

ASYMBIOTIC SEED GERMINATION OF *Vanda*: *IN VITRO* GERMINATION AND
DEVELOPMENT OF THREE HYBRIDS

By

TIMOTHY R. JOHNSON

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

© 2007 Timothy R. Johnson

To my wife, of course

ACKNOWLEDGMENTS

I would like to thank the members of my committee, Dr. Tom Sheehan and Dr. Dennis Gray, for helpful comments on experimental designs, as well as for their editorial feedback. I would like to thank my major professor, Dr. Michael Kane, for his continual dedication to all aspects of this project, as well as his dedication to my professional development. I also wish to acknowledge Daniela Dutra, Philip Kauth, Nancy Philman, and Scott L. Stewart for providing the type of insight that only friends and lab-mates can provide. Thanks also go out to Dr. Martin Motes and the Redland Professional Orchid Growers (Homestead, FL) for partial financial support of this project. Last but not least, I thank my wife, Danielle, for putting up with so many late nights and long trips, as well as my generally distracted demeanor over the past two years. We did it.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	4
LIST OF TABLES	7
LIST OF FIGURES	8
ABSTRACT	9
CHAPTER	
1 LITERATURE REVIEW	11
Introduction to the Family Orchidaceae	11
The Wholesale Orchid Market	13
Classification of Tribe Vandeeae	14
Seed Propagation of <i>Vanda</i>	15
Micropropagation of <i>Vanda</i>	16
Initiation of Tissue Cultures	18
Shoot tip explants	18
Inflorescence and flower bud explants	20
Leaf explants	20
Development and Rooting of Protocorm Like Bodies	22
Summary	23
Objectives	24
2 ASYMBIOTIC SEED GERMINATION OF <i>Vanda</i> HYBRIDS	26
Introduction	26
Materials and Methods	27
Results	29
Discussion	33
APPENDIX	
ATTEMPTS TO INITIATE <i>Vanda</i> TISSUE CULTURES	47
Introduction	47
Initiation of <i>Vanda</i> Tissue Culture Lines Using Newly Emerged Leaves of Flowering Size Plants	48
Materials and Methods	48
Results and Discussion	49
Initiation of <i>Vanda</i> Tissue Culture Lines Using Seedlings, Seedling Leaves, and Cut Protocorms in Liquid Culture	50
Methods	50
Results and Discussion	51

Initiation of <i>Vanda</i> Tissue Culture Lines Using Seedling Leaf Sections	52
Methods	52
Results and Discussion	53
LIST OF REFERENCES	56
BIOGRAPHICAL SKETCH	63

LIST OF TABLES

<u>Table</u>	<u>page</u>
1-1 List of interspecific and intergeneric <i>Vanda</i> hybrids referenced in text.....	25
2-1 Parentage of <i>Vanda</i> hybrids used for experimentation.....	37
2-2 Composition of asymbiotic media used to test the effect of photoperiod on germination and development of <i>Vanda</i> hybrids S005, S013, and S014.....	38
2-3 Developmental stages of <i>Vanda</i> hybrids	39

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
2-1 Germination of <i>Vanda</i> hybrids S005, S013, and S014.....	40
2-2 Protocorm development of <i>Vanda</i> hybrid S005	41
2-3 Protocorm development of <i>Vanda</i> hybrid S013	42
2-4 Protocorm development of <i>Vanda</i> hybrid S014	43
2-5 Scanning electron micrographs of <i>Vanda</i> hybrid S014.....	44
2-6 Thin section light micrographs of <i>Vanda</i> hybrid S014.....	45
2-7 Twelve month old cultures of <i>Vanda</i> hybrid S014.....	46
A-1 Tissue culture of <i>Vanda tessellata</i> × <i>Vanda</i> Arjuna	55

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

ASYMBIOTIC SEED GERMINATION OF *Vanda*: *IN VITRO* GERMINATION AND
DEVELOPMENT OF THREE HYBRIDS

By

Timothy R. Johnson

August 2007

Chair: Michael Kane

Major: Horticultural Science

The majority of potted flowering orchids sold in the United States are *Phalaenopsis* hybrids. However, as consumers become accustomed to growing orchids in the home, demand for other genera will undoubtedly increase. The major objective of this study was to compare the germination and development of three *Vanda* hybrids, a genus with mass market potential. Specifically, I evaluated whether hybrids with different pedigrees had significantly different culture requirements. After 12 weeks culture, differences in germination and development were found between the three hybrids screened. Significantly more seed of *Vanda* Motes Primrose × *Ascocenda* Tavivat (hybrid S014) germinated (82.0–95.5%) than seeds of *Vanda* Paki × (*Vanda tessellata* × *Vanda cristata*; hybrid S005) or (*Vanda* Joan Warne × *Vanda* Paki) × *Vanda* Loke (hybrid S013) on Knudson C (KC), half-strength Murashige & Skoog (½MS), or *Phyto*Technology Orchid Seed Sowing Medium (P723) under three photoperiods (8/16 h, 12/12 h, 16/12 h light/dark). During 12 weeks of observation, protocorms of S005 and S013 did not develop past Stage 3 (first leaf present), while S014 seeds developed to Stage 4 (one leaf and more than one root present; 0.5–0.6%) and Stage 5 (two leaves and more than one root present; 2.7–5.6%) when cultured on P723 under all photoperiods tested. All hybrids appeared to undergo similar early development (Stage 0–3). Limited advanced development of hybrids S005

and S013 may be due either to inadequate culture conditions or low seed vigor. These data indicate that commercial production of some *Vanda* hybrids may be limited by slow growth and development. Efforts to breed hybrids which germinate and develop rapidly may be required to improve the mass market potential of *Vanda* hybrids.

Attempts to initiate *Vanda* tissue cultures using explants from flowering size plants and seedlings were largely unsuccessful. Information on the protocols tested is included in the appendix.

CHAPTER 1 LITERATURE REVIEW

Introduction to the Family Orchidaceae

The family Orchidaceae is estimated to have about 25,000 species and 800 genera, which Dressler placed into five subfamilies (1993). Members of the family are highly variable in habitat and form. The vast majority of orchid species are epiphytic, but 4,000 known species are primarily terrestrial (Dressler, 1981). Orchids exhibit two distinct growth habits: sympodial and monopodial. The sympodial growth habit involves determinate stem growth and subsequent spreading by rhizomes while the more evolutionarily derived monopodial growth form is marked by continual apical growth with little or no axillary sprouting. Many modifications to these two general growth habits can be found, however most orchid species can be placed into one category or the other (Arditti and Ernst, 1992; Dressler, 1993).

The flowers of orchid species are as variable as the growth forms. Dressler (1981) hypothesized that the Orchidaceae has not yet undergone the degree of natural selection that older angiosperm families have undergone;

[M]any of the evolutionary stages of the [Orchidaceae] are still found in living members. If we had only the most primitive living representations of the family, they might be dismissed as rather peculiar lilies. But the vast array of not-missing links show these primitive orchids to be the first stages in the evolution of this distinctive family.

Orchid flowers are typically bilaterally symmetrical and trimerous with inferior ovaries (Dressler, 1993). Typically one petal is larger or more ornate than the others and is termed the lip or labellum (Arditti and Ernst, 1992; Dressler, 1993). Orchid flowers usually display resupination, twisting or bending of the pedicel, during development. Because of this, the labellum, which is adaxial in the flower bud, comes to rest in an abaxial position (Arditti and Ernst, 1992; Dressler, 1993). Another defining characteristic of orchids is that their stamens are all located on one side of the flower rather than being radially dispersed like those of other

angiosperm orders (Dressler, 1981). In addition, the style and filaments of orchid flowers are fused to form a column or gynostemium (Arditti and Ernst, 1992; Dressler, 1993). The column is marked by the presence of the rostellum, a modification to the column that separates the stigmatic surface from the anthers (Arditti and Ernst, 1992; Dressler, 1981), which are located at the end of the column (Arditti and Ernst, 1992; Dressler, 1981; 1993). While primitive orchids still exhibit powdery pollen, the pollen of more derived orchids is contained in discrete, hard packets or pollinia that are attached to a sticky disc termed the viscidium. The viscidium attaches to potential pollinators, resulting in removal of pollinia and (possibly) subsequent pollination (Arditti and Ernst, 1992; Dressler, 1993).

Darwin (1892) wrote extensively about the mechanisms by which orchid pollination occurs. While he studied and presented examples of specific pollinators to illustrate his theories about co-evolution, these may be the exceptions rather than the rules in the Orchidaceae (Dressler, 1993). Most orchids appear to be adapted for insect-mediated pollination (Dressler, 1981) and often exhibit “deceptive” pollinator attraction strategies (Arditti and Ernst, 1992; Cozzolino and Widmer, 2005; Dressler, 1981). As many as 400 species of orchids are sexually deceptive, offering some resemblance to the sexual partners of pollinator species, whereas one-third of all orchids are food deceptive (Cozzolino and Widmer, 2005). Deception takes many other forms in the Orchidaceae including simulation of prey, simulation of substrata and “resemblance to antagonists” (Arditti and Ernst, 1992). Attraction without reward (i.e. deception) is believed to be evolutionarily primitive in orchids, as it is a prevalent characteristic and may be a long-term adaptive strategy that limits inbreeding depression and excessive fruit set, both of which may reduce fitness (Cozzolino and Widmer, 2005).

The Wholesale Orchid Market

Since the USDA began keeping statistics on orchid production, sales of orchid cut flowers and potted plants have increased significantly in the United States; since 1996 there has been a 29% increase in the number of orchid growers (Jerardo, 2006; Nash, 2003). Wholesale values of potted flowering orchids reached \$100 million in 2001 with cut orchid flower sales adding an additional \$8.6 million. While cut flower sales in the United States have been declining for the last five years (approximately \$6.9 million in wholesale value during 2005), wholesales of potted flowering orchids rose to an estimated \$144 million in 2005, second only to that of poinsettias. Florida wholesalers alone made an estimated \$47 million net sales on orchid potted plants in 2005 (Jerardo, 2006).

The resurgence of orchids as popular house plants can be largely attributed to one genus, *Phalaenopsis* (Griesbach, 2002; 2003; Nash, 2003). In 2003, *Phalaenopsis* species and hybrids accounted for 85–90% of all orchids sold (Nash, 2003). At present, *Phalaenopsis* are being prominently featured in design and fashion magazines, business and hotel lobbies, and TV series' sets. *Phalaenopsis* hybrids (which are affectionately called “beginner’s orchids” for their relative ease of culture and willingness to rebloom) are new symbols of elegance and sophistication though the ease with which they are produced and grown has not always been feasible. *Cattleya*, *Cymbidium*, and *Paphiopedilum* were favored during the parlor plant craze of Victorian times. At that time, *Phalaenopsis* were considered difficult to culture since they were not suitable for the very hot rear parlor or very cool front parlor of Victorian style houses (Griesbach, 2002; 2003). In addition, *Phalaenopsis* plants proved difficult to ship during this era, as they do not have pseudobulbs to store water, and their soft leaves are susceptible to bacterial and fungal infection under humid, stagnant conditions (Griesbach, 2003).

Commercial orchid production has not yet met demand (Griesbach, 2002) as evidenced by the steadily increasing number of orchids sold annually (Jerardo, 2006). As consumers become knowledgeable about growing *Phalaenopsis*, they will undoubtedly become interested in growing other orchid genera. One genus that may be well suited for the growing orchid market is *Vanda*. Modern breeding programs are changing the archetype of *Vanda* hybrids being difficult to grow and slow to bloom (Grove, 1995; Motes, 1997). *Vanda* hybrids often exhibit large flowers, a floriferous habit, virtually pest free culture and a wide color pallet (Motes, 1997). *Ascocenda* hybrids (Table 1-1 contains a list of parentages for hybrids that are listed in this chapter), for example, are typically compact in stature, form numerous large flowers and are free blooming (Fitch, 2005). Other bigeneric hybrids like *Vandafinetia* offer an even more compact habit with unusual flowers. Such plants may be marketable for windowsill cultivation (Grove, 1995). Although *Vanda* hybrids have many qualities that make them highly marketable, the production of *Vanda* hybrids is currently confined to small-scale growing operations (personal observation).

Classification of Tribe Vandaeae

Classification of the vandaceous orchids has proven difficult and has lead to conflicting taxonomic visions of the group (Arditti and Ernst, 1992). Dressler (1981; 1993) placed *Vanda* and closely allied species into the largest of the Orchidaceae subfamilies, Epidendroideae, which includes *Calypso*, *Coelogyne*, *Cymbidium*, *Dendrobium*, *Epidendrum*, and *Maxillaria*. The common characteristics of this highly variable subfamily include epiphytic habit, pseudobulbs or corms, fleshy leaves with distichous arrangement (often caduceus), lateral inflorescence, and hard pollinia with caduceus anthers (Dressler, 1993).

Such a broad description of this subfamily warrants further taxonomic splitting, and so Dressler placed *Vanda* into the phylad Epidendroid (eight pollinia, often reduced to six, four, or

two), and the subclad Dendrobiod (1993). Tribes of the Dendrobiod subclad (including Dendrobieae, Podochileae, and Vandaeae) have distinctive “spherical silica” bodies, or stegmata, present in their cells and typically lack pseudobulbs (Dressler, 1993). Interestingly, this subclad contains both sympodial and monopodial genera. Dressler (1993) wrote that these differences are minor, as “[o]nly continued apical growth and rooting at the nodes is needed to convert [sympodial growth] to monopodial growth”.

Among the more recognized genera of the tribe Vandaeae are *Aerides*, *Phalaenopsis*, and *Vanda*. The Vandaeae can be further divided into the subtribes Aeridinae, Angraecinae and Aerangidinae (Dressler, 1993). The Aeridinae consist of 103 genera of monopodial orchids with a *Vanda*-type velamen (multilayered with larger epivelamen cells marked by helical thickenings), two or four pollinia, one or more stipes and a viscidium (Arditti and Ernst, 1992; Dressler, 1993). Flower structures in the subtribe are variable in both size and shape.

Seed propagation of *Vanda*

In the 1920s, Knudson (1922) demonstrated that orchid seed could be germinated without mycorrhizal fungi by growing seed *in vitro* on medium containing mineral nutrients and sugar. Prior to this, Noel Bernard and Hans Burgeff had presented evidence which supported the conclusion that orchid seeds could only be germinated symbiotically (discussed in Knudson, 1922). Seed germination studies have been conducted for several *Vanda* species and hybrids. Roy and Banerjee (2002) found that after three months culture, over 70% of immature *Vanda tessellata* seeds germinated on a variety of media. Bhaskar and Rajeevan (1996) reported fewer than 20% of immature *Vanda* John Club seeds germinated when sown on full-strength Murashige and Skoog (MS; Murashige and Skoog, 1962) and Knudson C (KC; Knudson, 1946) media, and 80% germination of seeds cultured on half-strength Murashige and Skoog medium (½MS). Devi et al. (1998) were able to germinate 90% of *Vanda coerulea* seeds using a wicking

system with liquid Vacin and Went medium (VW; Vacin and Went, 1949) supplemented with Murashige and Skoog vitamins (Murashige and Skoog, 1962). Recently, Kishor et al. (2006) reported that seed of *Ascocenda* Kangla germinated readily when sown on VW containing 2.3 μM kinetin and 0.5 μM NAA (90% germination).

The trend in *Vanda* hybridization programs is to breed increasingly more complex hybrids in an attempt to generate plants with superior flower form and color. Researchers have not yet compared the effect of media and photoperiod on seed germination of complex hybrids (those hybrids with more than two species in their heritage; i.e. greater than F1 hybrids) in side-by-side experiments. For commercial growers of *Vanda* hybrids, understanding the influence of photoperiod and germination media on the growth and development of complex *Vanda* hybrids is important. If problems with growth and development of complex hybrids are detected, the goals of breeders may need to be altered from focusing exclusively on flower characteristics. In order to make *Vanda* a commercially profitable crop, production of *Vanda* must be competitive with that of *Phalaenopsis*. Breeders may need to focus on characteristics such as vigor, time to first bloom, flower longevity, and floriferousness in order to minimize the cost of growing plants and capitalize on mass market consumption of flowering orchids.

Micropropagation of *Vanda*

While seed culture can be an effective means for producing plants in mass, it does not allow for the cloning of unique or highly prized specimens. In 1960, Morel published a procedure for cloning *Cymbidium* via tissue culture. The tissue culture method, which was more completely described by Wimber (1963), involved isolating shoot tips and culturing them aseptically in the presence of hormones and chemical nutrients until they “developed into a full plant” (Morel, 1960). The first successful cloning of *Cymbidium* was recognized as a means to produce virus-free clones from prized plants that were “entirely contaminated with virus...”. Morel (1960;

1964) and Wimber (1963) noted the inherent potential of this technique for the mass production of desirable plants for commercial sales.

Since this early work, orchids have been successfully propagated using roots, leaves, stems, flower buds and inflorescences as explant materials (Arditti, 1993; Arditti and Ernst, 1992). *Vanda* has been notoriously difficult genus to micropropagate (Arditti, 1993; Morel, 1964), though successful tissue culture protocols have been published. Micropropagation has been used extensively with intergeneric *Vanda* crosses such as *Aranda* and *Mokara*, both of which are grown extensively in the Indonesian flower markets (Arditti and Ernst, 1992; Lee et al., 1996).

Seed culture of *Vanda* is a productive way to mass produce plants, as previously discussed (Bhaskar and Rajeevan, 1996; Devi et al., 1998; Sharma, 1998), and seed derived protocorms can be used as explants to establish tissue culture lines (Mathews and Rao, 1979; Roy and Banerjee, 2002). However, the marketable quality of orchid seedlings is variable, making clonal propagation of awarded plants a more desirable method of multiplication, if possible. The vast majority of the published work on the micropropagation of *Vanda* species, interspecific *Vanda* hybrids, intergeneric *Vanda* hybrids and *Vanda* allies has used shoot tips as explants (Cheah and Sagawa, 1978; Ghani et al., 1992; Kanika and Vij, 2004; Kunisaki et al., 1972; Lakshmanan et al., 1995; Malabadi et al., 2004; Seeni and Latha, 1992; Teo et al., 1973; Van Le et al., 1999). When harvesting explants from sympodial orchids, sacrificing a shoot tip results in the loss of a single new growth, however, harvesting the apical meristem from monopodial orchids like *Vanda* may result in plant death (Goh and Wong, 1990; Intuwong and Sagawa, 1973; Sharma and Vij, 1997; Vij et al., 1986). In addition, it can be difficult to excise and sterilize such large explants.

Successful micropropagation of *Vanda* species and hybrids has been attempted using adult leaves (Lay, 1979; Sharma and Vij, 1997), axenic seedling leaves (Vij et al., 1986), undifferentiated inflorescence buds (Goh and Wong, 1990; Intuwong and Sagawa, 1973), differentiated flower buds (Valmayor et al., 1986), and inflorescence nodes (Decruse et al., 2003). Even with many reports of successful tissue culture of *Vanda* species and hybrids, it remains to be demonstrated that any one method can be applied to a wide range of *Vanda*. The numerous methods by which *Vanda* have been tissue cultured may reflect a problem that must be assessed species-by-species or hybrid-by-hybrid. Additionally, incidences of somaclonal variation and potential alterations to flower quality have not been studied in *Vanda*. Random mutation has been reported in woody plants (Hashmi et al., 1997; Pogany and Lineburger, 1990; Tremblay et al., 1999; Vendrame et al., 1999) as well as herbaceous plants (Al-Zahim et al., 1999; Paek and Hahn, 1999; Thomas et al., 2006). Researchers have also found somaclonal variation in *Phalaenopsis* hybrids (Chen et al., 1998; Tokuhara and Mii, 2001), indicating that unwanted mutation could be a problem in the tissue culture of *Vanda*.

Initiation of Tissue Cultures

Shoot tip explants

Modified VW medium supplemented with 15% (v/v) coconut water (CW) has been used to produce protocorm like bodies (PLBs) from cultured shoot tips of *Aranthera James Storei* (Cheah and Sagawa, 1978), *Vanda insignis* × *Vanda tessellate* (Teo et al., 1973), and *Papilionanthe* Miss Joaquim (synonym = *Vanda* Miss Joaquim; Kunisaki et al., 1972). While this may be an effective and highly applicable method for mass clonal propagation of *Vanda* hybrids, none of these studies compared VW medium supplemented with CW to other media.

The medium of Mitra et al. (MM; Mitra et al., 1976) has also been used for establishment of *Vanda* species and hybrids in culture. Kanika and Vij (2004) studied the effect of MM

supplemented with auxins and cytokinins. They found that medium supplemented with 2.3 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 μM 6-benzyladenine (BA), and 20% CW resulted in the formation of two to three PLBs in 100% of *Vanda coerulea* shoot tip explants. However, they did not examine the effects of other concentrations of 2,4-D and/or BA on initiation of regeneration. Seeni and Lathe (2000) used MM supplemented with 10% CW, 500 mg L^{-1} peptone, 30 g L^{-1} sucrose, 8.8 μM BA, and 4.1 μM NAA to compare the response of shoot tips derived from mature plants and *in vitro* plantlets of *V. coerulea*. Testing both liquid and solid (7 g L^{-1} agar) media, they found that explants obtained from *in vitro* plantlets were 15–20% more responsive than explants harvested from flowering size plants. However, a shortcoming of this study was the small sample size (only five explants were tested for each media).

Malabadi et al. (2004) studied the effects of thidiazuron (TDZ) on PLB production from shoot tips of *V. coerulea* cultured on VW basal medium supplemented with 7 g L^{-1} agar, 2.0 g L^{-1} casein hydrolysate, 0.5 g L^{-1} L-glutamine, 1.0 g L^{-1} meso-Inositol, 30 g L^{-1} sucrose, and 250 mg L^{-1} peptone. It was reported that the addition of 11.35 μM TDZ resulted in optimum PLB production (96% of 30 cultures responded). TDZ has also been proven effective for production of PLBs on thin sectioned leaf explants of *Doritaenopsis* (Park et al., 2002).

Lakshmanan et al. (1995) reported PLB formation from *Aranda Deborah* shoot tips and thin sectioned shoot tips. They noted that addition of BA reduced the number of PLBs produced on explants cultured on VW, KC, and MS media supplemented with various amounts of CW.

Valmayor et al. (1986) conducted a very extensive comparison of the effects of tissue culture media on regeneration from terminal flower buds of an *Ascocenda* hybrid, *Vanda lamellata* \times *Ascocenda Tropicana*. They screened 55 modified formulae of liquid KC, MS, VW media containing combinations of 2,4-D, BA, CW, and kinetin (KN) for regenerative effects on

terminal flower buds. Of all the explants tested, only the youngest flower buds initiated PLBs, and only when grown on KC with 15% CW and 4.65 μM kinetin.

Inflorescence and flower bud explants

Intuwong and Sagawa (1973) presented a process for propagating newly emerged inflorescences of *Ascofinetia* Cherry Blossom in liquid VW with 15% CW and 20 g L^{-1} . While they reported that other hybrids were also cultured, a single inflorescence of both *Vascostylis* Blue Fairy and *Neostylis* Lou Sneary were cultured, but failed to produce PLBs. No other media formulae were tested for initiation of PLBs.

Goh and Wong (1990) compared the effect of 0.2 μM BA and 4.7 μM KN alone and in combination on culture initiation using “inflorescence tips” of *Aranda* Deborah in modified formulae of KC and VW both with 15% CW and 20 g L^{-1} sucrose. They reported that none of the modified VW formulae were satisfactory for the production of PLBs or shoot induction. Explants cultured on KC containing CW, KN, and sucrose produced PLBs (32% of explants) or shoots (68% of explants). Explants cultured on KC supplemented with BA alone or with KN regenerated PLBs or shoots.

Leaf explants

Sharma and Vij (1997) reported PLB production using adult *Vanda* leaves. Newly emerged leaf explants of *Vanda cristata* cultured on MM containing 44.4 μM BA and 26.9 μM α -naphthaleneacetic acid (NAA) or 44.4 μM BA and 28.6 μM indole-3-acetic acid (IAA) produced PLBs only on media containing additional copper II ions (2.2 mg L^{-1}). Explants from leaves longer than 3 cm failed to regenerate. Only explants taken from the proximal one-third of each leaf formed PLBs. Seeni and Latha (1992) used solid MM supplemented with 20 g L^{-1} peptone and 20 g L^{-1} sucrose to test the response of newly emerged whole leaf explants of *Renanthera imschootiana* to NAA and BA alone and in combination for the production of PLBs. Inclusion

of 44.4 μM BA and 5.4 μM NAA in the medium promoted initiation of shoots in 80% of whole leaf explants.

Seeni and Latha (2000) tested the response of seedling leaf explants on MM containing 7 g L^{-1} agar, 10% CW, 500 mg L^{-1} peptone, 30 g L^{-1} sucrose, and various combinations of BA and NAA. They also tested the effect of leaf position on responsiveness. After 12 weeks culture, 96% of second position leaves produced an average 13.6 PLBs when cultured on media containing 8.8 μM BA and 4.1 μM NAA. Leaves at the first, third, fourth, and fifth positions were less responsive with respect to PLB production. Seeni and Latha's attempts to initiate PLBs on adult leaves resulted only in callus formation after 12 weeks culture.

In vitro derived plantlets have been used as leaf explant sources. Lay (1979) reported leaf bases of *Aranda Noorah* Alsagoff regenerated shoots when cultured on VW medium containing 50% CW and 20 g L^{-1} sucrose, or MS containing 15% CW, 1 mg L^{-1} glycine, 100 mg L^{-1} myo-Inositol, 0.25 mg L^{-1} nicotinic acid, 0.25 mg L^{-1} pyridoxine, 30 g L^{-1} sucrose, 1 mg L^{-1} thiamine, 9.05 μM 2,4-D, and 8.9 μM BA. Vij et al. (1986) induced initiation of *Vanda testacea* by culturing 0.5–1 cm thick leaf cross sections on MM, 2 mg L^{-1} peptone and either 2.9 μM gibberellic acid or 4.4 μM BA and 5.4 μM NAA.

Sheelavanthmath et al. (2005) reported on the effects of BA, KN, and TDZ on *Aerides crispum* leaf cross sections in MS medium with 10 g L^{-1} agar and 20 g L^{-1} sucrose. They were able to successfully initiate PLBs on 100% of leaf cross sections 3–5 mm thick on media containing 2 μM BA. Explants formed an average of 22 PLBs after eight weeks of culture. Explants grown in the presence of 0.5 and 1 μM TDZ produced PLBs on 83% of explants tested and an average 7.1 and 8.0 PLBs/explant, respectively.

Development and Rooting of Protocorm Like Bodies

Research on development and rooting of tissue cultured *Vanda* has focused on the effects of growth regulators and complex, undefined additives on shoot differentiation. While often not explicitly defined, shoot differentiation probably refers to successful conversion of PLBs into leaf and root bearing seedlings, or direct organogenesis of shoots. Complex nutrients like banana powder and CW contain inorganic salts, amino acids, plant hormones, sugars, and vitamins that effect plant development (Arditti and Ernst, 1992). Kunisaki et al. (1972) studied the effects of sucrose concentration and CW on *Papilionanthe* Miss Joaquim shoot tips cultured in solid VW medium and reported better differentiation when CW and 10 or 20 g L⁻¹ sucrose were present, but did not support their findings with any measurements of success.

Teo et al. (1973) reported that *Vanda insignis* × *Vanda tessellata* PLBs failed to differentiate and perished on solid VW supplemented with 15% CW and sucrose, but developed shoots on solid sugar-free VW with CW, and produced both shoots and roots on solid VW containing sucrose. Seeni and Latha (2000) modified their initiation medium (modified MM) for *Vanda coerulea* shoot tips with 35 g L⁻¹ ripe banana pulp, 30% CW and 1.08 μM NAA, but did not report whether these additives lead to increased shoot differentiation.

Decruse et al. (2003), studying *Vanda spathulata* reported on the effects of solid (6 g L⁻¹ agar) MM containing different concentrations of BA and IAA alone and in combination on initiation of direct shoots. They reported that after three months culture, explants cultured on medium supplemented with 4.4–22.2 μM BA produced one shoot. Higher BA concentrations (44.4–88.8 μM) resulted in the production of less than three shoots per explant. Explants cultured on medium containing 44.4 μM BA with 17.1 μM IAA and 66.6 μM BA with 28.5 μM IAA produced 12.6 and 12.1 shoots/explant, respectively. This indicates that the inclusion of both cytokinins and auxins may improve multiplication rates of *Vanda* cultures.

Valmayor et al. (1986) conducted the most extensive research on shoot differentiation from *Vanda lamellata* × *Ascocenda Tropicana* flower bud explants by testing the effects of three media (KC, MS, and VW) supplemented with various concentrations of complex, undefined nutrients, plant growth regulators (PGRs), and sucrose. Their results indicate that many media were effective at inducing shoots: VC with 20 g L⁻¹ sucrose, MS with 15% CW, KC with 15% CW, and KC amended with both 15% CW and 8.9 μM BA.

Malabadi et al. (2004) published one of the few studies on root differentiation of *Vanda* in which the effects of various auxins (IAA, indolebutyric acid [IBA], and NAA) on rooting of *in vitro* derived shoots of *Vanda coerulea* were compared. Optimal results (87% rooting) were observed on half-strength VW containing 11.42 μM IAA. VW basal medium with BA and IAA concentrations less than 26.9 μM and 24.5 μM, respectively, failed to induce root production. Seeni and Latha (1992) reported successful *in vitro* rooting of *Renanthera imschootiana* shoots in the presence of 5.4 μM NAA and 1% activated charcoal (the exact medium used is unclear, but presumed to be MM with 20 g L⁻¹ sucrose and 20 g L⁻¹ peptone, as was used for initiation).

Summary

To date, no studies have conclusively assessed the feasibility for using any one micropropagation protocol for the tissue culture of interspecific and intergeneric *Vanda* hybrids. Given the number of potential species involved in any one breeding program, as well as the various ways in which hybrids are crossed and backcrossed, it seems unlikely that any one protocol will work for all hybrids. Due to the limited breadth of any one particular *Vanda* micropropagation protocol and the seemingly hybrid-specific requirements of all *Vanda* tested to date, seed propagation may be a more reliable means of producing large quantities of plants with minimal input. If so, the question then becomes whether *Vanda* seeds from unique and diverse lineages will germinate and grow under common *in vitro* conditions?

Objectives

- OBJECTIVE I. Assess the effects of photoperiod and culture media on the germination and seedling development of *Vanda* hybrids of diverse breeding histories.
- OBJECTIVE II. Compare the seedling growth and development of *Vanda* hybrids using scanning electron microscopy and light microscopy.
- OBJECTIVE III. Interpret results in the context of a growing market for potted flowering orchids and assess the feasibility of using seed propagation for the mass market production of *Vanda* hybrids.

Table 1-1. List of interspecific and intergeneric *Vanda* hybrids referenced in text. Parentage of registered hybrids was found on the International Orchid Register (http://www.rhs.org.uk/plants/registration_orchids.asp).

Intergeneric hybrid names	Parentage
<i>Aranda</i>	<i>Arachnis</i> × <i>Vanda</i>
<i>Ascocenda</i>	<i>Ascocentrum</i> × <i>Vanda</i>
<i>Doritaenopsis</i>	<i>Doritis</i> × <i>Phalaenopsis</i>
<i>Mokara</i>	<i>Arachnis</i> × <i>Vanda</i> × <i>Ascocentrum</i>
Intergeneric hybrids	Parentage (seed parent × pollen parent)
<i>Ascofinetia</i> Cherry Blossom	<i>Neofinetia falcata</i> × <i>Ascocentrum ampullaceum</i>
<i>Aranda</i> Deborah	<i>Arachnis hookeriana</i> × <i>Vanda lamellata</i>
<i>Aranda</i> Noorah Alsagoff	<i>Arachnis hookeriana</i> × <i>Vanda</i> Dawn Nisimura
<i>Aranthera</i> James Storei	<i>Arachnis hookeriana</i> × <i>Renanthera storei</i>
<i>Ascocenda</i> Kangla	<i>Vanda coerulea</i> × <i>Ascocentrum auranticum</i>
<i>Ascocenda</i> Tropicana	<i>Ascocentrum curvifolium</i> × <i>Vanda</i> Betsy Sumner
<i>Neostylis</i> Lou Sneary	<i>Neofinetia falcata</i> × <i>Rhyncostylis coelestis</i>
<i>Vascostylis</i> Blue Fairy	<i>Ascocenda</i> Meda Arnold × <i>Rhynchostylis coelestis</i>
Interspecific hybrids	Parentage (seed parent × pollen parent)
<i>Papilionanthe</i> Miss Joaquim	<i>Papilionanthe hookeriana</i> × <i>Papilionanthe teres</i>

CHAPTER 2
ASYMBIOTIC SEED GERMINATION OF *Vanda* HYBRIDS

Introduction

Sales of potted flowering orchids in the United States have risen since data was first collected by the USDA in 1996. Wholesales of potted orchids reached \$100 million in 2001, and an estimated \$144 million in 2005 (Jerardo, 2006). It has been estimated that as many as 90% of all orchids sold are *Phalaenopsis* species and hybrids (Nash, 2003). However, as consumers become accustomed to growing and displaying orchids in residential and commercial settings, the demand for other orchid genera may also increase.

Extensive research on the physiology of *Phalaenopsis* flower induction (Blanchard and Runkle, 2006; Li et al., 2006; Su et al., 2001; Wang et al., 2002) has undoubtedly contributed to the commercial success of this genus by decreasing time to flowering, thus lowering per unit production costs and retail sale prices. *Vanda* hybrids may also have a profitable place in the expanding orchid market, though their production in the United States is at this time limited by small-scale production and the long time to flowering. The result is a relatively high production cost for *Vanda* hybrids compared to that of *Phalaenopsis*.

Vanda hybrids have many characteristics that are amenable to mass consumption including a variable color pallet, long lasting flowers, free-blooming habit, fragrant flowers and multiple inflorescences. In addition, some hybrids exhibit a compact stature and cold tolerance. Research on the growth and development of *Vanda* could lead to more efficient methods to produce salable (i.e. flowering) plants, thus making *Vanda* both profitable for large-scale production and affordable for consumers in the mass market.

While germination studies of *Vanda* species and hybrids have been conducted (Bhaskar & Rajeevan, 1996; Devi et al., 1998; Kishor et al., 2006; Roy and Banerjee, 2002), limited

information regarding protocorm and seedling development has been published. Asymbiotic seed germination studies of terrestrial orchids have revealed that germination alone may not be a reliable indicator of subsequent plant growth and development (Hoshi et al., 1994; Kauth et al., 2006; Stenberg and Kane, 1998). The use of distinct morphological stages (= developmental stages), can be used to more precisely compare growth and development of different orchid species, hybrids, and/or cultivars. The objective of this study was to describe and compare the growth and development of three complex *Vanda* hybrids asymbiotically cultured on a range of *in vitro* media and under different photoperiodic regimes.

Materials and methods

Seed source. Seeds of three *Vanda* hybrids (Table 2-1) were provided by Motes Orchids (Homestead, FL). Seeds were removed from mature, undehisced capsules on 2 February 2006, and stored at 5–10% relative humidity and $20^{\circ} \pm 5^{\circ}\text{C}$. Seeds were removed from dry capsules five weeks after capsules were harvested, then transferred to filter paper packets (Whatman No. 2) and stored over desiccant for four weeks prior to experimentation. A small sample of seeds (approximately 300) from each capsule was subjected to a tetrazolium (TZ) viability test (Lakon, 1949). Seeds were pretreated for 15 min in 5.0% $\text{Ca}(\text{OCl})_2$ before being soaked for 24 h in distilled deionized (DD) water at 25°C , followed by a 24 h soak in 1.0% TZ in darkness at 30°C . Seeds were observed with a dissecting microscope and scored as viable (pink or red embryo) or nonviable (white embryo). Percent viability was calculated by dividing the number of viable embryos by the total number of embryos scored.

Effect of asymbiotic media and photoperiod. Seed germination and subsequent protocorm development of *Vanda* hybrids were compared on three media (Table 2-2): Knudson C (KC), *PhytoTechnology* Orchid Seed Sowing Medium (P723) and half-strength Murashige & Skoog ($\frac{1}{2}\text{MS}$). All media were commercially prepared by *PhytoTechnology* Laboratories

(Shawnee Mission, KS). To standardize the concentration of activated charcoal (AC), agar, and sucrose in the screened media, the following modifications were made: 8 g L⁻¹ TC agar (*PhytoTechnology Laboratories*) and 1 g L⁻¹ AC were added to KC; 8 g L⁻¹ TC agar, 1 g L⁻¹ AC and 20 g L⁻¹ sucrose were added to ½MS. All media were adjusted to pH 5.7 with 0.1 M KOH before autoclaving at 121°C and 117.67 kPa. Medium was then dispensed as 30 mL aliquots into 9 cm diameter Petri plates.

Seeds were surface sterilized in a solution of ethanol:NaOCl (Clorox):sterile distilled deionized (DD) water (5:5:90) for two min followed by three rinses in sterile DD water. Surface sterilized seeds were placed back in sterile DD water, agitated with a vortex shaker to keep seeds suspended, and the volume of water adjusted until a 30 µL aliquot consistently yielded 25–35 seeds (average 30.9). Petri plates were then inoculated with three 30 µL aliquots of seed suspension from one of three *Vanda* hybrids and sealed with a single layer of NescoFilm (Karlan Research Products Corporation, Cottonwood, AZ). Each aliquot was treated as a subreplicate.

Petri plates containing one of three media and seeds from one of three *Vanda* hybrids were maintained at 23° ± 2°C under a 8/16 h, 12/12 h, or 16/8 h light/dark (L/D) photoperiod (3×3×3 factorial) provided by cool white fluorescent lights (General Electric F96T12, Fairfield, CT) at 50 µM m⁻² s⁻¹ for 12 weeks. Plates were observed every two weeks for signs of germination and subsequent protocorm development. Developmental stages (Table 3) were adapted from Stewart and Zettler (2002). Five replicates of each treatment were performed.

Percentage of seed/protocorms in each stage was calculated for each replicate by dividing the number of seeds/protocorms in each stage by the total number of seeds in each plate. Data on week 12 individual stage percentages was arcsine transformed to normalize data. Data was then analyzed using general linear modeling and least square mean separation ($\alpha = 0.05$). Time

course data was analyzed using general lineal modeling, standard error, and least square mean separation ($\alpha = 0.05$). Statistical analysis was completed with SAS v 9.1 (SAS Institute Inc., Cary, NC).

Scanning electron microscopy. S014 seeds and protocorms were used for examination by scanning electron microscopy (SEM) since other hybrids did not develop beyond Stage 3. All samples were fixed in 10% FAA under vacuum pressure for at least 24 h before dehydration in a graded ethanol series and subsequently critical point dried (CPD). Stage 1 protocorms were processed in methanol since samples dehydrated in ethanol tended to collapse during CPD. Samples were gold sputter coated for 30 s at 50 mA. Samples were then observed and digital images captured with a Hitachi S-4000 scanning electron microscope.

Light microscopy. Stage 4 and 5 seedlings from S014 were fixed in FAA as previously described before being embedded in paraffin wax and sectioned at 4 μm at the Molecular Pathology and Immunology Core Lab (University of Florida, Gainesville). Staining procedures were modified from Sakai (1973). Paraffin embedded longitudinal cross-sections were stained with 0.05% toluidine blue O for 15 min, rinsed with water, and air dried. Paraffin was then removed with two 60 sec rinses in Hemo-De (Scientific Safety Solvents; Keller, TX), a xylene substitute, before cover slips were mounted with Pro-Texx (American Scientific Products; McGaw Park, IL). Slides were observed with a Nikon Labophot-2 microscope. Digital images were captured with a Nikon Coolpix 990.

Results

Effects of asymbiotic media and photoperiod. After two weeks culture, seeds of all three *Vanda* hybrids had begun to germinate under all conditions tested (Fig. 2-1). Cumulative germination of S005 (34.1–43.5%) under all culture conditions of media and photoperiod was significantly lower than S013 (50.8–61.7%) and S014 (82.0–95.3%). S005 seeds cultured under

8/16 h L/D photoperiod initially germinated to a higher percentage than S005 seeds cultured under 12/12 h and 16/8 h L/D photoperiods, but cumulative differences in germination after 12 weeks were not significantly different between treatments (Fig. 2-1 A-C). Cumulative germination of S013 was similar under all culture conditions (Fig. 2-1 D-F). Cumulative germination of S014 on P723 under 8/16 h (95.5%) and 16/8 h (95.3%) L/D photoperiods was significantly higher than seeds cultured on KC and ½MS under all photoperiods (Fig. 2-1 G-H). Cumulative germination (93.7%) of S014 cultured on P723 under a 12/12 h L/D photoperiod was significantly greater than that of seeds cultured on ½MS under all photoperiods and seeds cultured on KC under 12/12 h and 16/8 h L/D photoperiods. In addition, maximum germination of S014 seeds cultured on P723 occurred by week eight, whereas seeds cultured on KC or ½MS did not reach maximum observed germination until 10–12 weeks of culture. TZ testing indicated that seeds were 53.5% (S005), 75.4% (S013) and 92.0% (S014) viable.

Embryos of S005 cultured on KC under 12/12 h or 16/8 h L/D photoperiods did not develop beyond Stage 1 (34.1% and 36.9%, respectively) in the 12 weeks they were observed (Fig. 2-2 A-C). Few Stage 2 protocorms (0.3%) developed under 8/16 h L/D photoperiod. S005 embryos developed to Stage 2 when cultured on ½MS (0.7–2.6%; Fig. 2-2 D-F) while seeds cultured on P723 (Fig. 2-2 G-I) developed to Stage 3 (0.7–2.7%) under all photoperiods screened, and Stage 5 under 12/12 h (0.2%) and 16/8 h (0.2%) L/D photoperiods. Stage 4 protocorms were not observed.

S013 embryos did not develop beyond Stage 2 when cultured on KC (0.4–1.8%; Fig. 2-3 A-C) and ½MS (6.1–8.1%; Fig. 2-3 D-F). However, seeds cultured on P723 developed significantly more Stage 2 protocorms (36.8–38.2%) under all photoperiods. For S013 seeds cultured on P723 (Fig. 2-2 G-I), Stage 1 protocorms were most abundant at week two (34.1–

45.1%). However, as Stage 1 protocorms continued to develop to more advanced stages, the percentage of Stage 1 protocorms decreased to 12.8% (8/16 h L/D), 15.2% (12/12 h L/D), and 16.1% (16/8 h L/D) by week 12. Stage 3 protocorms of hybrid S013 were observed on P723 under 8/16 h L/D (0.8%) photoperiod and were not significantly different than 0% when cultured on P723 under 16/8 h L/D (0.3%) photoperiod.

When cultured on KC (Fig. 2-4 A-C) and $\frac{1}{2}$ MS (Fig. 2-4 D-F), seeds of S014 did not develop beyond Stage 2 regardless of photoperiod. However, seeds cultured on P723 developed to Stage 5 (Fig. 2-4 G-I) under all photoperiods tested. Stage 1 protocorms (Fig. 2-5 B) were most abundant after two weeks culture on P723 under all photoperiods (8/16 h L/D, 51.5%; 12/12 h L/D, 54.2%; 16/8 h L/D, 44.7%). A majority of seeds cultured on P723 had developed to Stage 2 protocorms (Fig. 2-5 C) by week four with the highest percentage of Stage 2 seedlings observed during week six (8/16 h L/D, 60.7%; 12/12 h L/D, 61.9%; 16/8 h L/D, 68.2%). By week 10, S014 seeds had developed to Stages 3–5 (Fig. 2-5 D-F) on P723 under all three photoperiods tested. Final percentages of Stage 3 protocorms were not significantly different for seeds cultured on P723 under different photoperiods (8/16 h L/D, 13.1%; 12/12 h L/D, 14.2%; 16/8 h L/D, 14.2%). Significantly more Stage 4 protocorms were observed on P723 treatments under 12/12 h (5.0%) and 16/8 h (4.8%) than under 8/16 h L/D (0.3%) photoperiod. Significantly more Stage 5 seedlings were observed on P723 replicates cultured under 16/8 h L/D (7.9%) than those cultured under 8/16 h (2.7%) and 12/12 h L/D photoperiod. Visible contamination rate over all treatments was 8.9%.

Developmental sequence. Unimbibed seeds of the three *Vanda* hybrids (Fig. 2-5 A) used in this study differed greatly in size (approximate lengths: S005 – 132–142 μm ; S013 – 172–190 μm ; S014 – 190–265 μm). Some embryos of all hybrids imbibed to the point of testa rupture by

week two (Fig. 2-1; Fig. 2-5 B). Stage 1 protocorms were polarized with the suspensor end of the embryo comprised of much smaller cells than those in the apical region. By week four, some protocorms had developed to Stage 2 (Fig. 2-5 C) with slightly elongated apical regions defining the protomeristem. Rhizoids were occasionally present on Stage 2 protocorms. With further development, the protomeristem developed an angular opening from which the first true leaf emerged (Stage 3, Fig. 2-5 D). At least one true root typically emerged from protocorms at this time (Stage 4, Fig. 2-5 E) and prior to the emergence and elongation of a second true leaf (Fig. 2-5 F). Thick sections of Stage 5 seedlings revealed a collar through which leaves emerged (Fig. 2-6 A), which constituted a large portion of the protocorm body. The apical meristem (Fig. 2-6 B) was spade-shaped and located near the center of the protocorm. Lateral meristems (Fig. 2-6 C) were found along leaf axes in Stage 4 protocorms and Stage 5 seedlings.

Observations on subculture of *Vanda* hybrids. Three month old S014 seedlings were transferred to newly prepared P723 in sterile *PhytoTech* Culture Boxes (*PhytoTechnology* Laboratories; product # P700) and sealed with a single layer of NescoFilm. Approximately three months after transfer, a proportion of seedlings began to exhibit symptoms of decline (loss of chlorophyll, tissue translucency, cessation of growth; Fig. 2-7 A). Believing this to be the result of media depletion or ethylene accumulation, six month old seedlings that did not exhibit symptoms were transferred to fresh *PhytoTech* Culture Boxes containing P723. These vessels were not sealed with NescoFilm. Seedlings again displayed symptoms of decline following the second transfer. Culture indexing with liquid Leifert and Waites Sterility Test Medium (Leifert et al., 1989) indicated that tested seedlings did not harbor pathogens. Twelve months after seeds were sown, nearly all seedlings lost pigmentation, became transparent, and were considered

dead. A small number of seedlings formed callus (Fig. 2-7 B), some of which regenerated shoots and/or somatic embryos (Fig. 2-7 C).

Discussion

Few studies on germination and seedling production of *Vanda* species and hybrids have been conducted (Bhaskar and Rajeevan, 1996; Roy and Banerjee, 2002). Anatomical studies of *Vanda* seeds and protocorms have focused on embryo development (Swamy, 1942) and early protocorm ultrastructure (Ricardo and Alvarez, 1971). Ricardo and Alvarez (1971) included a diagram of a *Vanda* protocorm with emerging leaves, however this figure did not depict the presence of an apical dome or protocorm collar as illustrated herein.

This report constitutes the first comparison of germination and seedling development of *Vanda* hybrids under several different asymbiotic culture conditions. In this study, S014 seeds cultured on KC exhibited significantly greater percentage germination compared to S014 seeds cultured on $\frac{1}{2}$ MS. These results differ from those reported by Bhaskar and Rajeevan (1996) who reported that the hybrid *Vanda* John Club, germinated better on $\frac{1}{2}$ MS (80%) than on KC (20%). In addition, germination of *Vanda tessellata* seeds cultured on various modified formulations of $\frac{1}{2}$ MS and KC did not differ in final percent germination (Roy and Banerjee, 2002). Similar to hybrids S005 and S013, *Vanda tessellata* grew slowly and produced few advanced stage seedlings, even after three months culture. TZ based estimates of viability for hybrids S005 (53.5%) and S013 (75.4%) were considerably greater than observed maximum germination (43.5% and 61.7%, respectively). Viability estimates for hybrid S014 (92.0%) were comparable to observed germination on P723 (93.7–95.3%). It is possible that the sterilization protocol used in this study impaired the germination of hybrids S005 and S013 by damaging the embryos, however this hypothesis does not account for the concurrence between observed germination and estimated viability of hybrid S014. However, orchid seed viability estimates do not appear to be

good indicators for germinability (Lauzer et al., 1994; Shoushtari et al., 1994; Vujanovic et al., 2000). Therefore, germinability should be tested as well as viability.

Protocorm development of *Vanda* hybrid seeds cultured on $\frac{1}{2}$ MS was consistently more advanced than that of seeds cultured on KC. This may be due to greater total nitrogen or a higher $\text{NO}_3:\text{NH}_4$ ratio in $\frac{1}{2}$ MS compared to KC. Curtis and Spoerl (1948) reported that *Vanda tricolor* seeds cultured on media containing NO_3 developed more rapidly than seeds cultured in the presence of NH_4 when nitrogen concentrations were kept constant. This is in contrast to reports of the nitrogen preference of *Calopogon tuberosus*, *Cattleya*, and *Cymbidium*. These orchids were better able to utilize NH_4 than NO_3 during germination and early growth (Curtis and Spoerl, 1948; Kauth et al., 2006; Raghavan and Torrey, 1964). In these species the ability to utilize nitrate may be delayed until nitrate reductase is synthesized (approximately 60 days after imbibition for *Cattleya*; Raghavan and Torrey, 1964). Nitrate reductase in *Vanda* may be synthesized or activated sooner after imbibition than other studied species.

Protocorm development of all three *Vanda* hybrids was significantly greater on P723. This medium has also been shown to support high germination percentage and development of *C. tuberosus* (Kauth et al., 2006). P723 contains less total ammonium and nitrogen than either $\frac{1}{2}$ MS or KC, but contains additional organic nitrogen in the form of peptone. Peptone has also been shown to promote germination and development of *V. tricolor*, *Paphiopedilum* species, *Prosthecia cochleata*, and *Spathoglottis plicata* (Curtis, 1947).

Clear trends in the effect of photoperiod were not observed for hybrids S005 and S013, while advanced development of S014 was slightly greater for seeds cultured on P723 under longer photoperiods. S014 seeds cultured on P723 developed significantly more Stage 4 protocorms under 12/12 h and 16/8 h L/D photoperiods, as well as more Stage 5 seedlings under

a 16/8 h L/D photoperiod. Preliminary data indicated that *Vanda* hybrid seeds cultured in dark (0/24 h L/D) germinated poorly and embryos did not develop as rapidly as those cultured in lighted conditions. Although further study is needed, this may indicate that culturing seeds under longer photoperiods results in more rapid protocorm development.

It is unclear why subcultured S014 seedlings lost pigmentation and died following transfer to fresh media. Since *PhytoTech* culture boxes seal tightly, a possible cause of seedling decline is accumulation of ethylene in the head space. If accumulation of gaseous respiratory byproducts is the problem, *in vitro* culture of *Vanda* may be improved by aerating culture vessels (Lai et al., 1998), using photoautotrophic culture (Nguyen et al., 2001; Xiao et al., 2003), or incorporating ethylene inhibitors in the medium (Brar et al., 1999). Another possible explanation for seedling death is that transferred seedlings were not able to adjust to the sudden cultural change from depleted media to fresh media with relatively higher mineral salt concentrations. If the observed seedling death is linked to subculture, seedlings may benefit from being sown in large culture vessels containing enough media to support long-term growth. Alternatively, media that support germination and early development may not meet the nutritional requirements of older seedlings.

Callus formation from germinating seeds has been documented with *V. tessellata* (= *Vanda roxburghii*; Bose and Mukerjee, 1974) and *Vanda coerulea* (Devi et al., 1998). Screening for genotypes that produce callus was not an objective of this study. However, callus-forming genotypes may be useful for studying both the factors that induce callus formation of *Vanda* and the factors that promote shoot organogenesis, embryogenesis, and conversion.

When studying the growth and development of a genus as large and interbred as *Vanda*, comparing several genotypes, species, or hybrids can be valuable. Had hybrid S014 been excluded in this study, the mistaken conclusion could have been drawn that the media and/or

photoperiods tested in this study were inadequate for supporting advanced growth of *Vanda* hybrids since hybrids S005 and S013 did not develop to advanced stages under most conditions tested. However, since S014 seeds germinated rapidly and developed to advanced stages, it can not be ruled out that S005 and S013 have low seed vigor or that protocorms of these two hybrids exhibit symptoms of inbreeding depression. Such problems may contribute to slow growth and a long time to flowering of certain *Vanda*. This may be the result of *Vanda* breeders' tendency to breed for improved flower form, size, and color (features that are recognized and awarded by orchid societies) while disregarding the importance of rapid growth and development in a commercial setting. The current breeding trends, which cater to a hobbyist niche market, could be limiting the potential success of *Vanda* hybrids in the expanding orchid market. In addition, commonly employed breeding practices such as sibling mating and backcrossing (two techniques used to produce plants that are homozygous for recessive flower alleles) may lead to the buildup of deleterious alleles. Such practices could contribute to poor seedling germination and vigor.

Table 2-1. Parentage of *Vanda* hybrids used for experimentation (seed parent × pollen parent).
 Parentheses are used when a parent is itself an unnamed hybrid.

ID	Hybrid
S005	<i>Vanda</i> Paki × (<i>Vanda tessellata</i> × <i>Vanda cristata</i>)
S013	(<i>Vanda</i> Joan Warne × <i>Vanda</i> Paki) × <i>Vanda</i> Loke
S014	<i>Vanda</i> Motes Primrose × <i>Ascocenda</i> Tavivat

Table 2-2. Composition of asymbiotic media used to test the effects of photoperiod on germination and development of *Vanda* hybrids S005, S013, and S014. S005 – *Vanda* Paki × (*Vanda tessellata* × *Vanda cristata*), S013 – (*Vanda* Joan Warne × *V. Paki*) × *Vanda* Loke, S014 – *Vanda* Motes Primrose × *Ascocenda* Tavivat, KC – Knudson C, ½MS – half-strength Murashige & Skoog, P723-*Phyto*Technology Orchid Seed Sowing Media.

	KC	P723	½MS
<i>Macronutrients</i> (mM)			
Ammonium	13.8	5.15	10.31
Calcium	2.12	0.75	1.5
Chlorine	3.35	1.5	1.5
Magnesium	1.01	0.62	0.75
Nitrate	10.49	9.85	19.7
Potassium	5.19	5.01	10.02
Phosphate	1.84	0.31	0.63
Sulfate	4.91	0.71	0.86
Sodium	.	0.10	1.51
<i>Micronutrients</i> (µM)			
Boron	.	30	50
Cobalt	.	0.03	0.11
Copper	.	0.03	0.10
Iron	90	50	50
Iodine	.	1.20	2.50
Manganese	30	30	37.9
Molybdenum	.	26	0.52
Zinc	.	9.2	30
<i>Vitamins</i> (mg L ⁻¹)			
Myo-Inositol	.	100	.
Nicotinic acid	.	1	.
Peptone	.	2000	.
Pyridoxine	.	1	.
Thiamine	.	10	.
Total N (mM)	24.31	unknown	30.01
NH ₄ :NO ₃	1.32	0.52	0.52

Table 2-3. Developmental stages of *Vanda* hybrids.

Stage	Description
0	Ungerminated seed with embryo
1	Enlarged embryo, testa ruptured (= germination)
2	Appearance of protomeristem and/or rhizoids
3	Emergence and elongation of first leaf
4	One leaf and one or more roots present
5	Presence of two or more leaves, roots present (= seedling)

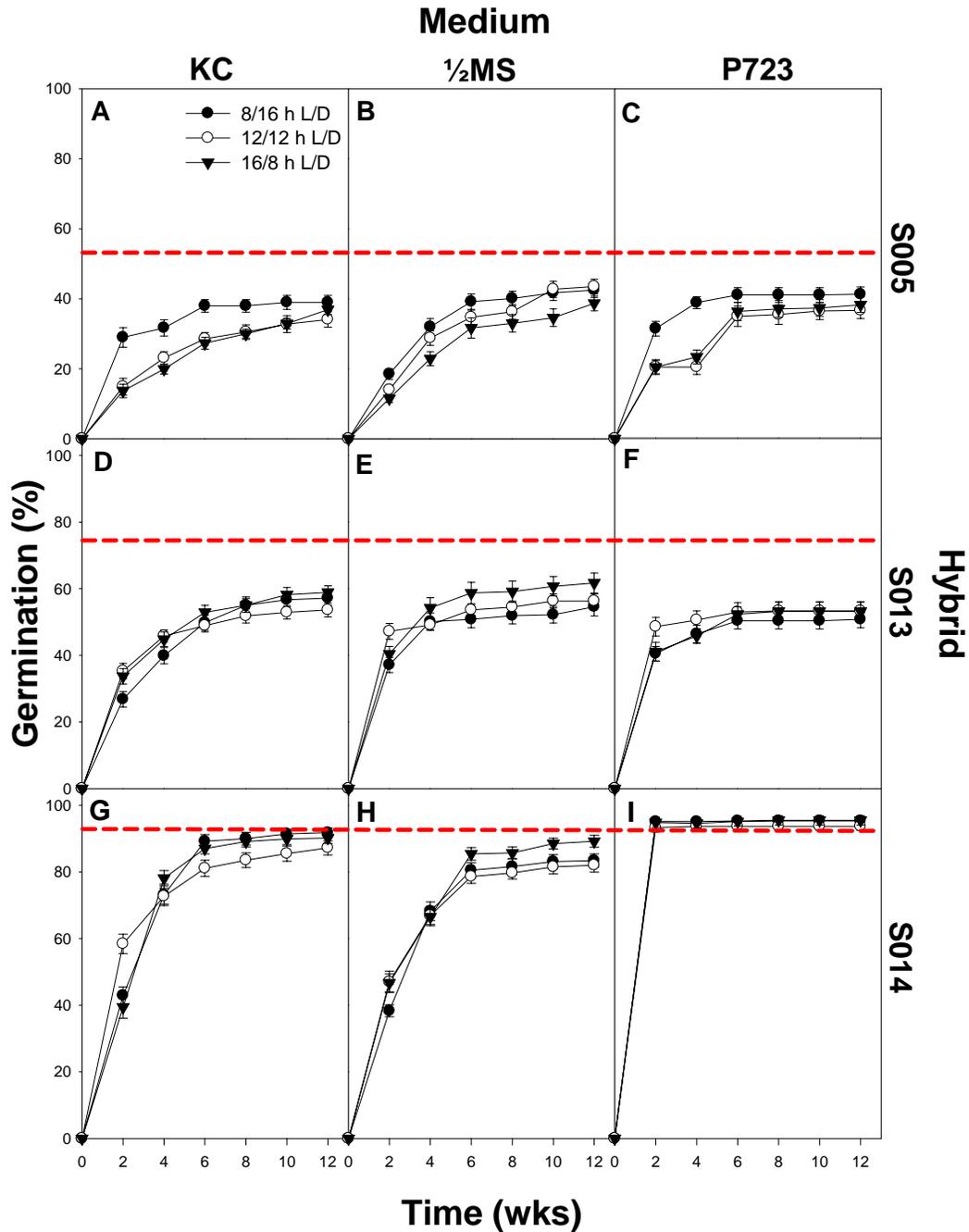


Figure 2-1. Germination of *Vanda* hybrids cultured on three different media under an 8/16 h, 12/12 h, or 16/8 h light/dark photoperiod. Red lines represent estimated viability based on tetrazolium staining. A) *Vanda* Paki × (*Vanda tessellata* × *Vanda cristata*) (S005) cultured on Knudson C Medium (KC). B) S005 cultured on half-strength Murashige and Skoog Medium (½MS). C) S005 cultured on *PhytoTechnology* Orchid Seed Sowing Medium (P723). D) (*Vanda* Joan Warne × *V. Paki*) × *Vanda* Loke (S013) cultured on KC. E) S013 cultured on ½MS. F) S013 cultured on P723. G) *Vanda* Motes Primrose × *Ascocenda* Tavivat (S014) cultured on KC. H) S014 cultured on ½MS. I) S014 cultured on P723.

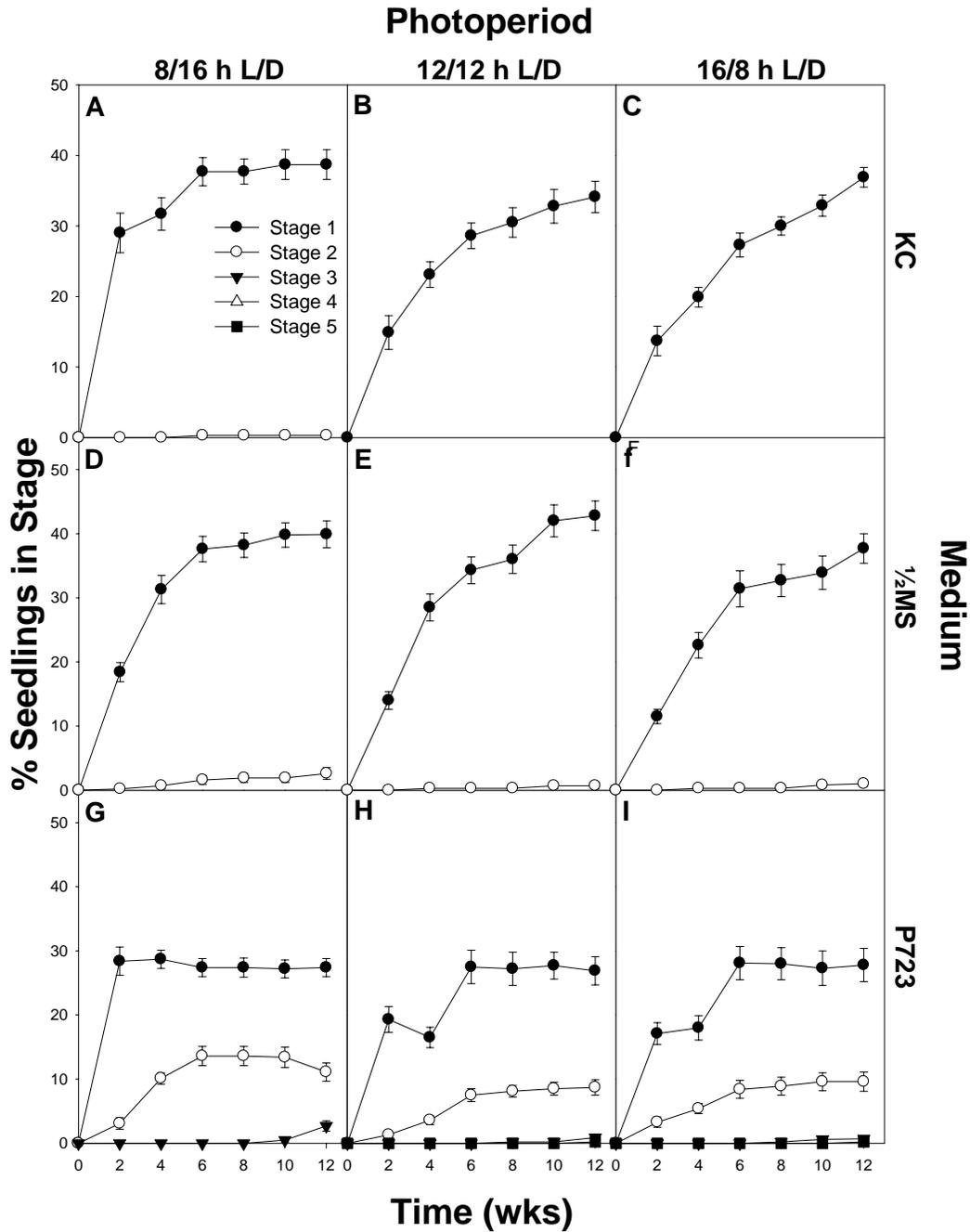


Figure 2-2. Protocorm development (see Table 2-3 for stage definitions) of *Vanda Paki* × (*Vanda tessellata* × *Vanda cristata*) (S005), cultured for 12 weeks on Knudson C Medium (KC), half-strength Murashige & Skoog Medium ($\frac{1}{2}$ MS), or *PhytoTechnology* Orchid Seed Sowing Medium (P723) under an 8/16 h, 12/12 h, or 16/8 h light/dark (L/D) photoperiod. A) KC, 8/16 h L/D. B) KC, 12/12 h L/D. C) KC, 16/8 h L/D. D) $\frac{1}{2}$ MS, 8/16 h L/D. E) $\frac{1}{2}$ MS, 12/12 h L/D. F) $\frac{1}{2}$ MS, 16/8 h L/D. G) P723, 8/16 L/D. H) P723, 12/12 h L/D. I) P723, 16/8 h L/D.

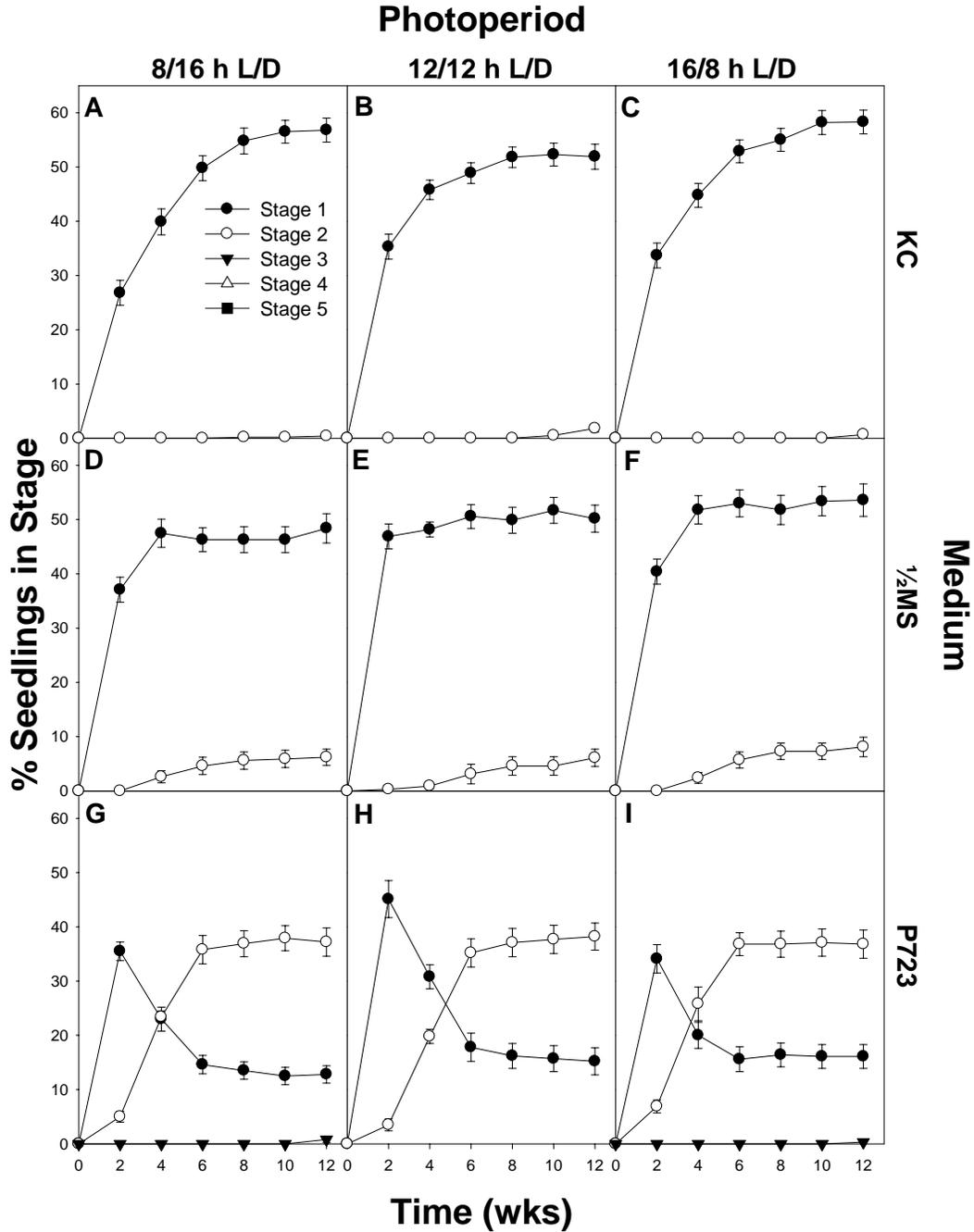


Figure 2-3. Protocorm development (see Table 2-3 for stage definitions) of (*Vanda* Joan Warne × *Vanda* Paki) × *Vanda* Loke (S013), cultured for 12 weeks on Knudson C Medium (KC), half-strength Murashige & Skoog Medium (1/2MS), or *Phyto*Technology Orchid Seed Sowing Medium (P723) under an 8/16 h, 12/12 h, or 16/8 h light/dark (L/D) photoperiod. A) KC, 8/16 h L/D. B) KC, 12/12 h L/D. C) KC, 16/8 h L/D. D) 1/2MS, 8/16 h L/D. E) 1/2MS, 12/12 h L/D. F) 1/2MS, 16/8 h L/D. G) P723, 8/16 L/D. H) P723, 12/12 h L/D. I) P723, 16/8 h L/D.

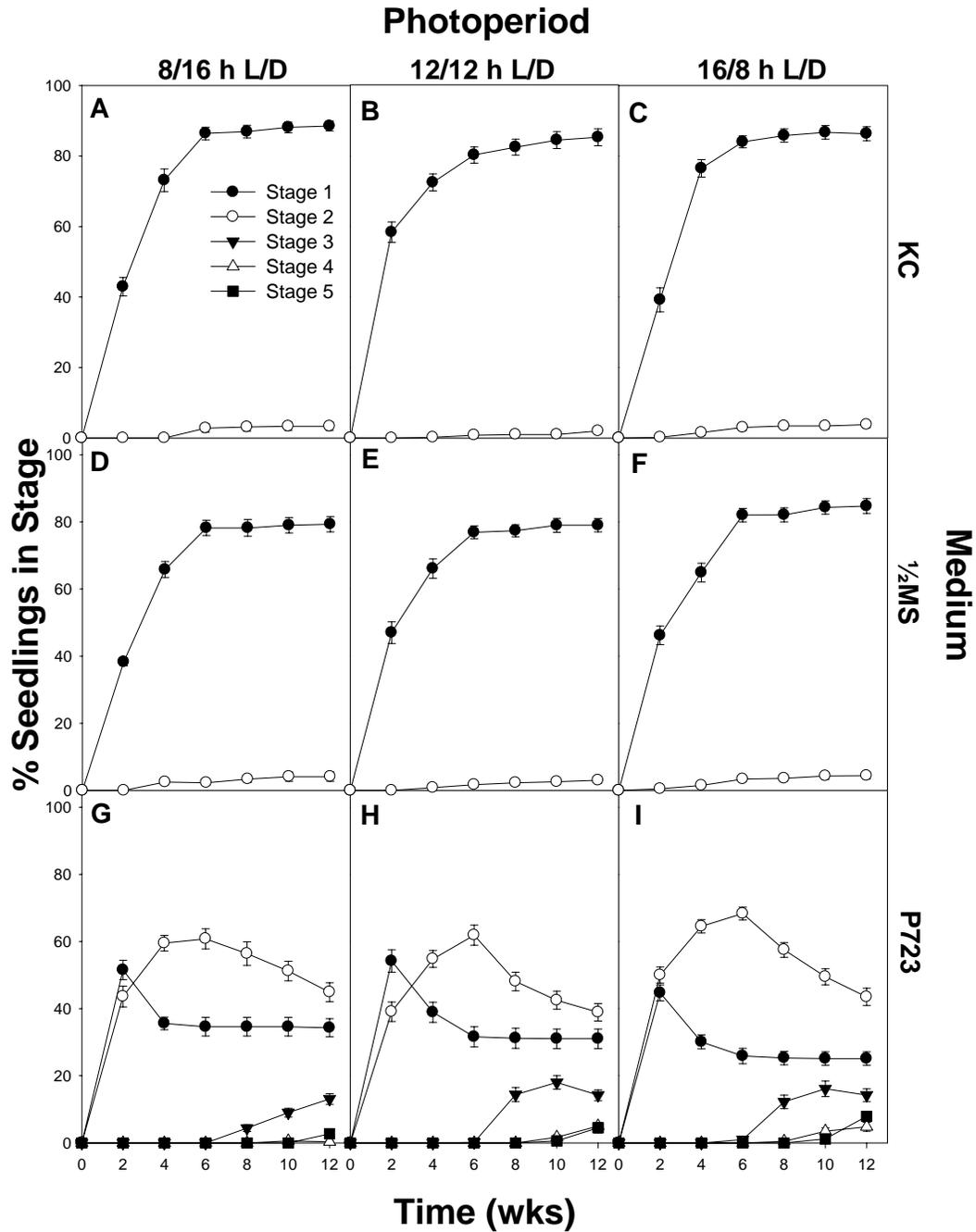


Figure 2-4. Protocorm development (see Table 2-3 for stage definitions) of *Vanda Motes Primrose* × *Ascocenda Tavivat* (S014), cultured for 12 weeks on Knudson C Medium (KC), half-strength Murashige & Skoog Medium ($\frac{1}{2}$ MS), or *PhytoTechnology* Orchid Seed Sowing Medium (P723) under an 8/16 h, 12/12 h, or 16/8 h light/dark (L/D) photoperiod. A) KC, 8/16 h L/D. B) KC, 12/12 h L/D. C) KC, 16/8 h L/D. D) $\frac{1}{2}$ MS, 8/16 h L/D. E) $\frac{1}{2}$ MS, 12/12 h L/D. F) $\frac{1}{2}$ MS, 16/8 h L/D. G) P723, 8/16 L/D. H) P723, 12/12 h L/D. I) P723, 16/8 h L/D.

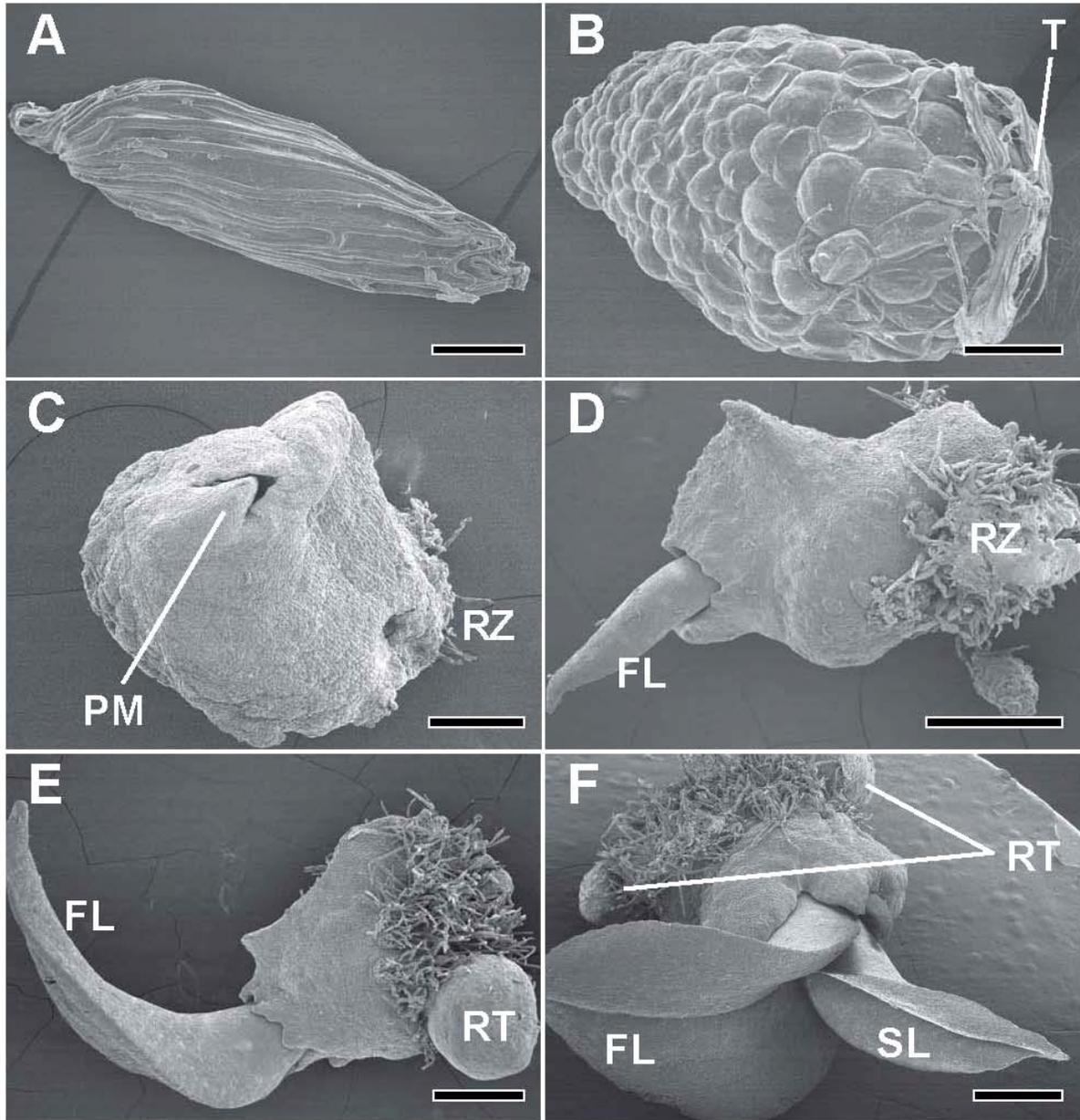


Figure 2-5. Scanning electron micrographs of the germination and early development of hybrid S014 (*Vanda Motes Primrose* × *Ascocenda Tavivat*) (see Table 2-3 for stage definitions). A) Stage 0 seed. Scale bar = 50 μ m. B) Stage 1 protocorm with ruptured testa (T). Scale bar = 100 μ m. C) Stage 2 protocorm with developing protomeristem (PM) and rhizoids (RZ). Scale bar = 0.5 mm. D) Stage 3 protocorm with emerging first leaf (FL). Scale bar = 1.0 mm. E) Stage 4 protocorm with emerging root (RT). Scale bar = 1.0mm. F) Stage 5 seedling with elongating second leaf (SL). Scale bar = 1.0 mm.

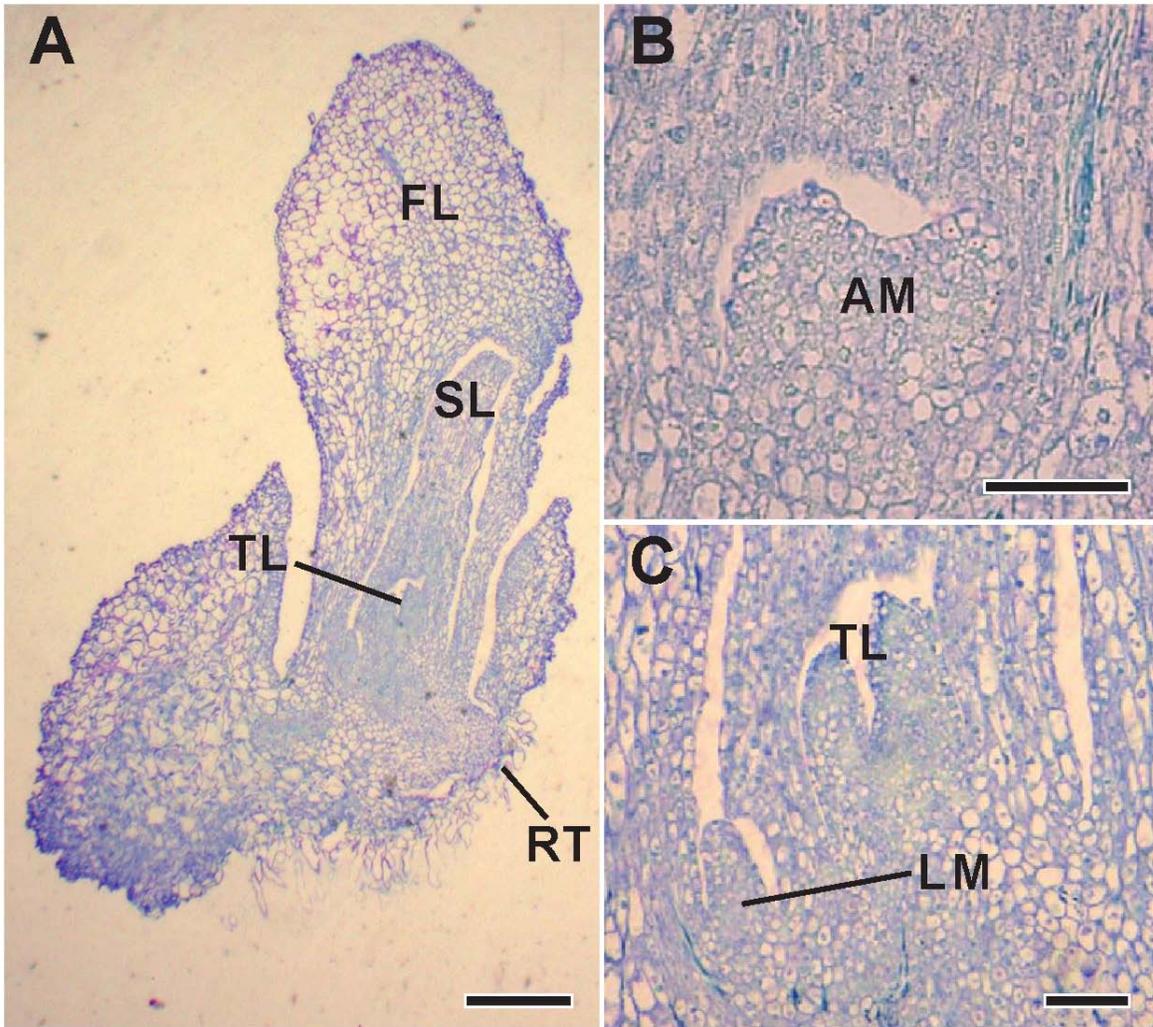


Figure 2-6. Thin section light micrographs of hybrid S014 (*Vanda Motes Primrose* × *Ascocenda Tavivat*). A) 4 μm thin section of Stage 5 seedling with first leaf (FL), second leaf (SL), third leaf (TL), and root (RT) present. Scale bar = 0.5 mm. B) High magnification image of a seedling apical meristem (AM). Scale bar = 200 μm. C) Thin section through the apical dome of a seedling containing a lateral meristem (LM). Scale bar = 100 μm.

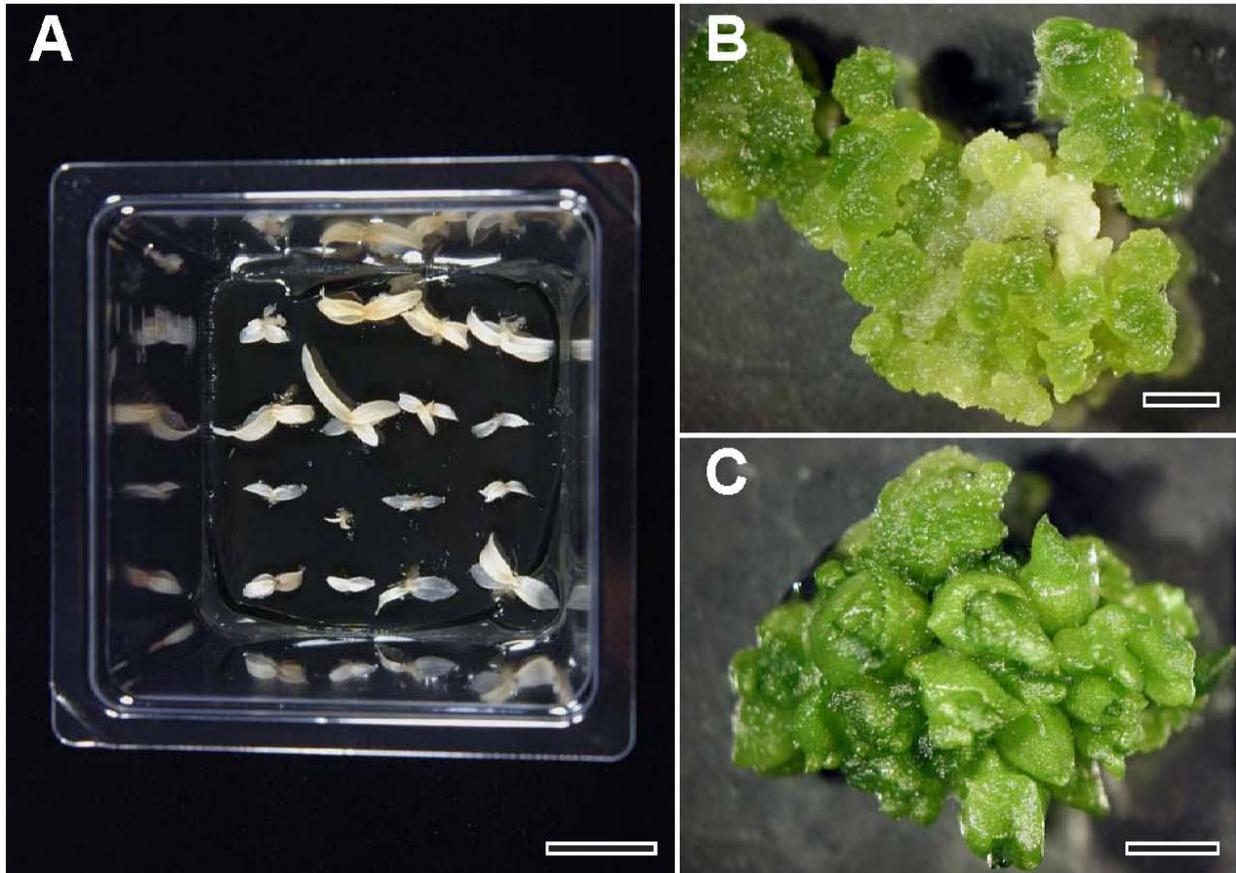


Figure 2-7. Twelve month old cultures of hybrid S014 (*Vanda Motes Primrose* × *Ascocenda Tavivat*) cultured on *PhytoTechnology* Orchid Seed Sowing Medium (P723). A) Typical culture of dead seedlings that have lost pigmentation and become transparent. Scale bar = 2 cm. B) Mass of tissue with globular masses, which appear to be protocorm like bodies. Scale bar = 2.5 mm. C) Embryogenic and/or organogenic callus with differentiating shoots. Scale bar = 2.5 mm.

APPENDIX
ATTEMPTS TO INITIATE *Vanda* TISSUE CULTURES

Introduction

Propagation of orchids from seed can be an efficient method of commercial production. However, the quality of individual seeds resulting from any single breeding event can be highly variable in both growth rates and flower characteristics due to recombination. It is only through clonal propagation that the unique genetics of an awarded specimen can be retained. Clones of unique and desirable plants are often in demand, but developing a salable crop of plants through traditional methods of plant division is a slow process. In such cases, growers and producers may be able to employ micropropagation techniques in order to rapidly and reliably produce plants of identical or nearly identical quality.

As discussed in Chapter 1, micropropagation of flowering size *Vanda* species and hybrids has been documented by many different researchers (Cheah and Sagawa, 1978; Decruse et al., 2003; Ghani et al., 1992; Goh and Wong, 1990; Intuwong and Sagawa, 1973; Kanika and Vij, 2004; Kunisaki et al., 1972; Lakshmanan et al., 1995; Lay, 1979; Le et al., 1999; Malabadi et al., 2004; Seeni and Latha, 1992; Sharma and Vij, 1997; Teo et al., 1973; Valmayor et al., 1986). Taken as a whole, the body of literature on *Vanda* micropropagation indicates that there are many suitable explants for initiating cultures, but the effectiveness of any one protocol appears to be species, hybrid, or genotype specific.

During the course of this research, several attempts were made to initiate cultures of various *Vanda* hybrids from both flowering size plants and seedlings. Attempts to initiate *Vanda* cultures from flowering size plants and seedling explants were conducted, but not in the context of a scientific experiment. An experiment on the effect of various plant growth regulators (PGRs) on PLB formation from seedling leaf explants was conducted and is also included in this

appendix. Attempts to establish cultures were largely unsuccessful, although a single culture of (*Vanda* Motes Honeybun × *Vanda* Rasri Gold) × *Vanda* Rasri Gold was established (Fig. A-1). The purpose of this appendix is to provide information on the initiation protocols that were attempted so as to provide information to future researchers.

Initiation of *Vanda* Tissue Culture Lines Using Newly Emerged Leaves of Flowering Size Plants

Explants from newly emerged leaves of *Vanda cristata* less than 3 cm in length cultured on modified MM regenerated PLBs when cultured in the presence of high concentrations of BA and NAA combined (44.4 µM and 26.9 µM, respectively), and BA and IAA combined (44.4 µM and 28.6 µM, respectively; Sharma and Vij, 1997). When culturing monopodial orchids such as *Vanda*, initiating tissue cultures from leaf explants is an attractive option since harvesting shoot-tips may result in plant death (Goh and Wong, 1990; Sharma and Vij, 1997; Vij et al. 1986) and can be difficult to sterilize (personal observation).

Materials and Methods

Plant material. Newly emerged leaves less than 4 cm in length were removed from flowering sized plants of *Vanda tessellata*, *Vanda* Arjuna, and *Ascocenda* Motes Burning Sands. Leaves were washed in running tap water for 15 min, rinsed in 70% ethanol for 30 s and surface sterilized in a solution of 1.2% NaOCl (v:v; 1:5; Clorox bleach: sterile distilled deionized [DD] water) for 12 min, then rinsed three times in sterile DD water for 60 s per rinse. Each surface sterilized leaf was cut in half along the midvein and cut into two or three sections, each approximately 1 cm². Each leaf half was treated as an explant and inoculated into a single Petri plate containing one of five media.

Culture media and culture conditions. Explants were cultured on one of five solid media: Knudson C Medium (KC) with 15% (v/v) coconut water (CW), Vacin and Went medium

(VW) with 15% CW, Mitra et al. medium (MM) with 15% CW, MM containing 22.5 μM 6-benzyladenine (BA) and 13.5 μM α -naphthaleneacetic acid (NAA), and MM containing 45 μM BA and 27 μM NAA. Basal media were adjusted to pH 5.7 with 0.1 M NaOH before autoclaving for 40 minutes at 121°C and 117.67 kPa. PGRs were filter sterilized and added after media was autoclaved and cooled. Autoclaved media were dispensed as 30 mL aliquots into 9 cm diameter Petri plates and allowed to harden. After inoculation, plates were sealed with a single layer of NescoFilm and cultured at $23^\circ \pm 2^\circ\text{C}$ under a 16/8 h light/dark (L/D) photoperiod provided by cool white fluorescent lights at $40 \mu\text{M m}^{-2} \text{s}^{-1}$.

Results and Discussion

Leaf explants from the three *Vanda* hybrids screened did not respond to any of the media tested. Contamination rates were high: approximately 75% of *Ascocenda* Motes Burning Sands cultures, 60% of *Vanda* Arjuna cultures and 10% of *Vanda tessellata* leaf explants were visibly contaminated. This may indicate that the surface sterilization procedure employed was not adequate. The fungal and/or bacterial load on *Vanda* explants may need to be reduced prior to explant harvest. Sharma and Vij (1997) soaked leaves of wild collected *Vanda cristata* in 0.1% streptomycin for 20 min followed by 15 min in 0.1% HgCl_2 , but did not report contamination rates. In addition, stage 1 contamination may be reduced by selecting pathogen-free donor plants (Leifert and Cassells, 2001), selecting plants with lower bacterial/fungal loads, or by treating donor plants with antibiotics or fungicides prior to explant harvest (Nagy et al., 2005).

Within two months of culture initiation, all explants from the three hybrids screened turned black and were presumed dead. The screened culture media or culture conditions may not have been conducive to regeneration. Explants from newly emerged leaves of *V. cristata* cultured on modified MM regenerated PLBs when cultured in the presence of high concentrations of BA (44.4 μM), NAA (26.9 μM) and a combination of BA and indole-3-acetic acid (44.4 μM and

28.6 μM , respectively; Sharma and Vij, 1997). Leaves from adult *Renanthera imshootiana* plants also required a relatively high concentration of BA combined with NAA (44.4 μM and 4.1 μM , respectively) for maximum shoot regeneration (80% response; 38 shoots/explant; Seeni and Latha, 1992). It is unknown whether similar protocols can be used for *Vanda* hybrids.

Initiation of *Vanda* Tissue Culture Lines Using Seedlings, Seedling Leaves, and Cut Protocorms in Liquid Culture

The establishment of orchid tissue cultures from seedling explants is well documented (see Chen and Chang, 2006; Hoppe and Hoppe, 1988; Kuo et al., 2005; Lu, 2004; Park et al., 2002; Sheelavantmath et al., 2000; Vaz et al., 2004; Yan, 2005). Sterile seedlings of many orchid genera can be easily produced using asymbiotic germination. Establishing cultures this way may bypass difficulties associated with sterilizing seasoned plant material. In addition, young plant material has been shown more responsive than more mature explants in many non-orchid species (Becerra et al, 2004; Hoque and Mansfield, 2004; Molina et al., 2002). However, the genotype and plant quality of cultures initiated from seedlings is unknown. The objective of this protocol was to assess the regenerative potential of various explants from hybrid *Vanda* seedlings.

Materials and Methods

Plant material. Four month old seedlings of *Vanda tessellata* \times *Vanda* Arjuna served as explants. Seeds were sterilized as described in Chapter 2 and sown onto *Phyto*Technology Orchid Seed Sowing Medium (P723). Seeds were first cultured under 8/16 h, 12/12 h, or 16/8 h L/D photoperiod for four weeks, then transferred to a common growth chamber under a 16/8 L/D hour photoperiod for six months. Four different explants were used in this protocol: intact protocorms, bisected protocorm halves, excised intact leaves, and bisected leaf halves.

Culture media and culture conditions. Two different media were used for this investigation: KC with 15% CW (v/v) and Orchid Multiplication Medium (*Phyto*Technology cat.

No. P793). Media were adjusted to pH 5.7, dispensed as five mL aliquots into 25 mL screw cap test tubes, and sterilized at 121°C and 117.67 kPa for 10 min. Test tubes containing sterile media were capped and sealed with NescoFilm. Test tubes were placed in a roller drum (Bellco Glass, Inc., Vineland, NJ) at 10 rpm under 16/8 h L/D photoperiod. Illumination was provided by cool florescent lights at a maximum of 58 $\mu\text{M m}^{-2}\text{s}^{-1}$.

Protocol Design. All replicates contained three subreplicates. Seven replicates of all explant \times media combinations were completed. Explants were observed for signs of regeneration after four weeks culture.

Results and Discussion

A few leaf explants and bisected protocorms cultured in KC with 15% CW showed signs of regeneration. Responding cultures were transferred to 250 mL Erlenmeyer flasks containing 50 mL of sterile KC with 15% CW (v/v) and sealed with aluminum foil and NescoFilm. Cultures were placed on a G10 Gyrotory Shaker (New Brunswick Scientific, Edison, NJ) and agitated at 60 rpm. Regenerating leaf explants became necrotic under these conditions and many were discarded. Three responding bisected protocorm explants continued to grow and produced between two and five shoots after approximately four weeks culture. Cultures began to show signs of necrosis after approximately four months culture and several transfers to fresh liquid medium. Explants were eventually transferred to KC with 15% CW solidified with 0.8% TC[®] agar. One culture responded positively to solid media (Fig. A-1 A), while two cultures became necrotic. The proliferating culture produced numerous shoots and was subsequently subcultured (Fig. A-1 B).

Liquid culture has been shown to be an efficient method of propagating agronomic crops such as potato (Akita and Ohta, 1998; Jiménez et al., 1999; Piao et al., 2003), and various ornamental crops (Adelberg and Toler, 2004; Paek et al., 2001; Prasad and Gupta, 2006)

including *Phalaenopsis* (Young et al., 2000). Such a method may also be effective in multiplication of *Vanda* cultures.

Initiation of *Vanda* Tissue Culture Lines Using Seedling Leaf Sections

Leaf explants have several advantages over shoot tips as donor material for tissue culture. Leaves are easier to excise from plants than shoot tips (especially when working with seedlings), and excision of leaves is less traumatic to donor plants. Finally, the surface area of a leaf offers the potential for very efficient and rapid rates of multiplication given the probable single cell origin of PLBs, provided that a suitable protocol can be developed. Leaf explants from terrestrial orchid species and several epiphytic genera including *Vanda* have been used to establish *in vitro* cultures (Chen et al., 1999; 2003; Chung et al., 2005; Churchill et al., 1973; Kuo et al., 2005; Park et al., 2002; Seeni and Latha, 2000; Yam and Weatherhead, 1991).

Much tissue culture research has been done with *Phalaenopsis*, as it is the most important genus of orchids commercially available (Griesbach, 2003). Researchers have demonstrated that thidiazuron, in concentrations ranging from 3–9 μM , is an effective PGR for inducing PLBs from *Phalaenopsis* seedling leaf explants (Chen and Chang, 2006), *Doritaenopsis* seedling leaf explants (Park et al, 2002), and 18 month old *Phalaenopsis* leaf explants (Kuo et al, 2005). The objective of this experiment was to test the effects of cytokinin type and concentration on PLB production from *Vanda* hybrid leaf explants.

Materials and Methods

Plant material. Seeds of (*Vanda* Motes Honeybun \times *Vanda* Rasri Gold) \times *Vanda* Rasri Gold were surface sterilized and cultured on P723 at $23^\circ \pm 2^\circ\text{C}$ under a 16/8 h L/D photoperiod provided by cool white fluorescent lights at $40 \mu\text{M m}^{-2} \text{s}^{-1}$. Leaves at least 0.5 cm in length were harvested from sterile four month old seedlings. For each replicate, one whole leaf was cut in

half, separating the base and tip, and both sections were inoculated into a single culture vessel containing one of 13 cytokinin treatments.

Culture media and culture conditions. P723 was used as the basal medium in this experiment. Medium was modified with the addition of 1, 10, or 50 μM of BA, 6- γ,γ dimethylallylamino purine, kinetin, or TDZ. Basal medium without PGRs was used as the control. Media was adjusted to pH 5.7 with 0.1 M NaOH. Ten mL aliquots were then dispensed into 20 mL glass scintillation vials. Scintillation vials were autoclaving for 10 min at 121°C and 117.67 kPa.

Each replicate consisted of the tip and base sections of a single leaf (adaxial side in contact with media). Inoculated vessels were capped and sealed with a single layer of NescoFilm. Cultures were wrapped in two layers of aluminum foil to exclude light (0/24 h L/D) and incubated at $23^{\circ} \pm 2^{\circ}\text{C}$ for four weeks. At this time, cultures were examined for signs of tissue response and replicates were transferred to fresh media containing the same media treatment that they were originally cultured on. Cultures were again covered with aluminum foil and incubated at $23^{\circ} \pm 2^{\circ}\text{C}$ for an additional four weeks.

Experimental design. Five replicates of each treatment were used and the experiment was repeated twice. Treatment effects on explant response were observed after four and eight weeks culture.

Results and Discussion

Visible contamination for this experiment was 0%. Explants did not respond to any of the treatments after eight weeks culture, however, the majority of leaf explants were still green by the end of eight weeks culture. Visible phenol exudates were not observed in the medium of any treatments. In a study by Seeni and Latha (2000), it was found that 70% of *Vanda coerulea* seedling leaves cultured in the presence of 8.8 μM BA combined with 4.1 μM NAA produced

PLBs. It is possible that leaf explants would respond to PGRs used in combination, though they did not respond to any single PGR tested in this experiment.

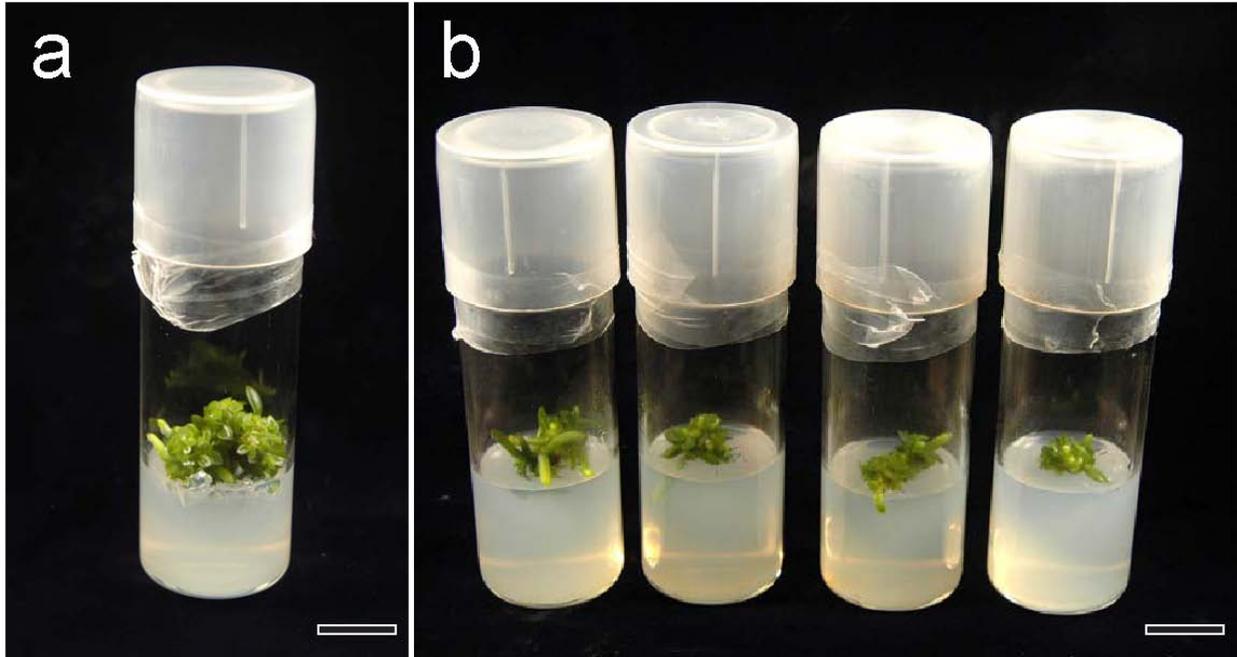


Figure A-1. Tissue culture of *Vanda tessellata* × *Vanda Arjuna* initiated in liquid Knudson C with 15% coconut water (v/v). A) Regenerated shoots growing on original explant. Scale bar = 2 cm. B) Division and subsequent regeneration of cultures. Scale bars = 2 cm.

LIST OF REFERENCES

- Adelberg J, Toler J (2004) Comparison of agar and an agitated, thin-film, liquid system for micropropagation of ornamental elephant ears. *Hortscience* 39:1088-1092
- Akita M, Ohta Y (1998) A simple method for mass propagation of potato (*Solanum tuberosum* L.) using a bioreactor without forced aeration. *Plant Cell Rep* 18:284-287
- Al-Zahim MA, Ford-Lloyd BV, Newbury HJ (1999) Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. *Plant Cell Rep* 18:473-477
- Arditti J (1993) *Micropropagation of orchids*. John Wiley and Sons, Inc.: New York
- Arditti J, Ernst R (1992) *Fundamentals of orchid biology*. John Wiley and Sons, Inc.: New York
- Becerra DC, Forero AP, Góngora GA (2004) Age and physiological condition of donor plants affects *in vitro* morphogenesis in leaf explants of *Passiflora edulis* f. *flavicarpa*. *Plant Cell Tiss Org Cult* 79:87-90
- Bhaskar J, Rajeevan PK (1996) Embryo culture of *Vanda* 'John Club'. *S Indian Hort* 44:36-38
- Blanchard MG, Runkle ES (2006) Temperature during the day, but not during the night, controls flowering of *Phalaenopsis* orchids. *J Exp Bot* 15:4043-4049
- Bose TK, Mukherjee (1974) Effect of growth substances on seedling growth and differentiation from callus of *Vanda* *in vitro* culture. *Orchid Rev* 82:148-149
- Brar MS, Moore MJ, Al-Khayri JM, Morelock TE, Andersen EJ (1999) Ethylene inhibitors promote *in vitro* regeneration of cowpea (*Vigna unguiculata* L.) *In Vitro Cell Dev Biol Plant* 35:222-225
- Cheah KT, Sagawa Y (1978) *In vitro* propagation of *Aranda* Wendy Scott and *Aranthera* James Storei. *Hortscience* 13:661-662
- Chen JT, Chang WC (2006) Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*. *Bio Plant* 50:169-173
- Chen JT, Chang C, Chang WC (1999) Direct somatic embryogenesis on leaf explants of *Oncidium* Gower Ramsey and subsequent plant regeneration. *Plant Cell Rep* 19:143-149
- Chen TY, Chen JT, Chen WC (2003) Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. *Plant Cell Tiss Org Cul* 76:11-15
- Chen WH, Chen TM, Fu YM, Hsieh RM, Chen WS (1998) Studies on somaclonal variation in *Phalaenopsis*. *Plant Cell Rep* 18:7-13
- Chung HH, Chen JT, Chang WC (2005) Cytokinins induce direct somatic embryogenesis of *Dendrobium* Cheingmai Pink and subsequent plant regeneration. *In Vitro Cell Dev Biol* 41:765-769

- Churchill ME, Ball EA, Arditti J (1973) Tissue culture of orchids: I. methods for leaf tips. *New Phytol* 72:161-166
- Cozzolino S, Widmer A (2005) Orchid diversity: an evolutionary consequence of deception? *Trends Ecol Evol* 20:487-494
- Curtis JT (1947) Studies on the nitrogen nutrition of orchid embryos I. complex nitrogen sources. *Am Orchid Soc Bull* 16:654-660
- Curtis JT, Spoerl E (1948) Studies on the nitrogen nutrition of orchid embryos II. comparative utilization of nitrate and ammonium nitrogen. *Am Orchid Soc Bull* 17:111-114
- Darwin C (1892) The various contrivances by which orchids are fertilized by insects. 2nd ed. University of Chicago Press: Chicago
- Decruse SW, Gangaprasad A, Seeni S, Menon VS (2003) Micropropagation and ecorestoration of *Vanda spathulata*, an exquisite orchid. *Plant Cell Org Tiss Cult* 72:199-2003
- Devi CG, Damayanti M, Sharma GJ (1998) Aseptic embryo culture of *Vanda coerulea* Griff.. *J Orchid Soc India*. 12:83-87
- Dressler RL (1981) The orchids, natural history and classification. Harvard University Press: Cambridge, MA
- Dressler RL (1993) Phylogeny and classification of the orchid family. Cambridge University Press: Melbourne, AU
- Fitch CM (2005). Ascocentrums. *Orchids*. 74:20-25
- Ghani AKBA, Haris H, Hajiujung NB (1992) Production of *Renantanda* plantlets from shoot tips *in vitro*. *Lindleyana*. 7:3-6
- Goh CJ, Wong PF (1990) Micropropagation of monopodial orchid hybrid *Aranda* 'Deborah' using inflorescence explants. *Sci Hort* 44:315-321
- Griesbach RJ (2002) Development of *Phalaenopsis* orchids for the mass-market. In Janick J and Whipsey A (eds) Trends in new crops and new uses. ASHS Press: Alexandria, VA
- Griesbach RJ (2003) Orchids emerge as major world floral crop. *Chron Hort* 43:6-9
- Grove D L (1995) Vandas and Ascocendas and their combinations with other genera. Timber Press: Portland
- Hashmi G, Huettel R, Meyer R, Krusberg L, Hammerschlag F (1997) RAPD analysis of somaclonal variation derived from embryo callus cultures of peach. *Plant Cell Rep* 16:624-627
- Hoppe AG, Hoppe HJ (1988) Tissue culture of *Ophrys apifera*. *Lindleyana* 3:190-194

- Hoque ME, Mansfield JW (2004) Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of *Indica* rice genotypes. *Plant Cell Tiss Org Cult* 78:217-223
- Hoshi Y, Kondo K, Hamatani S (1994) *In vitro* germination of four Asiatic taxa of *Cypripedium* and notes on the nodal micropropagation of American *Cypripedium montanum*. *Lindleyana* 9:93-97
- Intuwong O, Sagawa Y (1973) Clonal propagation of Sarcanthine orchids by aseptic culture of inflorescences. *Am Orchid Soc Bull* 42:209-215
- Jerardo A (2006) Floriculture and nursery crop outlook. USDA FLO-05
- Jiménez W, Pérez N, de Feria M, Bardón R, Capote A, Chávez M, Quiala E, Pérez JC (1999) Improved production of potato microtubers using a temporary immersion system. *Plant Cell Tiss Org Cult* 59:19-23
- Kanika, Vij SJ (2004) Micropropagation of *Vanda coerulea* (Orchidaceae) through shoot-tip culture. *Harayana J Hort Sci* 33:227-228
- Kauth PJ, Vendrame WA, Kane ME (2006) *In vitro* seed culture and seedling development of *Calopogon tuberosus*. *Plant Cell Tiss Org Cult* 85:91-102
- Kishor R, Sha Valli Khan PS, Sharma GJ (2006) Hybridization and *in vitro* culture of an orchid hybrid *Ascocenda* 'Kangla'. *Sci Hort* 108:66-73
- Knudson L (1922) Nonsymbiotic germination of orchid seeds. *Bot Gaz* 73:1-25
- Knudson L (1946) A new nutrient solution for the germination of orchid seed. *Am Orchid Soc Bull* 15:214-217
- Kunisaki J, Kim K, Sagawa Y (1972) Shoot-tip culture of *Vanda*. *Am Orchid Soc Bull* 41:435-439
- Kuo HL, Chen JT, Chang WC (2005) Efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* 'Little Steve'. *In Vitro Cell Dev Biol* 41:453-456
- Lai CC, Yu TA, Yeh SD, Tang JS (1998) Enhancement of *in vitro* growth of papaya multishoots by aeration. *Plant Cell Tiss Org Cult* 53:221-225
- Lakon G (1949) The topographical tetrazolium method for determining the germination capacity of seeds. *Plant Physiol* 24:389-394
- Lakshmanan P, Loh CS, Goh CJ (1995) An *in vitro* method for rapid regeneration of a monopodial orchid hybrid *Aranda* Deborah using thin section culture. *Plant Cell Rep* 14:510-514

- Lauzer D, St-Arnaud M, Barabé D (1994) Tetrazolium staining and *in vitro* germination of mature seeds of *Cypripedium acaule* (Orchidaceae). *Lindleyana* 9:197-204
- Lay FFM (1979) Studies on the tissue culture of orchids 2: clonal propagation of *Aranda*, *Ascocenda*, *Cattleya* by leaf tissue culture. *Orchid Rev* 87:343-346
- Le VB, Phuong NTH, Hong LTA, Van KTT (1999) High frequency shoot regeneration from *Rhynchostylis gigantean* (Orchidaceae) using thin cell layers. *Plant Growth Reg* 28:179-185
- Lee YH, Wong SM, Tan WK, Goh CJ (1996) Breeding vandaceous orchids for commercial cut-flowers in Singapore: an overview. *Euphytica* 89:235-241
- Leifert C, Cassells AC (2001) Microbial hazards in plant tissue and cell cultures. *In Vitro Cell Dev Biol Plant* 37:133-138
- Leifert C, Waits WM, Nicholas JR (1989) Bacterial contamination of micropropagated cultures. *J Appl Bacteriol* 67:353-361
- Li GS, Duan J, Chen ZL, Zeng SJ, Jiang YM, Joyce DC (2006) $KClO_3$ application affects *Phalaenopsis* orchid flowering. *Sci Hort* 110:362-365
- Lu MC (2004) High frequency plant regeneration from callus culture of *Pleione formosana* Hayata. *Plant Cell Tiss Org Cult* 78:93-96
- Malabadi RB, Mulgand GS, Nataraja K (2004) Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. *Plant Cell Tiss Org Cult* 76:289-293
- Mathews VH, Rao PS (1980) *In vitro* multiplication of *Vanda* hybrids through tissue culture technique. *Plant Sci Letters* 17:383-389
- Mitra GC, Prasad RN, Roychowdury A (1976) Inorganic salts and differentiation of protocorms in seed-callus of an orchid and correlated changes in its free amino acid content. *Indian J Explor Biol* 14:350-351
- Molina DM, Aponte ME, Cortina H, Moreno G (2002) The effect of genotype and explant age on somatic embryogenesis of coffee. *Plant Cell Tiss Org Cult* 71:117-123
- Morel GM (1960) Producing virus-free Cymbidiums. *Am Orchid Soc Bull* 29:495-497
- Morel GM (1964) Tissue culture—a new means of clonal propagation of orchids. *Am Orchid Soc Bull* 33:473-478
- Motes MR (1997) *Vandas their botany, history and culture*. Timber Press: Portland.
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15:437-497

- Nagy JK, Sule S, Sampaio JP (2005) Apple tissue culture contamination by *Rhodototula* spp.: identification and prevention. *In Vitro Cell Dev Biol Plant* 41:520-524
- Nash N (2003) *Phalaenopsis* primer: A beginner's guide to growing moth orchids. *Orchids*. 72:906-913
- Nguyen QT, Kozai T, Heo J, Thai DX (2001) Photoautotrophic growth response of *in vitro* cultured coffee plantlets to ventilation methods and photosynthetic photon fluxes under carbon dioxide enriched condition. *Plant Cell Tiss Org Cult* 66:217-225
- Paek KY, Hahn EJ (1999) Variation in African violet 'Crimson Frost' micropropagated by homogenized leaf tissue culture. *HortTech* 9:625-627
- Paek KY, Hahn EJ, Son SH (2001) Application of bioreactors for large-scale micropropagation systems of plants. *In Vitro Cell Dev Biol Plant* 37:149-157
- Park SY, Yeung EC, Chakrabarty D, Paek KY (2002) An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture. *Plant Cell Rep* 21:46-51
- Piao XC, Chakrabarty D, Hahn EJ, Paek KY (2003) A simple method for mass production of potato microtubers using a bioreactor system. *Curr Sci* 84:1129-1132
- Pogany MF, Lineburger RD (1990) Phenotypic variation during micropropagation of the chimeral *Rhododendron* 'President Roosevelt'. *Plant Cell Tiss Org Cult* 21: 201-209.
- Prasad VSS, Gupta SD (2006) *In vitro* shoot regeneration of gladiolus in semi-solid agar versus liquid cultures with support systems. *Plant Cell Tiss Org Cult* 87:263-271
- Raghavan V, Torrey JG (1964) Inorganic nitrogen nutrition of the seedlings of the orchid, *Cattleya*. *Am J Bot* 51:264-274
- Ricardo Jr. MJ, Alvarez MR (1971) Ultrastructural changes associated with utilization of metabolite reserves and trichome differentiation in the protocorm of *Vanda*. *Amer J Bot* 58:229-238
- Roy J, Banerjee N (2002) Optimization of *in vitro* seed germination, protocorm growth and seedling proliferation of *Vanda tessellata* (Roxb.) Hook. Ex G. Don. *Phytomorph* 52:167-178
- Sakai WS (1973) Simple methods for differential staining of paraffin embedded plant material using toluidine blue O. *Stain Tech* 48:247-249
- Seeni S, Latha PG (1992) Foliar regeneration of the endangered Red Vanda, *Renanthera imschootiana* Rolfe (Orchidaceae). *Plant Cell Tiss Org Cult* 29:167-172
- Seeni S, Latha PG (2000) *In vitro* multiplication and ecorehabilitation of the endangered Blue Vanda. *Plant Cell Tiss Org Cult* 61:1-8

- Sharma J (1998) Studies on *Vanda*: effects of age of capsules (pods) on *in vitro* seed germination. J Orchid Soc India 12:43-45
- Sharma V, Vij SP (1997) Effect of CuSO₄.5H₂O on *in vitro* regenerative capacity of foliar explants excised from mature *Vanda cristata* Lindl. plants. Phytomorph 47:203-208
- Sheelavanthmath SS, Murphy HN, Hema BP, Hahn EJ, Paek KY (2005) High frequency of protocorm like bodies (PLBs) induction and plant regeneration from protocorm and leaf sections of *Aerides crispum*. Sci Hort 106:395-401
- Sheelavantmath SS, Murthy HN, Pyati AN, Kumar HGA, Ravishankar BV (2000) *In vitro* propagation of the endangered orchid, *Geodorum densiflorum* (Lam.) Schltr. through rhizome section culture. Plant Cell Tiss Org Cult 60:151-154
- Shoushtari BD, Heydary R, Jogson GL, Arditti J (1994) Germination and viability staining of orchid seed following prolonged storage. Lindleyana 9:77-84
- Stenberg ML, Kane ME (1998) *In vitro* seed germination and greenhouse cultivation of *Encyclia boothiana* var. *erythronioides*, an endangered Florida orchid. Lindleyana 13:101-112
- Stewart SL, Zettler LW (2002) Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens*, *H. quinquiseta*, *H. macroceratitis*) from Florida. Aquat Bot 72:25-35
- Su WR, Chen WS, Koshioka M, Mander LN, Hung LS, Chen WH, Fu YM, Huang KL (2001) Changes in gibberellin levels in the flowering shoot of *Phalaenopsis hybrida* under high temperature conditions when flower development is blocked. Plant Physiol Biochem 39:45-50
- Swamy BGL (1942) Morphological studies in three species of *Vanda*. Curr Sci 11:285-286
- Teo CKH, Kunisaki JT, Sagawa Y (1973) Clonal propagation of strap-leaved *Vanda* by shoot-tip culture. Am Orchid Soc Bull 42:403-405
- Thomas J, Vijayan D, Joshi SD, Lopez SJ, Kumar PR (2006) Genetic integrity of somaclonal variation in tea (*Camellia sinensis* (L.) O Kuntze) as revealed by inter simple sequence repeats. J Biotech 123:149-154
- Tokuhara K, Mii M (2001) Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae). In Vitro Cell Dev Biol Plant 37:457-461
- Tremblay L, Levasseur C, Tremblay FM (1999) Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic stability. Am J Bot 86:01373-1381
- Vacin E, Went F (1949) Some pH changes in nutrient solutions. Bot Gaz 110:605-613

- Valmayor HL, Pimentel ML, Martinez MT (1986) Callus formation and plantlet morphogenesis in *Vanda*. *Malayan Orchid Rev* 20:22-30
- Van Le B, Phuong NTH, Hong LTA, Van KTT (1999) High frequency shoot regeneration from *Rhynchostylis gigantean* (Orchidaceae) using thin cell layers. *Plant Growth Reg* 28:179-185
- Vaz APA, Figueiredo-Ribeiro RC, Kerbauy GB (2004) Photoperiod and temperature effects on *in vitro* growth and flowering of *P. pusilla*, an epiphytic orchid. *Plant Physiol Biochem* 42:411-415
- Vendrame WA, Kochert G, Wetzstein HY (1999) AFLP analysis of variation in pecan somatic embryos. *Plant Cell Rep* 18:853-857
- Vij SP, Sood A, Sharma M (1986) In vitro segment culture of *Vanda testacea* (Lindl) Reichb F (= *V. parviflora* Lindl) (Orchidaceae). *Curr Sci* 55:1100-1101
- Vujanovic V, St-Arnaud M, Barabé D, Thibeault G (2000) Viability testing of orchid seed and promotion of coloration and germination. *Ann Botany* 86:79-86
- Wang WY, Chen WS, Chen WH, Hung LS, Chang PS (2002) Influence of abscisic acid on flowering in *Phalaenopsis hybrida*. *Plant Physiol Biochem* 40:97-100
- Wimber DE (1963) Clonal multiplication of Cymbidiums through tissue culture of the shoot meristem. *Am Orchid Soc Bull* 32:105-10
- Yam TW, Weatherhead A (1991) Leaf-tip culture of several native orchids of Hong Kong. *Lindleyana* 6:147-150
- Yan N, Hu H, Huang J, X K, Wang H, Zhou Z (2005) Micropropagation of *Cypripedium flavum* through multiple shoots of seedlings derived from mature seeds. *Plant Cell Tiss Org Cult* 84:114-118
- Young PS, Murthy HN, Yoeup PK (2000) Mass multiplication of protocorm-like-bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. *Plant Cell Tiss Org* 63:67-72
- Xiao YL, Lok YH, Kozai T (2003) Photoautotrophic growth of sugarcane plantlets *in vitro* as affected by photosynthetic photon flux and vessel air exchange. *In Vitro Cell Dev Biol Plant* 39:186-192

BIOGRAPHICAL SKETCH

Tim Johnson's research experience began as an undergraduate at the University of Wisconsin-Eau Claire (UWEC) where he worked with Dr. Wilson Taylor on the evolution of early land plants. Tim graduated from the UWEC in 2003 with a BS in biology and minor in anthropology. His experience as a student researcher and his interest in orchids lead him to the Plant Propagation, Conservation and Biotechnology Lab at the University of Florida, where he worked on the propagation of commercial orchids, while cultivating an interest in the conservation of Florida's native orchids. Tim will begin working towards a Ph.D. in Environmental Horticulture at the University of Florida in the fall of 2007. He plans to study the seed physiology, seed ecology, and reintroduction science of native orchids. He is dedicated to improving the scientific understanding of orchid ecology and educating the public about the need for protecting wildland plant communities. In his spare time, Tim enjoys reading, traveling, gardening, running, being lazy with his family, and fishing (which he doesn't get to do nearly enough).