The Microprogation and Reintroduction into an Open Environment of Sterile *Plumeria rubra* Adult and Seedling Tissue©

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Plumeria, a tropical woody ornamental with attractive fragrant flowers, has been transported around the world and has recently become a collector's plant. New seed-derived cultivars are being introduced wherever the plant can be grown, but increase tends to be slow despite the ease of rooting of cuttings and grafting. Early efforts to tissue culture adult plant material were frustrated by microbial contamination. This study was initiated to determine efficient ways to micropropagate seedling and adult tissues. Six media based on the Murashige and Skoog medium with various amendments were evaluated. Juvenile tissue from seedlings derived from seed that had been surface sterilized and planted into sterile medium was used as an explant source, while adult tissues were derived from meristems dissected from axillary buds that were forced by decapitation of the main stem. Juvenile tissues were successfully subcultured in a Murashige and Skoog medium supplemented with Gamborg vitamins, 1 mg·L^{-1} benzylaminopurine, 1 mg·L^{-1} $AgNO_3$, 500 mg·L^{-1} casein hydrolysate, 20 g·L^{-1} glucose, and 5 g·L^{-1} agar. Rooting was also achieved in this medium. Adult tissue was slower to develop but demonstrated growth on a medium containing 1 mg·L^{-1} kinetin. The use of $AgNO_3$ in the medium appeared to inhibit callus production and enhance shoot regeneration. Upon transfer into nonsterile conditions 82% of plantlets survived.

INTRODUCTION

Plumeria, a plant native to the New World tropics, has been successfully propagated, enjoyed, and spread worldwide by growers, aficionados, and scientists (Eggenberger and Eggenberger, 2000). The fragrant delicate blooms are the product desired by most, although other cultural and medicinal uses for plumeria have been noted such as the anesthetic qualities of *Plumeria rubra* sap (Chak and Patnaik, 1972).

Propagation of these plants has traditionally been through seedpod production, grafting, air-layering, and rooting of cuttings. In vitro tissue culture adds a new option by which growers, aficionados, and scientists can propagate and study this incredible genus. Studies of the plant can be traced back to collections of *P. filifolia* tissue out of Cuba by Mr. C. Wright (1856), one of which is on display on the web at the New York Botanical Garden. One hundred and forty-nine years later, study continues with enthusiasm and interest in the cultivation of plumeria has launched a thriving business in cutting and plant sales.

Early in vitro studies on plumeria occurred in India with Bhaumik et al. (1975) looking at the effects of plant growth regulators on the pH of the cells of bark, eventually arriving at an ideal pH of 5.8 for the cells. Later, attempts were made elsewhere to initiate growth of plumeria tissue in vitro, but those proved unsuccessful due to contaminates (Kunisaki, personal communication). Cytokinins, long used in tissue culture to stimulate shoot production, also enhanced branching of pruned stems (Kwon and Criley, 1991).

Miller's preliminary studies in 2003 helped confirm Bhaumik's research on pH and plumeria cells and provided the methods to acquire and clean tissue for sterile culture. The purposes of this study were to demonstrate the effects of six different media upon seedling and adult meristem tissue and to demonstrate a method for reintroduction of rooted plantlets.

MATERIALS AND METHODS

Media. A prepared mixture of the basal salts for the Murashige and Skoog medium (Murashige and Skoog, 1962) was obtained from Phytotech Laboratories, as were the other ingredients that were employed in the different treatments.

A. Murashige and Skoog basal salts (1.48 $g \cdot L^{-1}$) with Gamborg vitamins, 1 $mg \cdot L$1 benzylaminopurine, 1 $mg \cdot L^{-1}$ silver nitrate, 500 $mg \cdot L^{-1}$ casein hydrolysate, 20 $g \cdot L^{-1}$ anhydrous glucose, and 5 $g \cdot L^{-1}$ tissue culture grade agar. Seedling tissue.
B. Murashige and Skoog basal salts (1.48 $g \cdot L^{-1}$) with Gamborg vitamins, 1 $mg \cdot L^{-1}$ benzylaminopurine, 500 $mg \cdot L^{-1}$ casein hydrolysate, 20 $g \cdot L^{-1}$ anhydrous glucose, and 5 $g \cdot L^{-1}$ tissue culture grade agar. Seedling tissue.
C. Murashige and Skoog basal salts (1.48 $g \cdot L^{-1}$) with Gamborg vitamins, 1 $mg \cdot L^{-1}$ kinetin, 500 $mg \cdot L^{-1}$ casein hydrolysate, 20 $g \cdot L^{-1}$ anhydrous glucose, and 5 $g \cdot L^{-1}$ tissue culture grade agar. Two additional media containing 1.5 and 2 $mg \cdot L^{-1}$ kinetin were also prepared. Seedling and adult tissue.
D. Murashige and Skoog basal salts (1.48 $g \cdot L^{-1}$) with Gamborg vitamins, 500 $mg \cdot L^{-1}$ casein hydrolysate, 20 $g \cdot L^{-1}$ anhydrous glucose, and 5 $g \cdot L^{-1}$ tissue culture grade agar. Seedling tissue.

Media Preparation. All ingredients, except agar were mixed into deionized water. The pH was adjusted to 5.8 and agar added. The mixture was heated to 88 °C while stirring and then poured into culture vessels. All culture vessels (20 mm × 120 mm) were filled approximately 20%, capped, and autoclaved for 15 min at 15 psi.

Adult Tissue Sterilization. Recently formed tissue was selected showing neither cracks nor necrotic areas to avoid possible contamination from fungal spores. Stem tissue was collected from developing axillary buds from topped plants. These stems were approximately 4 cm in length. Prior to surface sterilizing, all expanded leaves were removed, the petioles being excised as close to the stem as possible.

The tissue was surface-sterilized by agitation for 35 min in a 10% sodium hypochlorite bleach solution with a few drops of surfactant added. It was then rinsed twice with sterile water and inserted approximately 2 cm into the medium, which had previously been aseptically broken with forceps. The culture vessels were sealed with Parafilm™ to reduce atmospheric gas exchange.

All culture vessels were placed in racks in an area maintained between 21–24 ºC under broad-spectrum fluorescent lamps set for 14-h light periods. Those were checked periodically for contaminates and necrotic/abscised petiole tissue was removed as needed.

Seed Sterilization. Seed was soaked in deionized water for 8 h, then sterilized in a 5% sodium hypochlorite bleach solution with a few drops of surfactant added and agitated for ~5 min or until the seed coat became somewhat transparent. Those were then sterile rinsed twice and set radicle side down onto cotton bridges moistened by a solution containing half-strength Gamborg B-5 basal salts with 20 $g \cdot L^{-1}$ anhydrous glucose. All germination vessels (same size as culture vessels), prior to setting of seed, had been autoclaved for 15 min at 15 psi.

Germination vessels were maintained between 21–24 ºC under broad-spectrum fluorescent lamps set for 14-h light periods. Periodic checks for contaminates occurred, and observations for problems with the seed leaves pushing out of the seed coat were made, assisting the tissue as needed. After the germinated seedling tissue had developed at least four nodes, tips were sub-cultured onto various media.

Transplanting of Rooted Seedling and Adult Plantlets. After the tissue had developed into a plantlet with roots, it was extracted from the culture vessel and the roots were rinsed in sterile water and placed into a sterile growing medium consisting of equal parts of 1 sand : 1 perlite : 1 vermiculite (by volume) in 10-cm pots, placed into gallon-sized zip-type bags. The medium was moistened with deionized water, and the bags were then sealed and maintained between 21–24 ºC under broad-spectrum fluorescent lamps set for 14-h light periods.

After 1 month, plantlets that had produced more than four nodes were transplanted into a medium containing 1 perlite : 3 potting soil (v/v), placed back into their respective bags, sealed, and maintained between 21–24 ºC under broad-spectrum fluorescent lamps set for 14-h light periods. After one week the bags containing plants with potting soil were opened slightly to allow for gas exchange. As the plants matured, they were transplanted into 15-cm pots containing the same medium and moved outdoors.

Statistical analysis was performed upon plantlets placed upon Media A and B using a Wilcoxon Signed Rank Test at the 95% confidence level for bud break. Statistical analysis was also performed for callus production (on a scale of 0 to 5, 0 = no callus 5 = abundant callus) on plantlets set in media (1, 1.5, or 2.0 $mg \cdot L^{-1}$ in Medium C containing kinetin using ANOVA at the 95% confidence level.

RESULTS

Adult Tissue. Of the adult stem tissue that was placed on Medium C during the 60-day study period, all stem tissue placed survived and two-thirds had leaf primordial into elongated leaves.

Seedling Tissue. Medium A had the best overall effect upon seedling tissue by inhibiting callus formation and inducing buds to grow. Medium B had the worst overall effects upon seedling tissue by inducing heavy callus formation causing the tissue to quickly perish. Medium C, regardless of kinetin concentration, was intermediate in the effects it had upon both tissues, inducing callus formation, but not as heavily as that presented by Medium B. Medium D demonstrated results similar to that of Medium C.

Rooting was noted in both Media A and C. Medium A demonstrated an ability to induce elongation of dormant buds. In a comparison between Media A and B, five replicates each of seedling tissue, all tissue perished on Medium B, while the tissue placed upon Medium A survived the 60-day test period. Two stem sections that had dormant buds on Medium A produced one shoot each while one plantlet, which had a meristem, produced two additional shoots. Analysis showed a p value of 0.059 (Fig. 1). No statistical analysis was performed upon the vitality of seedling tissue between Media A and B, as all those in Medium B perished.

As seedling tissue was, for the most part, restricted to Medium C (except as noted in the previous paragraph) for propagation, no statistical analysis was performed to compare treatment differences between variants of this medium and Media A and B. However, two-month readings presented a statistical difference in callus production between Medium D and Medium C that contained 1.5 $mg \cdot L^{-1}$ kinetin (Fig. 2). Seedling tissue greatly out-produced adult tissue during the 60-day study period producing 49 plantlets from the 14 sets, whereas the single explants remained as set.

Transplanting from a sterile environment presented four fatalities out of 22 attempted; fatalities were associated with non-sterile pots.

DISCUSSION

Seedling tissue replicates with ease, grows rapidly, and can be topped and sub-cultured multiple times. This would be especially important in the propagation of rare *Plumeria* species, such as *P. alba*, *P. filifolia*, *P. inodora*, *P. pudica*, *P. stenopetala*, and *P. stenophylla*. As in vitro culture presents the ability to maintain sterility, tissue can be multiplied and subcultured under controlled conditions, theoretically for an indefinite period of time.

Adult tissue culture does not yet replicate with the ease of seedling tissue. It is slower growing and larger in size, and topping/sub-culture has not yet happened. Two adult stems that have been transplanted from a sterile environment share the same ease for transplanting as demonstrated by seedling tissue.

The effects shown by Medium B may be related to the production of ethylene by the tissue, which then causes a "run-away" effect, and have been seen with media containing kinetin as well. Ethylene can diffuse through the intercellular spaces of a plant, and studies have shown that callus formation is

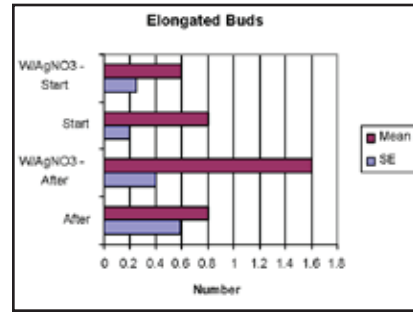

Figure 1. Average number of meristems for plantlets set upon mediums (a – no $AgNO_3$) and (b – with $AgNO_3$) after 60 days.

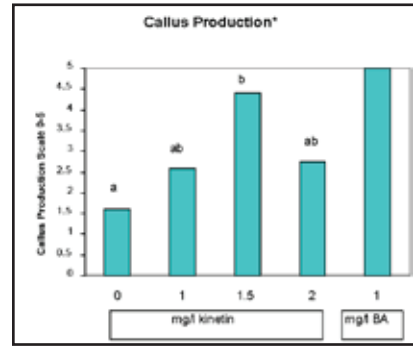

Figure 2. Callus production in response to kinetin and BA after 60 days.

*Bars with different letters denote significant difference at the .05 level.

correlated to the amount of ethylene being produced by the tissues in culture and that ACC, an ethylene precursor, is responsible for the formation of callus (Tadeo et al., 1995). Zobel (1987) demonstrated the effects of gaseous compounds upon tissue contained within sealed vessels and that ethylene can rapidly accumulate to phytoxic concentrations. The effects shown by Medium B suggest that this must be taken into account.

The addition of silver nitrate, an ethylene antagonist, appears to corroborate studies that have shown it significantly enhanced embryogenic callus production and increased shoot regeneration (Fei et al., 2000). By adding silver nitrate to the medium, the action of ethylene is slowed, which inhibits callus formation and cell enlargement, allowing for more normal growth. Initial studies demonstrated an extremely fast "run-away" effect upon the tissue with the addition of the auxin 2,4-D. As the tissue appears to root readily enough without the addition of an auxin and noting the deleterious effects that 2,4-D had upon the tissue, such agents were omitted. Sharad Tiwari (2003) pointed out the necessity to have levels of plant growth regulators that provide for good tissue growth and how the addition of silver nitrate helped maintain embryogenic status of androgenic calli in durum wheat more effectively than other media with 2,4-D alone.

Various challenges presented by the in vitro culture of plumeria are not impossible to overcome. Contaminates can still be a problem if strict adherence to aseptic technique is not followed. Responses to chemical stimuli are variable. Ethylene will be a problem for closed vessels, and plumeria tissue appears to readily produce this gas in vitro. Assuming control for the production of ethylene, maintenance of sterility of the tissue and culture vessel, and supply of nutrients in the media, one can theoretically multiply plumeria seedling tissue indefinitely.

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