii's anthurium breeding program uses a conventional flask system in the production of microplants. This system uses foil enclosed 125 ml flasks and a rotary shaker to facilitate aeration. The program has adopted a media protocol developed to avoid somaclonal variation. The protocol uses low concentrations (0.2 mg/L) of cytokinin (6-

Benzylaminopurine) and limited the exposure time (2 subcultures) of explants during culture (Kunisaki, 1980). Liquid media are known to improve growth rates in numerous species compared to solid media by allowing rapid uptake of nutrients and dilution of exudates (Preene, 2010). The use of liquid media also allows for options such as automation and semi-automation (Berthouly and Etienne, 2005). The conventional flask system and Kunisaki's 1980 media protocol have been used in the successive propagation of all new cultivars developed by the breeding program and for supplying growers with in vitro stock plants as propagation materials.

Although, Kunisaki's (1980) protocol reduces the incidence of somaclonal variation during micropropagation, it has not capitalized on the advantages given by liquid culture systems (i.e. conventional flask systems) due to low concentrations of BA required to maintain the microplants' genetic stability. A single shoot may develop in 12 to 18 months depending on the variety (Kamemoto and Kuehnle, 1996; Matsumoto and Kuehnle, 1997). It takes around 3 years to establish enough microplants for varietal testing (Kamemoto and Kuehnle, 1996) and 1- 1.5 years to produce the 25,000-35,000 microplantlets needed for an acre of commercial planting (E. Tanouye and T.T Neo pers. comm.). The slow growth rate is a bottleneck for breeding and commercial production.

To increase microplant production, other methods must be explored. One such method is the use of bioreactors which can exploit liquid culture systems and allow for precise control of microenvironments (Watt, 2012; Georgiev et al., 2014). There are four main classes of bioreactors: 1) the liquid phase bioreactors, 2) gas-phase bioreactors, 3) temporary immersion systems (TIS) and 4) hybrids. Most bioreactors submerge plant tissues continuously in liquid medium, which often lead to abnormalities caused by asphyxia and hyperhydricity (Debnath, 2011).

Temporary immersion systems (TIS) avoid negative effects brought about by submerged conditions such as asphyxiation and hyperhydricity by exposing tissues periodically to the liquid medium and then allowed to rest or be exposed to the gaseous environment within vessels (Debanath, 2011; Georgiev et al., 2014). The TIS has the capability of providing the most natural environment to in vitro cultures (Steingroewer et al., 2013).

One of the most common temporary immersion systems used in commercial micropropagation is the *R´ecipient `an immersion Temporaire Automatique* or RITA® (CIRAD and Vitropic, France). The RITA® has a simple design compared to other TIS; it forces ambient air into the vessel through sterile air vents using a pump where as other TIS require bulky shakers (e.g. tilting and rocker types) and complex control blocks (e.g. TIS with media renewal mechanism). The RITA® bioreactor is compatible with most pumps. In addition, the manifold that is attached to the bioreactors can fit up to 20 units and can be fabricated. Manifolds can be adjusted to fit 5-20 units allowing for flexibility from small scale to large scale production. The smaller vessel volume (500 ml) of the RITA® compared to other larger bioreactors (e.g. SETIS-6L) lessens the risk of losing large numbers of microplants to contamination (Georgiev et al., 2014).

In this thesis, the aim was to compare the RITA® bioreactor system with the conventional flask system to assess production capacity. The use of the RITA® on anthurium was first reported by Ruffoni and Savona (2005) but lacked optimizations for the system, therefore we also aimed to optimize RITA® culture settings (e.g. immersion frequency, volume, and resting cycles). These objectives were addressed in Chapter 3.

In addition, the comparison and measure of the variability of the in vitro responses of ten anthurium genotypes under the RITA® bioreactor system were assessed. Developing an

optimized protocol for different genotypes may be challenging because of the variable growth responses in in vitro conditions. Different genotypes are likely to show varying responses under the same culture conditions. Measuring and identifying variability of in vitro responses, followed by cross-referencing results with existing pedigree information provided baseline information that can be used for protocol development. These objectives were addressed in Chapter 4.

CHAPTER 2: LITERATURE REVIEW

Anthurium

Taxonomy and Systematics

Anthurium is the largest genus in Araceae with 1,690 described species, of which 950 are published, and with new species still being discovered in the Andes, the number of species could easily exceed 2000 (Croat, 2015; Boyce and Croat, 2011-2018). Majority of the species are still poorly characterized. Previous sectional classifications relied on the Lucid multichotomous key for *Anthurium* which distinguished 18 clades (Croat and Scheffer, 1983). Molecular studies realigned some of the sections within the genus e.g. section *Schizoplacium* to section *Dactylophyllium* (Croat and Carlsen, 2013).

A consensus between the traditional sectional classification and molecular phylogeny has not been reached, most notably due to the highly homoplasious (characters that evolved independently in different lineages which have similar function due to species occurring in similar environment) morphological characters used in the traditional sectional classification within the genus *Anthurium.* Realignment studies for the genus is still underway and to date, the genus is classified into 18 clades with two series. Among the clades, seven are monophyletic (i.e. *Andiphilum (*Schott) Croat, *Calomystrium* (Schott) Engl., *Dactylophyllium* (Schott) Engl., *Leptanthurium* (Schott) Engl., *Polyphyllium* Engl., *Tetraspermium* (Schott) Engl., and *Multinervia* (Croat) Carlsen & Croat (Carlsen and Croat, 2019).

Morphology

Anthuriums are herbaceous monocotyledonous plants with several forms. They are mostly epiphytes with some hemiepiphytic forms, or they can occur as terrestrials (Croat, 1983). Some unique forms are the epilithic anthuriums which grow on rock surfaces and the rheophytes which can survive in fast moving water or they can occur as both e.g. *A. andicola, A. rupicola, A. sytsmae* and *A. antioquense* (Croat, 1988). In the genus *Anthurium*, the growth habit (Figure 2.1) can either be monopodial or sympodial (Henriquez, 2015). The stem and internodes can range from short to elongate with numerous aerial roots at each node. Cataphylls, the small lanceolate leaf-like structures, can be persistent or deciduous. The true leaves are attached to a firm petiole that is either stiff or flexible and often sheathed at the base. These leaves often cluster at the end of the stem. The leaf blade is simple. Leaf shape can vary in form; it can be ovate, elliptic, or lanceolate, cordate or digitately lobed and can either be fused or divided. The leaves have netted venations and a stout midrib. Its inflorescence (spadix) can be pendulate, usually occurring one per node with a subtending spathe inserted on the peduncle. The spathe is usually flat and spreading or reflexed, erect and hooding or cup-shaped (Croat, 1983). The flowers closely aggregate into a spiral and truncate at the apex (Croat, 1983; Kamemoto and Kuehnle, 1996). They are perfect and protogynous in nature, which make them a primarily outcrossing species (Kamemoto and Kuehnle, 1996). The fruit produces 1-2 seeds per berry (Croat, 1983; Kamemoto and Kuehnle, 1996). The berry shape ranges from ovoid, oblong-ovoid, oblong or obovoid. They vary in coloration and have two cells containing one seed per cell (Croat ,1983).

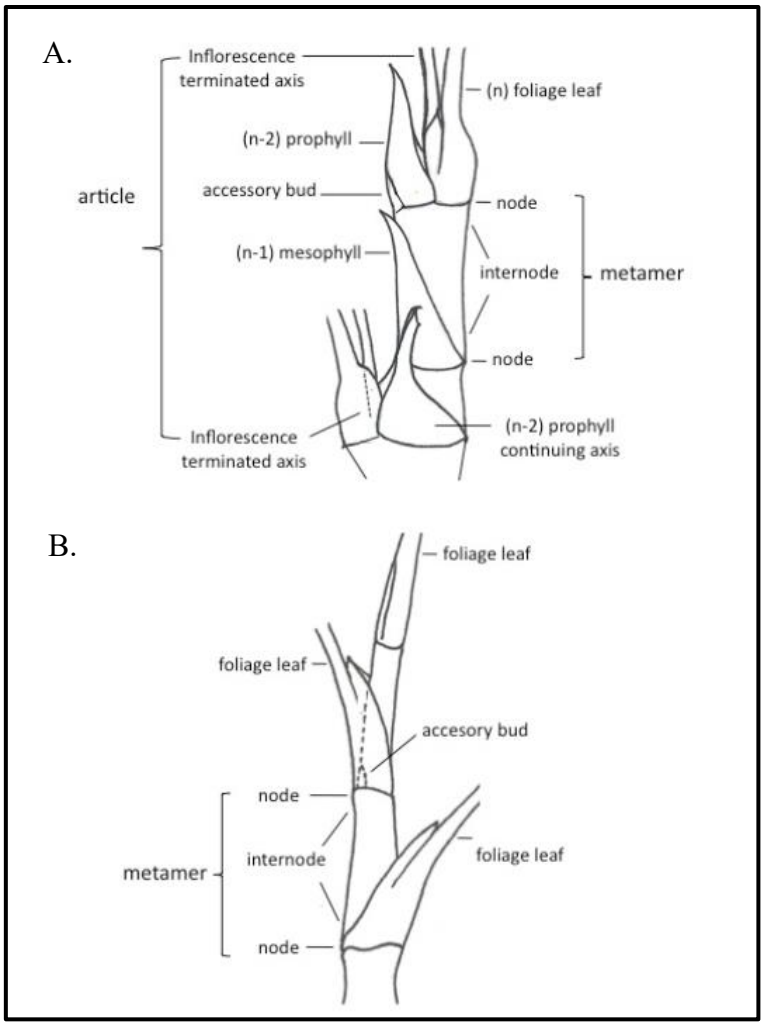

Figure 2.1. Type of growth in *Anthurium*. A.) Sympodial and B.) Monopodial (Henriquez, 2015).

Domestic and International Anthurium Market Trends

At present, the Netherlands is the leader for the global market for anthurium. It is both the top producer and importer of anthuriums as cut flower, cut foliage, and potted plants (Pizano, 2005; Sheela, 2009; CBI Market Survey, 2017). The Netherlands has developed the anthurium into an iconic plant for tropical ornamentals while expanding its uses. Yellow dyes have been incorporated into the spathes of potted anthuriums to market for Easter. The varieties of color are only limited by the availability of dyes (Peeters, 2015).

In addition, the onset of the DIY (Do-It-Yourself) movement has also influenced marketing of anthurium internationally. In 2015, DIY plant kits were sold as an outlet for customer creativity, in which consumers were able to design their own potted plant (Tazelaar, 2015). In 2019, two anthurium hybrids, *Anthurium* 'Jungle Queen,' an *Anthurium schlechtendalii*, hybrid and *Anthurium* 'Queen of Hearts', an *Anthurium watermaliense* hybrid, were identified as candidates for the next trend for indoor potted plants (Mather, 2019).

With the introduction of the new design concept Tropical Nouveau (Figure 2.2) tropical and temperate flowers blend to create new ways of complementing botanicals of opposing origins (Garcia, 2019). Anthuriums are able to fill a novel niche in floral design which leads to higher demand for the cut flower.

Figure 2.2 Tropical Nouveau design trend. Design: Hitomi Gilliam, AIFD. Photo: Jaclyn Nicole Uy

Production and Cultivation

Seed

The first anthurium to arrive in Hawaii was a shell -pink anthurium (*Anthurium andraeanum*) which was introduced by estate owner S.M. Damon in 1889. The anthurium was purchased from London and was brought back to Oahu. Hobbyists and nurserymen quickly took interest in the unique plant and started propagating them through seeds (Neal, 1965). At present, conventional methods of producing anthurium include seeds, divisions, top cuttings, and in vitro methods. Growing anthuriums from seed to first bloom may take up to three years (Croat, 1979; Chandler, 1991; Higaki et al., 1995).

Berries are collected from hand or open pollinated flowers and the seeds (1-2 seeds) are squeezed out of the pulp. Once extracted, the seeds can be planted in shredded hapu'u *(Cibotium chamissoi* or tree fern fibers) and kept under 75 to 80% shade (Higaki et al., 1995). The germination time for seeds ranges from 1-2 weeks to 3 months (Chandler, 1991; Higaki et al., 1995). Transplanting is done 4 to 6 months after sowing (Higaki etal., 1995). Due to the protogynous nature of anthurium flowers which favor cross-pollination, the resulting seedlings are highly heterogenous, particularly for flower quality, color, yield and time to first flowering (Geier,1990; Kamemoto and Kuehnle, 1996; Matsumoto and Kuehnle, 1997).

Divisions and Cuttings

Commercial propagation is mainly done through divisions and top cuttings. Divisions refer to the developing lateral shoots taken from the basal portion of the plant, while top cuttings are the uppermost portion of the stems that are cut off from the plant which contain at least 2-3 leaves. The cutting is then rooted in well-aerated media where roots develop within 2-3 weeks. The rooted top cutting develops flowers in approximately 6 months depending on the cultivar

(Matsumoto and Kuehnle, 1997). Lateral shoots develop on the remaining portion of the plant, referred to as “gobo” by growers in Hawaii. The number of lateral shoots that develop in anthurium cultivars are highly variable.

The application of 6-Benzylaminopurine (BA) has been observed to stimulate lateral shoot development in anthuriums. Application of 1000 ppm BA on the cut portion of topped basal stems led to increased lateral shoot production in the anthurium cultivar ‘Ozaki Red’ which produced 3.6 lateral shoots per plant (Higaki and Rasmussen, 1979). In juvenile anthurium plants (10-15 cm in height) foliar application of 1000 ppm BA on topped plants lead to an increase in lateral shoot production (4 lateral shoots/plant) compared to topped anthuriums without BA application (1.8 lateral shoots/ plant) (Imamura and Higaki, 1988). In ‘Nitta’ 5.7 lateral shoots were produced per plant and bud initiation occurred at 253.8 days (Maitra and Roychowdhury, 2014).

Micropropagation

Propagation through divisions and top cuttings is relatively slow and may take years for growers to reach the number of plantlets required for full production capacity. Diseases such as bacterial blight caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* and anthurium decline caused by the burrowing nematode, *Radopholus similis* result in poor vigor of top cuttings from overgrown field plantings (Uchida et al., 2003). Additionally, latent infection in in vitro cultures (Norman and Alvarez, 1994) necessitates triple indexing of in vitro stock (Tanabe et al, 1990) to produce disease-free planting materials.

The majority of anthurium production in Hawaii is reliant on micropropagation for the supply of planting materials. Several in vitro techniques are available including seed culture, callogenesis, somatic embryogenesis and apical or axillary bud culture. Leaf, petiole and axillary

buds are used to initiate callus or shoots and are continuously subcultured for further multiplication. Micropropagated stock plants are then sent to specialty propagators (contracted tissue culture facilities) for mass production of rooted microplants (Kamemoto and Kuehnle, 1996). Due to the heterogeneity of anthurium seeds, in vitro culture using seeds is mostly limited to conservation of endangered or rare anthuriums and expanding the breeding pool by bulking up on unknown phenotypes (Tanabe et al., 1989). Successful micropropagation using seeds as explants has been done in *Anthurium parvispathum* (Atta-Alla et al., 1998) and *Anthurium antioquiense* Engl (Murillo-Gómez et al., 2014).

Other techniques such as callogenesis and somatic embryogenesis are employed for clonal propagation but due to prolonged exposure to high concentrations of plant growth regulators required for callus initiation, somaclonal variation may occur (Geier, 1990; Kuehnle and Sugii, 1991). Callus induction and plantlet regeneration were found to be genotype specific in anthuriums (Yang et al., 2002).

In 1993, a survey was sent out to estimate the extent of Anthurium micropropagation in the USA. Seven major commercial facilities were identified and estimates of the plants produced from these laboratories reached 3.8 M. These laboratories primarily use axillary bud and apical bud culture to avoid somaclonal variation (Matsumoto and Kuehnle, 1997). However, somaclonal variation in axillary buds has been reported. Some nodal-derived plants of anthurium cultivars ‘Rudolf’ (21%) and UH1003 (2%) developed severely stunted shoots or shortening of internodes after 3-4 years in vitro culture. Callus-derived (12-13-month-old) plants from ‘Rudolf’ (2%) and UH1060 (1%) showed stunting, abnormal leaf shape and variegation (Kuehnle and Sugii, 1992).

Since micropropagation is still the fastest method for increasing clones, it is the preferred method for providing planting materials. Laboratories in the Netherlands produce micropropagated plantlets and micro-cuttings (i.e. unrooted tissue cultured shoots), transported without agar in sterile plastic containers, for export and field planting (Bleiswijk, 2016). In the US, Florida produces in vitro grown plantlets for potted anthurium production. The in vitro grown plantlets are transferred in multi-cavity liner trays with a 1:1:1 peat, perlite, and bark mix media and then transferred to pots or to the field (Chen et al., 2015).

To avoid somaclonal variation, the University of Hawaii anthurium breeding program uses an axillary bud culture protocol (Kunisaki, 1980). This protocol uses a low concentration (0.2 ppm) of BA and limited the exposure time (two subcultures) of explants during culture. This method has been adapted in the propagation of all new cultivars developed by the breeding program.

Two-node segments (one axillary bud per node) are initiated in a 125 mL flask containing liquid media with 0.3 MS + 0.2 ppm BA+ 15% coconut water for 45 days. After initiation, primary shoots are excised from the axillary bud mass after which the bud mass is allowed to proliferate for another 45 days to promote growth additional shoot in an 0.5 MS medium supplemented with 15% coconut water and 20 g/L sucrose without growth regulators and solidified with 2g/L gellan gum (CultureGel™,Phytotech). These secondary shoots are excised and transferred to the same solid media for further development (Figure 2.3).

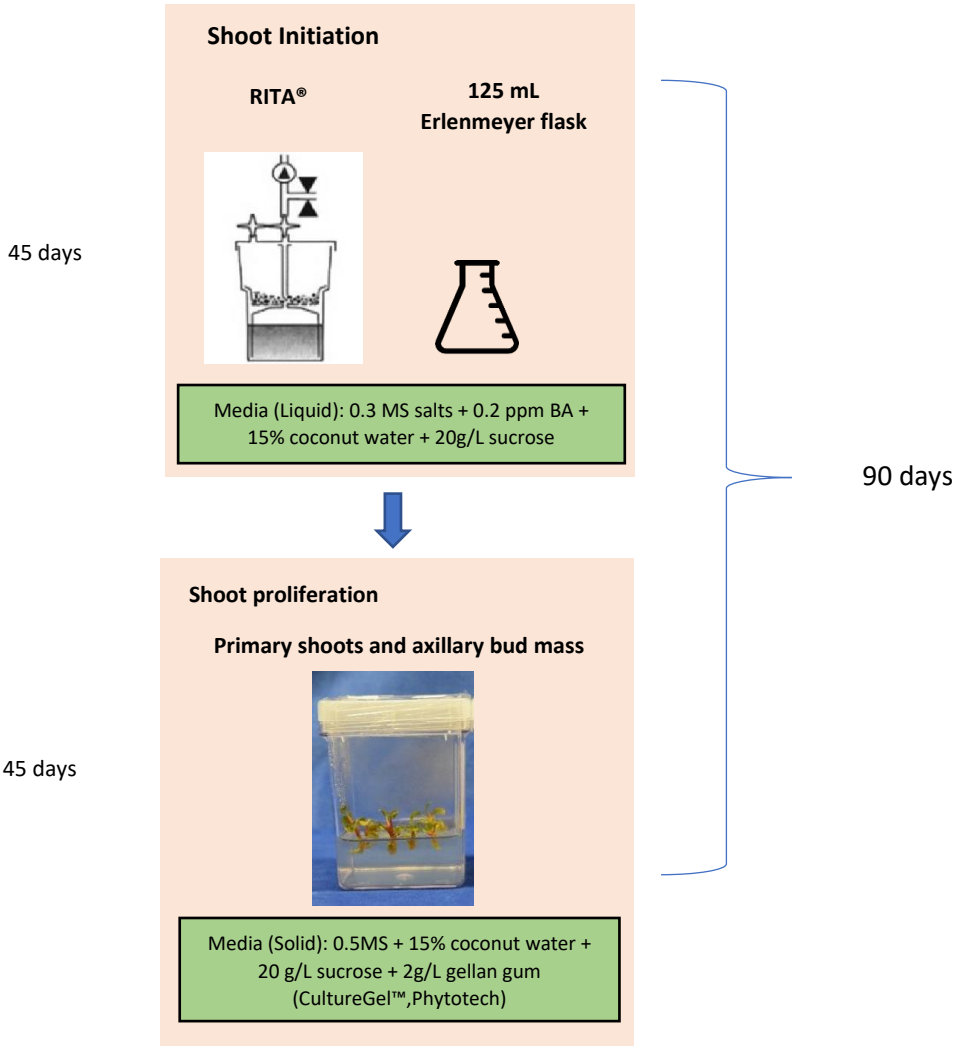

Figure 2.3 Axillary bud culture protocol (Kunisaki, 1980).

Anthurium Breeding

Anthuriums have bisexual and protogynous flowers which enable them to cross pollinate easily and thus resulting in a wide diversity in offspring phenotypes. There is relatively little variation in spathe color within wild species but cultivated *A. andraeanum* shows a wide variety of color, size and shape. The cultivated *A. andraeanum* is presumed to be of hybrid origin (Kamemoto and Kuenhle, 1996). *A. andraeanum* can undergo interspecific hybridization with other *Anthurium* species such as *A. amnicola* for production of miniatures and purple spathes, *A. antioquense* for bacterial blight resistance, *A. armeniese* for fragrance , *A. formosum, A. hoffmanii, A. lindenianum,* and *A. nymphaeifolium* for cup-shaped spathes (Kamemoto and Kuehnle, 1996).

The Anthurium breeding program in Hawaii provided evidence of the hybrid nature of the cultivated *A. andraeanum* Hort. The species from the wild, *A. andraeanum* Linden ex André, has an orange spathe. Interspecific hybrids produced between cultivated *A. andraeanum* and other *Anthurium* species such as *A. lindenianum, A. hoffmanii and A. nymphaepholium* produced progenies with spathe colors that differed from the parents. A wide range of recombinants was also observed in the F2 hybrids with the heart-shaped spathe typical of *A. andraeanum* suggesting the hybrid nature of one parent. Backcrosses resulted in the loss of the characteristics of the non-*A. andreanum* parent in the progeny (Kamemoto and Kuenhle, 1996).

Anthuriums are bred for cut flower or potted plants. In Florida, the primary focus of breeding is for potted plant production (Henny et al., 2017). In Hawaii, breeding has focused on cut flower production, although there have been cultivars developed for potted plants. Cut flower breeding in *Anthurium* was primarily targeted to three types: the standards, the obakes and the tulip types. The standards (Figure 2.4 A.) have glossy, broad, symmetrical, and heart-shaped

spathes with overlapping lobes and one uniform color. The obakes (Figure 2.4 B.) which is the Japanese word for ghost or change, are characterized by dual colorations of green and their respective spathe color. They vary in size, shape, and color. Some obakes remain as standard types during their younger phase of development and only turn into their dual coloration at maturity. The tulip-types (Figure 2.4 C) are characterized by upright and cupped spathes (Kamemoto and Kuehnle, 1996).

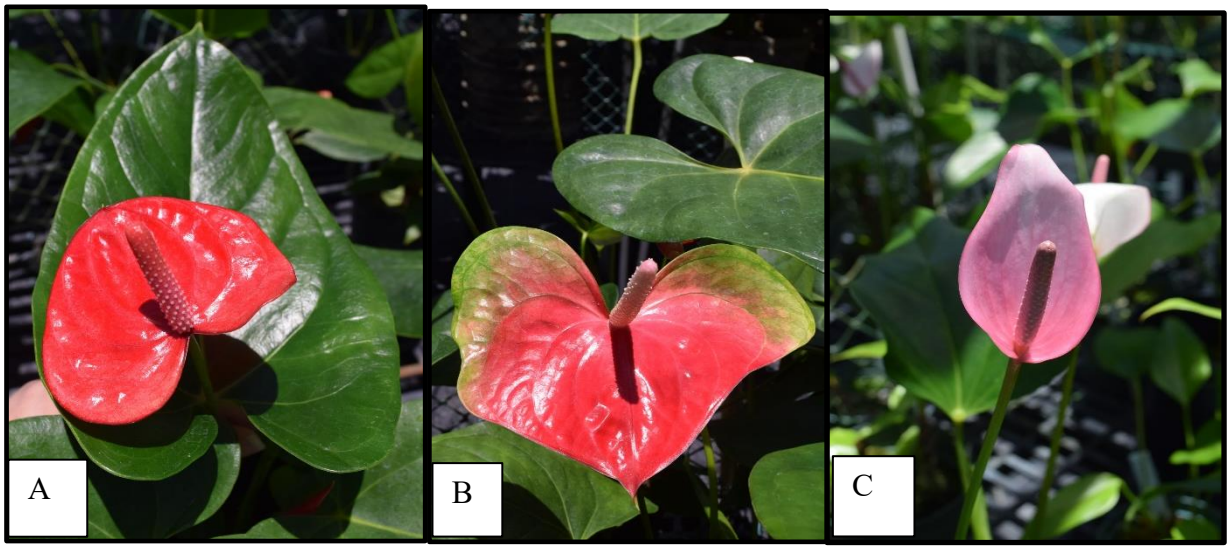

Figure 2.4 Types of Anthurium. A.) Standard, B.) Obake and C.) Tulip-types.

The breeding objectives for anthurium have changed over the years. In the 1990s, breeding for color was primarily focused on five major groups, red, orange, pink, coral and white with some unique novel colors such as purple, green, brown and mottled patterns (Kamemoto and Kuehnle, 1996). Current trends for colors such as pastels and pinks have shifted breeding towards softer tones of orange and coral with emphasis on blush types (Figure 2.5) and bright pinks. Grey and muted tones have also gained the interests of floral designers (pers. comm. Hitomi Gilliam, AIFD and Louis Hiranaga, AIFD). Focus on spadix color was also less evident

in the 1990s. In the majority of anthurium cultivars, spadix coloration contrasts with the spathe color. However, current designs require subtle and non-contrasting coloration that help unify the design (pers. comm. Hitomi Gilliam, AIFD). As such, selecting for non-contrasting coloration of the spadix has also been initiated.

Figure 2.5 Standard anthurium with blush.

In addition, breeding for spathe shape was primarily geared towards heart-shaped, flat and uniform spathes with a 30º to 45º carriage (angle from which the flower is attached to the peduncle) to facilitate ease of shipment (Kamemoto and Kuenhle, 1996). Demand for more texture and depth in flowers has shifted toward selecting for wider variations in shape, spathe curvature and spathe carriage that is more perpendicular (90º) to obtuse (130º to 150º).

Preference for other characteristics remain the same such as in yield, brittleness of the peduncle, internode length, vase life, resistance to anthracnose (*Colletotrichum gloeosporioides*), bacterial blight (*Xanthomonas axonopodis* pv. *dieffenbachiae*) and burrowing nematode (*Radopholus similis*).

Flower yield is influenced by temperature, illumination, water availability, nutrition, genetics and occurrence of pests and diseases. An acceptable yield is 6 flowers per plant stem per year. Long, straight, and sturdy peduncles that carry flowers above the leaves are also desired. (Kamemoto and Kuenhle, 1996).

It takes approximately 13-14 years (pollination to cultivar release) to release a new cultivar of anthurium. Stigmas become receptive when spathes unfurl, after which the pollen are shed when the stigma has lost its receptivity. Pollination is accomplished by rubbing the length of the spadix with the index finger. The pollen collected are then deposited onto the spadix of a receptive flower. After 6-7 months, the berries mature. The berries are then collected, and the pulp is separated from the seed. Seeds are then germinated on fern fibers or other types of media. After 3-4 months the seedlings are transplanted and in another 8-10 months the plants are transferred to 6-inch pots. Flowering takes approximately 18 months from sowing. Upon flowering, individual seedlings are selected, then the progenies are carefully screened for 24-36 months for desired attributes. Selected plants are mass produced through micropropagation (36 months) for field testing (4-5 years) (Kamemoto and Kuehnle, 1996). Selection and evaluation take up considerable time in cultivar development. The availability of micropropagated plants for field testing is a major bottleneck that highly affects the timeline for cultivar release. Improving the micropropagation system will hasten selection and evaluation, and ultimately variety release.

Bioreactors

Commercial production of plants through micropropagation presents distinct advantages over the conventional methods of propagation: production of disease-free planting material and production of large quantities of true to type plants in short amounts of time and in limited

spaces. With the development of new semi-automated systems such as plant-based bioreactors, micropropagation technology has vastly improved. It has opened avenues for secondary metabolite production, rapid propagation of hard to root cultivars and genetically modified plants.

Bioreactors are specialized containment vessels that are designed for intensive and scaled up production of microplants. They usually consist of a culture vessel which could contain biological units such as bacteria, algae, plant or animal cells, and whole plants along with a compartment for the nutrient solution. The nutrient solution could either be separated or contained within the same container. They maintain their own microenvironment and capitalizes on liquid culture and an inflow and outflow system (Takayama, 2011). Bioreactors have an automated or semi-automated controller blocks that regulate conditions such as temperature, agitation, illumination regime, pH, dissolved O_2, CO_2 concentrations, composition of the gaseous environment, and volume of the liquid medium within it (Watt, 2012; Georgiev et al, 2014).

Bioreactors are categorized into four main types: 1) the liquid phase bioreactors, 2) gas-phase bioreactors, 3) temporary immersion systems (TIS) and 4) hybrids. Of the four bioreactor types, the TIS is considered the most effective in plants since it closely mimics conditions found in the plant's natural environment such as free gas exchange and intermittently available water (Steingroewer et al., 2013).

Intermittent flooding conditions and free gas exchange allow the microplant to avoid abnormalities caused by asphyxia and hyperhydricity (Debnath, 2011). The most well-known TIS, R´ecipient `an immersion Temporaire Automatique or RITA® (CIRAD and Vitropic, France), has been used in the commercial production of many high value crops such as vanilla

(Ramírez-Mosqueda and Iglesias-Andreu, 2016; Ramos-Castellá et al., 2014), stevia (Ramírez-Mosqueda et al., 2016) and apple (Zhu, Li and Welander, 2005).

The RITA® system is a 500 mL vessel system with two separate compartments (Figure 2.6 A). The upper compartment is the culture chamber which has a support mesh that holds the explant units. The lower compartment contains the medium storage tank. Ambient air is pumped into air vents which filter the air and keep the whole vessel sterile. The pumped air then travels down to the medium storage tank through a small pipe. The pressure that builds up in the media storage tank forces the nutrient solution up to the culture chamber, irrigating and aerating the whole system (Figure 2.6 B).

The RITA® has two main phases of activity, the immersion phase and the resting phase. During the immersion phase, the nutrient solution is introduced to the plants, usually for less than 30 minutes (immersion time) for most protocols. The second activity is the resting phase (also referred to as immersion interval, resting interval, resting cycle or cycle) which can range from a few minutes to 24 hours.

An immersion sequence, which consists of four events. First, the resting step in which the RITA® is at rest. Gas exchange and flooding of the upper compartment are absent. The second step is pressure build up. Ambient air is pumped in to build pressure and facilitate gas exchange. In the third step, flooding occurs. Positive pressure is applied to the lower compartment to push the nutrient solution up into the upper compartment thus irrigating the explants. The fourth step is the draining of the nutrient solution. Air flow stops, and the upper compartment is drained of the nutrient solution allowing the system to return to the resting phase (Vitropic, n.d.).

The use of RITA® in plant micropropagation has changed the outlook of mass production of in vitro plants. It was first used in banana micropropagation using a 20-minute

immersion time at a 2-hour resting cycle (Alvard et al., 1993). Shoot production was greater compared to the conventional method. In vanilla, a three-fold increase in multiplication rate was observed after using an immersion time of 2 minutes with a resting cycle of 4 hours (Ramos-Castellá et al., 2014).

In *Stevia rebudiana*, the highest number of shoots formed on nodal segments subjected to an immersion time of 2 minutes and a resting time of 8 hours (Ramírez-Mosqueda et al., 2016). Ruffino and Savona (2005) used RITA® to proliferate in vitro plantlets (1.5 cm) of *Zantedeschia aethiopica* and *Anthurium andraeanum* using an immersion time of 3 minutes for every 3 hours. However, their paper did not report any optimization experiments.

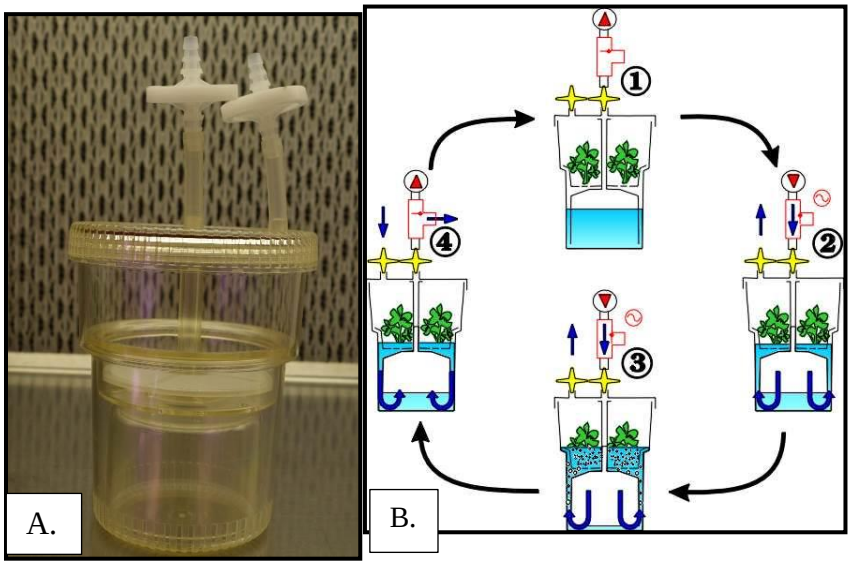

Figure 2.6. Temporary immersion system A.) RITA® and B.) immersion process. Image retrieved: https://vitropic.pagesperso-orange.fr/rita/en/accueil.htm

Genotype Effect during Tissue Culture

Multiple factors affect plant response under in vitro conditions such as plant age, explant type or tissue type, nutrition, levels of endogenous and exogenous growth hormones, light,

temperature, and gases in headspace of containers. One of the most important factors challenging plant researchers and tissue culturists all over the world is genotype. The effect of genotype has been documented in many in vitro studies whether in protocol development or plant transformation. Its effect has been most notable in the plant's regeneration potential, the ability to regenerate from calli. For example, Mathias and Simpson (1986) investigated the effect of coconut water on callus induction in eight hexaploid lines of wheat. Significant varietal differences were found in shoot regeneration capacity in media with or without coconut water.

When eight rice cultivars were subjected to a callus induction medium (MS medium + 4 ppm TDZ), significant varietal differences were observed in percent regeneration which ranged from 65-90%, while mean shoot number produced per explant ranged from 2.1-9.3 (Dey et al., 2012). The average shoot length (4.2-9.6 cm) also showed differences between cultivars.

In eggplant, regeneration efficiency, percent responsive explant (67-83%), the number of shoot produced per explant (1.27-2.48) and the percentage of shoots that rooted (60-100%) was also found to be significantly different among five cultivars (Muktadir et al., 2016).

Genotype has also been observed to affect callus induction in ten interspecific crosses of gerbera, significant differences in shoot regeneration rates (57-90%) and callus induction rates (5-43%) were observed in transverse thin cell layer cultures (Nhut et al., 2007).

Another component of in vitro culture that is affected by genotype is culture establishment which includes survival, browning or production of phenolics and contamination. In *Eucalyptus dunnii*, ten genotypes were evaluated, and five media treatments were assessed. Different responses among genotypes were observed regarding in vitro establishment parameters such as survival rates (6.6-70%), phenol oxidation (13.32-73.28%), and contamination rates (6.67-60%) (Navroski et. al., 2014).

Kunisaki (1992) reported that *Anthurium andraeanum* Hort. cultivars varied in their response to basal MS salt concentrations in vitro. 'Marian Seefurth' grew well in full strength MS, 'Anuenue' grew poorly in full strength MS, but grew well in 1/2MS. 'Ozaki' grew poorly in 1/2MS but grew well in 1/3MS.

Genotype is a major consideration in protocol development. Most protocols were developed using a select few varieties. When the in vitro growth response of a newly introduced or acquired genotype is poor, re-evaluation of the propagation protocol is necessary to achieve acceptable growth. Improvements in micropropagation protocols are beneficial not only for large scale plant production, but also for basic research such as genetic engineering. Plant transformation often requires the use of certain genotypes as preferred hosts. However, these preferred hosts may not be as amenable to the previously developed protocols as tissue culture is the primary method of mass propagating transformants. Even with the advent of new gene-editing technologies such as CRISPR-Cas9 (Doudna and Charpentier, 2014), base editing (Rees and Liu, 2018) and prime editing (Anzalone et al., 2019), tissue culture is still an essential vehicle for producing gene-edited plants (Mishra et al., 2020).

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CHAPTER 3: OPTIMIZATION OF THE RECIPIENT FOR AUTOMATED TEMPORARY IMMERSION (RITA®) SYSTEM PARAMETERS FOR IN VITRO SHOOT MULTIPLICATION OF *ANTHURIUM ANDRAEANUM* HORT. 'NEW PAHOA RED'

Abstract

Anthurium andraeanum Hort. 'New Pahoa Red' is the most widely grown cultivar in Hawaii. As such, propagules for this cultivar are in great demand by growers. Current production systems for anthurium rely on the availability of microplants, which are the primary source of propagating materials. Conventional methods for micropropagation include multiplication of axillary buds in liquid culture, which could take 2-3 years to generate adequate numbers for field planting. The use of temporary immersion bioreactors such as the RITA® system has been adapted for the commercial micropropagation of many crops due to its ease of use, ability to produce high volumes of microplantlets and to bypass physiological disorders caused by submerged culture. This study compared the conventional flask system and the RITA® bioreactor system and optimized culture variables (immersion time, media volume and resting interval) for the RITA® system. Shoot initiation (3-4fold) and proliferation rates (1.6-2.6fold) in *Anthurium andraeanum* Hort. 'New Pahoa Red' were significantly higher in the RITA® bioreactor system compared to the conventional system. The treatment combination of 5 minutes immersion time and media volume of 20mL per explant had the largest total axillary bud mass volume per RITA® (9 cm^3) and the highest total number of secondary shoot per RITA® (56 secondary shoots) compared to other treatment combinations. Meanwhile when different resting intervals were compared, a resting interval of 2 hours produced the largest total axillary bud mass volume per RITA® (8 cm^3) and the highest total number of secondary shoot per RITA® (47 secondary shoots) compared to other resting intervals. Inclusion of the RITA® bioreactor

system to current anthurium micropropagation protocols can significantly supplement and efficiently increase the micropropagule production.

Introduction

Anthuriums are conventionally propagated using seeds, top cuts, divisions and micropropagation. Seeds were used as the primary means of propagation in the late 1930s until the 1940s. However, seedlings are highly heterogenous particularly for traits such as flower quality, color, yield and time to first flowering (Geier, 1990; Kamemoto and Kuehnle, 1996; Matsumoto and Kuenhle, 1997). Berries are collected from hand pollinated or open pollinated fruits and then seeds are extracted from the pulp. The germination time ranges from 1 to 2 weeks to 3 months depending on the cultivar (Chandler, 1991; Higaki et al., 1995) and seedlings may take 2 to 3 years to reach first bloom (Croat, 1979; Chandler, 1991; Higaki et al., 1995).

Propagation from top cuttings and division is slow. The number of lateral shoots that develop on the remaining portion of the mother plant from which top cuttings are taken are also highly variable and are dependent on the size of the remaining mother plant. Even with the foliar application of 6 -Benzylaminopurine (BA) on the basal portion from which the top cutting was obtained, lateral shoot production only increased by 3.6 lateral shoots per plant in ‘Ozaki Red’ (Higaki and Rasmussen, 1979), and 5.67 lateral shoots in ‘Nitta’ (Higaki and Rasmussen, 1979; Maitra and Roychowdhury, 2014).

Diseases such as anthurium blight (*Xanthomonas axonopodis* pv. *diffenbachiae*) can readily spread through cuttings and division compared to micropropagation. Moreover, burrowing nematodes (*Radopholus similis*) cause severe stunting in plants so cuttings from infected plants cannot be used (Uchida et al., 2003). Consequently, commercial production of

anthuriums in both Hawaii and worldwide is dependent on in vitro derived planting materials (microplants and microcuttings) (Matsumoto and Kuehnle, 1997; Bleiswijk, 2016).

To minimize somaclonal variation during micropropagation the University of Hawaii's anthurium breeding program relies on an axillary bud culture protocol (Kunisaki, 1980) which uses low concentrations (0.2 ppm) of BA with limited subculturing. This method has been adopted to clone all new hybrids selected for evaluation and field testing with cooperators. Since this protocol employs a low concentration of BA, shoot initiation can be relatively slow. It can take up to 16-18 months for the first shoots to develop depending on cultivar and 36 months to mass produce selected plants for field testing (Kamemoto and Kuehnle, 1996; Matsumoto and Kuehnle, 1997). Thus, availability of micropropagated plants for field testing and cultivar release has been identified as a major bottleneck for cultivar development.

One approach that has not been widely used due to limited studies is the application of semi-automated bioreactors such as the Recipient for Automated Temporary Immersion (RITA®) which uses liquid culture and temporary immersion. RITA® was first used and developed for banana micropropagation where shoot production was higher using an immersion time of 5 minutes and 2 hour resting cycle compared to the conventional method (Alvard et al., 1993). RITA® has also been used in the micropropagation of high value crops such as vanilla (Ramos-Castellá et al., 2014; Ramírez-Mosqueda and Iglesias-Andreu, 2016), stevia (Ramírez-Mosqueda et al., 2016) and apple (Zhu, Li and Welander, 2005). The use of the RITA® bioreactor system in anthurium was first reported by Ruffino and Savona (2005) where they were able to proliferate 1.5 cm plantlets using an immersion time of 3 minutes for every 3 hours. However, their study did not report any optimization on RITA® immersion variables such as immersion time, media volume (volume/explant) and resting intervals.

Anthurium andraeanum Hort. ‘New Pahoa Red’ is the most widely grown cultivar in Hawaii. Propagules for this variety are in great demand by commercial growers. Hence, this variety was selected for the present study. To determine

e whether the use of the RITA® bioreactor system can increase in vitro shoot production, we compared the RITA® bioreactor system to the conventional flask system (foil covered 125 mL Erlenmeyer flask on rotary shakers) used as the standard for anthurium micropropagation. Secondly, to optimize the RITA® bioreactor system, we investigated the effects of different immersion times, volumes per explant and resting intervals on in vitro shoot production.

Materials and Methods

Stock Plant Priming

In vitro stock plants (2-3 years old) from the germplasm of the University of Hawaii's anthurium breeding program were used in all the experiments. To rejuvenated germplasm stocks, in vitro stock plants of *Anthurium andraeanum* Hort. ‘New Pahoa Red’ were primed through pretreatment with 0.3X MS liquid medium supplemented with 15% coconut water and 20 g/L sucrose for 15 days. This was done for all experiments to standardize stock plant quality.

Plant Material and Explant Preparation

Two-node segments (one axillary bud per node) were excised from the primed in vitro stock plants. Leaf sheaths were peeled away from the nodes to expose the axillary buds. The two-node segments were placed into the culture vessels (RITA® or 125 mL Erlenmeyer flask). Each culture vessel has 10 two-node segments. This was done for all experiments.

Media Composition

Two types of growth media based on Kunisaki (1980) protocol were used for the experiments. A liquid medium containing 0.3X MS salts supplemented with 0.2 mg/L BA, and 15% coconut water with 20 g/L sucrose was used for shoot initiation. The second medium of half- strength MS salts supplemented with 15% coconut water with 20 g/L sucrose and solidified with 2g/L gellan gum (CultureGel™,Phytotech)was used for shoot proliferation. The pH of both culture media used in the experiments was adjusted to 5.8 with 0.1 N NaOH before autoclaving at 121 °C and 103 kPa for 20 minutes.

A different media aliquot was used for the shoot initiation medium in each experiment. For the comparison between the conventional flask system and the RITA® bioreactor system, each 125 mL Erlenmeyer flask had an aliquot of 50 mL and each RITA® bioreactor had an aliquot of 200 mL. For the effect of immersion time and media volume, treatment combinations with volumes of 10 mL per explant had an aliquot of 100 mL per RITA® bioreactor while treatment combinations with volumes of 20 mL per explant had an aliquot of 200 mL per RITA® bioreactor. For the effect of the resting interval each RITA® bioreactor had an aliquot of 200 mL. For the shoot proliferation medium, each vessel (GA-7; Magenta™) had an aliquot of 90 mL.

Culture Conditions

All cultures were maintained in a culture room with temperatures ranging from 22-27°C under a 16 hour photoperiod. The cultures were illuminated with a combination of a 40W fluorescent cool white light (Linear T-12; Philips) and a wide spectrum fluorescent light (GrowLux® Sylvania) with a light intensity of 45 µmol/m^2/s and a daily light integral of 3.89 µmol/m^2/d.

Nodal Culture Protocol

Ten two-node segments were placed inside culture vessels (125 mL flask or RITA® bioreactor) and cultured in the shoot initiation medium for 45 days. After 45 days in the shoot initiation medium the explants (primary shoots with axillary bud masses) were taken out of the culture vessels to excise the primary shoots from the axillary bud mass to break apical dominance. The primary shoots were placed in the shoot proliferation medium for further growth and used as new stock plants in the in vitro germplasm collection. Axillary bud masses were also placed in Magenta boxes in the shoot proliferation medium to develop secondary shoots for 45 days. After 45 days of culture in the shoot proliferation medium secondary shoots with at least 2 leaves were excised and placed in fresh shoot proliferation medium for storage and eventual distribution to growers. This protocol was used in all experiments.

Comparison between the Conventional Flask System and the RITA® Bioreactor System

Ten two-node segments were placed in 125 mL Erlenmeyer flasks containing 50 mL of shoot initiation medium. The 125 mL Erlenmeyer flasks were sealed with aluminum foil and were placed on top of rotary shakers (100 rpm) for 45 days. Ten two node segments were also placed in the RITA® bioreactor containing 200 mL of shoot initiation medium. The RITA® setting used for this set up was the standard immersion time and resting interval for banana micropropagation which is a setting of 20 minutes every 2 hours (Alvard et al., 1993). Each treatment had 5 replicates. The experiment was repeated once.

The percentage of explants with shoots, the average number of primary shoots per explant, the average shoot length (cm), and the average number of primary shoots per vessel were calculated 45 days after culture in the shoot initiation medium. Data for the average number

of secondary shoots per vessel were recorded 45 days after culture in the shoot proliferation medium.

Effect of Immersion Time and Media Volume

To determine the effect of RITA® immersion variables on shoot initiation and shoot proliferation, two factors , immersion time (5 minutes, 10 minutes, and 20 minutes) and media volume (10 mL per explant and 20 mL per explant) were compared. The 2-hour resting interval from the previous experiment was used. Ten two-node segments were placed in each RITA® bioreactor and each treatment was replicated thrice.

The percentage of explants with shoots, the average number of primary shoots per explant, the average shoot length (cm), and the average number of primary shoots per vessel were calculated 45 days after culture in the shoot initiation medium. In addition, axillary bud biomass was assessed through water displacement method, the total axillary bud mass volume (cm^3) was also taken 45 days after culture in the shoot initiation medium. Data for the average number of secondary shoots per vessel were recorded 45 days after culture in the shoot proliferation medium.

Effect of Resting Interval

To determine the effect of the resting interval on shoot initiation and shoot proliferation, three intervals were compared: 2 hours, 4 hours and 8 hours. The immersion time of 5 minutes and media volume of 20 mL/ explant were used based from the results of the experiment on the effect of immersion time and media volume. Each RITA® bioreactor contained 200 mL of the shoot initiation medium. Each bioreactor had 10 two-node segments and each treatment was replicated thrice.

The percentage of explants with shoots, the average shoot length (cm), and the average number of primary shoots per vessel were calculated 45 days after culture in the shoot initiation medium. In addition, the total axillary bud mass volume (cm^3) was also taken 45 days after culture in the shoot initiation medium. The average number of secondary shoots per vessel was calculated 45 days after culture in the shoot proliferation medium.

Statistical Analysis

For the comparison between the RITA® bioreactor system and the conventional flask system, the experiment was laid out in in a Completely Randomized Design (CRD) and was repeated once. The data assumptions were tested using Levene´s test for equality of variances and Shapiro–Wilk test for normality. Means of the repeated experiments were compared for significant differences using the independent samples T-test.

For the evaluation of the effect of immersion time and media volume, the experiment was laid out in a Completely Randomized Design (CRD). The univariate data were subjected to 2-way ANOVA, and when an interaction between factors was indicated, a Simple Effect Analysis was done comparing main effects to the interaction effects. When no interaction was indicated, the group means were compared for significant differences by Tukey's HSD test.

For determining the effect of the resting intervals, the experiment was laid out in a Completely Randomized Design (CRD). The data were subjected to the one-way analysis of variance (ANOVA), followed by a comparison of group means via the Tukey's HSD test.

All statistical tests were evaluated at a significance level of 5%. Data were analyzed using IBM SPSS Statistics **(**version 26.0; IBM, Anorak, NY).

Results

Comparison between the Conventional Flask System and the RITA® Bioreactor System

The RITA® bioreactor system and the conventional flask system were compared in two experiments. Independent t-test showed significant difference ($p<0.05$) for the percentage of explants with shoots ($p= 0.0003$; Appendix Table 3.1), number of primary shoots per explant ($p=0.0003$; Appendix Table 3.2), shoot length in cm ($p=0.0005$; Appendix Table 3.3), the number of primary shoots per vessel ($p=0.0008$; Appendix Table 3.4) and the number of secondary shoots per vessel ($p=0.003$; Appendix Table 3.5). Therefore, the two experiments were treated separately.

Significant differences ($p<0.05$) were observed between the RITA® bioreactor system and the conventional flask system (Table 3.1). In Experiment 1, the percentage of explants with shoots was higher in the RITA® bioreactor (74%) compared to the conventional flask system (42%). A higher number of primary shoots per explant was also observed in the RITA® bioreactor (3) compared to the conventional flask system (2). The number of primary shoots per RITA® bioreactor was higher with 4.0-fold (25) increase relative to the conventional flask system (6). The number of secondary shoots was also higher in the RITA® bioreactor (18) compared to the conventional flask system (11).

Similar trends were observed for Experiment 2, where the RITA® bioreactor system also had a higher average for growth response during shoot initiation and shoot proliferation compared to the conventional flask system. The percentage of explants with shoots was higher in the RITA® bioreactor (90%) compared to the conventional flask system (32). A higher number of primary shoots per explant was also observed in the RITA® bioreactor (3) compared to the conventional flask system (1). The number of primary shoots per RITA® bioreactor was higher

with 3.0-fold (17) increase relative to the conventional flask system (6). The number of secondary shoots was also higher in the RITA® bioreactor (37) compared to the conventional flask system (14).

During shoot initiation, bud initials (Figure 3.1 A; B) started to develop 14 days into culture in both the RITA® and conventional flask system. At 14 days in the shoot initiation medium, there was no observable difference between the two systems. By 45 days, considerable differences in the axillary bud masses were observed (Figure 3.1 C). Explants under the RITA® system developed larger basal masses with numerous axillary buds (Figure 3.1 C). Differences inthe coloration of the petioles was observed. The petiole of the explants from the RITA® system changed from green to red while the ones from the conventional flask system remained green (Figure 3.1 C).

To suppress apical dominance during shoot proliferation, the primary shoots were excised 45 days after culture in the initiation medium, and the remaining axillary bud masses were transferred to a solid medium to promote further development. More secondary shoots were produced per vessel in the RITA® bioreactor system (Table 3.1; Figure 3.1 D). These shoots developed from axillary bud masses that were larger (Figure 3.1 C) at the end of the 45-day shoot initiation period.

Table 3.1. Comparative performance of *Anthurium andraeanum* Hort. 'New Pahoa Red' under the RITA® bioreactor system and the conventional flask system.

Experiment 1					
	45 days after culture in shoot initiation medium*				**45 days after culture in shoot proliferation medium**
Culture system	**Percentage of explants with shoots[a]**	**Number of primary shoots per explant[a]**	**Shoot length (cm)[a]**	**Number of primary shoots per vessel[a]**	**Number of secondary shoots produced per vessel[a]**
RITA®	74 a	3 a	3 a	25 a	18 a
Flask	42 b	2 b	1 b	6 b	11 b
Experiment 2					
	45 days after culture in shoot initiation medium *				**45 days after culture in shoot proliferation medium**
Culture system	**Percentage of explants with shoots[a]**	**Number of primary shoots per explant[a]**	**Shoot length (cm)[a]**	**Number of primary shoots per vessel[a]**	**Number of secondary shoots produced per vessel[a]**
RITA®	90 a	3 a	2 a	17 a	37 a
Flask	32 b	1 b	1 b	6 b	14 b

[a] -indicates parameter with significant difference between the two experiments using an independent sample t-test at $\alpha < 5\%$; n=10.

* Primary shoots were excised to break apical dominance and axillary bud masses were transferred to solid shoot proliferation medium.

Means within trials followed by the same letter in a column are not significantly different using independent sample t-test at $\alpha < 5\%$; n=5.

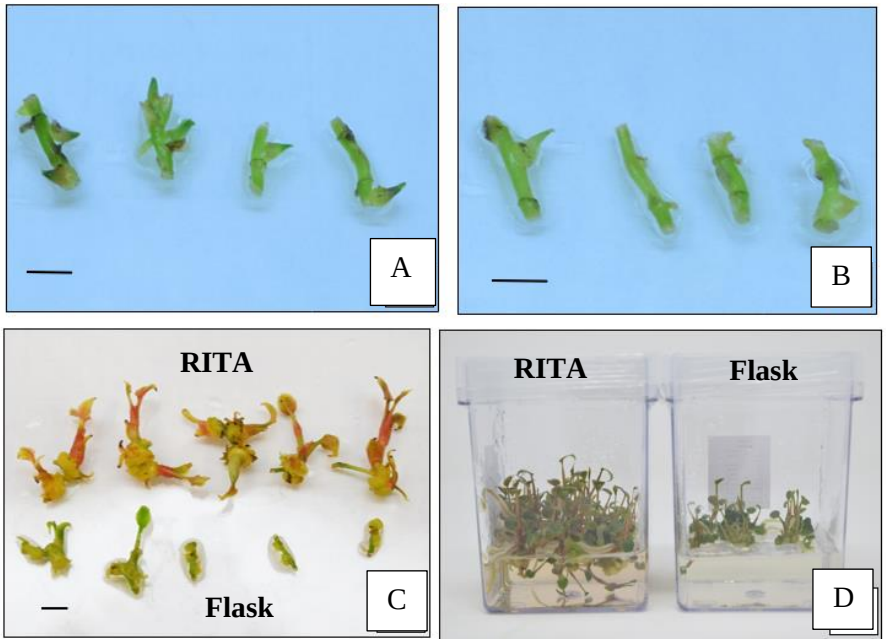

Figure 3.1. Comparison of in vitro responses of *Anthurium andraeanum* cv. 'New Pahoa Red' under the conventional flask system and the RITA® bioreactor system. Emergence of bud initials after 14 days under A.) the RITA® bioreactor system and B) the conventional flask system; C.) shoot development 45 days after culture in the RITA® bioreactor system and in the conventional flask system; D.) proliferation of shoots in solid media, 45 days after shoot initiation from RITA® bioreactor system and from the conventional flask system. Bar= 1 cm

Effect of Immersion Time and Media Volume in RITA® Bioreactors

Immersion time (p= 0.0001; Appendix Table 3.8; Appendix Table 3.9) was the primary factor that affected the number of primary shoots per explant. Interactions between independent factors immersion time and media volume with respect to dependent variables number of primary shoots per explant (p=0.182; Appendix Table 3.8) and shoot length (p=0.922; Appendix Table 3.9) were not significant. An immersion time of 10 minutes produced the highest number of primary shoots per explant (2.1) (Table 3.2) compared to immersion times of 5 minutes or 20 minutes. Shoot length was not significantly affected by immersion time.

Table 3.2. Effect of immersion time on average number of primary shoots per explant and average shoot length, 45 days after culture in the shoot initiation medium.

Immersion time (min)	Number of primary shoots per explant*	Shoot length (cm)**
5 minutes	1.6 b	1.3 a
10 minutes	2.1 a	1.1 a
20 minutes	1.7 b	1.2 a
Main Effect (p>0.05)	0.0001	0.004

*Interaction effect p=0.182 at $\alpha < 5\%$

** Interaction effect p=0.922 at $\alpha < 5\%$

- Means followed by the same letter in a column are not significantly different using Tukey's HSD test at $\alpha < 5\%$; n=6.

Interaction effects between immersion time and media volume were significant for percentage of explants with shoots (p=0.024; Appendix Table 3.10), number of primary shoots produced per RITA® (p=0.002; Appendix Table 3.11), total axillary bud mass volume per RITA® (p=0.035; Appendix Table 3.12) and number of secondary shoots produced per RITA® (p=0.00002; Appendix Table 3.13). Treatments were therefore considered as combinations of the two factors, immersion time × media volume. Means between the treatment combinations were then compared by simple effect analysis (p<0.05) (Table 3.3). An immersion time of 10 minutes

in a media volume of 20 mL produced the highest percentage of explants (97%;) and the highest number of primary shoots per RITA® (20).

The total axillary bud mass volume (cm^3) of 10 axillary bud masses was measured to quantify the size of the axillary bud mass. The largest total axillary bud mass volume per RITA® was obtained with the treatment combination 5 minutes x 20 mL (9 cm^3), followed by 5 minutes x 10 mL (7 cm^3), 10 minutes x 20 mL (4 cm^3), 10 minutes x 10 mL (4 cm^3), 20 minutes x 10 mL (4 cm^3) and 20 minutes x 20 mL (3 cm^3). The total axillary bud mass volume per RITA® was generally proportional to the initial axillary bud mass (Figure 3.2) that that develops during the shoot initiation period.

Table 3.3. Effect of immersion time and media volume on the growth response of Anthurium cultivar 'New Pahoa Red' under the RITA® bioreactor system

Immersion time (minutes)	**Media volume (mL)**	**45 days after culture in shoot initiation medium***			**45 days after culture in shoot proliferation medium**
		Percentage of explants with shoots (%)	**Number of primary shoots per RITA®**	**Total axillary bud mass volume per RITA® (cm^3)**	**Number of secondary shoots per RITA®**
5	**10**	80 b	13 b	7 b	45 b
	20	77 b	12 b	9 a	56 a
10	**10**	73 b	14 b	4 c	15 d
	20	97 a	20 a	4 c	18 d
20	**10**	70 b	12 b	4 c	20 c
	20	73 b	12 b	3 c	8 e

*Primary shoots were excised to break apical dominance and axillary bud masses were placed on solid shoot proliferation medium.

Means followed by the same letter in a column are not significantly different using Tukey's HSD test at $\alpha <$ 5%; n=3.

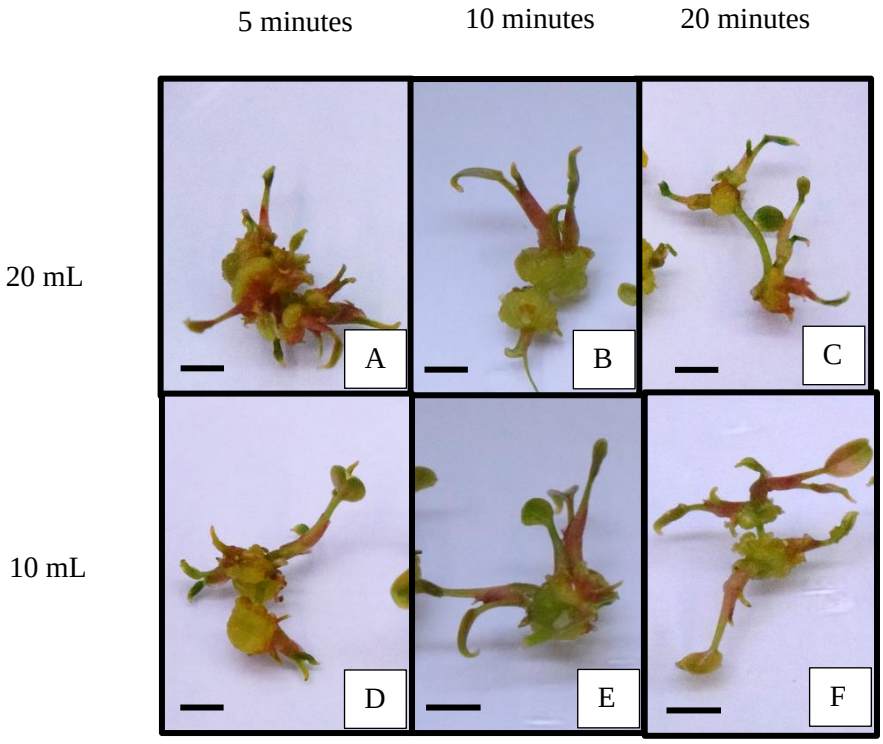

Figure 3.2 Primary shoots and axillary bud mass of *Anthurium andraeanum* Hort. 'New Pahoa Red' under the RITA® bioreactor system 45 days after culture in shoot initiation medium. Immersion time (column) and media volume (row). A.) 5 minutes x 20 mL; B.)10 minutes x 20 mL; C.) 20 minutes x 20 mL; D.) 5 minutes x 10 mL; E.) 10 minutes x 10 mL and F.) 20 minutes x 10 mL. Bar=0.5 cm

The largest total axillary bud mass (9 cm^3; Figure 3.2 A) was in the 5-minute x 20 mL treatment combination. After excision of the primary shoots, the axillary bud masses proliferated a total of 56 secondary shoots (Table 3.3; Figure 3.3 A) on solid medium at the end of 45 days on solid medium. A similar trend was also observed regarding the number of secondary shoots produced per RITA® (Table 3.3). A larger total axillary bud mass volume per RITA® produced a larger number of secondary shoots per RITA® during shoot proliferation (Figure 3.4). In general, the treatment combination of 5-minute x 20 mL was the optimum immersion shoots combination based on total axillary bud mass volume per RITA® and the number of secondary produced per RITA®.

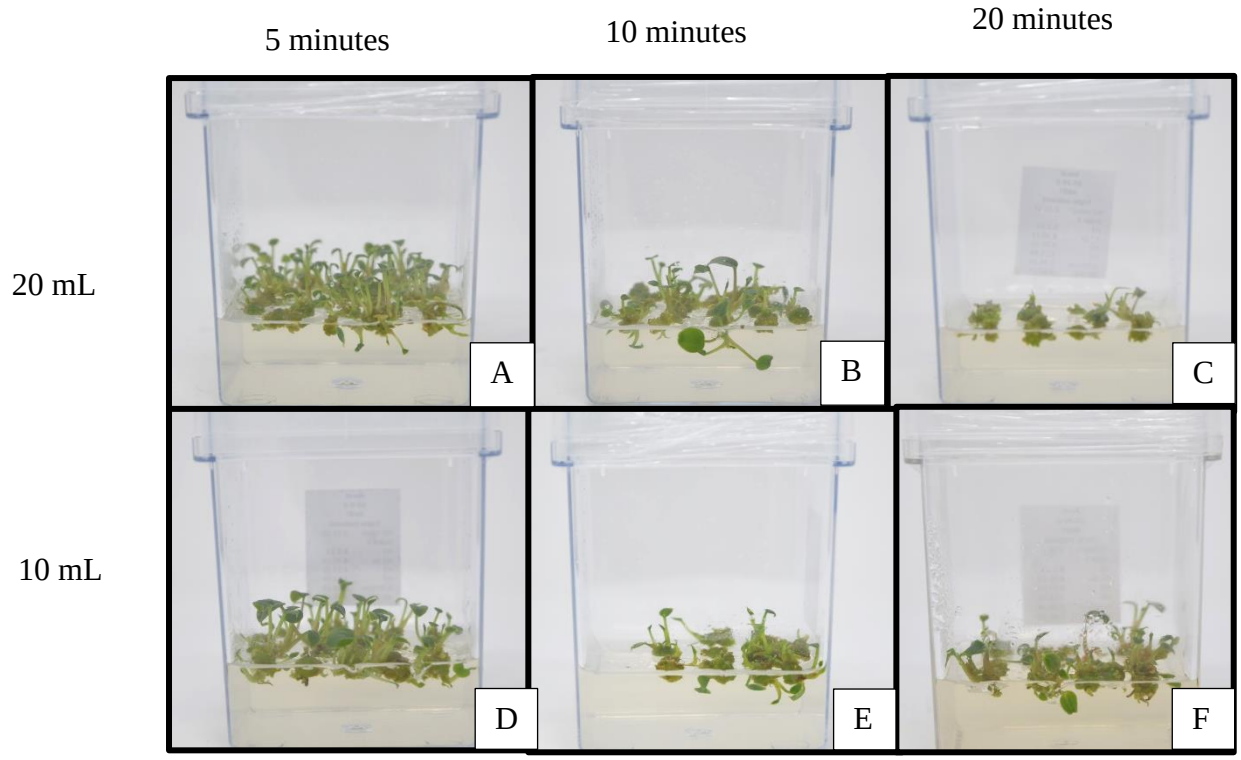

Figure 3.3. Shoot proliferation of *Anthurium andraeanum* Hort. 'New Pahoa Red' axillary bud mass previously cultured in RITA® bioreactors and then subsequently transferred to media containing 1/2 MS basal salts + 15% coconut water + 20g/L sucrose + 2g/L Gellan gum (CultureGel^{TM}). Immersion time (column) and media volume (row). A.) 5 minutes x 20 mL; B.)10 minutes x 20 mL; C.) 20 minutes x 20 mL; D.) 5 minutes x 10 mL; E.) 10 minutes x 10 mL and F.) 20 minutes x 10 mL.

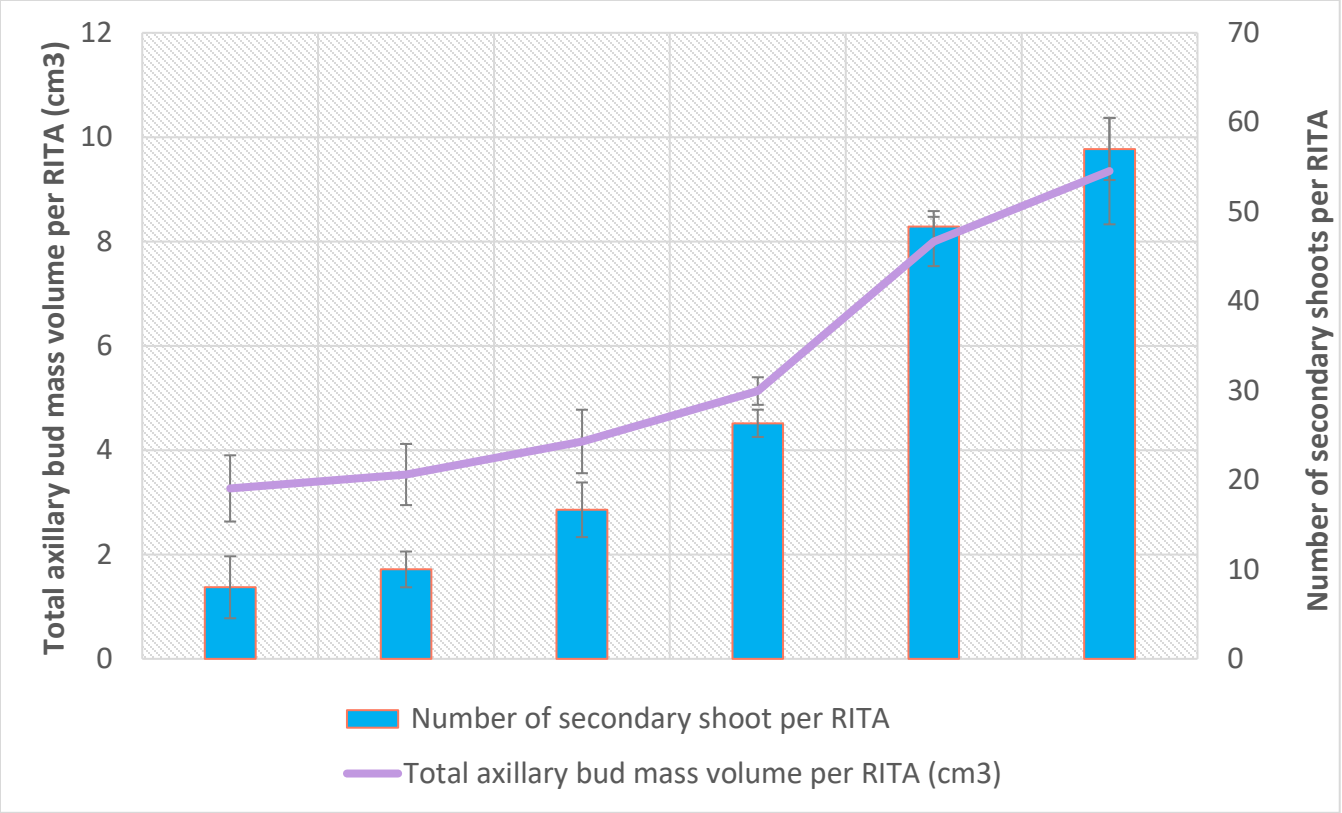

Figure 3.4. Effect of immersion time and media volume: relation between axillary bud mass volume and the number of secondary shoots produced by *Anthurium andraeanum* Hort. 'New Pahoa Red'. Error bars represent standard errors.

Effect of Resting Interval in RITA® Bioreactors

The resting interval is the period between two immersion times or the period when the bioreactor is at rest. This was another factor taken into consideration for optimization. The optimum immersion time (5 minutes) which yielded the highest number of secondary shoots per RITA® from the previous experiment was used for this study.

Significant differences were observed in terms of shoot length (p=0.005; Appendix Table 3. 15.), total axillary bud mass volume per RITA® (p=0.0001; Appendix Table 3.17.) and number of secondary shoots per RITA® (p=0.0001; Appendix Table 3.18). The longest shoot length was 2 cm, which was observed in the treatment with a 2-hour resting interval. The highest

total axillary bud mass volume per RITA® was also in the 2-hour resting interval (8 cm^3), followed by the 4-hour (5 cm^3) resting interval and the 8-hour resting interval (2 cm^3). The number of secondary shoots produced per RITA® followed a similar response (Table 3.4). Larger axillary bud masses (Figure 3.5 A-C) produced more secondary shoots (Figure 3.5 D-F).

Table 3.4. Effect of resting interval between 5-minute immersions on the growth response of Anthurium cultivar 'New Pahoa Red' under the RITA® bioreactor system.

Resting interval (hours)	**45 days after culture in shoot initiation medium***					**45 days after culture in shoot proliferation medium**
	Percentage of explants with shoots (%)	**Number of primary shoots produced per RITA®**	**Number of primary shoots per explant**	**Shoot length (cm)**	**Total axillary bud mass volume per RITA® (cm^3)**	**Number of secondary shoots produced per RITA®**
2	100 a	22 a	2 a	2 a	8 a	47 a
4	97 a	20 a	2 a	1 b	5 b	31 b
8	100 a	22 a	2 a	1 b	2 c	30 b

*Primary shoots were excised to break apical dominance and axillary bud masses were placed on solid shoot proliferation medium.

- Means followed by the same letter in a column are not significantly different using Tukey's HSD test at $\alpha <$ 5%; n=3.

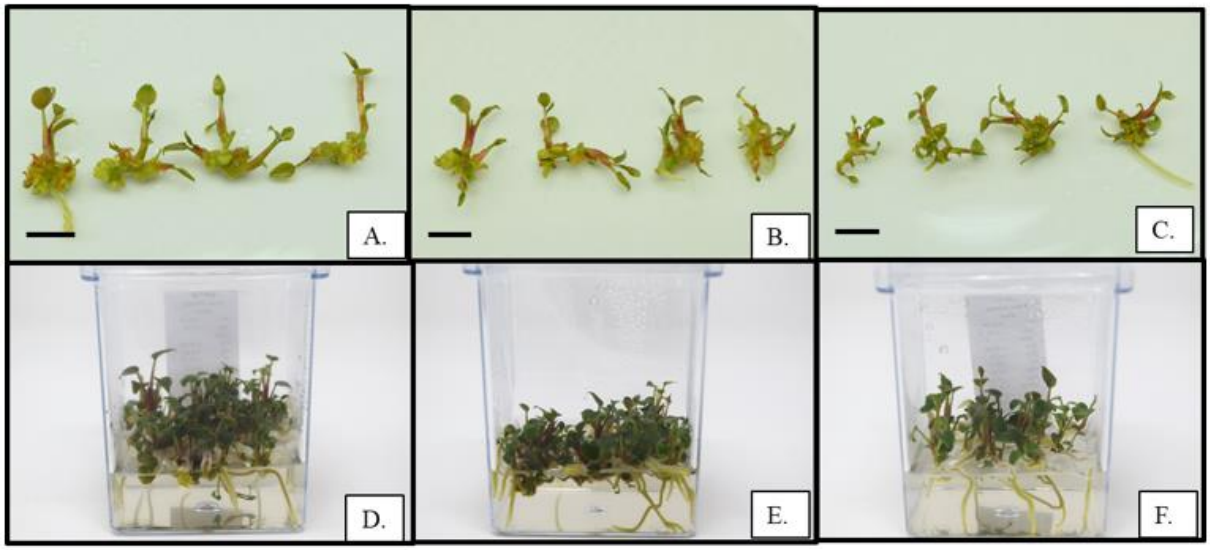

Figure 3.5. Primary shoots and axillary bud mass (Top - 45 days after culture in shoot initiation medium) and secondary shoots (Bottom - 45 days after culture in shoot proliferation medium) of Anthurium cv. 'New Pahoa Red' under different resting intervals. Primary shoots: A.) 2 hours; B.) 4 hours and C.) 8 hours. Secondary shoots: D.) 2 hours; E.) 4 hours and F.) 8 hours. Bar=0.5 cm.

Discussion

Adapting liquid culture to micropropagation has been known to promote higher multiplication and proliferation rates than the conventional gelled cultures. Multiple speculations on how liquid culture improves growth and performance of plants under in vitro conditions have been proposed. Enhanced proliferation was due to the improved availability of nutrients caused by higher diffusion rates encountered when in a liquid interphase in comparison to solid medium (Singha, 1982 cited by Avila et al., 1996). More uniform distribution of nutrients and growth hormones is achieved in liquid culture (Gawel and Robacker, 1990), and enhanced with agitation (Jackson et al., 1991; Liu et al., 2002).

Another factor thought to enhance proliferation in liquid culture is the dispersion of inhibitory metabolites. In gelled cultures, theses metabolites which are usually phytotoxic in

large amounts, accumulate near areas surrounding the explant, and thus inhibit growth. The effect of these factors is not universal; explant type and genotype also come into play (Ascough and Fennell, 2004). While liquid culture systems promote multiplication rates, they also present several disadvantages such as oxygen deficiency and hyperhydricity i.e., a vitrified or glass-like state (Ziv 1991a).

In this study, the RITA® bioreactor system (20 minutes for every 2 hours; Alvard, et al., 1993) exhibited higher shoot initiation and proliferation than the conventional flask system for liquid culture (Table 3.1). Similar observations were seen in willow (Regueira, et al., 2018) and chestnut (Vidal, et al., 2015) where shoots cultured in plantform^{TM} and RITA® generated higher proliferation than in semisolid medium. In *Anthurium* cv. Rosa, nodal explants subjected to the Ebb-and-Flow system (1L) generated a higher number of shoots (31.5) than partial immersion (7.25) and semisolid media (4.5), along with improved rooting and survival rates during acclimatization (Martinez-Estrada et al., 2019).

Temporary immersion bioreactors have been used to alleviate hyperhydricity while maintaining the benefits offered by liquid culture (Berthoughly and Etienne, 2005; Georgiev et al., 2014). Hyperhydricity was not evident in this study, but leaf epidermal imprints from *in vitro* plantlets did show differences in terms of stomatal opening (Appendix Figure 3.1). Plants in the conventional flask system displayed stomates that remained open during nighttime (Appendix Figure 3.1 A), which is not usual for plants with C3 metabolism, such as *Anthurium* (Luttge et al., 1993; Mardegan, et al., 2011). Stomates from microplants in the RITA® bioreactors remained closed at night which is seen in most plants with the C3 metabolism. Whether or not this difference in stomatal opening between the plantlets in the RITA® bioreactor system and the conventional flask system contributed to the increase in growth response remains inconclusive.

The increase in multiplication rates in temporary immersion systems may be attributed to recurring gas exchanges that occur in the vessels. Submerged conditions present a major constraint on the available CO_2 for photosynthesis. Diffusion of CO_2 in water is approximately 10,000 times slower than in air due to water's greater density (Raven, 1970). This slower diffusion causes the rate constant (k 1/2) for CO_2 uptake (100 to 200 μmol L^{-1}) to be 6 to 11 times higher than the air equilibrium concentrations (Maberly and Madsen, 1998) and results in a transport limitation on photosynthesis. As a result, lower multiplication rates occur when plantlets are submerged, as seen in the conventional flask system (Table 3.1)

In addition, spike in temperatures and RH that occurred during the comparison between the RITA® bioreactor system and conventional flask system could have contributed to the difference between the repeated experiments. Experiment 1 was conducted from December 2018 to March 2019 (Appendix Figure 3.2). On January 13, 2019- January 16, 2019 temperatures and RH were above tolerable temperatures (30º) and could have negatively impacted in vitro growth at the time.

Immersion time primarily affected the growth of axillary bud masses and secondary shoots. Although interactions were observed between media volume and immersion time, treatment combinations (Table 3.3) with shorter immersion times (5 minutes) generally generated larger total axillary bud masses (9 cm^3) which subsequently formed higher numbers of secondary shoots (56 shoots; Appendix Figure 3.4) during shoot proliferation. Similar responses were observed in hops (*Humulus lupulus* L. cv. Tettnanger), where a shorter immersion time (1 minute) resulted in higher number of shoots (197 shoots) and higher multiplication rate (3.9-fold) compared to longer immersion times (4 minutes) which had a lower number of shoots (149 shoots) and lower multiplication rates (2.5-fold) (Gatica-Arias and Weber, 2013).

Resting interval also affected axillary bud mass growth and secondary shoots formation. A shorter resting interval (2 hours) produced larger total axillary bud mass volume and higher number of secondary shoots compared to the 4-hour or 8-hour intervals (Table 3.4). Shorter resting intervals (6 hours) in *Guadua angustifolia* resulted in a higher number of shoots (3.5 shoots) compared to longer resting intervals (8 hours) which had a lower number of shoots (2 shoots) (Gutierrez et al., 2016). Shorter immersion times (1 minute) and resting intervals (4 hours) in coffee stimulated higher embryo production (3,081 embryos per bioreactor) and higher embryo regeneration (2,094 plantlets per bioreactor) compared to longer immersion times (15 minutes) and resting intervals (24 hours) which produce less embryos (480 embryos per bioreactors) and less embryo regeneration (428 plantlets per bioreactor) (Albarran et al., 2005). However, no significant differences in terms of growth responses were observed to result from different resting intervals in *Anthurium* cv. Rosa (Martinez-Estrada et al., 2019). Differences in multiplication rates and percent rooting were observed in ten clones of chestnuts under culture conditions combining an immersion time of 3 minutes and an interval of 6 hours. The multiplication rate of the 10 clones ranged from 3.1- to 8.2-fold, while percent rooting ranged from 40% to 75% (Vidal et al., 2015).

Responses to immersion time and resting intervals are thus not universal and could differ between genotypes. The varying response to different immersion variables could be problematic for optimization.

Conclusion

We successfully improved in vitro shoot production of *Anthurium andraeanum* Hort. 'New Pahoa Red' with the use of the RITA® bioreactor system and by optimizing RITA® protocol developed for banana micropropagation by Alvard et al. (1993). The RITA® bioreactor setting of 20-minute immersion every 2 hours (Alvard et al, 1993) significantly enhanced shoot initiation and proliferation rates in *Anthurium andraeanum* Hort. 'New Pahoa Red' in comparison to the conventional flask system. The optimum the treatment combination of 5 minutes of immersion x 20-mL media volume with a resting interval of 2 hours enhanced axillary bud mass and secondary shoot development during shoot proliferation. Increase in multiplication rate in the RITA® bioreactors system was attributed to recurring gas exchange in the vessel during each cycle. Thus, the RITA® bioreactor system could be a useful addition to current anthurium micropropagation protocols.

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CHAPTER 4: COMPARATIVE ANALYSIS OF IN VITRO RESPONSES OF TEN ANTHURIUM CULTIVARS UNDER TEMPORARY IMMERSION (RITA®): A PRELIMINARY STUDY

Abstract

One of the most challenging factors that plant researchers and tissue culturists encounter under in vitro conditions is the effect of genotype. Assessing proliferative variation under in vitro conditions could provide guidelines for future protocol development. The objective of this study was to assess anthurium accessions from the University of Hawaii anthurium breeding program under the RITA® temporary immersion system and subject them to diversity analysis. To evaluate shoot initiation, ten accessions of anthurium were placed in RITA® bioreactors supplemented with a liquid medium containing 0.3X MS salts with 0.2 mg/L BA, 15% coconut water and 20 g/L sucrose. Primary shoots were excised after 45 days to allow axillary buds to develop into secondary shoots. Percentages of explants with shoots, number of primary shoots per RITA®, shoot length, total axillary bud mass volume (cm^3) were recorded. Bud masses (trimmed explant bases) were placed on a solid medium containing ½ MS salts with 15% coconut water, 20 g/L sucrose and 2g/L gellan gum (CultureGel™, Phytotech) to observe shoot proliferation and growth. The degree of bud formation and number of secondary shoots per RITA® were assessed after another 45 days. Significant differences in in vitro response between the genotypes were observed. The percentage of explants with shoots among the genotypes ranged between 3-93% with A697 and UH1244 having the highest percentages (93%) while A213-2 had the lowest percentage at 3%. The number of primary shoots per RITA® among the genotypes ranged between 0-23 with A697 having the highest number of primary shoots (23) and A213-2 with the lowest number of primary shoots (0). The total axillary bud mass volume per RITA® among the genotypes ranged between 3-9 cm^3 with 'Midori' having the largest total

volume (9 cm^3) and A213-2 had the smallest total volume (3 cm^3). For the degree of basal mass formation, four genotypes had basal masses that were greater than 6 mm, 5 genotypes had basal masses that were between 4-6 mm and 1 genotype had a basal mass size that was between 1-3 mm. The number of secondary shoots per RITA® among the genotypes range between 6-70 with UH2409 having the highest number of secondary shoots and UH1145 with the lowest number of secondary shoots. Analyses of the parameters revealed low to moderate diversity/variability for the in vitro responses. Cluster analysis of quantitative and qualitative parameters revealed five clusters and that growth habit affected secondary shoot proliferation. In addition, cross-referencing clusters with existing pedigrees revealed similarities within the lineages of the genotypes. Genotypes under Cluster 3 had *A. amnicola, A. formosum, A. kamemotoanum* and *A. antioquense* in their background and were observed to produce a moderately high number of primary shoots and a low number of secondary shoots. Anthurium genotypes with the *A. amnicola, A. formosum, A. kamemotoanum* and *A. antioquense* lineages are expected to perform similarly to the genotypes under Cluster 3. Genotypes under Cluster 4 had an *A. andraeanum* hybrid and A697 as one of the parents. Genotypes under Cluster 4 also produced moderately high numbers of primary shoots and a high number of secondary shoots. Anthurium genotypes with the *A. andraeanum* lineage or A697 progenies are expected to perform similarly to genotypes under Cluster 4. Genotype was also found to exert a greater influence on secondary shoot production than growth habit. This indicates that referencing breeding data and historical records could be valuable resources for predicting or creating response profiles for in vitro performance in anthurium.

Introduction

Commercial production of anthuriums has geared breeding schemes towards higher flower yield, disease resistance, longer vase life and ease of packaging and shipment. Focus was given to developing the three conventional types of anthurium: 1) standards, that have broad, symmetrical heart-shaped spathes with overlapping lobes and have one uniform color; 2) The obakes, characterized by dual colorations of greens and their respective spathe color. and 3) tulip types which have upright and cupped spathes (Kamemoto and Kuehnle, 1996).

Breeding objectives for anthurium spathe color have dramatically changed from the 1990s, shifting focus from the five major color groups (red, orange, pink, coral and white) to different shades of pinks, pastels and coral. Blush types, grey and muted tones have also gained the interest of floral designers (Hitomi Gilliam, AIFD and Lois Hiranaga, AIFD, pers. comm.). With novel design concepts like Tropical Nouveau, where tropical and temperate flowers are combined to fashion ways of complementing botanicals of opposing origins (Garcia, 2019), anthuriums are able to fit in novel niches in floral design leading to higher demand for more unique and novel flower types. This increased demand for new varieties also pushes breeding into fast tracking development of unique varieties that would suit present consumer's taste.

Development of new cultivars takes approximately 13-14 years from pollination to release. Selection and field testing take 4-5 years, about 30-35% of the time to develop and release a new cultivar. Selected plants are increased through micropropagation and take about 36 months to build up an adequate amount for field testing. The availability of micropropagated plants for field testing has been identified as a major bottleneck that highly affects the timeline for cultivar release (Kamemoto and Kuehnle, 1996).

Genotype has been a major consideration for in vitro protocol development. The effect of genotype has been documented in vitro studies (Mathias and Simpson, 1986; Nhut, et al., 2007; Dey et al., 2012; Navroski et al., 2014; Muktadir et al., 2016) but has yet to be fully assessed in anthurium. The earliest report on genotype dependency in in vitro cultured anthurium was observed by Pierik et al., 1974, where genotypes of mature plants responded differently on a callus induction media containing BCM salts enriched with 1 mg/L 6-(benzylamino)-9-(2-tetrahydropyranyl) 9H-purine (PBA). One-third of the genotypes were able to develop calli. A later study revealed growth rates were also genotype specific (Pierik, 1975). Meanwhile, plant regeneration also varied based on genotype. The lamina culture of nine anthurium genotypes resulted in percent plant regeneration that ranged between 0% - 8.3% (Yang et al., 2002). Anthurium cultivars 'Choco', 'Pistache' and 'Tropical' also significantly differed in shoot regeneration rates (4.3 shoots per explant, 3.5 shoots per explant and 10.1 shoots per explant, respectively) (Nhut et al., 2006).

Assessment of genotype dependency in anthurium is needed for expediting cultivar release and for providing baselines for protocol development. Most protocols are effective for a few select varieties or are developed to work for a specific genotype (Benson, 2000Garcia-Gonzales et al., 2010;). When tissue culturists are presented with new genotypes, re-evaluation of culture protocols/conditions must be done due to differences in response.

Germplasm repositories, such as gene banks and breeding programs, house large quantities of germplasm and are time consuming and costly to maintain. Limited resources often leave curators and breeders with little option for optimization. The University of Hawaii's anthurium breeding program houses 175 accessions in their in vitro collection including species such as *A. antioquiense, A formosum, A. nymphaefolium,* and *A. standleyi,* and interspecific

hybrids. Analysis of proliferation differences among the anthurium germplasm accessions under in vitro conditions have yet to be measured. Assessing phenotypic diversity under in vitro conditions could provide guidelines for future propagative protocol development and genetic studies.

Comparative analysis of in vitro growth responses in bioenergy sorghum parental lines, i.e. sorghum parents used for the development of biofuel lines, and the common grain sorghum (Tx430) revealed varying responses of plant regeneration and types of calli produced. These types of comparative analyses could provide new resources and tools to identify and access genetic loci, candidate genes and allelic variants for their role in in vitro responsiveness (Flinn et al., 2020). These analyses could also provide guidelines for transformation studies. Since genome editing still requires the initial step of transformation of the edited construct into the genotype of interest, identifying genotypes that are not suitable for transformation and often fail to regenerate afterwards is of major importance (Botella, 2019).

The objectives of the study were to assess the in vitro growth response of ten accessions from the University of Hawaii anthurium breeding program under the RITA® temporary immersion system through methods used for diversity analysis and to cross-reference results with their pedigrees.

Materials and Methods

Stock Plant Priming

In vitro stock plants (2-4 years old) of ten anthurium accessions (Appendix Table 4.1) from the germplasm of the University of Hawaii's anthurium breeding program were identified as candidates for phenotypic analysis based on the known response to previous conventional protocols. To rejuvenate stocks, in vitro stock plants were primed through pretreatment with 0.3X MS liquid medium supplemented with 15% coconut water and 20 g/L sucrose for 15 days.

Plant Material and Explant Preparation

Two-node segments (one axillary bud per node) were excised from the primed in vitro stock plants. Leaf sheaths from the two-node segments were removed from the nodes to expose the axillary bud. The peeled two-node segments were then placed in a 150 mm x 20 mm petri dish with approximately 50 mL of sterile deionized water to keep the segments moist, and then placed into the RITA® bioreactor. Each bioreactor contained 10 two-node segments.

Media Composition

Two types of growth media modified from the Kunisaki (1980) protocol were used for the experiments. A 200 mL volume of shoot initiation liquid medium containing 0.3X MS salts supplemented with 0.2 mg/L BA, 15% coconut water with 20 g/L sucrose was used in the RITA® bioreactor. A 90 mL volume of shoot proliferation medium with half strength MS salts supplemented with 15% coconut water with 20 g/L sucrose and solidified with 2g/L gellan gum (CultureGel™,Phytotech was placed inside GA-7 vessels (Magenta™). The pH of the all culture media used in the experiments was adjusted to 5.8 with 0.1 N NaOH before autoclaving at 121 °C and 103 kPa for 20 min.

Culture Conditions

All cultures were maintained in a culture room with temperatures ranging from 22 to 27°C under a 16 hr photoperiod. The cultures were illuminated with a combination of a 40W fluorescent cool white light (Linear T-12; Philips) and a wide spectrum fluorescent light (GrowLux® Sylvania) with a light intensity of 45 μmol/m^2/s and a daily light integral of 3.89 μmol/m^2/d.

Comparison of In Vitro Responses

The ten two-node segments (one axillary bud per node) from ten anthurium accessions were excised from the established in vitro stock plants and placed in shoot initiation medium cultured in RITA® bioreactors for 45 days. After 45 days in the shoot initiation medium the whole explants (primary shoots with axillary bud masses) were taken out and the primary shoots were excised from the axillary bud mass to break apical dominance. The primary shoots and axillary bud masses were then transferred to the shoot proliferation medium. The primary shoots were returned to the in vitro germplasm collection to serve as new stock plants. Axillary bud masses were transferred to the shoot proliferation medium for 45 days to allow further development of secondary shoots. After 45 days of culture in the shoot proliferation medium, secondary shoots with at least 2 leaves were excised and placed in fresh shoot proliferation medium for storage. Unequal replication (3-5) of accessions was unavoidable due to limited availability of some in vitro stock plants. An immersion time of 5 min and a resting interval of 2 hours was used in this experiment based on previously optimized conditions for *A. andraeanum* Hort. 'New Pahoa Red' (as detailed in Chapter 3).

Data Gathered and Statistical Analysis

The growth habit, whether monopodial or sympodial, was determined during explant preparation. Responses to the shoot initiation medium were assessed 45 days after culture in the shoot initiation medium. The parameters measured were the number of explants with shoots, the number of primary shoots produced per RITA, the shoot length in cm, the total axillary bud mass volume per RITA® (cm^3) and the degree of basal mass formation. Basal mass formation was rated using the following scale: 1= 1-3 mm; 2= 4-6 mm and 3= >6 mm. Responses to shoot proliferation were assessed 45 days after removal of the primary shoot and placing axillary basal masses into the shoot proliferation medium. The total number of secondary shoots from the 10 explants per vessel was gathered 45 days after culture in the shoot proliferation media.

The experiments were laid out in a Completely Randomized Design (CRD). The data were subjected to the Welch's analysis of variance (ANOVA) (Welch, 1947), followed by a comparison of group means via Games-Howells test (Toothaker, 1991) for unequal variances and replicates.

In vitro responses were subjected to the Shannon-Weaver Diversity Index, using the following formula:

$$H' = -\Sigma H' = -\Sigma pi(\log_2 pi)/\log_2 N)$$

where, *pi* is the proportion of the genotypes that fall under an in vitro response class and *N* is the total number of in vitro response class. The values were also normalized to keep the H' values between 0-1. In this analysis, we used a reciprocal index of 1/H'(NIST, 2016) , so that high values indicate high diversity and low values indicate low diversity (NIST, 2016) The scale for the indices (H') was patterned from an arbitrary index used for *Oryza sativa* (Rabara et al., 2014)

and was assigned as 1= maximum diversity, 0.99-0.75 = high diversity, 0.74-0.51 = moderate diversity, 0.50-0.41= low to moderate diversity, 0.40-0.01= low diversity and 0 = no diversity.

Accessions and cultivars were clustered through two-step cluster analysis (combination of hierarchical and k-means cluster analysis) using IBM SPSS Statistics **(**version 26.0; IBM, Anorak, NY). Similarities were compared using Log-likelihood distance measure. Silhouette measure of cohesion and separation analysis (Rousseeuw, 1987) was used to determine number of cluster and quality. Clusters were then plotted against the number of primary and secondary shoots per RITA®. Resulting clusters were cross-referenced with existing pedigrees and growth habit to find similarities among the genotypes.

All statistical tests were evaluated at a significance level of 5%. Data and clusters were analyzed and generated using IBM SPSS Statistics **(**version 26.0; IBM, Anorak, NY).

Results

Comparative Analysis of Quantitative In Vitro Growth Responses of Ten Genotypes of *Anthurium*

Welch's analysis of variance (ANOVA) and Game-Howells test were chosen for comparisons among the ten anthurium genotypes due to the unequal replications and the likelihood of unequal variances (Table 4.1). Distinct differences ($p<0.05$) were observed in the percentage of explants with shoots ($p=0.002$). A697 and UH1244 had the highest percentage of explants that developed shoots at 93 % while A213-2 had the lowest percentage at 3 %. Significant differences were also observed in the number of primary shoots per RITA® ($p=0.003$). A697 had the highest number of primary shoots (23), followed by UH1145 (17) and UH1244 (16), while A213-2 had the lowest (0). The total axillary bud mass volume per RITA® also showed significant differences ($p=0.008$) among the ten genotypes. The largest total axillary

bud mass volume per RITA® was observed in 'Midori' (8.92 cm^3), followed by A697 (7 cm^3), UH1145 (6 cm^3) and UH1067 (6 cm^3). The smallest total axillary bud mass volume per RITA® was observed in the genotype A213-2 (3 cm^3). Significant differences between genotypes were also observed in the number of secondary shoots produced per RITA® ($p=0.011$), the greatest being in UH2409 (70), while UH1145 (6) had the least. In contrast, the shoot length among the ten genotypes were similar ($p=0.052$) to each other and ranged from 0.50 cm to 1.95 cm.

Basal mass formation differed among the ten genotypes (Table 4.1; Figure 4.1) Genotypes 'Midori', A697, UH2271 and UH2409 had basal mass sizes that were greater than 6 mm. UH1067, A213-2, UH1244, UH2282 and UH1145 had basal mass sizes between 4-6 mm. A888 had a basal mass size between 1-3 mm.

Table 4.1. Comparison of quantitative in vitro growth response of ten genotypes of *Anthurium* under the RITA® bioreactor system using Welch-ANOVA and Games-Howells test for means comparison.

Genotype	45 days after culture in initiation medium*					45 days after culture on shoot proliferation medium
	Percentage of explants with shoots (%)	**Number of primary shoots per RITA®**	**Shoot length (cm)**	**Total axillary bud mass volume per RITA® (cm^3)**	**Degree of basal mass formation****	**Number of secondary shoots per RITA®**
UH1067	68 a	12 b	2 a	6 a	2	8 e
'Midori'	75 a	11 b	1 a	9 a	3	29 d
A213-2	3 c	0 c	1 a	3 c	2	8 e
A697	93 a	23 a	2 a	7 a	3	49 abc
UH2271	70 a	10 b	1 a	6 ab	3	46 c
UH1244	93 a	16 ab	1 a	5 ab	2	15 de
UH2282	48 b	7 b	2 a	6 ab	2	15 de
A888	82 a	13 ab	1 a	4 b	1	10 e
UH2409	60 a	9 b	2 a	5 ab	3	70 ab
UH1145	90 a	17 ab	2 a	6 a	2	6 e
Welch's ANOVA ($p<0.05$)	0.002	0.003	0.053	0.008	N/A	0.011

* Emergence of primary shoots. Primary shoots were excised to break apical dominance

**Degree of basal mass formation: 1-3 mm -1, 4-6 mm -2; >6 mm -3

Means followed by the same letter in a column are not significantly different using Games-Howells test for unequal variances at $\alpha < 5\%$. N=3-5

Figure 4.1. Comparison of the primary shoots and axillary bud masses of ten *Anthurium* genotypes under the RITA® bioreactor system 45 days after culture in the shoot initiation medium. A.) UH1067, B.) 'Midori', C.) A213-2, D.) A697, E.) UH2271, F.) UH1244, G.) UH2282, H.) A888, I.) UH2049 and J.) UH1145. White arrows indicate-axillary buds. Bar = 0.5 cm

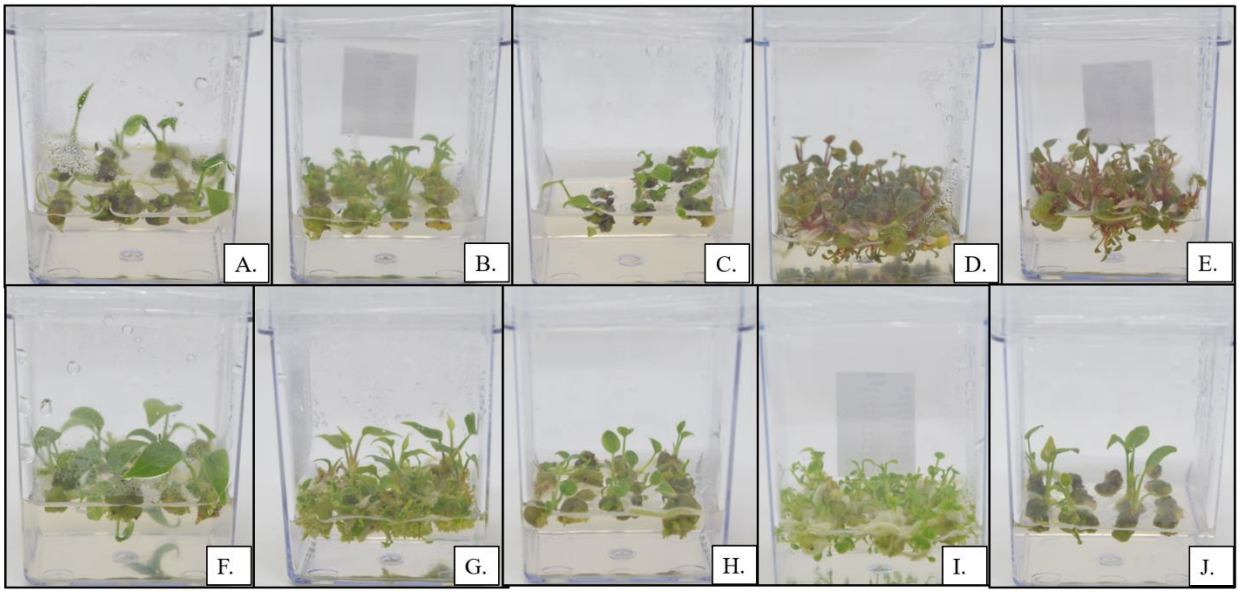

Figure 4.2. Comparison of the secondary shoots of ten *Anthurium* genotypes under the RITA® bioreactor system 45 days after culture in shoot proliferation medium. A.) UH1067, B.) 'Midori, C.) A213-2, D.) A697, E.) UH2271, F.) UH1244, G.) UH2282, H.) A888, I.) UH2049 and J.) UH1145.

Assessment of In Vitro Growth Responses of Ten Genotypes of *Anthurium* under the RITA® Bioreactor System using Shannon-Weaver Diversity Index

The in vitro responses of the ten anthurium genotypes were evaluated using the Shannon-Weaver Diversity Index (Table 4.2) to assess variation of response under the current RITA® protocol. Moderate diversity was observed between the genotypes for percentage of explants with shoots (0.538) and the number of primary shoots per RITA® (0.536). Similarly, the diversity index for the degree of bud mass formation (0.525) among genotypes was moderately diverse. Shoot length among the ten genotypes had a low-moderate diversity (0.413). The diversity indices for the total axillary bud mass volume per RITA® and the number of secondary shoots per RITA® among the genotypes were found to be low (0.356 and 0.372, respectively). Overall, in vitro responses among the ten genotypes range between slightly to moderately diverse.

Table 4.2. Shannon-Weaver diversity index for the in vitro growth response of ten genotypes of *Anthurium* under the RITA bioreactor system

In vitro Growth Response	H'	Diversity
Shoot initiation		
Percentage of explants with shoots (%)	0.538	Moderate
Number of primary shoots per RITA®	0.536	Moderate
Shoot length (cm)	0.413	Low-Moderate
Total axillary bud mass volume per RITA® (cm^3)	0.356	Low
Degree of bud mass formation	0.525	Moderate
Shoot proliferation		
Number of secondary shoots per RITA®	0.372	Low

Two-Step Cluster Analysis of Ten *Anthurium* Genotypes base on Qualitative and Quantitative In Vitro Response Variables

Cluster determination was based on five quantitative characteristics and two qualitative variables. The quantitative variables were the percentage of explants with shoots, shoot length, the number of primary shoots per RITA®, number of secondary shoots per RITA®, and the total axillary bud mass volume per RITA®. The two qualitative variables were growth habit and degree of basal mass formation.

Two step cluster analysis revealed five distinct clusters (Figure 4.3), and Silhouette measure of cohesion and separation analysis revealed that cluster quality was fair (Appendix Figure 4.1). Cluster 1 is composed of A213-2, an anthurium species, *A. nymphaeifolium.* Cluster 2 is composed of 'Midori', a commercial *A. andraeanum* Hort. cultivar of unknown parentage. Cluster 3 is composed of anthurium hybrids, UH1145 (Appendix Figure 4.2) and UH1067 (Appendix Figure 4.2), UH1244 (Appendix Figure 4.3), UH 2282 (Appendix Figure 4.4) and A888, *A.* ×*ferriense*. UH1145 and UH1067 are selections from the same cross and are thus siblings. Cluster 4 has UH2409 (Appendix Figure 4.5) and UH2271 (Appendix Figure 4.6). Both selections are hybrids of 'New Pahoa Red'. Cluster 5 is composed of another *A. andraeanum* Hort. commercial variety known as 'New Pahoa Red' (A697).

The ten genotypes were evaluated based on the number of primary shoots per RITA® and the number of secondary shoots per RITA®, since these two variables were identified as important parameters for commercial micropropagation (Figure 4.3). Cluster 1 which is composed of only A213-2 produced no primary shoots and few axillary buds (Figure 4.1 C). A213-2 also produced fewer secondary shoots (8). Cluster 2 which is only composed of 'Midori' had a moderately high number of primary shoots per RITA® (11) as well as a moderately high number of secondary shoots per RITA® (29). The numbers of primary shoots per RITA® for

genotypes in Cluster 3 ranged from 7-17 while the number of secondary shoots per RITA® ranged between 6-15. Meanwhile, Cluster 4 includes UH2271 and UH2409 which have moderately high numbers of primary shoots per RITA® that ranged from 9-10 and a high number of secondary shoots per RITA® that ranged from 46-70. The final group, Cluster 5, has only one genotype, A697 which has a high number of primary shoots per RITA® (23) and a high number of secondary shoots per RITA® (49).

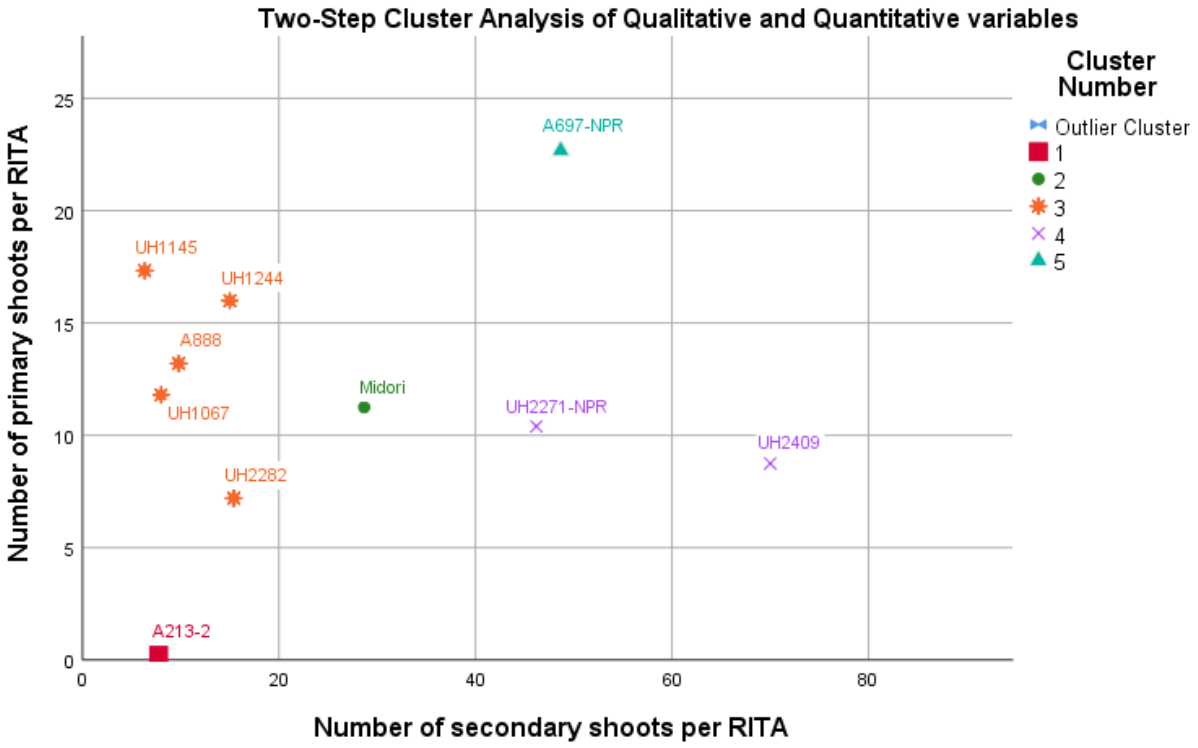

Figure 4.3. Two-Step cluster analysis of qualitative and quantitative variables evaluated against the number of primary shoots per RITA®, number of secondary shoots per RITA® and genotype.

In addition to evaluating the clusters against the number of primary shoots per RITA® and the number of secondary shoots per RITA®, the growth habits of the ten genotypes were classified as monopodial or sympodial (Figure 4.4). Growth habit could affect growth response

since it was observed to influence development of shoots in meristem and leaf cultures (J. Kunisaki and T. Amore, pers. comm.).

Growth habit seemed to have no influence on the number of primary shoots. Some monopodial genotypes responded with high numbers of primary shoots per RITA® such as A697 (23) while other monopodial genotypes such as UH2282 (Cluster 3), 'Midori' (Cluster 2), UH2271 (Cluster 4) and UH2409 (Cluster 4) produced moderately high numbers of primary shoots . Sympodial genotypes produced moderately high to low numbers of primary shoots per RITA® (Figure 4.4).

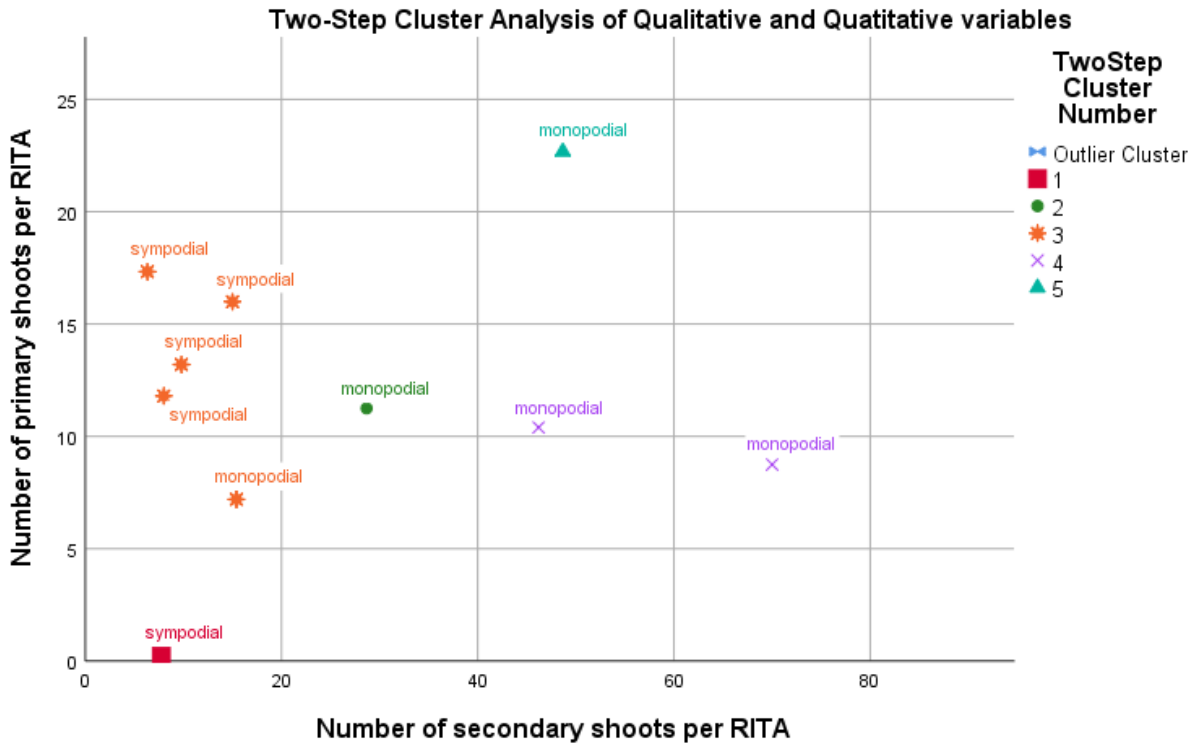

Figure 4.4. Two-Step cluster analysis of qualitative and quantitative variables evaluated against the number of primary shoots per RITA®, the number of secondary shoots per RITA® and growth habit (monopodial and sympodial).

Growth habit was observed to have influence on the production of secondary shoots during proliferation. Monopodial genotypes exhibited moderately high to high numbers of secondary shoots per RITA®, except for UH2282 which belongs to Cluster 3. Sympodial genotypes displayed low numbers of secondary shoots per RITA® (Figure 4.4), including genotypes in Cluster 3 and Cluster 1.

Discussion

Genotype has been observed to have profound effects on in vitro response, especially in shoot/plant regeneration (Mathias and Simpson, 1986; Dey et al., 2012; Muktadir et al., 2016) , callus formation (Nhut et al., 2007) and in vitro establishment (Navroski et al., 2014; Flinn et al., 2020). In ten anthurium cultivars, genotype was observed to play an important role in the callus formation of leaf segments cultured in MS+ 1 mg/L BA + 0.08 mg/L 2-4,D. Percent callus formation range between 0-65%, and two of the ten cultivars had no response (Nhut et al., 2006).

Genotype effect on in vitro response has also been directly observed in corn where a highly regenerable Type II friable germplasm was developed to increase production of type II callus and transformation efficiency. Lines from B73 and A188 were crossed to develop the Hi II germplasm (Armstrong et al., 1991) which is widely used as the model corn system for transformation studies and commercial maize transformation (Yadava et al., 2017; Du et al., 2019).

Newly introduced genotypes often require reassessment of protocol or re-optimization under in vitro conditions. These differences in response can be a hindrance when optimizing protocols for certain species as most protocols were developed for selected varieties (Benson, 2000; Garcia-Gonzales et al., 2010;). This presents a bottleneck for production systems and

transformation/genetic studies. Establishing relationships between genotype and in vitro response through clustering and cross-referencing can be a helpful tool for creating benchmark profiles for optimization and transformation studies.

Most in vitro studies base their conclusion on ANOVA (not including MANOVA) and a follow up post hoc comparison, an approach which has statistical limitations. ANOVA and post hoc comparisons cannot give an estimation of the degree of variation in responses (Good and Lunneborg, 2006; Norman and Streiner, 2014). In addition, ANOVA requires samples to be simple random samples (SRS) which are samples that are taken from large data pools (Norman and Streiner, 2014). In this study, samples were taken from the available germplasm found in the repository which does not provide a very large sample pool. The amount of germplasm housed in repositories is often limited by space and manpower. These facilities typically carry 5-10 working samples (active samples) and 10 stock samples (storage samples). In addition, ANOVA requires that samples must be independent from each other or that samples are not directly affected by each other (Good and Lunneborg, 2006). This means that the analytical capacity of ANOVA cannot fully accommodate organisms that may experience crowding effect or community effect. This community effect is often observed in plants that perform well in community pots such as orchids (Yee, 1983; Kuehnle, 2007) and anthuriums (Higaki et al., 1995; Bejoy et al., 2008; Bhattacharya et al., 2015). ANOVA also assumes equal standard deviations and equal sample sizes, although it can tolerate moderate deviations (Norman and Streiner, 2014).

Replicates ranged from 3 to 5 and were limited by explant availability. To account for unequal standard deviations, Welch's ANOVA, a non-parametric ANOVA designed for unequal variances and samples, was used. The analysis of the in vitro responses revealed significant

differences between some of the genotypes except for the shoot length. No significant difference was observed among genotypes when the shoot length was assessed.

In the previous study, where immersion variables of the RITA® bioreactor system were optimized for the anthurium cultivar 'New Pahoa Red' (Chapter 3), larger total axillary bud mass volume per RITA® were observed to produce more secondary shoots during shoot proliferation due to the presence of more axillary buds (Uy, 2020 unpublished). In the present study, a larger total axillary bud mass volume did not translate to more secondary shoots for some genotypes (Table 4.1). 'Midori' had the largest total plant volume per RITA® (9 cm^3) but only produced 29 secondary shoots per RITA®, which is significantly lower compared to genotypes with similar total axillary bud mass volumes, such as UH2409, UH2271 and A697 that produced greater numbers of secondary shoots (70, 49 and 46, respectively). Moreover, UH1145 (6 cm^3) and UH1067 (6 cm^3), which had total plant volumes that did not significantly differ from 'Midori', showed fewer secondary shoots (6 and 8, respectively). Apart from 'Midori', variation in secondary shoot production is correlated (r =0.84) with the degree of basal mass formation. Genotypes that exhibited basal masses greater than 6 mm, such as A697, UH2271, and UH2409, also showed higher numbers of secondary shoots (Table 4.1). These genotypes may have developed more axillary buds (Figure 4.1 D, E, & I) compared to 'Midori' (Figure 4.1 B). In addition, genotypes with smaller basal masses such as UH1067, A213-2, UH1244, UH2282, A888 and UH1145 were observed to have fewer secondary shoots (8, 8, 15, 15, 10 and 6, respectively). They also developed fewer axillary buds (Figure 4.1 A, C, F, G, H & J). These observations suggest that genotypes that developed large basal masses with more axillary buds (Figure 4.1 D, E, & I) would have led to higher numbers of secondary shoots (Figure 4.2 D, E,

&I), while genotypes with smaller basal masses (Figure 4.1 A, C, F, G, H & J) that have fewer axillary buds developed lower numbers of secondary shoots (Figure 4.2 A, C, F, G, H & J).

The Shannon-Weaver Diversity Index was initially designed to measure predictability in strings of text (Shannon and Weaver, 1949). Now it is widely used to measure diversity in ecology (Clarke and Warwick, 2001) and trait variability in conservation and breeding (Upadhyaya et al., 2003; Rabara et al., 2014; Gashaw et al., 2016). The Shannon-Weaver diversity index is derived from the idea that the higher the difference in letters and the more equal their abundance in the string of interest, the higher the difficulty of prediction of the next character within the string (Shannon and Weaver, 1949). The formula is as follows:

$$H' = -\Sigma H' = -\Sigma pi(\log_2 pi)/\log_2 N$$

where *pi* is the proportion of characters belonging to the *ith* type of letter present in the string of text (Shannon and Weaver, 1949) while in ecology, *pi* is the proportion of individuals belonging to the *ith* species in the population of interest (Clarke and Warwick, 2001). Meanwhile in this study, the *pi* is the proportion of genotypes belonging to the *ith* class of *n*-class character, where *n* is the number of phenotypic classes of an in vitro response. In this study the reciprocal formula preferred by some analysts: 1-H' (NIST, 2016) was used to make the H' indices reflective of their scale. Therefore H' with larger values now have a high diversity. Larger inequalities in the frequencies of the class now leads to a larger diversity index. If the frequencies are concentrated to one class and the other classes are uncommon regardless of their frequency, the diversity index approaches zero (Clarke and Warwick, 2001).

The Shannon-Weaver diversity index revealed low to moderate variability/diversity in in vitro responses (Table 4.2) among the ten genotypes. This low to moderate variability suggests

more clustered responses among the ten genotypes, which in turn make protocol adjustment more manageable compared to if high variability was observed. Highly variable responses make protocol optimization difficult due to a more scattered spread of responses. This implies that development of more protocols is needed to fulfill growth requirements of different genotypes. Low variability does not necessarily describe excellent or poor performance but with clustered responses a more predictable response profile is achieved thereby facilitating simpler protocol adjustments.

A Two-Step cluster analysis was performed to produce clusters. The clusters were then evaluated against variables (the number of primary shoots per RITA® and the number of secondary shoots per RITA®) identified as important parameters considered for commercial micropropagation. This was done to determine if genotype could affect in vitro performance.

Resulting clusters were cross-referenced with the existing pedigrees from the anthurium program's breeding records. Cross-referencing the genotypes in Cluster 3, which produced moderately high numbers of primary shoots and low numbers of secondary shoots (Figure 4.3) also revealed similarities in some of their lineages. The genotypes UH1145 (Appendix Figure 4.2), UH1067 (Appendix Figure 4.2), UH1244 (Appendix Figure 4.3) and UH2282 (Appendix Figure 4.4) exhibit an *A. amnicola, A. formosum* and *A. kamemotoanum* in their background. These species are hard to establish under in vitro conditions. Leaf cultures of *A. amnicola, A. formosum and A. kamemotoanum* exhibit extreme tissue browning caused by phenolics (T. Amore, pers. comm.). In addition, UH1244 has an *A. antioquense* hybrid (Appendix Figure 4.3) as one of its parents. *A. antioquense* was also identified to be recalcitrant to in vitro plant establishment (J. Kunisaki and T. Amore, pers. comm.). The background of UH2282 is composed of 25% *A. amnicola, A. formosum,* and *A. kamemotoanum* crosses and 75% *A.*

andraeanum (Appendix Figure 4.4). Although UH2282's background is composed of predominantly *A. andraeanum,* it produced a low number of secondary shoots, suggesting that anthurium genotypes with *A. amnicola, A. formosum, A.antioquense* and *A. kamemotoanum* in their background, or crosses with these species regardless of the other parent, may exhibit similar responses as those genotypes found in Cluster 3.

Genotypes in Cluster 4, UH2409 (Appendix Figure 4.5) and UH2271, (Appendix Figure 4.6) shared A697 or 'New Pahoa Red.' as a similar parent. The genotype A697 was also included in this study and was observed to be have the highest number of primary and secondary shoots per RITA® (Figure 4.3). Both UH2409 and UH2271 also produced high numbers of secondary shoots. In addition, the parents of UH2271 and UH2409 are both *A. andraeanum* hybrids (Appendix Figure 4.5 and Appendix Figure 4.7). One of the parents of UH2409, a fast-growing variety, 'Apapane' (UH1651) has about 70% *A. andraeanum* in its background (Appendix Figure 4.6). 'Kaumana' which is one of UH2271's parents, and A697 are known *A. andraeanum* Hort. varieties without available heritage records. This suggests that anthurium genotypes with *A. andraeanum* lineages and resulting progenies from A697 may perform similarly to the genotypes found in Cluster 4.

Assessment of different genotypes can also help create response profiles for predicting in vitro performance. In sorghum, tissue browning caused by phenolic oxidation was identified as a reliable indicator of poor embryogenic response during culture (Sato et al., 2004).

In this study, the growth habit (monopodial or sympodial) was initially thought to have an effect on shoot production, since observation in the field and axillary bud cultures seemed to suggest that cultivars that have certain growth types follow a particular trend of growth (J. Kusnisaki and T. Amore, pers. comm.). Upon assessing 10 genotypes, growth habit was

observed to exert some influence on secondary shoot production, but a larger pool of genotypes is needed to fully ascertain this.

One of the monopodial genotypes (UH2282) displayed a low number of secondary shoots (15) compared to other monopodial genotypes (Figure 4.3 and Figure 4.4). UH2282 displayed a lower number (15) of secondary shoots compared to the average 42 secondary shoots per RITA® of the five monopodial genotypes. It is then probable that genotype exerts a greater influence over secondary shoot production than growth habit since UH2282 share common lineages (*A. amnicola, A. formosum,* and *A. kamemotoanum*) with the sympodial genotypes under Cluster 3.

Generating response profiles for in vitro performance using pedigree or historic records could prove to be useful for future genetic and functional studies. It could also be used as indicators for secondary metabolite production and transformation efficiency.

Certain requirements are needed to create reliable baselines and profiles, such as the existence of an established protocol and the availability of pedigree/historic records to be used for cross-referencing. In addition, gene-environment interaction also plays a role in in vitro growth. Therefore, if these same genotypes were to be subjected to a different medium or protocol/system, a different response may be expected.

Conclusion

Significant differences were seen in the in vitro responses among the ten genotypes except in terms of the shoot length. Size of axillary basal masses and the number of axillary buds affect secondary shoot proliferation. The Shannon-Weaver Diversity index revealed low to moderate variability among the genotypes which indicates more clustered responses among the ten genotypes. These clustered responses lead to the development of more predictable response profiles that can simplify protocol adjustments. The cluster analysis of quantitative and qualitative variables generated five clusters which were evaluated against the type of growth and shoot production parameters. After cross-referencing clusters with existing pedigrees, genotypes in Cluster 3 were found to share *A. amnicola, A. formosum, A. kamemotoanum* and *A. antioquense* in their background. These genotypes produce a moderately high number of primary shoots and a low number of secondary shoots. Anthurium genotypes which possess the *A. amnicola, A. formosum, A. kamemotoanum* and *A. antioquense* lineages are likely to respond similarly to the genotypes under Cluster 3. Genotypes found in Cluster 4 share an *A. andraeanum* hybrid and A697 as one of their parents. These anthurium genotypes produced a moderately high number of primary shoots and a high number of secondary shoots. Anthurium genotypes with the *A. andraeanum* lineage or progenies of A697 are likely to respond similarly to the genotypes found in Cluster 4. Cross-referencing clusters with existing pedigrees also revealed that genotype exerts a greater influence over secondary shoot production compared to growth habit. The monopodial genotype UH2282 performed similarly to those sympodial genotypes in Cluster 3 and share *A. amnicola, A. formosum, A. kamemotoanum* lineages with them. Parentage was found to affect the secondary shoot proliferation of some accessions

indicating that breeding data help with predicting or creating response profiles for in vitro performance.

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CHAPTER 5: GENERAL SUMMARY AND CONCLUSION, RECOMMENDATIONS, FUTURE PERSPECTIVES

General Summary and Conclusion

The RITA® bioreactor system increased secondary shoot production (37) compared to the conventional flask system (14). The increase in vigor and multiplication rates of microplants produced in bioreactors is attributed to reduced physiological disorders such as hyperhydricity and asphyxiation (Berthoughly and Etienne, 2005; Georgiev et al., 2014). Physiological abnormalities caused by hyperhydricity were not observed in this study. The increase in multiplication rate was attributed to frequent gas exchange or reduced asphyxiation.

The opening of stomates at night is not typically observed in C3 plants such as those in the genus *Anthurium*. Leaf epidermal imprints taken from in vitro plantlets of the RITA® bioreactor system and the conventional flask system showed differences on the timing of stomatal opening. The stomates on the leaves of the in vitro shoots in the conventional flask system remained open during nighttime (9:00 pm) while stomates from in vitro shoots in the RITA® bioreactors were closed. It remains unclear if this difference in the time of stomatal opening contributed to the overall increase in growth response observed in the RITA bioreactors.

Shorter immersion times (i.e., 5 minutes) increased secondary shoot production (56) compared to longer immersion times (i.e., 20 minutes) in plants which had fewer secondary shoots (20). Shorter immersion times (i.e., 5 minutes) also had larger total axillary bud mass volumes (9 cm^3) compared to longer immersion times (i.e., 20 minutes) which produced smaller total axillary bud mass volumes (4 cm^3)

Additionally, shorter resting intervals (i.e., 2 hours) also resulted in increased secondary shoot production (47) compared to longer resting intervals (i.e., 8 hours) in which there were fewer secondary shoots (30). Shorter resting intervals (i.e., 2 hours) also resulted in a larger total axillary bud mass volume (8 cm^3) compared to longer resting intervals (i.e., 8 hours) resulted in a smaller total axillary bud mass volume (2 cm^3). Shorter intervals translate to more frequent gas exchange in a day.

The in vitro responses of ten genotypes showed significant differences in terms of the percentage of explants with shoots ($p=0.002$), number of primary shoots per RITA® ($p=0.003$), total axillary bud mass volume per RITA® ($p=0.008$), number of secondary shoots produced per RITA® ($p=0.011$), while shoot length (cm) ($p=0.052$) showed no significant differences.

Initial observations during the RITA optimization for *A. andraeanum* Hort. 'New Pahoa Red' suggested that a larger total axillary bud mass volume (cm^3) would lead to a higher number of secondary shoots. However, when ten genotypes were evaluated under the optimized RITA® setting of 5 minutes immersion time and a 2 hour resting interval, a larger total axillary bud mass volume did not translate to a higher number secondary shoots for some genotypes. The discrepancy between the two studies may be due to the presence of more axillary buds found in some of the genotypes.

To quantify the differences in in vitro responses among the ten genotypes, the Shannon-Weaver Diversity Index was used. The index revealed low to moderate variability in terms of in vitro responses. The low to moderate variability suggests more clustered responses among the ten genotypes, indicating that protocol adjustment may be more manageable. A higher variability would make optimizing protocols for multiple genotypes difficult because of a more scattered spread of responses. This suggests that development of more protocols is needed to satisfy the

growth requirements of different genotypes. Meanwhile low variability does not describe the quality of in vitro performance (excellent or poor performance) but with clustered responses it is possible to expect more predictable response profiles thereby facilitating simpler protocol adjustments.

Two-step clustering of the genotypes based on in vitro responses resulted into five distinct clusters. Cluster 1 contains *A. nymphaeifolium,* A213-2. Cluster 2 is comprised of 'Midori', a commercial *A. andraeanum* Hort cultivar. Cluster 3 is composed of anthurium hybrids, UH1145, UH1067, UH1244, UH 2282 and *A.* × *ferriense* A888. Cluster 4 contained UH2409 and UH2271. Cluster 5 is composed of another *A. andraeanum* Hort commercial variety known as 'New Pahoa Red' (A697). Genotypes in Cluster 3 produced moderately high numbers of primary shoots and low numbers of secondary shoots. Cluster 4 produced high numbers of secondary shoots. Similarities in genetic background was found after clusters were cross-referenced with available pedigree records. The hybrid genotypes in Cluster 3 were found to have *A. amnicola, A. formosum* and *A. kamemotoanum* in their backgrounds. Meanwhile the genotypes in Cluster 4 were mainly composed of *A. andraeanum* lineages.

Cross-referencing the genotypes with their pedigree has demonstrated that the parentage affected in vitro performance. This was extremely notable in UH2282 where its background is predominantly 75% *A. andraeanum* and 25% *A. amnicola, A. formosum* and *A. kamemotoanum.* Based on the pedigree records of the genotypes under Cluster 4 which is mainly *A. andraeanum,* UH2282 should perform similarly to UH2409 and UH2271. However, UH2282 produced a low number of secondary shoots even though its background is predominantly composed of the *A. andraeanum* lineages. The low number of secondary shoots observed in UH2282 suggests that genotypes with *A. amnicola, A. formosum,* and *A. kamemotoanum* in their background or hybrids

resulting from these species regardless of the other parent may exhibit similar responses as those in Cluster 3.

Monopodial and sympodial growth habits were initially thought to influence plant growth under in vitro conditions. Assessment of the genotypes revealed that growth habit had less influence on secondary shoot production compared to lineage. UH2282, a monopodial genotype under Cluster 3 displayed a low number of secondary shoots (15) compared to mean of the five monopodial genotypes which is 42 secondary shoots. Pedigree records show that UH2282 shares common lineages (*A. amnicola*, *A. formosum*, and *A. kamemotoanum*) with the sympodial genotypes under Cluster 3 which suggests that genotype is able exert a greater influence over secondary shoot production than growth habit

The inclusion of the RITA bioreactors to current micropropagation systems/protocols and the assessment of genotype dependency in terms of in vitro responses will enhance microplantlet production and fast track varietal release in anthurium. In addition to pedigrees and historic records, comparative analysis of in vitro growth responses could provide baselines for protocol optimization and future genetic and functional studies.

Recommendations

The RITA® bioreactor system employs a relatively simple system which makes use of a pneumatic pump (Georgiev et al, 2014). The atmospheric environment in the vessel is renewed by pumping in ambient air filtered through 0.22 μm filters. During the gas ambient air exchange, CO_2, O_2 and other gas levels are undetermined but are assumed to also change, thus negating asphyxiation effects encountered in fully submerged culture systems (Bertouly and Eteinne, 2005). Since the RITA® bioreactor system uses a pneumatic pump to push air through the

bioreactors, it is also possible to enrich CO_2 levels in the system by feeding the pump with CO_2. Elevated CO2 levels have been known to increase plant yield and biomass (Ainsworth, 2008; Thompson et al., 2017; South et al., 2019). CO_2 level enrichment in the RITA® bioreactor system could therefore be used to further increase shoot production.

Other temporary immersion bioreactors such as such as the "Box in Bag" bioreactors (10L) and the We V-box (1.3L) and the SETIS™ (6L) have larger headspace and offer media renewal compared to the RITA® (Georgiev et al., 2014). Comparison of the performance of the RITA® with other TIS can help identify which system would be appropriate for commercial micropropagation schemes.

Comparative analysis of in vitro responses of different anthurium genotypes has shown that anthurium parentage can influence performance under the in vitro conditions. Hybrids with *A. andraeanum* and *A. amnicola, A. formosum,* and *A. kamemotoanum* lineages were sufficiently represented, and inferences were made possible. Other hybrids with the *A.* × *ferriense* and *A. nymphaefolium* lineages were poorly represented due to unavailability of in vitro stock plants. Inclusion of other hybrids with the *A.* × *ferriense* and *A. nymphaefolium* lineage along with other genotypes used in breeding program in the analysis could further shed information on influence of these species' in vitro performance.

Future Perspective

Inclusion of the RITA® bioreactor system in current micropropagation schemes would boost anthurium production and hasten cultivar release since generation of sufficient quantities of microplants (1000 per selection for every cooperator and site) for cultivar selection takes 36 months. The RITA system is compatible for large scale and small scale micropropagation labs.

Estimates based on results from the optimized RITA® setting (5 minutes immersion at a 2 hour resting interval and media volume of 20 ml per explant) for 'New Pahoa Red' microplant production suggests a projection of 100 shoots for the initiation phase (45 days after culture in shoot initiation medium) and 300 shoots during the multiplication/proliferation phase (45 days after culture in shoot proliferation medium) using five bioreactors. A total of 400 shoots can be produced in a 90 day production cycle using the RITA® system (Figure 5.1 A) in comparison to the conventional flask system that generates an estimated 150 shoots in a 90 day production cycle (Figure 5.1 A).

In conjunction with pedigree and historical records such as passport data and field performance data, comparative analysis of in vitro responses provides valuable information that could help develop response profiles for anthurium. Additionally, these types of analysis could also pave the way towards the development of genetic markers that can be used to screen for genetic stability and embryogenesis by differentiating somaclonal variation caused by genetic mutations or epigenetic events (Miguel and Marum, 2011).

Comparative analysis of in vitro responses could help identify genotypes that perform well under in vitro conditions. When high performing genotypes are identified, they can be used in the breeding program to introgress ease of propagation in cultivar development.

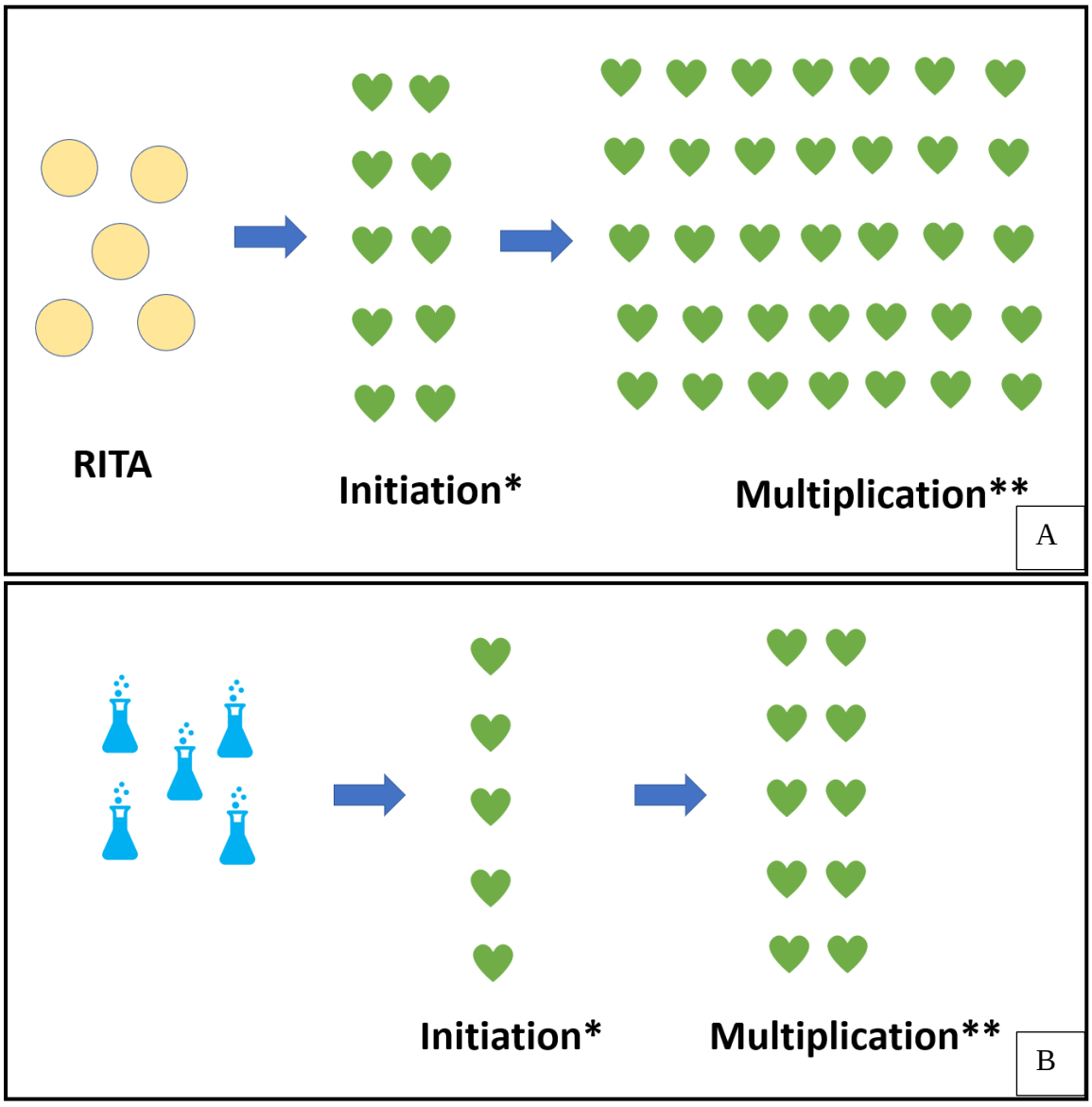

Figure 5.1. Microplant production scheme for 'New Pahoa Red'. A.) RITA® bioreactor system and B.) Conventional flask system. One heart represents 10 shoots. *-45 days after culture in shoot initiation medium. **-45 days after culture in shoot proliferation.

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APPENDICES

Appendix Table 3.1. Independent Samples T-test for Experiment 1 and 2: Percentage of explants with shoot.

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage of explants with shoot	Equal variances assumed	0.511	0.495	5.842	8	0.0003	32.00000	5.47723	19.36950	44.63050

Appendix Table 3.2. Independent Samples T-test for Experiment 1 and 2: Number of primary shoots per explant.

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Number of primary shoots per explant	Equal variances assumed	1.274	0.292	8.356	8	0.0003	1.35200	0.16181	0.97887	1.72513

Appendix Table 3.3. Independent Samples T-test for Experiment 1 and 2: Shoot length in cm

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
								Std. Error	95% Confidence Interval of the Difference	
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Difference	Lower	Upper
Shoot length in cm	Equal variances assumed	3.106	0.116	14.342	8	0.0005	1.70600	0.11895	1.43169	1.98031

Appendix Table 3.4. Independent Samples T-test for Experiment 1 and 2: Number of primary shoots per vessel

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
								Std. Error	95% Confidence Interval of the Difference	
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Difference	Lower	Upper
Number of primary shoots per vessel	Equal variances assumed	2.982	0.122	7.296	8	0.0008	18.80000	2.57682	12.85784	24.74216

Appendix Table 3.5. Independent Samples T-test for Experiment 1 and 2: Number of secondary shoots per vessel.

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Number of secondary shoots per vessel	Equal variances assumed	0.097	0.763	4.161	8	0.003	6.20000	1.48997	2.76413	9.63587

Appendix Table 3.6. Independent samples t-test for Experiment 1: Comparison of the RITA® bioreactor system and the conventional flask system.

Independent Samples Test										
Source		**Levene's Test for Equality of Variances**		**t-test for Equality of Means**						
		F	**Sig.**	**t**	**df**	**Sig. (2-tailed)**	**Mean Difference**	**Std. Error Difference**	**95% Confidence Interval of the Difference**	
									Lower	**Upper**
Percent explants with shoots	Equal variances assumed	0.511	0.495	5.842	8	0.0004	32.00000	5.47723	19.36950	44.63050
Number of primary shoots per explant 1	Equal variances assumed	1.274	0.292	8.356	8	0.0000	1.35200	0.16181	0.97887	1.72513
Shoot length in cm	Equal variances assumed	3.106	0.116	14.342	8	0.0000	1.70600	0.11895	1.43169	1.98031
Number of secondary shoot per vessel	Equal variances assumed	0.097	0.763	4.161	8	0.0032	6.20000	1.48997	2.76413	9.63587

Appendix Table 3.7. Independent samples t-test for Experiment 2: Comparison of the RITA® bioreactor system and the conventional flask system.

Independent Samples Test										
Source		**Levene's Test for Equality of Variances**		**t-test for Equality of Means**						
		F	**Sig.**	**t**	**df**	**Sig. (2-tailed)**	**Mean Difference**	**Std. Error Difference**	**95% Confidence Interval of the Difference**	
									Lower	**Upper**
Percent explants with shoots	Equal variances assumed	0.122	0.736	6.328	8	0.000	58.00000	9.16515	36.86512	79.13488
Number of primary shoots per explant 2	Equal variances assumed	0.796	0.398	7.719	8	0.000	1.54000	0.19950	1.07995	2.00005
Shoot length in cm	Equal variances assumed	0.001	0.978	3.283	8	0.011	0.32636	0.09942	0.09710	0.55561
Number of secondary shoots per vessel	Equal variances assumed	1.944	0.201	11.833	8	0.000	22.20000	1.87617	17.87355	26.52645

Appendix Table 3.8. Effect of immersion time and media volume: Analysis of variance of average number of primary shoots per explant.

Between-Subjects Effects					
Dependent Variable: Average number of primary shoots per explant					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	0.856[a]	5	0.171	9.065	0.001
Intercept	55.827	1	55.827	2955.559	0.000
Immersion time (A)	0.781	2	0.391	20.676	0.0001
Media volume (B)	0.001	1	0.001	0.029	0.867
Immersion time x Media Volume (AXB)	0.074	2	0.037	1.971	0.182
Error	0.227	12	0.019		
Total	56.910	18			
Corrected Total	1.083	17			

a. R Squared = 0.791 (Adjusted R Squared = 0.703)

Appendix Table 3.9. Effect of immersion time and media volume: Analysis of variance of average shoot length.

Between-Subjects Effects					
Dependent Variable: Average shoot length (cm)					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	0.080[a]	5	0.016	0.612	0.693
Intercept	25.534	1	25.534	977.215	0.0001
Immersion time (A)	0.044	2	0.022	0.842	0.455
Media volume (B)	0.032	1	0.032	1.214	0.292
Immersion x Media volume (AXB)	0.004	2	0.002	0.082	0.922
Error	0.314	12	0.026		
Total	25.927	18			
Corrected Total	0.394	17			

a. R Squared = 0.203 (Adjusted R Squared = -0.129)

Appendix Table 3.10. Effect of immersion time and media volume: Analysis of variance of percentage of explants with shoots.

Between-Subjects Effects					
Dependent Variable: Percentage of explants with shoots					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	13.833[a]	5	2.767	4.980	0.011
Intercept	1104.500	1	1104.500	1988.100	0.000
Immersion time	5.333	2	2.667	4.800	0.029
Media volume	2.722	1	2.722	4.900	0.047
Immersion time x Media volume	5.778	2	2.889	5.200	0.024
Error	6.667	12	0.556		
Total	1125.000	18			
Corrected Total	20.500	17			

a. R Squared = 0.675 (Adjusted R Squared = 0.539)

Appendix Table 3.11. Effect of immersion time and media volume: Analysis of variance of number of primary shoots per RITA®.

Between-Subjects Effects					
Dependent Variable: Number of primary shoots produced per RITA®					
Source	**Type III Sum of Squares**	**Df**	**Mean Square**	**F**	**Sig.**
Corrected Model	163.778[a]	5	32.756	15.118	0.000
Intercept	3472.222	1	3472.222	1602.564	0.000
Immersion time (A)	107.111	2	53.556	24.718	0.000
Media volume (B)	10.889	1	10.889	5.026	0.045
Immersion time x Media volume (AxB)	45.778	2	22.889	10.564	0.002
Error	26.000	12	2.167		
Total	3662.000	18			
Corrected Total	189.778	17			

a. R Squared = 0.863 (Adjusted R Squared = 0.806)

Appendix Table 3.12. Effect of immersion time and media volume: Analysis of variance of total axillary bud mass volume per RITA®

Between-Subjects Effects					
Dependent Variable: Total axillary bud mass volume per RITA®					
Source	**Type III Sum of Squares**	**Df**	**Mean Square**	**F**	**Sig.**
Corrected Model	84.916[a]	5	16.983	41.535	0.000
Intercept	513.067	1	513.067	1254.784	0.000
Immersion time (A)	80.314	2	40.157	98.211	0.000
Media volume (B)	0.934	1	0.934	2.284	0.157
Immersion time x Media volume (AxB)	3.668	2	1.834	4.485	0.035
Error	4.907	12	0.409		
Total	602.890	18			
Corrected Total	89.823	17			

a. R Squared = 0.945 (Adjusted R Squared = 0.923)

Appendix Table 3.13. Effect of immersion time and media volume: Analysis of variance of number of secondary shoots per RITA®.

Between-Subjects Effects					
Dependent Variable: Number of secondary shoots per RITA®					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	5442.444[a]	5	1088.489	153.069	0.000
Intercept	13014.222	1	13014.222	1830.125	0.000
Immersion time (A)	5031.444	2	2515.722	353.773	0.000
Media volume	2.000	1	2.000	0.281	0.606
Immersion time x Media volume (AXB)	409.000	2	204.500	28.758	0.000
Error	85.333	12	7.111		
Total	18542.000	18			
Corrected Total	5527.778	17			

a. R Squared = 0.985 (Adjusted R Squared = 0.978)

Appendix Table 3.14. Effect of resting interval: Analysis of Variance (ANOVA) of percentage of explants with shoots.

ANOVA

Percentage of explants with shoots

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		22.222	2	11.111	1.000	.422
	Linear Term	Contrast	0.000	1	0.000	0.000	1.000
		Deviation	22.222	1	22.222	2.000	0.207
Within Groups			66.667	6	11.111		
Total			88.889	8			

Appendix Table 3.15. Effect of resting interval: Analysis of Variance (ANOVA) of average shoot length in cm.

ANOVA

Dependent Variable: Average shoot length in cm

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		0.042	2	0.021	14.984	0.005
	Linear Term	Contrast	0.040	1	0.040	28.812	0.002
		Deviation	0.002	1	0.002	1.156	0.324
Within Groups			0.008	6	0.001		
Total			0.050	8			

Appendix Table 3.16. Effect of resting interval: Analysis of variance (ANOVA) of average number of primary shoots per RITA®.

ANOVA

Average number of primary shoots produced per RITA®

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		12.667	2	6.333	1.075	0.399
	Linear Term	Contrast	0.167	1	0.167	0.028	0.872
		Deviation	12.500	1	12.500	2.123	0.195
Within Groups			35.333	6	5.889		
Total			48.000	8			

Appendix Table 3.17. Effect of resting interval: Analysis of variance (ANOVA) of total axillary bud mass volume per RITA®

ANOVA

Dependent Variable: Total axillary bud mass volume

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		50.027	2	25.013	222.891	0.000
	Linear Term	Contrast	49.307	1	49.307	439.366	0.000
		Deviation	0.720	1	0.720	6.416	0.045
Within Groups			0.673	6	0.112		
Total			50.700	8			

Appendix Table 3.18. Effect of resting interval: Analysis of variance (ANOVA) of number of secondary shoots per RITA®.

ANOVA

number of secondary shoots per RITA®

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		590.889	2	295.444	49.241	0.000
	Linear Term	Contrast	468.167	1	468.167	78.028	0.000
		Deviation	122.722	1	122.722	20.454	0.004
Within Groups			36.000	6	6.000		
Total			626.889	8			

Appendix Table 4.1 Welch Analysis of variance (ANOVA) for the number of primary shoots per RITA.

Robust Tests of Equality of Means

Number of primary shoots per RITA®

	Statistic[a]	df1	df2	Sig.
Welch	57.132	9	11.021	0.003

a. Asymptotically F distributed.

Appendix Table 4.2 Welch Analysis of variance (ANOVA) for the percentage of explants with shoot.

Robust Tests of Equality of Means				
Percentage of explants with shoots				
	Statistic[a]	df1	df2	Sig.
Welch	53.173	9	11.592	0.002

a. Asymptotically F distributed.

Appendix Table 4.3 Welch Analysis of variance (ANOVA) for the shoot length in cm

Robust Tests of Equality of Means				
Average shoot length in cm				
	Statistic[a]	df1	df2	Sig.
Welch	6.169	9	10.795	0.053

a. Asymptotically F distributed.

Appendix Table 4.4 Welch Analysis of variance (ANOVA) for total axillary bud mass volume

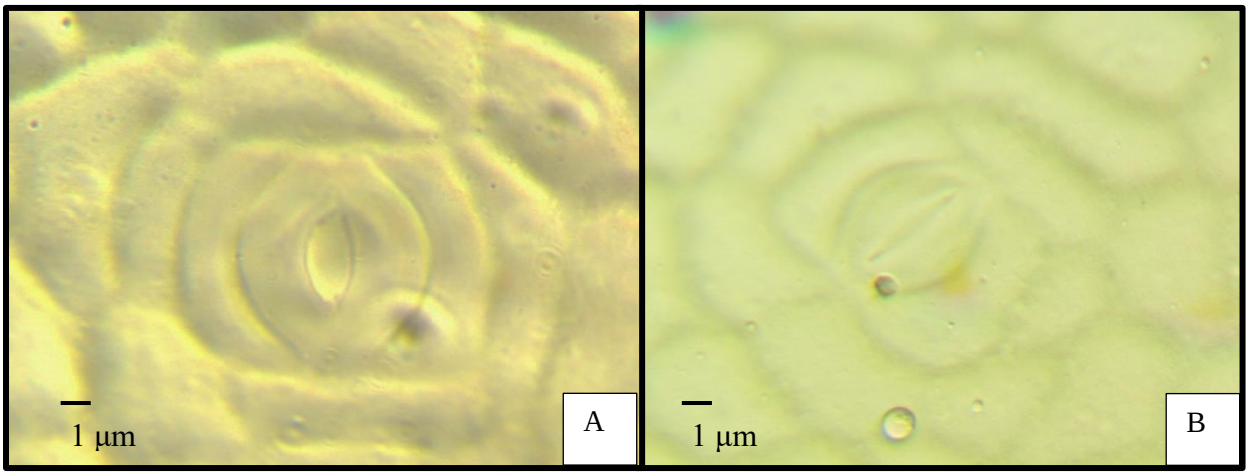
Robust Tests of Equality of Means				
Total axillary bud mass volume				
	Statistic[a]	df1	df2	Sig.
Welch	39.202	9	11.627	0.008

a. Asymptotically F distributed.

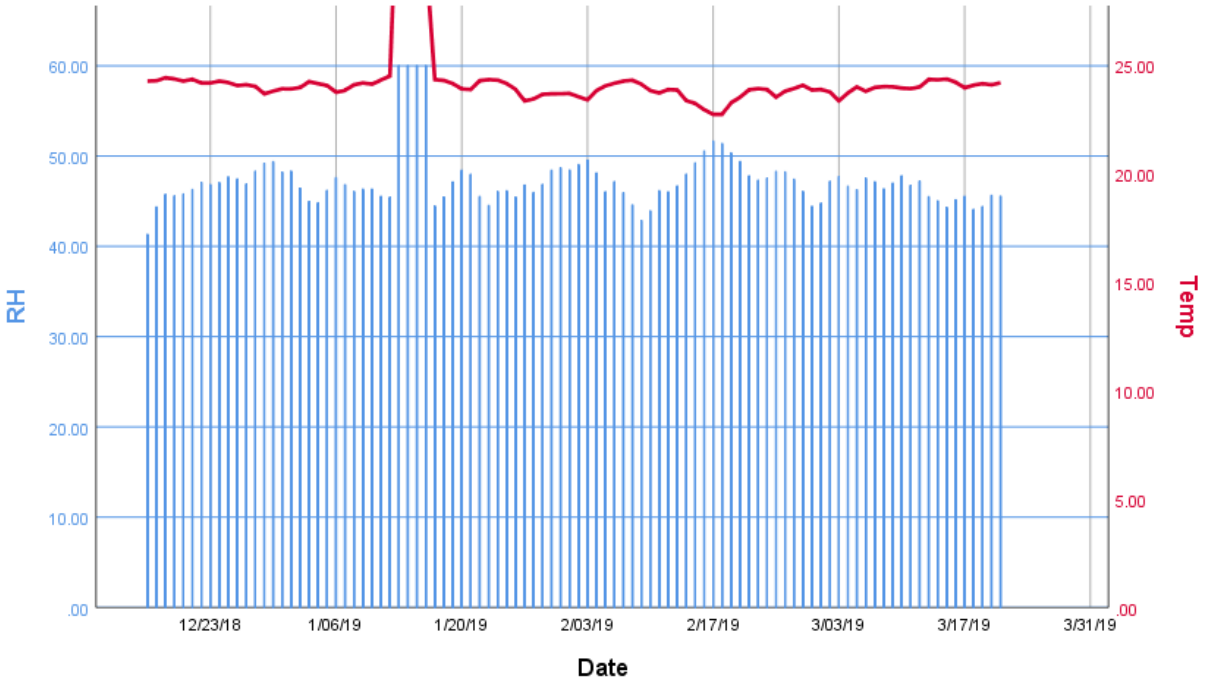
Appendix Table 4.5 Welch Analysis of variance (ANOVA) for average number of secondary shoots per RITA

Robust Tests of Equality of Means				
Average number of secondary shoots per RITA®				
	Statistic[a]	df1	df2	Sig.
Welch	225.701	9	11.105	0.011

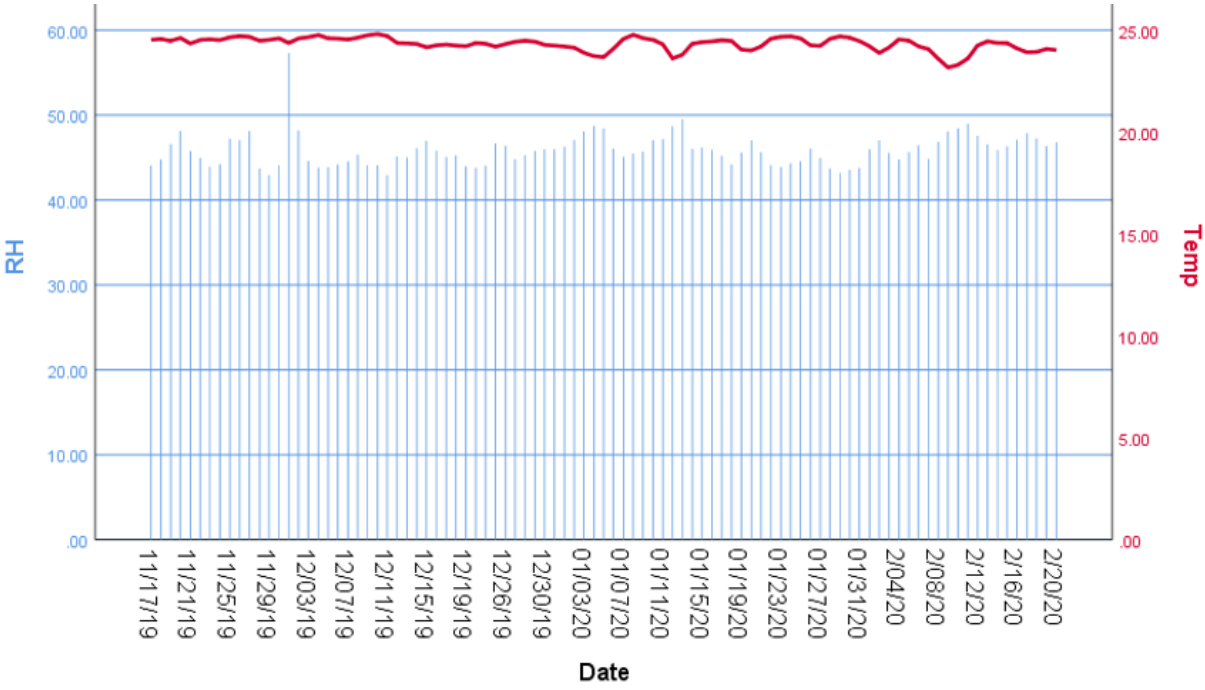
a. Asymptotically F distributed.



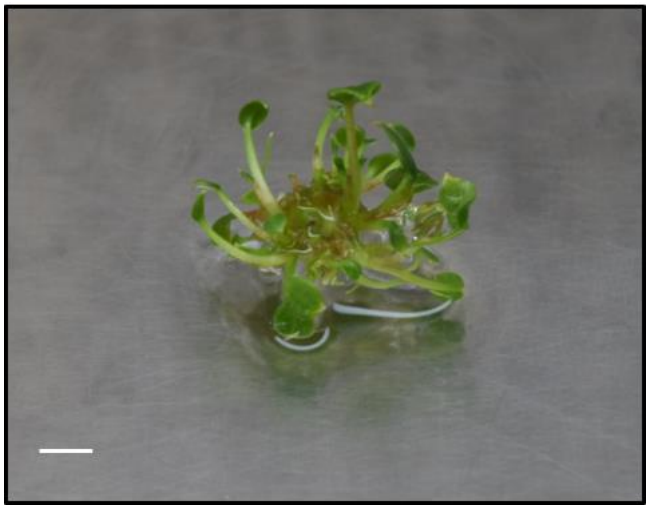
Appendix Figure 3.1. Abaxial leaf epidermal imprint of in vitro cultured *Anthurium andraeanum* Hort. cv. 'New Pahoa Red' taken during the night (9:00 pm). A.) Conventional flask system (flooded condition; stomates are constantly open) and B.) RITA® bioreactor system (temporarily flooded condition, stomates are closed).



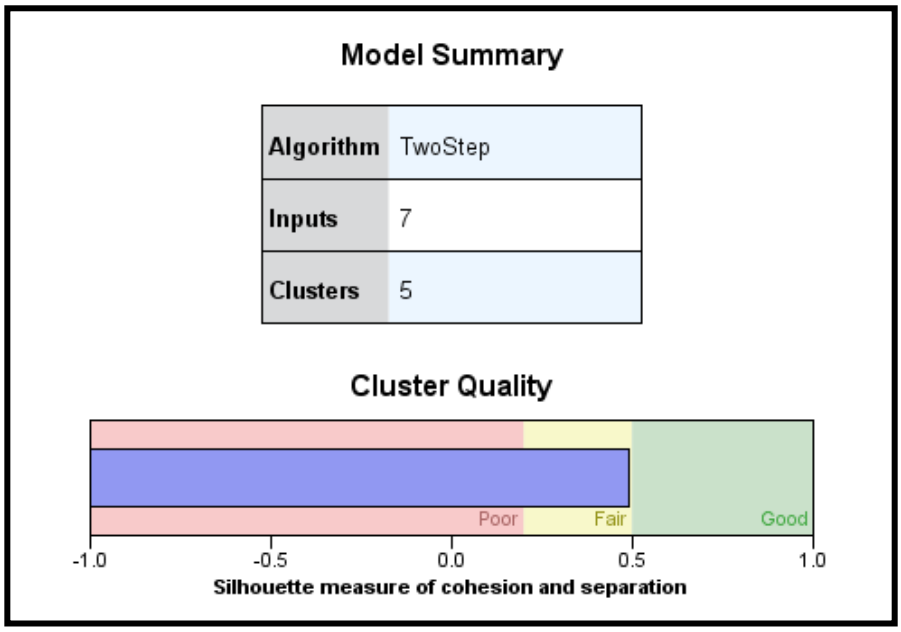
Appendix Figure 3.2 Average daily room temperature and relative humidity for the Amore laboratory, from December 2018 to March 2019.



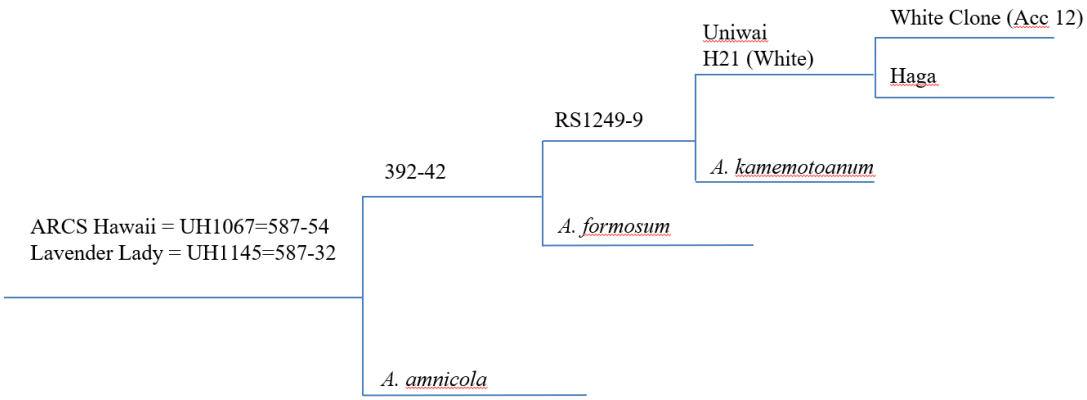
Appendix Figure 3.3 Average daily room temperature and relative humidity for the Amore laboratory, from November 2019 to February 2020.



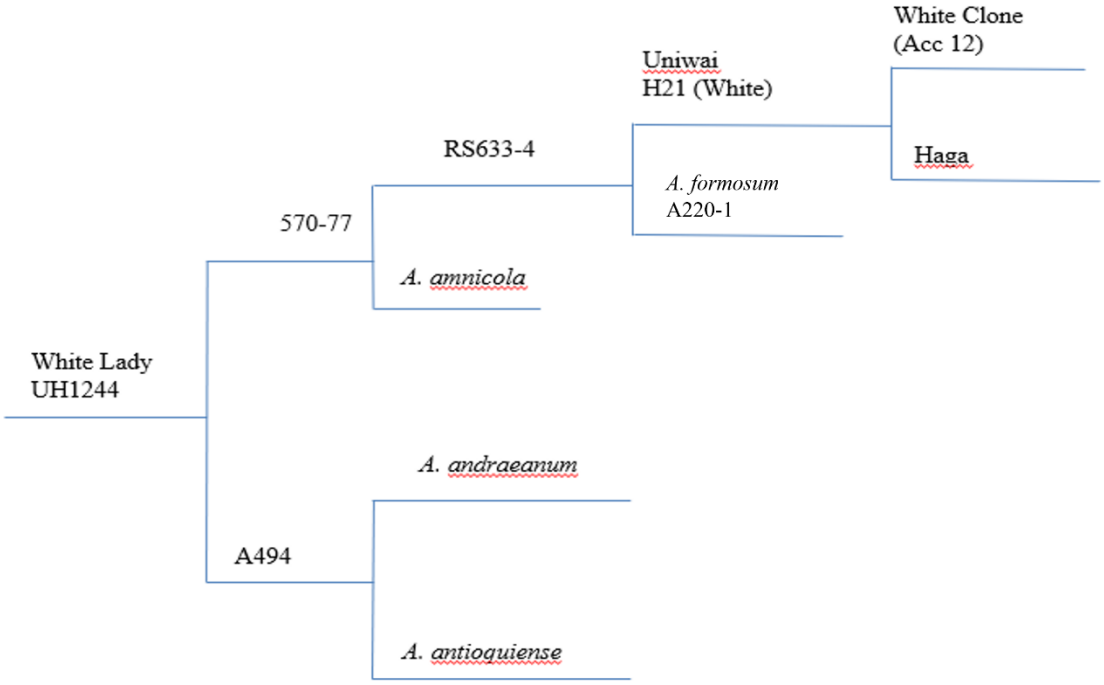
Appendix Figure 3.4. Secondary shoots arising from axillary bud mass of *Anthurium andraeanum* Hort. cv. ‘New Pahoa Red’ 45 days after initiation (shoot proliferation) under the immersion treatment of 5 min and 20 ml. Bar= 0.5 cm



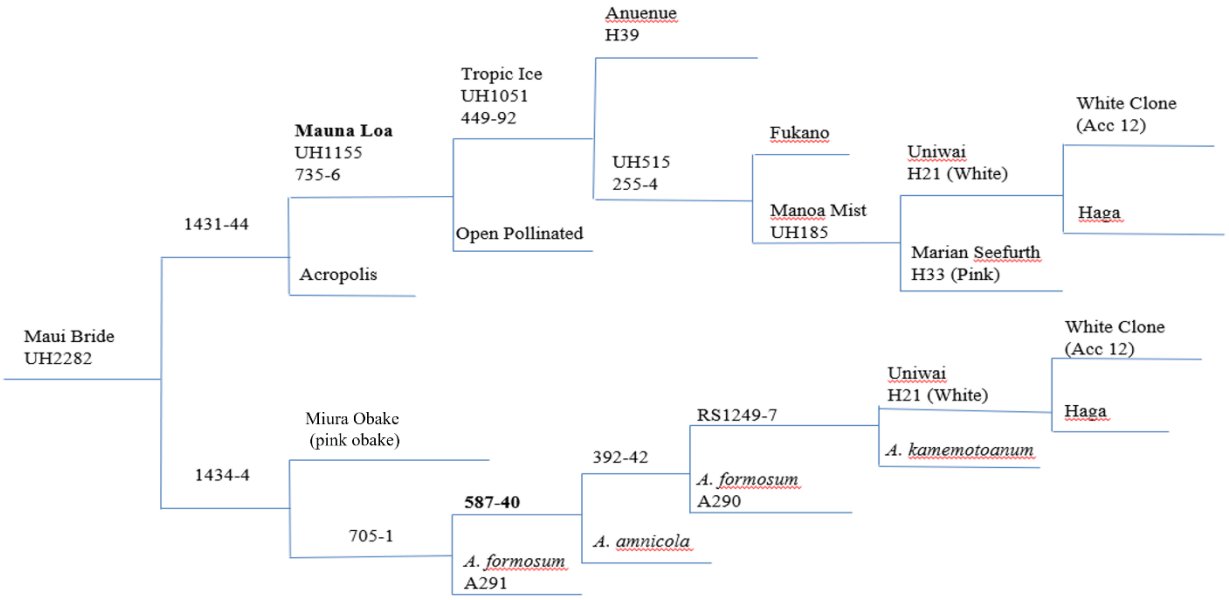
Appendix Figure 4.1. Silhouette measures of cohesion and separation of clusters according to quantitative and qualitative variables of the 10 anthurium genotypes



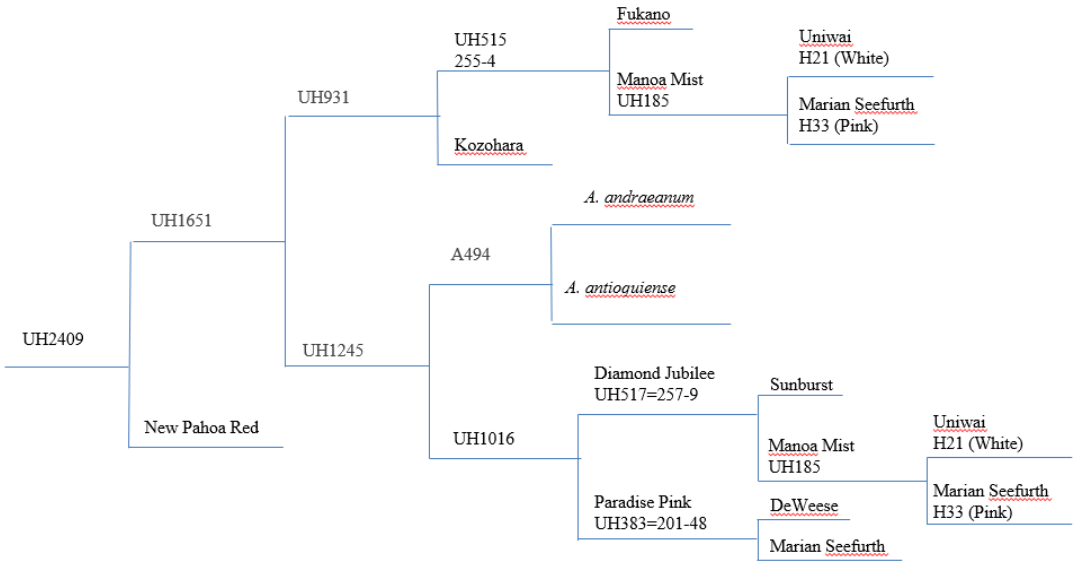
Appendix Figure 4.2 ARCS Hawaii and Lavender Lady lineage. Growth habit: sympodial.



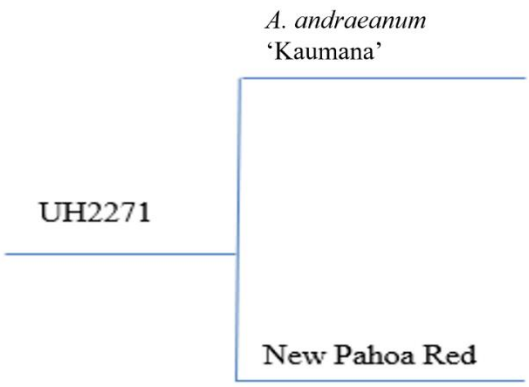
Appendix Figure 4.3 White Lady lineage. Growth habit: sympodial



Appendix Figure 4.4. Maui Bride lineage. Growth habit: monopodial.



Appendix Figure 4.5 UH2409 lineage. Growth habit: monopodial



Appendix Figure 4.6 UH2271 lineage. Growth habit: monopodial.