REGENERATION OF *PHILODENDRON MICANS* K. KOCH THROUGH PROTOCORM-LIKE BODIES AND IMPROVEMENT OF PLANT FORM USING GROWTH REGULATORS

By

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To my parents

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Figure page

LIST OF ABBREVIATIONS

B-Nine®	butanedioic acid mono (2,2-dimethylhydrazide)
BA	6-benzyladenine
BM	basal medium
Bonzi®	β-[(4-chlorophenyl)methyl]-α-(1,1-dimethylethyl)-1*H*- 1,2,4-triazole-1-ethanol
CPPU	N-(2-chloro-4-pyridyl)-N-phenylurea
Cycocel®	(2-chlorethy) trimethylethanaminium chloride
Florel®	(2-chloroethyl) phosphonic acid
NAA	1- naphthalene acetic acid
PGR	plant growth regulator
PLBs	protocorm-like bodies
TDZ	*N*-phenyl-*N'* -1,2,3- thiadiazol-5-ylurea

Abstract of Thesis Presented to the Graduate School
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Philodendron micans K. Koch, commonly known as velvet philodendron, has both a soft foliar texture and attractive colors that could make it an important genetic resource for breeding new ornamental traits and introducing improvements to the genus *Philodendron. P. micans* rarely flowers in nature and thus far no breeding program has utilized this plant for interspecific hybridization. However, in addition to traditional breeding, new cultivars could be developed using somaclonal variation produced during in vitro propagation. As a step toward this direction, bud, leaf, petiole, and internode explants were cultured on Murashige and Skoog (MS) medium supplemented with BA (N^6-benzyladenine), CPPU [N-(2-chloro-4-pyridyl)-N- phenylurea], or TDZ (*N*-phenyl-*N′* -1,2,3- thiadiazol-5-ylurea) with NAA (1-naphthaleneacetic acid). Bud explants showed the greatest regenerative potential by producing both callus and globular structures. Callus was friable and unable to produce adventitious shoots; while globular structures differentiated and produced shoots and roots. Histological analysis suggested that the globular structures were protocorm-like bodies (PLBs), a novel pathway for plant regeneration. Further experiments showed that 75% bud explants of *P. micans* cultured

on MS medium supplemented with 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA produced PLBs within 8 weeks. Each explant produced more than 50 shoots that subsequently rooted. The survival rate of plantlets regenerated through this protocol was 98%. Analysis of 20 randomly selected plantlets using DNA flow cytometry showed that two were mixoploid. Such a high frequency of ploidy change may suggest the occurrence of somaclonal variation. However, regenerated plants phenotypically resembled mother plants. This was the first in vitro study of *P. micans*, and a new and reliable protocol has been developed for efficient regeneration of this species. Growth regulators to improve appearance and marketability of *P. micans* were also examined. Four growth regulators, Bonzi® {β-[(4-chlorophenyl)methyl]-α-(1,1-dimethylethyl)-1*H*- 1,2,4-triazole-1-ethanol}, B-Nine® (butanedioic acid mono (2,2-dimethylhydrazide), Cycocel® [(2-chlorethy) trimethylethanaminium chloride], and Florel® [(2-chloroethyl) phosphonic acid] were applied as foliar sprays or soil drenches in attempt to shorten internode length and reduce a leggy appearance. Bonzi® application was the most effective treatment and resulted in plants with a more compact growth form.

CHAPTER 1
LITERATURE REVIEW

The Genus *Philodendron*

The genus *Philodendron* contains approximately 700 species, making it the second largest genus in the family Araceae (Croat 1997). Philodendrons are native to tropical America and comprise a conspicuous component of the native flora because of their abundance, different growth styles, and attractive, durable leaves. Based on their growth habits, philodendrons were divided into three groups by McColley and Miller (1965). The first group is the vining or scandent type, such as *P. scandens* K. Koch & Sello (heartleaf philodendron). The second group has a self-heading and upright growing style represented by *P. wendlandii* Schott. The third group is the erect-arborescent or tree type, such as *P. bipinnatifidum* Endl., which appear self-heading when they are young, but assume a more woody and treelike shape as they are mature.

Philodendrons are popular ornamental foliage plants, particularly the vining types, which are grown as either hanging baskets or potted plants where vines are supported by totem poles (Chen et al. 2005). Because of its ease in production, low maintenance and great durability indoors, *P. scandens* dominated all other genera of tropical ornamental foliage plant production, accounting for 50% of the national wholesale value of foliage plants in the U.S. in 1950 and 36% in 1967 (McConnell et al. 1989; Chen and Henny 2008).

Recently, another vining type philodendron, *Philodendron micans*, commonly known in the trade as velvet philodendron or red philodendron, has been re-introduced to the foliage plant industry and has claimed a significant market share. *P. micans* is easily recognized by cordate leaves that are velvet bronze on the upper surface and

reddish to bronze on the lower surface. Taxonomically, it is unclear if *P. micans* is a distinct species. It has been documented as *P. micans* K. Koch (Marchan 1970), *P. hederaceum* (Jacq.) Schott (Croat 1997), and *P. scandens* forma *micans* (K. Koch) G. S. Bunting (Huxley 1994). Nevertheless, *P. micans* is closely related to *P. scandens* as both exhibit a vining growth style and have small chromosomes with $2n = 32$ (Marchan 1970).

Ornamental Value of *P. micans* and Potential for Improvement

Due to its unique foliar colors ranging from burgundy to bronze and copper, velvet philodendron is gaining popularity and offers consumers variety in foliage assortments as small containers or as climbers supported by totem poles, or as hanging baskets. The growth form of *P. micans* is often regarded as having a leggy appearance due to long internodes. This could be improved by introducing new cultivars with a more compact growth style. *P. micans* could be an important genetic resource for plant breeders seeking to add color and foliage texture into lines of vining *Philodendron* types. However, *P. micans*, similar to heartleaf philodendron, rarely flowers in nature. To date, no plant breeding program has targeted this plant for hybridization,

New cultivars can also be developed from somaclonal variation which is referred to as the phenotypic variation observed among plants regenerated after passage through a tissue culture stage (Larkin and Scowcroft 1981). During the tissue culture process, there are two main pathways of plant regeneration: organogenesis and somatic embryogenesis. Somaclonal variation may occur in plants regenerated from either pathway, and selected variants can be evaluated for cultivar development in floriculture crops. More than 82 cultivars of floricultural crops currently in the marketplace were developed through the selection of somaclonal variants (Chen and

Henny 2008). Another approach for modifying plant growth form is the use of plant growth regulators. There are many growth regulators commonly used by ornamental foliage plant growers to shorten internodes or improve branching of many foliage plant genera (Chen et al. 2005). The leggy growth habit of *P. micans* could be improved by growth regulator application.

In the foliage plant industry, *P. micans* is propagated exclusively by single eye cuttings; an economically feasible micropropagation system using tissue culture has not yet been established. Chen and Henny (2006) proposed that the continuous vegetative propagation of asexual-propagated plants may have allowed an accumulation of unexpressed somatic mutations not eliminated by the meiotic sieve. Thus, in vitro culture of *P. micans* may provide an opportunity for mutated cells to become expressed if plants arise from single cells that contain mutations. In addition to mutated cell expression, the process of in vitro culture itself can also induce mutations (Larkin and Scowcroft 1981; Lee and Phillips 1988; Chen and Henny 2008). Selection, characterization, and evaluation of the subsequent variants could result in new *P. micans* cultivars.

Plant Regeneration Pathways

Shoot Organogenesis

Shoot organogenesis is the regeneration of adventitious shoots from explants without pre-existing meristems and subsequent rooting of the shoots (Schwarz and Beaty 2000). There are direct and indirect shoot organogenesis depending on the origin of shoots. Direct shoot organogenesis is the production of shoots from single explants without any callus phase, while indirect organogenesis refers to the formation of shoots from an intermediary callus stage instead of directly from explants (Hicks 1980, Kane et

al. 1994). In practice, it is not always possible to distinguish between the two methods. Directly-formed meristems may proliferate to form a regenerative tissue similar in appearance to callus and may also become surrounded by callus so that it is difficult to ascertain its origin (George et al. 2008).

In general, three phases of organogenesis are recognizable, namely competence, determination, and finally adventitious shoots (Sugiyama 1999). Competence is defined as a state of cells which have retained the capacity for a particular kind of cellular differentiation. This includes the capability to respond to extra-cellular signals under stimulus that could be plant growth regulators or other alternative changes in culture environment. Determination is the state of previously competent cells committing to a differentiation pathway of shoot organogenesis after induction. Adventitious shoots form and develop into buds after the differentiation stage. The dedifferentiation phase refers to the span of time that cells are becoming competent and becoming able to response to growth regulating chemicals (Sugiyama 1999).

The nature of the internal factors governing cell competence is largely unknown. However, the study on *Nicotiana tabacum* by Attfield and Evans (1991) showed that a 1-2 day exposure to basal medium without plant growth regulator first before placing explants on IBM would maximize the rate of shoot formation, which indicates that 2 days was the time explants required to gain competence and growth regulators were not necessary in this period. During the induction phase, a competent cell or a group of competent cells become committed to a unique developmental fate on the stimulus of an inducing signal. At the end of the induction phase, cells become fully determined and

capable of shoot organogenesis even with the removal of the inducing signals (Schwarz and Beaty 2000).

Hu et al. (2005) studied morphogenesis in petiole derived callus of *Amorphophallus rivieri* Durieu and observed that compact callus consisted of 3 components: epidermis, subepidermis, and inner parenchyma cells. Cells in the subepidermis started to divide to give rise to the formation of a long and narrow meristematic zone after 1 week of culture on differentiation medium. During direct organogenesis, adventitious buds may not derived immediately from explants. There is evidence that a single epidermal cell may sometimes give rise to meristematic centers from one of which up to 22 identical shoots may arise (Broertjes et al. 1976; Shen 2007). Similarly, shoots on cultured explants of *Nicotiana tabacum* leaves were found to arise indirectly from nodules at the edges of the explants, which were mainly formed by divisions of palisade mesophyll cells around the edge of the explants (Attifield and Evans 1991).

Somatic Embryogenesis

Somatic embryogenesis or non-zygotic embryogenesis is a process whereby somatic cells differentiate into embryos, and somatic embryo germination, like zygotic embryos, produces seedlings (Gray 2000; Merkle et al. 1990). Since the first report of somatic embryogenesis in carrot cell culture in 1958 (Steward et al. 1958), in vitro somatic embryogenesis has been reported in more than 100 species (Krishnaraj and Vasil 1995). Somatic embryos can be either induced from the explant without any intervening callus phase, called direct somatic embyrogenesis or indirectly after a callus phase, commonly known as indirect somatic embryogenesis (Willams and Maheswaran, 1986).

There are three developmental stages in somatic embryogenesis in vitro: induction, development, and maturation (Zimmerman 1993). Explants are composed of heterogeneous cell types. During the induction stage, undifferentiated cells from explants can be induced to obtain an embryogenic determination and somatic embryos can be obtained directly from these cells via direct embryogenesis (Merkle 1997). Differentiated cells from explants can undergo dedifferentiation, meristemation, and redifferentiation into embryogenic cells. In this case, calli are usually formed on the explant tissue, and somatic embryos form on the callus, a process which is referred to as indirect somatic embryogenesis (Kohlenbach 1985). Somatic embryogenic cells can be distinguished from non-embryogenic cells because embryogenic cells usually contain dense cytoplasm, prominent nuclei, higher starch contents, thickened cell walls, and are less vacuolated (Mooney and van Staden 1987).

During the development of somatic embyros, a single embryogenic cell undergoes a series of transverse and longitudinal divisions, passing through globular, torpedo, and cotyledonary stages for dicots or globular, scutellar and coleoptilar stages for monocots. Finally, it forms a bipolar structure with a root and a shoot on opposite ends of the same axis with the capacity to reproduce entire plants (Arnold et al. 2002). During the maturation stage, somatic embryos accumulate a reserve and achieve desiccation tolerance, which is very important for successful germination and growth (Ammirato 1974). The maturation stage in somatic embryogenesis has just been recognized recently due to observations that the rate of germination and regeneration is often very poor even with well-developed somatic embryos (Bhojwani and Razdan 1996). A period of reversible arrested growth is necessary for proper embryo germination; without such

developmental stages, somatic embryos would germinate precociously and finally die (Gray and Purohit 1991).

Protocorm-like Body Formation

Term of protocorm-like bodies (PLBs) was first coined by Morel (1960) when the shoot apex of *Cymbidium* was cultured for producing virus-free plants, during which protocorms were formed from apical meristems rather than from seeds. A protocorm is the tuber-like swollen part of an orchid seed, which appears during the early stage of germination. Protocorm-like bodies are composed of many meristematic centers that are able to differentiate into shoots and roots (da Silva et al. 2000), which resemble somatic embryos. However, the shoots and roots of regenerated plantlets from PLBs are not on the same axis. Thus, there are two schools of thought regarding the identity of PLBs: one considers that PLBs are somatic embryos (Steward and Mapes 1971; Begum et al. 1994; Ishii et al. 1998; Chen and Chang 2000) and the other believes PLBs differ from somatic embryos (Norstog 1979; da Silva et al. 2000; Cui et al. 2008; Tian et al. 2008). The claim favoring the somatic embryo scenario is largely based on the evidence derived from in vitro regeneration of orchids. In fact, usage of the term 'PLB' had been initially restricted to orchids (Ishii et al. 1998). However, PLBs have been identified in a wide range of other plant genera including *Anthurium* (Yu et al. 2009), *Colocasia* (Abo El-Nil and Zettler 1976), *Heliconia* (Goh et al. 1995), *Lilium* (Nhut et al. 2001), *Musa* (Venkatachalam et al. 2006), *Pinellia* (Liu et al. 2009), *Rosa* (Tian et al. 2008), and *Syngonium* (Cui et al. 2008). This research hypothesis assumes that PLBs could be an independent pathway in plant regeneration. PLBs are distinguished from somatic embryos by the lack of a single embryonic axis (Norstog 1979) and also different from shoot organogenesis by the direct formation of abundant PLBs that are

able to produce plantlets with both shoots and roots resembling the characteristics of somatic embryogenesis.

Histological Analysis of Regeneration Processes

Histological analysis is critically important for identifying regeneration pathways. In shoot organogenesis, shoots are usually formed first. There is a well-developed vascular connection between shoots and maternal explants. Roots are then induced from the base of shoots, which result in plantlets with shoots and roots not in the same axis. In somatic embryogenesis, a somatic embryo is an independent entity; there is no vascular connection between somatic embryos and parental explants (Chengalrayan 2001). As mentioned above, the development of a single embryogenic cell undergoes a series of transverse and longitudinal divisions, passing through globular, torpedo, and cotyledonary stages for dicots or globular, scutellar and coleoptilar stages for monocots. Somatic embryo germination, like seed germination, results in shoots and roots on a bipolar structure. PLBs resemble somatic embryos morphologically, but PLBs have a vascular connection with the maternal explants and lack the bipolar structure. Depending on plant species and explant types, many PLBs can be produced per explant, and PLBs can produce shoots and roots directly (Cui et al. 2008; Tian et al. 2008).

In theory, plantlets can be regenerated from each of the three pathways, but different pathways result in different numbers of regenerated plantlets and the degree of somaclonal variation observed (Chen and Henny 2006). Additionally, for genetic transformation or germplasm conservation, one pathway may have more advantages than another. Thus, histological analysis is essential for ascertaining the plant regeneration pathway employed on a case by case basis.

Somaclonal Variation

Somaclonal variation refers to the heritable genetic variation in plants regenerated from tissue culture (Larkin and Scowcroft 1981). Among factors affecting somaclonal variation, plant genotype is probably the most important. Some cultivars show high variation rates while others are highly stable (Najaran and Walton 1987; Bouman and de Klerk 1997). Regeneration pathways also make significant differences in the somaclone occurrence. Plants regenerated from a pathway with callus phase (i.e. indirect shoot organogenesis or indirect somatic embryogenesis) are often high in somalconal variation (Merkle 1997). Plants regenerated from direct shoot organogenesis or direct somatic embryogenesis generally have a low rate of somaclonal variation because these processes have no callus phase (Merkle 1997). Regeneration through PLBs without the occurrence of callus in theory is also low in somaclonal variation. Plants produced from pre-existing meristematic cells are usually true-to-type. In addition, somaclonal variation generally increases with the time that a culture has been maintained in vitro, especially callus culture (Skirvin et al. 1994: Bouman and de Klerk 1997).

Somaclonal variation could be derived from pre-existing genetic variation in explants being cultured or induced during the tissue culture process (Evans 1989). To distinguish the source of variation has been experimentally difficult because the appearance of somaclonal variants relies on in vitro culture. However, with the advance of molecular genetics, it has become clear that somaclonal variation is not the result of a single genetic mechanism (Kaeppler et al. 2000). Changes at the chromosome level, variation of DNA sequences including activation of transposable elements, and

epigenetic effects have been shown to be the underlying mechanisms of somaclonal variation (Lee and Phillips 1988; Chen and Henny 2006).

Somalconal variants can be identified through morphological, cytological, and molecular means. Morphological characteristics such as plant form (height, width, internode length, leaf number, leaf size), foliar variegation patterns, leaf colors, flower color if any, and petiole color are compared between the variants and parental plants (Chen and Henny 2006). Only those variants that show distinct differences in one or more characteristics from parental plants are then selected and maintained for further evaluation. For research purposes, further evaluation may include molecular marker and cytological analyses. Cytological assay includes chromosome counts and DNA flow cytometry analysis. Among the available molecular marker techniques, amplified fragment length polymorphisms (AFLP) has been widely used for somaclonal and naturally occurring sport evaluation (Chao et al. 2005; Chen et al. 2004). AFLP can be used to detect variation on the DNA level and has proven to be extremely effective in distinguishing closely related genotypes (Chen et al. 2006).

CHAPTER 2
FACTORS INFLUENCING REGENERATION OF *PHILODENDRON MICANS*

Introduction

Although different species of *Philodendron* have been used as either landscape or potted ornamental plants, information regarding micropropagation of philodendrons is limited. Vardja and Vardja (2001) reported that shoots were produced from *P. tuxlanum* and *P. erubescens* cultured on MS (Murashige and Skoog 1962) medium containing N6-benzyladenine (BA) at concentrations from 2 to 4 mg L^{-1} with indole-3- acetic acid (IAA) at 0.1 mg L^{-1}. Blanco and Valverde (2004) micropropagated *P. corcovadense* using MS medium containing BA, N6-(Δ2-isopentenyl) adenine (2iP), IAA, and naphthaleneacetic acid (NAA) in 53 combinations. The highest rate of shoot multiplication, 3 shoots every four weeks, was achieved on MS medium containing 7 mg L^{-1} BA, and 3 mg L^{-1} kinetin. A shoot culture method was established by Gangopadhyay et al. (2004) for micropropagation of *P.* 'Xanadu' on MS medium supplemented with 5.0 mg L^{-1} BA and 0.5 mg L^{-1} IAA. Thus far, all reports on in vitro culture of philodendrons were via shoot culture and BA was identified to be the most effective cytokinin for micropropagation. However, there were no reports on in vitro regeneration of *P. micans.*

It has been well documented that species differ in regeneration capacity. Sears (1982) reported that variability occurred in different wheat genotypes for callus induction, regenerable callus formation, response to subculture, and plant regeneration potential. Similar results were shown by Mathias (1986) in wheat where the initiation of callus from immature embryos was different among eight breeding lines. Somatic embryogenesis is also strictly genetically controlled among species or closely related varieties levels. In an evaluation of somatic embryogenesis from leaves of

Dendranthema grandiflora, 12 out of 23 cultivars tested produced somatic embryos, but only five regenerated plants. Likewise, variation in somatic embryogenic capacity ranging from 4.8 % to 72.7% was found among 15 genotypes of coffee (*Coffea arabica*) (Molina et al. 2002).

Explant source including petals, sepals, anthers, carpels, ovules, placenta, endosperm, microspores, shoot apices, axiliary buds, hypocotyls, mesocotyls, roots, leaves, and stems has been shown to affect somatic embryogenesis (Merkle et al. 1990). Somatic embryogenesis in the 'Nabali' olive (*Olea europea* L.) was shown to be 46% for root, 30% for cotyledon, 26% for hypocotyle, 20% for petiole, and 10% for leaf explants (Shibli et al. 2001). The ability to induce somatic embryogenesis decreases with the ageing of explants and they can mature to a point where they completely lose their ability to induce somatic embryogenesis (Parrott et al. 1995). In general, explants from less differentiated, immature or meristematic tissues, such as immature zygotic embryos, cotyledons, and apical meristems, are more easily induced and established via somatic embryogenesis or organogenesis in vitro. Callus only initiates from young leaves, seed embryos or nodes, but never from mature leaves or stems in the Poaceae (George et al. 2008). Direct embryogenesis usually takes place on explants taken from cotyledon sections and embryonic axes (Plata and Vieitez 1990), whereas indirect somatic embryogenesis is derived from well-differentiated tissues and might require higher growth regulator concentrations in the medium (Hartmann and Kester 1983).

In addition to plant genotypes and explants types, growth regulators used for induction are also important for regeneration. Induction of somatic embryogenesis by cytokinins alone is relatively rare; however, adventitious shoots can be induced on

media containing only BA (Simmonds 1984). Although an auxin is necessary for the induction of somatic embryogenesis, it has an inhibitory effect on subsequent differentiation and development of somatic embryos (Terzi and LoSchiavo 1990). The ratio of cytokinins to auxins is critical in determining shoot versus root formation. A high cytokinin to auxin ratio promotes shoot meristem formation, while callus or root meristems are formed when the cytokinin to auxin ratio is low. It has been reported that different combination of auxins and cytokinins at 0, 1, 2 mg L^{-1} levels resulted in either direct adventitious shoot formation, callus formation, or indirect adventitious shoot formation (Makunga et al. 2005).

Dark treatment for newly inoculated explants can enhance morphogenesis. For example, keeping segments of apple leaves in the dark for the first three weeks of culture enhanced the subsequent regeneration of adventitious shoots and embryo-like structures (Welander 1988). Similarly, an initial period of dark culture increased somatic embryogenesis in anthers or anther-derived callus (Nair 1983). The highest frequency of white embryogenic tissue formation and the most normal embryoids of *Triticum aestivum* were obtained in the dark on MS medium, compared to the light treatment when other factors were same (Ozias-Akins 1983).The optimun shoot regeneration could be achieved by germinating embryos in darkness before preparing cotyledon explants of watermelon was reported by Compton (1999). Numerous report exist in the literature where the pretreatment of plant tissue in darkness improves adventitious shoot regeneration from cytoledons, nodal tissue, leaf cells, petioles (Punja et al. 1990; Leblay et al. 1991; Mohamed et al. 1992). The exact mechanism of how dark pretreatments stimulate subsequent organogenesis under light is not completely

understood. In some reports, the dark treatment was considered to increase endogenous auxin in the explants, which would promote callus formation or rooting (Huxter 1981). Except for the endogenous hormone regulation, dark-grown tissue produces much more ethylene than light-grown (Huxter 1981). The instances that ethylene would enhance organogenesis and organ development spread in a variety of culture systems (Kumar 1998).

Because there has been no previous knowledge on regeneration of *P. mican*, the first portion of this project was to evaluate responses of different types of explants to selected combinations and concentrations of growth regulators as well as to compare dark versus light culture on regeneration of *P. micans*.

Materials and Methods

Plant Materials and Explants

P. micans stock plants were grown in 15-cm diameter pots in a shaded greenhouse under a maximum photosynthetic photon flux density of 300 µmol m^{-2} s^{-1} at the University of Florida's Mid-Florida Research and Education Center in Apopka, FL. Nodes, 10 mm in length containing buds, petioles, leaves, and the internode sections were collected from vines and immersed in 70% ethanol for 1 min by agitation. Explants of nodes, petiole and internode sections (10 mm in length), and leaf sections (10 mm^2) were cut in sterile Petri dishes and placed in sterilized bottles containing 100 ml solution of 1.0% sodium hypochlorite (16%, v/v) and 1 to 2 drops of Tween-20 added as a surfactant. The bottles with explants and Clorox solution were agitated on a rotary shaker for 30 min at 120 rpm. To rinse the explants of the Clorox solution, the explants were immersed in 100 ml sterile distilled water and agitated for 5 min on the shaker at 120 rpm. After the rinsing two more times, the explants were ready for culture initiation.

Culture Medium

Murashige and Skoog (MS) mineral salts and vitamins (Murashige and Skoog 1962) with 2.8% (w/v) sucrose and 0.66% (w/v) agar (PhytoTechnology Laboratories, Shawnee Mission, KS) were used as a basal medium. The pH of the medium was adjusted to 5.8 with 1 M NaOH before autoclaving at 121oC for 25 min. Plant growth regulator solutions of BA, CPPU [N-(2-chloro-4-pyridyl)-N- phenylurea], TDZ (thidiazuron or N-phenyl-N′-1,2,3- thiadiazol-5-ylurea), and NAA were filter-sterilized and added to autoclaved basal medium based on the requirements of the following four experiments when the temperature dropped to 50oC. The medium was then poured in 100 x 15 mm sterile Petri dishes (Fisher Scientific, Pittsburgh, PA) with 20 mL each.

Experiments and Cultural Conditions

Four experiments were conducted with different explants. The first experiment was the culture of node explants on MS basal medium for three weeks. Buds were excised and cultured on MS medium supplemented with 1.0, 2.0, and 3.0 mg L^{-1} TDZ with 0.5 g L^{-1}NAA, respectively; 2.0 or 4.0 g L^{-1} BA with 0.5 g L^{-1} NAA; and 2.0 g L^{-1} CPPU with 0.5 g L^{-1} NAA. Each Petri dish was inoculated with four buds, six dishes per treatment. The bud explants were cultured under 16-h photoperiod provided by cool-white fluorescent tubes at a photon flux density of 8 µmol m^{-2} s^{-1}.

The second experiment was internode explants cultured on MS basal medium supplemented with 1.0, 2.0, and 3.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA, respectively and 4.0 g L^{-1} BA with 0.5 mg L^{-1} NAA. The explants were placed horizontally, four each on each medium-filled Petri dish with six dishes per treatment, which were cultured under 16-h photoperiod with a photon flux density of 8 µmol m^{-2} s^{-1}.

The third experiment was the culture of leaf and petiole explants on MS medium containing 1.0 and 2.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA, respectively. Sterilized leaf explants were placed with the adaxial surface up, and petiole explants were placed horizontally. There were four explants per Petri dish with six dishes per treatment for both leaf and petiole explants. Leaf and petiole explants were cultured under 16-h photoperiod with a photon flux density of 8 μmol m^{-2} s^{-1}.

The fourth experiment was the culture of bud explants on MS basal medium supplemented with 1.0, 2.0, and 3.0 mg L^{-1} TDZ with 0.5 mg L^{-1}NAA, respectively in the dark for 8 weeks. Again, there were four buds per dish with six dishes per treatment.

Data Collection and Analysis

The four experiments were arranged in a completely random design. Each Petri dish was considered an experimental unit, and each treatment had six replications. Explants that showed a response, such as swelling, formation of callus and/or globular structures, were recorded in each Petri dish, and frequencies of the responses were calculated. Data were subjected to analysis of variance (SAS GLM, SAS Institute, Cary, N.C.), and means separations were determined using Tukey's honest significant difference (HSD) at the 5% levels.

Results

Bud Explants

Fungal and bacterial contaminations were common to explants collected from the greenhouse-grown *P. micans*. Before the outlined four experiments, several tests had shown an intensive chemical sterilization, i.e. 1.0% sodium hypochlorite (16%, v/v) with 1 to 2 drops of Tween-20 and agitation on a rotary shaker for 30 min at 120 rpm, was required to minimize contamination. However, such sterilization often caused the

bleaching of young explants. Further tests showed that internodes and the node at or below the third leaf (counting back from the shoot tip) were more durable for the chemical sterilization.

The six combinations of growth regulators induced the expansion of lateral buds and production of callus and globular structures (Table 2-1). However, the frequencies in inducing bud expansion, callus, and globular structures varied significantly among treatments. The highest frequencies in producing swollen buds, callus, and globular structure were those explants induced by TDZ at 1.0, 2.0, and 3.0 mg L^{-1} with NAA at 0.5 mg L^{-1} NAA, respectively. Calli were formed from one side of bud explants (Figure 2-1A), and were yellowish and in friable form (Figure 2-1B) and eventually died. Globular structures were formed from the other side (Figure 2-1B). On the other hand, globular structures were green, in solid and nodular form, and were able to differentiate to produce shoots with roots (data not shown).

Table 2-1. Bud explants of *Philodendron micans* responding to in vitro culture on MS medium supplemented with TDZ, BA, or CPPU with NAA for 8 weeks under a 16 h light photoperiod at 40 µmol m^{-2} s^{-1}.

TDZ	NAA	BA	CPPU	Swollen explants (%)	Explants producing necrotic callus (%)	Explants producing globular structures (%)
1.0	0.5	0	0	83±5.3ab	33±5.3b	75±7.6a
2.0	0.5	0	0	92±5.3a	75±8.9a	54±9.8ab
3.0	0.5	0	0	96±4.0a	83±5.3a	42±5.3ab
0	0.5	4.0	0	47±5.1b	10±6.0b	42±4.0ab
0	0.5	2.0	0	41±6.0b	6.3±6.2b	36±10.0b
0	0.5	0	2.0	65±6.3b	55±4.8b	35±8.8b

Different letters within a column represent significant difference among treatments tested by Tukey's honest significant difference (HSD) at the 5% level.

Internode Explants

Table 2-2 show the response of internode explants to the three concentrations of TDZ with 0.5 mg L^{-1} NAA as well as 4.0 mg L^{-1} BA with 0.5 mg L^{-1} NAA. Many internodes failed to respond the induction (Figure 2-1A) or died (Figure 2-1B). The highest frequencies in explant expansion and callus formation occurred in those cultured on MS medium supplemented with 2.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ with 0.5 g L^{-1} NAA, respectively. The calli, however, quickly became yellow and died (Figure 2-1C). There was no occurrence of globular structures in all the treatments except for a low frequency among explants treated by 3.0 mg L^{-1} TDZ with 0.5 g L^{-1} NAA.

Leaf and Petiole Explants

Leaf and petiole explants were cultured on MS medium containing 1.0 and 2.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA. About 70% of cultured leaf and petiole explants were swollen (Figure 2-1 F, G, and I), but no leaf explants produced callus on medium supplemented with 1.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA and only 4% of leaf explants produced callus on medium containing 2.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA (Table 2-3; Figure 2-1H). Additionally, globular structures were not formed from leaf explants. There were 46% and 26% of petiole explants that formed callus when cultured on medium containing 1.0 or 2.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA (Figure 2-1J and K). However, the frequency for globular structures was only 8% from explants induced by 2.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA (Table 2-3).

Table 2-2. Responses of internode explants of *Philodendron micans* to in vitro culture on MS medium supplemented with TDZ or BA with NAA for 8 weeks under a 16 h light photoperiod at 40 µmol m^{-2} s^{-1}.

TDZ	NAA	BA	Swollen explants (%)	Explants producing necrotic callus (%)	Explants producing globular structures (%)
1.0	0.5	0	43±2.3ab	14±4.5b	0a
2.0	0.5	0	77±8a	15±8.5b	0a
3.0	0.5	0	31±2.8b	68±5.1a	5±4.9a
0	0.5	4.0	6.3±6.3c	10±6.3b	0a

Different letters within a column represent significant difference among treatments tested by Tukey's honest significant difference (HSD) at the 5% level.

Table 2-3. Responses of leaf and petiole explants of Philodendron micans to in vitro culture on MS medium supplemented with TDZ with NAA for 8 weeks under a 16 h light photoperiod at 40 µmol m^{-2} s^{-1}.

Explant	TDZ	NAA	Swollen explant (%)	Explant producing necrotic callus (%)	Explant producing globular structures (%)
Leaf	1.0	0.5	72±9.5a	0a	0a
	2.0	0.5	74±7.1a	4±3.9a	0a
Petiole	1.0	0.5	73±2.5a	46±5.5a	0a
	2.0	0.5	71±10.a	26±3.0a	8±5.2a
Explant significance			NS	**	NS
Treatment significance			NS	NS	NS

NS = No significant difference between explant types or treatments at P <0.05; ** = Significant difference at P< 0.01.
Different letters within a column represent significant difference among treatments tested by Tukey's honest significant difference (HSD) at the 5% level.

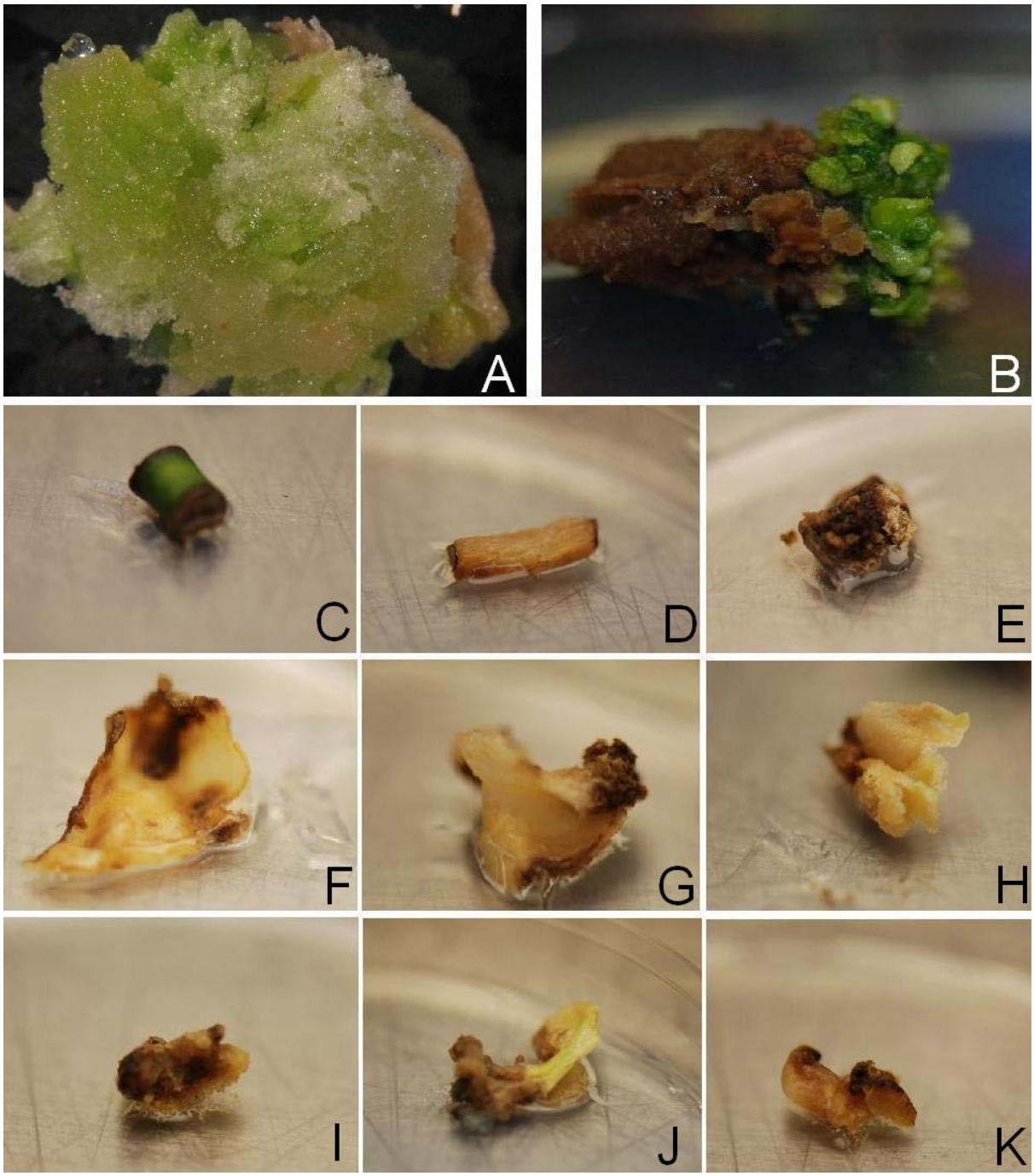

Figure 2-1. Different Philodendron micans explants were necrotic after 8 weeks of culture. Bud explants produced calli that were yellowish and friable (A) and one side of a bud explant produced calli that died and globular structures produced on the other side were alive (B). Internode explants did not respond to induction (C), died (D), and produced calli but died (E). Leaf explants became swollen (F and G) or produce calli (H), but died later. Petiole explants were swollen (I) and produced calli (J and K); the calli died after about 10 weeks.

Table 2-4. The frequency of swollen explants, necrotic callus, and formation of globular structures of bud, internode, leaf, and petiole expalnts cultured on MS medium regardless of growth regulator combinations or concentrations evaluated.

Explant type	Swollen explants (%)	Explants producing necrotic callus (%)	Explants producing globular structures (%)
Bud	90a	64a	75a
Petiole	71b	35bc	5b
Internode	49c	34c	2b
Leaf	73ab	2d	0b
Significance	**	**	**

** = Significant difference at $P < 0.01$.
Different letters within a column represent significant difference among treatments tested by Tukey's honest significant difference (HSD) at the 5% level.

Table 2-5. The frequency of swollen explants, necrotic callus and formation of globular structures produced by cytokinins of TDZ, BA, and CPPU regardless of the type of explants tested.

Cytokinin Type	Swollen explant (%)	Explant producing necrosis callus (%)	Explant producing globular structures (%)
TDZ	90a	64a	57a
BA	44c	8.15b	39a
CPPU	65bc	55ab	35b
Significance	**	**	NS

NS = No significant difference between explant types or treatments at P <0.05; ** = Significant difference at P< 0.01.
Different letters within a column represent significant difference among treatments tested by Tukey's honest significant difference (HSD) at the 5% level.

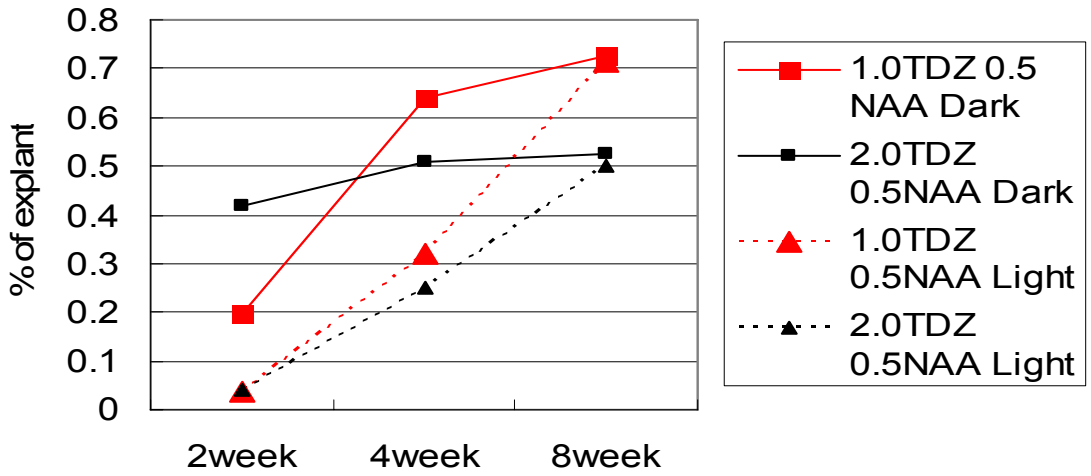

Figure 2-2. The frequency of globular structures formed under dark or 16 h light culture for 2, 4 and 8 weeks. There were significant differences between dark and light treatment with $P < 0.05$.

Bud Explants Cultured in the Dark

Bud explants cultured on MS medium containing 1.0 or 2.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA and held in the dark had significantly higher frequencies of globular structures 2 and 4 weeks after inoculation compared to those cultured under 16-h photoperiod with a photon flux density of 8 µmol m^{-2} s^{-1} (Figure 2-2). However, the frequencies for globular structure formation were similar between dark and light cultures for explants inoculated 8 weeks on the same growth regulator treatments. Additionally, the dark culture, like the light culture, produced both callus and globular structures.

Discussion

Initial investigation of *P. micans* showed that bud, internode, leaf, and petiole explants differed significantly in response to in vitro culture. Growth regulator combinations and concentrations resulted in different frequencies in callus and globular structure formation. In addition, the dark culture of bud explants greatly promoted the formation of globular structures at 2 and 4 weeks after culture.

Effects of Explants

Bud explants, regardless of growth regulator combinations or concentrations, exhibited the highest frequencies in explant expansion (90%), callus formation (64%), and production of globular structures (75%) in this study (Table 2-4). Internode and petiole explants had significantly lower frequencies in callus and globular structure formation. Leaf explants were inferior in formation of both callus and globular structures. The explant differences in response to in vitro culture could suggest that cells from internode, leaf, and petiole explants were less responsive to growth regulator induction. Another possibility could be that growth regulator combinations and/or their

concentrations screened were not optimal for induction of internode, leaf, and petiole explants.

Effects of Growth Regulators

With the NAA concentration used in the first three experiments set at 0.5 mg L^{-1}, TDZ was found to be a more effective cytokinin at inducing callus and globular structures, whereas BA produced globular structures and CPPU stimulated the most callus formation (Table 2-5). In addition there were two trends for bud explants: with an increase of TDZ concentration, callus formation frequency increased; meanwhile, the production frequency for globular structures decreased.

TDZ has also been found to induce callus in grape (Lin et al. 1989; Kartonmysheva et al. 1983), peanut (Gill 1999), orchid (Huan 2004), and several woody species (Huetteman 1993). However, there is little information concerning what type of callus was induced by TDZ. In the present study, the callus initiated was friable and loosely adhered on the surface of explants. The callus died after being transferred to a fresh medium. Continuing culture of explants with the callus, however, resulted in the death of explants. Therefore callus had to be removed from explants to secure survival of globular structures. Since the increased concentrations of TDZ enhanced callus formation, TDZ concentrations at or above 3.0 mg L^{-1} should be avoided in regeneration of *P. micans*.

Effects of Culture in the Dark

Maintaining cultures of explants in darkness enhanced the formation of globular structures during the first two to four weeks. After 2 weeks, most bud explants in the dark culture environment were swollen at the base of buds, which was followed by the appearance of light yellow or white globular structures (Fig 2-2). However, this

enhancement diminished once cultures approached 8 weeks in the dark (Figure 2-2). It is unknown if an initial 4-week dark culture followed by culture under lighted conditions would accelerate the occurrence of globular structures. Future research to determine the effect of this proposed practice is warranted.

Although the exact mechanism underlying dark-culture mediated organogenesis is not completely understood, dark treatments were reported to promote adventitious shoots and embryo-like structure (Welander 1988), somatic embryogenesis (Ozias-Akins 1983), as well as an increase in auxin and ethylene in the explants (Huxter 1981). Microscopic examination comparing dark and light cultured bean (*Phaseolus vulgaris*) showed more parenchyma cells and a higher proportion of less differentiated tissue in plantlets grown in the dark compared to the light (Herman and Hess 1963). In this study, where the globular structures observed consisted of parenchyma cells in the center area of bud explants, the dark conditions could stimulate the regeneration of those parenchyma cells and accelerate the formation and enlargement of globular structures.

CHAPTER 3
ESTABLISHMENT OF AN EFFECTIVE PROTOCOL FOR REGENERATION OF *PHILODENDRON MICANS*

Introduction

The previous study in Chapter 2 demonstrated that TDZ was effective in induction of globular structures in *P. micans*, but it was uncertain whether the globular structures were somatic embryos or PLBs. The use of TDZ in regeneration of dicot plants has been widely reported (Huetteman and Preece 1993; Lu 1993; Murthy et al. 1998). The success in regeneration of *P. micans* was still one of a few examples of globular structures induced by TDZ in monocot plants. Other regenerated monocots by TDZ or TDZ with auxins include wheat, barley, *Epipremnum*, (Qu et al. 2002; Zhang et al. 2005), *Phalaenopsis* (Shan et al. 2000; Chen et al. 2000), and *Syngonium* (Zhang et al. 2006).

TDZ is a plant growth regulator with cytokinin-like activity (Zhang et al., 2001; Landi and Mezzetti 2006), but Victor (1999) claimed that changes induced via TDZ undergo a different morphological route of development than that those induced by purine-cytokinins. TDZ has been shown to provide sufficient stimulus for induction of somatic embryos or can substitute for the combined auxin and cytokinin requirements in a variety of plant species, including peanut, tobacco, and geranium (Gill and Saxena 1992, 1993; Visser et al. 1992). Additionally, TDZ can promote the accumulation of purine cytokinins (Thomas and Katterman 1986) and/or the inhibition of cytokine oxidase activity (Kaminek and Armstrong 1990), which may also enhance its effectiveness in somatic embryogenesis. Preece et al. (1991) showed that TDZ was effective at concentrations as low as 10 pM and stimulated regeneration under a relatively short period exposure (Visser et al. 1992; Hutchinson and Saxena 1996). In

the present study, a lower concentration of TDZ (1 mg L^{-1}) was better on inducing globular structures of *P. micans* compared with other cytokinins studied.

The present study was intended to further evaluate TDZ effects on in vitro culture of bud explants of *P. micans*. The objectives were to identify the optimal concentration of TDZ and NAA in producing globular structures, to investigate the capability of globular structures for plantlet production, and also to perform histological analysis to determine whether the globular structures were PLBs or somatic embryos.

Materials and Methods

Plant Materials and Explants

Stems, 3rd lateral bud and below and 10-15 cm in length with lateral buds, were cut from the stock plants of P. micans grown in the shaded greenhouse. Leaves and roots around the nodes were removed. The stems were immersed in 70% ethanol for 1 min by agitation. Nodal explants, 10 mm in length, were cut in sterile Petri dishes and placed in a sterilized bottle containing 100 ml solution of 1.0% sodium hypochlorite (16%, v/v) and 1 to 2 drops of Tween-20 added as a surfactant. The bottle with explants and Clorox solution was agitated on a rotary shaker table for 30 min at 120 rpm. To rinse the Clorox solution, the explants were immersed in 100 ml sterile distilled water and agitated for 5 min on the shaker table at 120 rpm. Rinsing was repeated twice.

Culture Medium and Conditions

MS mineral salts and vitamins with 2.8% (w/v) sucrose and 0.66% (w/v) agar (PhytoTechnology Laboratories, Shawnee Mission, KS) were used as a basal medium. The pH of the medium was adjusted to 5.8 with 1 M NaOH before autoclaving at 121oC for 25 min. TDZ and NAA stock solutions were filter-sterilized and added to autoclaved basal medium when the temperature dropped to 50oC. The medium was then poured in

100 x 15 mm sterile Petri dishes (Fisher Scientific, Inc., Pittsburgh, PA) with 20 mL each, which was used as basal medium for inducing lateral bud growth. Another batch of MS medium supplemented with TDZ at 0.5, 1.0, and 2 mg L^{-1} with NAA at 0.0, 0.5, and 1.0 mg L^{-1}, respectively were used for culture of bud explants for globular structure induction. Additional treatments included MS basal medium without growth regulator or containing 3.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA for culture of bud explants. Thus, there was a total of 11 treatments for inducing globular structure using bud explants.

Nodal explants were cultured under 16-h photoperiod provided by cool-white fluorescent tubes at a photon flux density of 8 µmol m^{-2} s^{-1} with a temperature of 25 ± 2 °C. Three weeks after culture, lateral buds had grown to approximately 0.5 mm in length and were excised from nodes. After cutting bud tips off, the bud explants were cultured on the same MS medium with the respective 11 treatments. There were four bud explants per Petri dish and six dishes per treatments. Four weeks later, the bud explants were subcultured on the same medium with respective treatments. Data including survival rate, callus formation, and globular structures, and shoots numbers were recorded 8 weeks after bud culture.

Root Development and *Ex Vitro* Plantlet Establishment.

Shoots produced from globular structures were able to produce roots in MS medium supplemented with TDZ and NAA. For better shoot growth and rooting, globular structures with shoots were transferred to baby food jars containing MS basal medium without growth regulators or MS medium supplemented with 0.5 mg L^{-1} IBA. Regenerated plantlets were separated, washed free of agar using tap water, and transplanted into a soilless medium with sphagnum peat, vermiculate, and perlite at a 2:1:1 ration based on volume. Potted plants were grown in a shaded greenhouse under

a maximum photosynthetic photon flux density of 200 µmol m^{-2} s^{-1}, temperature range of 20 to 28^{o}C, and ambient relative humidity of 70 to 90%. Survival rates of plantlets in the shaded greenhouse were recorded two months after transplanting.

Histological Analysis

To determine the identity of the globular structures, bud culture samples were collected at different growth periods for histological examination. Globular structures, varying in size, were fixed in FAA solution (formalin: glacial acetic acid: 70% ethanol at 5:5:90 by volume) for 3 days. Samples were dehydrated in a series of ascending aqueous ethanol solutions at 70%, 80%, 95% for 2 hours each, and at 100% for 1 hour twice. To increase the transparency, samples were treated with a xylene:ethanol (1:1 by volume) solution for 2 hours, followed by pure xylene for 1 hour. The specimens were infiltrated by moving them into a vessel that contained paraffin wax maintained in 40-56 °C until complete saturation. Finally, the specimens were embedded in the paraffin wax for at least 24 h. Sections (10 µm) were cut using a rotary microtome, and mounted on glass slides. The sections were de-waxed in xylene, xylene:ethanol (1:1 by volume), 100%, 95%, 85%, 70% ethanol solution, stained with Heindenhain's haematoxylin and then covered by the cover slips with a drop of neutral balsam before examination under microscope. All sections were observed under a Nikon OPTIPHOT microscope and photographed using a Canon S3 IS digital camera.

Experimental Design and Data Analysis

All experiments were established in a completely randomized design with six replications. The frequency of bud explants in response to different concentrations of TDZ and NAA in the formation of globular structures was analyzed by SAS (SAS Inc. 1999). Mean separation was achieved by least significant difference (LSD) test at 95%

level. Additionally, a multiple linear regression model was developed for prediction of the frequency of globular structure formation from *P. micans* when TDZ and NAA were used for induction of bud explants.

Results

Induction of Globular Structures

Buds sprouted from nodal explants and reached a length about 0.5 cm three weeks after culture on MS basal medium. Eight weeks after the buds were cultured on MS medium supplemented with TDZ and NAA under 16 h lighting, dark green and globular structures were initiated at the basal of bud explants (Figure 3-1A). Those globular structures had a solid surface that differed from callus and more closely resembled somatic embryos as they could be removed as individuals from explants (Figure 3-1B). PLBs differentiated to form shoots (Figure 3-1C-F). TDZ was necessary for formation of globular structures. TDZ at 0.5, 1.0, and 2.0 without NAA resulted in 37, 58, and 46% of bud explants producing globular structures, respectively (Table 3-1). However, the highest frequency in globular structure formation was 75%, which occurred in MS medium supplemented with 1.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA. A frequency of 67 and 62% occurred in MS medium containing 2.0 and 1.0 mg L^{-1} TDZ with 1.0 mg L^{-1} NAA, respectively. Similar to the results in Chapter 2, bud explants showed increased frequencies in callus formation when cultured with elevated concentration of TDZ with 0.5 mg L^{-1} NAA. The calli had to be removed; otherwise, the browning of calli caused death of globular structures.

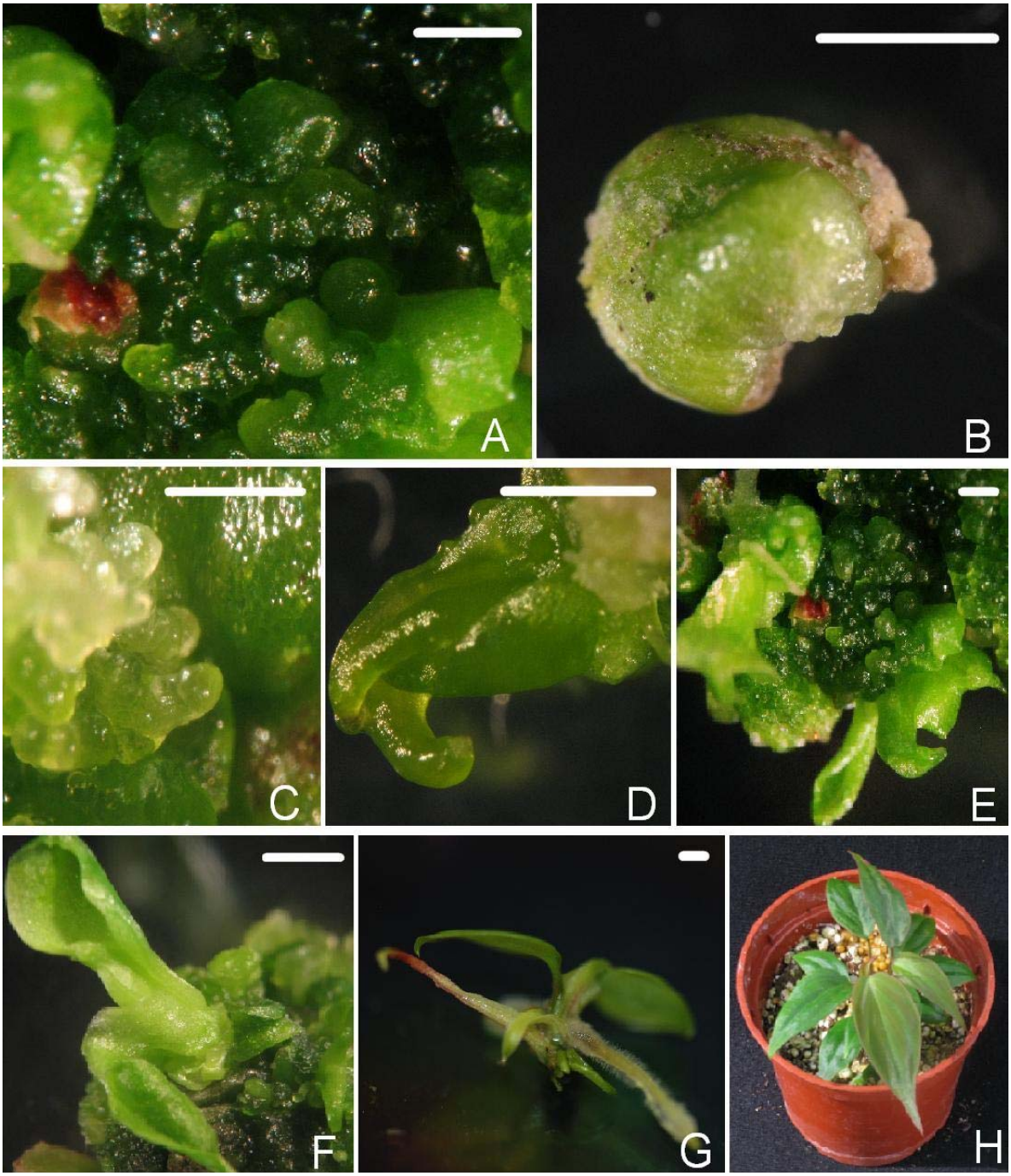

Figure 3-1. Dark green and globular structures were initiated at the basal of bud explants (A); PLBs were removable as individuals from explants (B); differentiation of PLBs produced shoots (C-F), shoots produced roots but the shoot and root were not in the same axis (G); and regenerated plantlets grown in soilless potting medium in a shaded greenhouse with a survival rate at 98%(H). Bar=2mm

Table 3-1. Effect TDZ and NAA at different concentrations on explant survival, production of necrotic callus, induction of protocorm-like bodies (PLBs) and shoots from Philodendron micans buds cultured on MS medium for 8 weeks in a 16 h light photoperiod at 40 µmol m^{-2} s^{-1}.

TDZ	NAA	Explants survived (%)	Explants producing necrosis callus (%)	Explant producing PLBs (%)	Shoot numbers per explant
0.0	0.0	95±3.9	0±0f	0±0c	-
0.5	0.0	79±3.2	13±8.5ef	37±5.6b	+
0.5	0.5	88±5.7	17±5.3ef	54±7.7ab	++
0.5	1.0	92±5.3	50±6.5bcd	50±6.5ab	++
1.0	0	83±8.2	20±4.2def	58±5.3ab	++
1.0	0.5	92±5.3	33±5.3cde	75±9.1a	+++
1.0	1.0	83±8.2	87±5.6a	62±8.5ab	+++
2.0	0	79±7.8	33±8.3cde	46±7.7ab	+++
2.0	0.5	75±9.0	75±9.1ab	54±7.7ab	+++
2.0	1.0	96±4.1	63±10.7abc	67±5.3ab	+++
3.0	0.5	67±8.3	83±5.2a	42±5.3b	+++
TDZ		NS	**	**	**
NAA		NS	**	*	**
TDZ*NAA		NS	**	NS	NS

NS = No significant difference between explant types or treatments at $P < 0.05$; * = significance at $P < 0.05$ level; and ** = significant difference at $P < 0.01$.
Different letters within a column represent significant difference among treatments tested by Tukey's honest significant difference (HSD) at the 5% level.
+ indicates <20 plantlets, ++ 20-50 plantlet, +++ >50 plantlet per explant

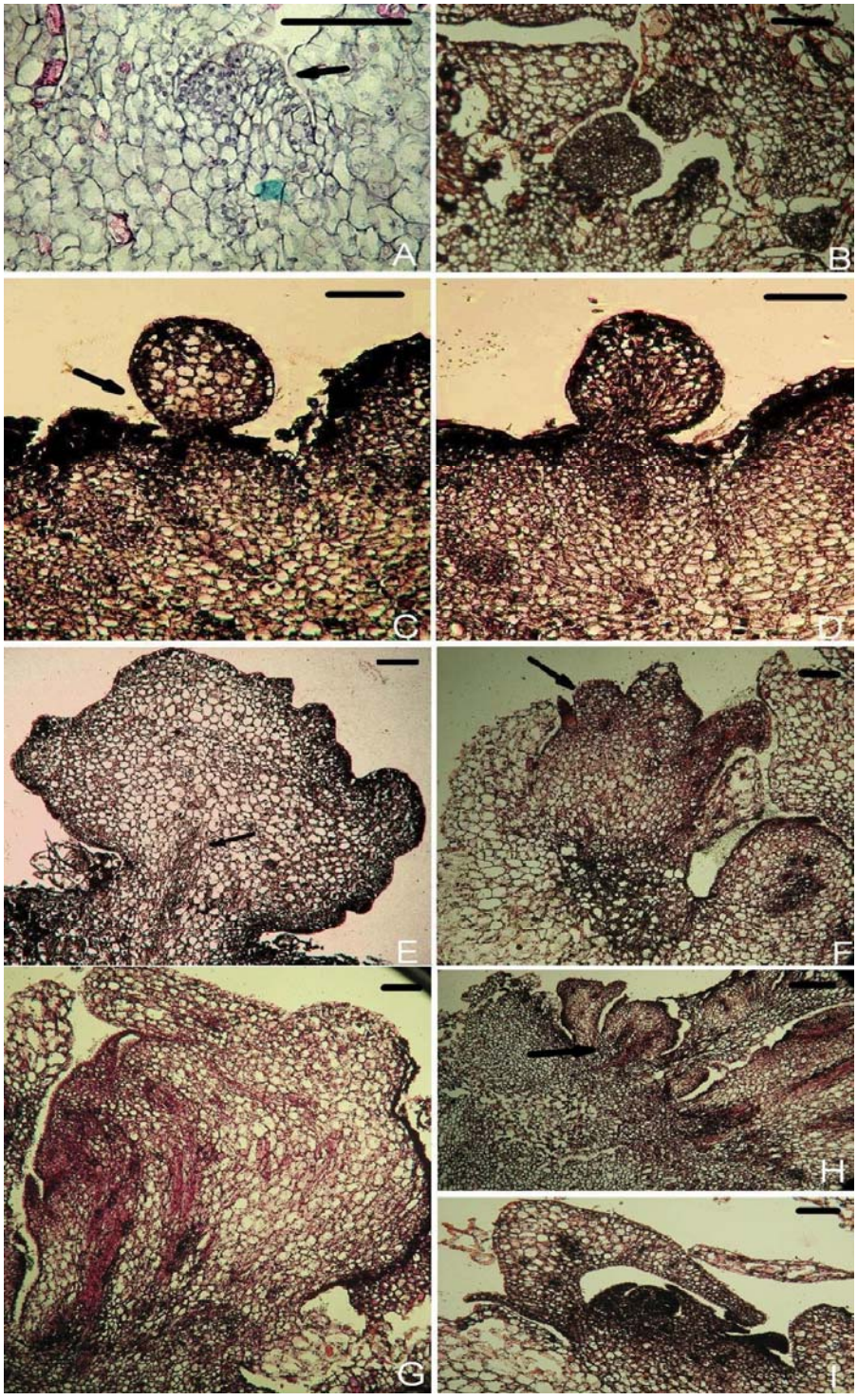

Figure 3-2. Histological analysis of the formation of PLBs. Cell differentiation (A) and formed globular structures (B); the globular structure resembled somatic embryos (C), but there was vascular connection between explants and the globular structure (D); development of PLB produced multiple shoot meristems (E-I). Bar=100μm.

A Model for Predicting Globular Structure Formation

A multiple linear regression model: $Y = 0.284x_1 - 0.43x_2 - 0.18x_1/x_2 + 0.91$, was developed. Where Y was the frequency of globular structure, x_1 was TDZ concentration, and x_2 was NAA concentration and should not be equal to 0. This model revealed the relationship between TDZ and NAA concentrations influencing the frequency of the formation of globular structures with the significance at $P < 0.10$.

Histological Analysis

The globular structures were first observed from bud explants on MS medium supplemented with TDZ and NAA after 28 days of culture. There were generally three types of cells observed under light microscopy: regenerative cells which were smaller in size and compact with more densely stained cytoplasm and more prominent nuclei; parenchyma cells which were larger with thin cells and less compact; non-regenerative smaller cells with thick cell walls and less stained cytoplasm (Figure 3-2A). Cell differentiation resulted in the formation of formed globular structures (Figure 3-2B); the globular structure resembled somatic embryos (Figure 3-2C), but there was vascular connection between explants and the globular structure (Figure 3-2D), suggesting they were PLBs. The meristematic mass, derived from those regenerative epidermal cells, formed several more meristematic domes that represented apical meristems after a few days (Figure 3-2E-I).

Shoot and Root Formation and *Ex Vitro* Transplanting

After 12 weeks of culture on MS medium supplemented with TDZ and NAA, shoots appeared from PLBs (Figure 3-2). Shoot numbers per explant varied depending on TDZ and NAA concentrations (Table 3-1). Explants with more than 50 shoots were

those cultured on MS medium at 1 mg L^{-1} TDZ with 0.5 or 1.0 mg L^{-1} NAA; 2 mg L^{-1} TDZ with 0, 0.5, or1.0 mg L^{-1} NAA, and 3.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA.

Shoots produced roots on MS medium with TDZ and NAA, also on MS medium with 0.5 mg L^{-1} IBA or without growth regulators. The roots were initiated from the base of the shoots. However, the shoot and root were not on the same axis (Figure 3-2). Plantlets were easily acclimatized (Figure 3-2). The survival rate in soilless medium was 98%.

Discussion

PLB Occurrence

The globular structures formed from bud explants were initially thought to be somatic embryos. But histological analysis showed that the structures clearly had a vascular connection with explants. Additionally, no polar structures were identified. These results suggest the globular structures were not somatic embryos. The globular structures were also different from callus, as they were solid with a smooth surface and once individual globular structures removed from explants were able to produce shoots when cultured on MS basal medium without growth regulators. On the other hand, PLBs are similar to somatic embryos but with vascular connection with explants (Norstog 1979; Cui et al., 2008; Tian et al. 2008). PLBs were able to produce more than 50 plantlets, and the plantlets produced roots. Thus, histological analysis along with the characteristics in regeneration of plantlets suggested the globular structures were actually PLBs formed from bud explants of *P. micans*.

PLB Induction by TDZ

It is interesting to note that TDZ alone was able to induce PLBs at a frequency of 46% when bud explants were cultured on MS containing TDZ at 2.0 mg L^{-1}. This could

be additional evidence supporting that TDZ can substitute for the combined auxin and cytokinin requirements of morphogenesis. TDZ alone has been demonstrated to induce somatic embryogenesis in a variety of plant species, including peanut, tobacco, and geranium (Gill and Saxena 1992, 1993; Visser et al. 1992).

The highest frequency in PLB formation in *P. micans*, however, occurred in bud explants induced by TDZ in combination with NAA. The concentrations and the ratios of TDZ and NAA appeared to be significantly important (Table 3-1). However, a simple increase or decrease of TDZ or NAA did not result in a linear increase of PLB formation frequency. Thus, a model was developed for describing their relationships. It reveals that PLB formation frequencies will increase with increased concentrations of TDZ but will be limited by NAA concentrations and TDZ and NAA ratios. Conversely, PLB formation frequencies will increase with decreased concentrations of NAA but will be affected by TDZ concentrations and TDZ and NAA ratios. Based on the average value inequality, the frequency of PLBs would be maximized when 0.43 x TDZ concentration = 0.18 x TDZ/NAA ratio, which resulted in the value of TDZ/NAA^2 is equal to 2.4. Although significance of the developed equation requires further testing, it suggests that for high frequency of PLB induction, it is essential to control the concentrations of TDZ and NAA and keep their ratio in appropriate ranges.

The Significance of the Established Regeneration System

As mentioned previously, efficient regeneration systems for *Philodendron* have not been well established. This is the first established regeneration system for *Philodendron* and it is through PLBs. Nodal explants were cultured on MS basal medium to produce bud explants in three weeks; bud explants then cultured on MS medium supplemented with 1.0 mg L^{-1} TDZ with 0.5 mg L^{-1} NAA resulted in PLB formation in 4-8 weeks, and

PLBs produced multiple shoots in another 4 weeks. More than 50 plantlets were produced from single explants. The resultant plantlets were readily grown in soilless substrate with 98% survival rate. Thus, this established system can be used for effective micropropagation of *P. micans*. Additionally, this protocol could also be readily used for stable genetic transformation (Chai et al. 2002), mass multiplication using bioreactors or synthetic seed production (Ara et al. 2000; Young et al. 2000), and for cryopreservation as described for orchids (Nikishina et al. 2007).

CHAPTER 4
ANALYSIS OF REGENERATED *P. MICANS* USING DNA FLOW CYTOMETRY

Introduction

P. micans after being established in soilless media as potted plants were morphologically and phenotypically stable and were identical to the parent plant and exhibited elongated internodes with velvet foliage. This suggested that regeneration through PLBs, similar to the regeneration through direct somatic embryogenesis, can produce plants with genetic stability. This stability is attributed in part to the fact that the formation of PLBs had no intervening callus phase . As discussed previously, although callus was formed from bud explants, it was independent from PLB formation. The callus had no capability for organogenesis and had to be removed for PLB proliferation. As a result, regenerants through PLBs exhibited little somaclonal variation.

Recent evidence, however, has shown that direct somatic embryogenesis can have a multi-cellular origin that may cause chromosomal alterations and ploidy changes (Endemann et al. 2001; Tremblay et al. 1999; Wilhelm 2000). Although it was unclear whether the PLBs of *P. micans* had resulted from single cells or multiple-cellular origin, an attempt to examine the ploidy level was important. However, due to the small size of chromosomes in *P. micans* (Marchan 1970), chromosome counting could be difficult.

Recently, DNA flow cytometry analysis has been shown to be a quick and effective method for determining ploidy levels (Johnston et al. 1999; Dolezel and Bartos 2005) including regenerated plants (Cui et al. 2009). The objective of this study was to determine the ploidy levels of randomly selected regenerated *P. micans* plants using DNA flow cytometry.

Materials and Methods

A stock plants of *P. micans* grown in the shaded greenhouse and 20 regenerated young plants were collected. Young leaves of the 21 plants were chopped and analyzed using a PARTEC PA cytometer (Partec GmbH, Münster, Germany) based on the procedures described below. How many of each plant Young leaves of each plant were chopped with a new razor blade in a Petri dish containing 0.5 ml ice-cold Partec CyStain® UV Ploidy solution (Partec GmbH, Münster, Germany) supplemented with 0.1% mercaptoethanol and 2.0% polyvinylpyrrolidone. An additional 1.5 ml of the same solution was added to the Petri dish and incubated on ice for 2 minutes. The suspension was filtered through a Partec 50 µm CellTrics disposable filter and analyzed using the PARTEC PA cytometer for determining the mean sample nuclei fluorescence intensity. The DNA histograms of nuclei from stock leaf tissue of *P. micans* were compared to those of the 20 regenerants to determine the possible occurrence of polyploidy.

Results and Discussion

P. micans has small chromosomes and a diploid number that has been reported as $2n = 32$ (Marchan 1970). Flow cytometry analysis showed stock plants and 18 out of the 20 regenerated plants had a single peak, indicating that there was no ploidy variation among the 18 plants. However, plants numbered 9 and 16 showed two peaks, which meant they were mixoploids. Since the two mixoploid plants are still young, just potted in a soilless medium, their morphological characteristics will be determined when they become mature. The histograms of the stock plants, and regenerated plants number 9, 16, and 22 are presented in Figure 4-1. Flow cytometric analysis indicated that 10% of the regenerated plants had a ploidy change which is a high percentage of chromosome variation among the regenerated population.

Such a high percentage of regenerated plants with chromosome alternation is surprising considering the fact the plants were regenerated from PLBs that had no intervening callus stage. Somaclonal variation therefore should be theoretically lower compared to regeneration through indirect shoot organogenesis although prolonged time in culture has also been suggested to increase the incidence of polyploidization (Endemann et al. 2001). The duration in regeneration of *P. micans* from bud explants to plantlets only required 12 weeks. It is unlikely that such a short duration could cause this high variation rate.

Another likely possibility could be due to the pre-existing somatic variation. *P. micans* does not flower under production or interiorscape settings and its commercial propagation is through eye cuttings. Continuous vegetative propagation of highly heterozygous plants may allow an increase in variation through the accumulation of somatic mutations because of the missing meiotic sieve (Buss 1983). In vitro regeneration differs from traditional vegetative propagation by allowing single or a few cells from pieces of explants to differentiate in vitro and develop into plantlets, which provides a greater chance of uncovering mutated cells.

This study demonstrated that chromosome alternation occurred in the regenerated population of *P. micans*. Further evaluation of the regeneranted plants may reveal that some may have a ploidy level such as tetraploidy and hexaploidy. Polyploidization is often associated with morphological changes such as robust growth, larger and thicker leaves, and resistance to biotic and abiotic stresses (Levin 1983). These desired traits could expand genetic diversity and thus can be used for new cultivar development of *P. micans.*

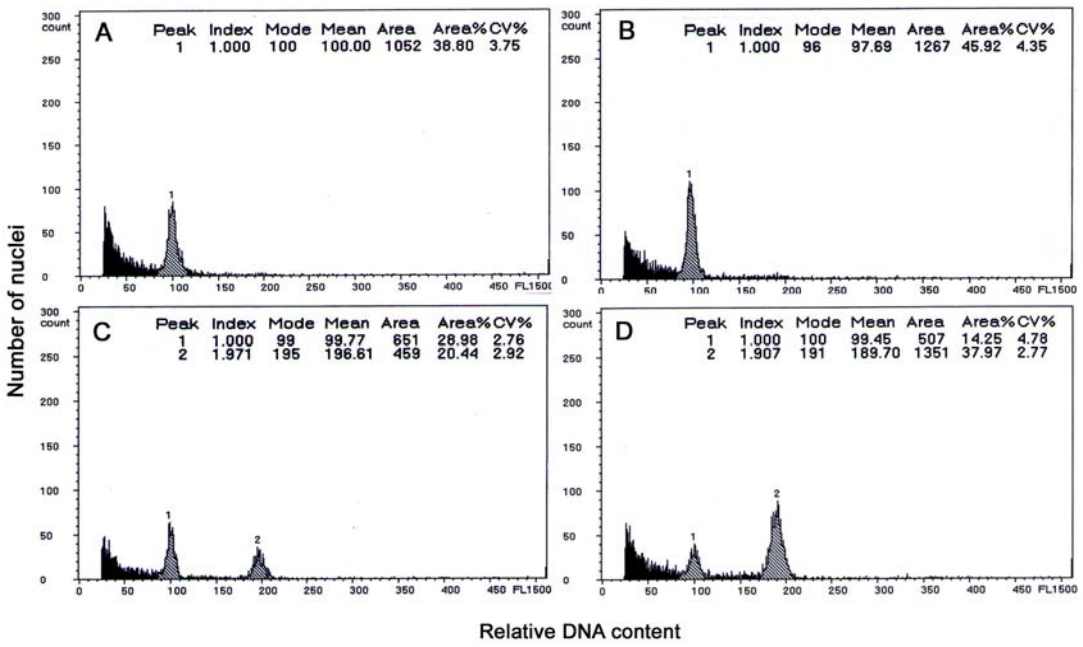

Figure 4-1. Histograms of relative fluorescence intensity obtained through PARTEC PA Flow Cytometer. The peak represents nuclei at G_1 phase of *P. micans* stock plant (A), regenerated plants No. 19 (B), No. 9 (C), and No. 16 (D).

CHAPTER 5
APPLICATION OF PLANT GROWTH REGULATORS FOR IMPROVING GROWTH FORM OF *P. MICANS*

Introduction

Tissue culture plantlets often have desirable growth habits when compared to plants propagated by standard methods. For example, *Anthurium*, *Dieffenbachia*, *Spathiphyllum*, and *Syngonium* develop multiple basal shoots when grown from tissue culture, exhibit more compact growth and produce finished plants that are fuller than plants produced by other methods (Conover 1985). However, *P. micans* plants regenerated through PLBs maintained their leggy growth style with elongated internodes after being produced in soilless potting medium in a shaded greenhouse, which suggests that additional breeding efforts, such as hybridization or creation of induced mutations, will be needed for improving the growth style of *P. micans*.

An alternative approach for reducing internode length is to use antigibberelin growth regulators since gibberellins promote stem elongation. In the floriculture industry, plant growth retardants have been used as a foliar spray or soil drench (Davis 1987). Bonzi® or Paclobutrazol [β-(4-chlorophenyl)methyl-α-(1,1-dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol] has been shown to control the height of *Caladium* x *hortulanum* Bird., *Codiaeum variegatum* (L.) Blume, *Schefflera actinophylla* Endl., *Euphorbia pulcherrima* Wind., and *Impatiens wallerana* (L.) Hook. f. (Barrett et al. 1994) as well as *Ficus. benjamina* (Barrett and Nell 1983). B-Nine® or daminozide ; [butanedioic acid mono (2,2-dimethylhydrazide] was used to reduce the height of *Mussaenda* L., a tropical ornamental shrub (Cramer and Bridgen 1998). Plant Height suppression was reported in Canna lily (*Canna* x *generalis* 'Florence Vaughan') by B-Nine® application and Cycocel® [(2-chlorethy) trimethylethanaminium chloride] (Bruner et al. 2001).

Application of foliar spray of Florel® [(2-chloroethyl) phosphonic acid] reduced internode elongation of *Gynura aurantica* (Blume) DC (Chen et al. 2002).

The study in Chapter 5 was intended to determine if growth regulators Bonzi®, B-Nine®, Cycocel®, and Florel® applied to *P. micans* as a foliar spray or as a soil drench could suppress internode elongation and improve the growth form of *P. micans.*

Materials and methods

Plant Material and Plant Growth Condition

Single node cuttings of *P. micans*, 4 to 5 cm in length, were rooted in 15-cm diameter pots filled by Fafard 2 Mix (Conrad Fafard Inc., Apopka, FL) at 5 cuttings each. Cuttings were grown in a shaded greenhouse under a maximum light level of 284 µmol m^2 s^{-1}, a temperature range of 20-28 C°, and a relative humidity of 60-100%. Three weeks after rooting, each pot received 5 grams of a 15N-7P_2O_5-15K_2O controlled-release fertilizer, Multicote with an 8-month longevity at a temperature of 21 °C (Haifa Chemicals Ltd., Haifa Bay, Israel). Plants were watered one or two times per week as needed. One month later, uniform 3 rooted cuttings were selected from the original 5 and the other 2 cuttings were pulled and discarded. The vines of selected plants were 20-25 cm in length.

Growth Regulators and Their Application

A total of 250 potted *P. micans* were prepared. The plants were subjected to either foliar spray or drenching treatment on August 3, 2009. Solutions of daminozide (B-Nine® SP, Uniroyal Chemical Co., Middlebury, CT) at 1,250, 2,500, and 5,000 mg L^{-1}; paclobutrazol (Bonzi®, Uniroyal Chemical Co.) at 15, 50, and 100 mg L^{-1}; chlormequat chloride (Cycocel®, Olympic Horticultural Products, Mainland, PA) at 500, 1,000, and 2,000 mg L^{-1}; and ethephon (Florel®, Monterey Lawn and Garden Products, Fresno, CA)

at 250, 500, and 1,000 mg L^{-1} were made using deionized water. The solutions, along with a deionized water as a control, were immediately foliar sprayed until runoff, approximating 25 mL per plant.

Another group of the plants were subjected drenching treatment on the same day. Solutions of daminozide (B-Nine®) at 625, 1250, and 2500 mg L^{-1}; paclobutrazol (Bonzi®) at 7, 15, and 50 mg L^{-1}; chlormequat chloride (Cycocel®) at 250, 500, and 1000 mg L^{-1}; and ethephon (Florel ®) at 125, 250, and 500 mg L^{-1} were prepared using deionized water. The solutions were applied as soil drenches at 125 mL per pot.

After both foliar spray and soil drenching, a hole was punched in the youngest leaf of each vine. Four weeks later, the same treatments, spray and drenching, were repeated to the same plants. Immediately following the second treatment, the shoot length from the marked leaf to the tip, the number of nodes from the marked leaf to the tip, largest leaf length and width were measured and the average stem length for each vine were calculated.

The experiment was arranged in a completely randomized design with 10 replications for each treatment. Internode length, the largest leaf length and width were recorded one month after the treatments. Data were analyzed by analysis of variance (SAS Institute 1999), and means separations were determined using Tukey's honest significant difference (HSD) at the 5% level. Additionally, data between growth regulator treated plants were compared to those of control plants using Tukey's Studentized Range at $P < 0.05$.

Results

Effects of Growth Regulator Foliar Spray

Internode lengths of plants sprayed by growth regulators regardless of types and concentrations significantly varied from the control plants (Table 5-1). However, among the growth regulator treated plants, there were no significant differences in internode length except for those sprayed by Florel® at 500 and 1000 mg L^{-1}. Internode length were 5.3 and 4.3 cm respectively for the two concentrations compared to 8.8 cm of the control plants. Foliar spray of Florel® at concentrations of 500 and 1000 mg L^{-1} resulted in defoliation. Leaf length and width were significantly reduced by the sprays of Florel® at 250 mg L^{-1}. Additionally, shoot tips of vines sprayed by Florel® grew upright compared to the other all other treated and control plants which grew horizontally. In general, there was a slight reduction in leaf size for *P. micans* plants sprayed with growth regulators, but the reduction was not significant (Figure 5-1, Table 5-1).

Effects of Growth Regulator Soil Drenching

Similar to the results of foliar spray, internode lengths of *P. micans* plants drenched with growth regulators differed significantly from the control plants (Table 5-2). There was some variation in internode length among all growth regulator drenched plants, however, the variation was non-significant except the plants drenched with 50 mg L^{-1} Bonzi® whose internode lengths were 6.4 compared to 8.8 cm of the control plants. Contrary to foliar spray, soil drenching of Florel®did not result in defoliation of plants; and soil drenched with Florel® did not induce upright growth of shoot tips. Leaf length and width were decreased by soil drenching treatments, but the effects were not significant except for those treated by Cycocel® at 1000 mg L^{-1} and Florel® at the three concentrations (Figure 5-2, Table 5-2).

Table 5-1. Average internode length, largest leaf length and leaf width of *Philodendron micans* after foliar spraying with B-nine®, Bonzi®, Cycocel®, and Florel® for one month.

Growth regulator	Concentration (mg L^{-1})	Internode length (cm)	Largest leaf length (cm)	Largest leaf width (cm)
Control	0	8.8	9.6	5.0
B-nine®	1250	7.1a*	8.8a	4.9
	2500	7.0a*	8.7a	5.0
	5000	6.4a*	8.9a	5.15
Bonzi®	15	7.6a*	8.7a	4.6
	50	7.4a*	9.2a	4.7
	100	6.4a*	9.0a	4.7
Cycocel®	500	7.7a*	8.5*	4.5
	1000	7.2a*	9.1	4.8
	2000	7.4a*	9.2	4.9
Florel®	250	7.2a*	6.6*	3.3*
	500	5.3b*	-	-
	1000	4.3b*	-	-

* Indicates significant difference between growth regulator treated plants and control plant tested by Tukey's Studentized Range at $P < 0.05$; -,
Different letters within a column represent significant difference among treatments tested by Tukey's honest significant difference (HSD) at the 5% level.

Table 5.2. Average internode length(AIL), the largest leaf length (LLL) and width wildth (LLW) of *Philodendron micans* after soil drenching of B-nine®, Bonzi®, Cycocel®, and Florel®.

Growth regulator	Concentration (ppm)	AIL(cm)	LLL (cm)	LLW(cm)
Control	0	8.8	9.6	5.0
B-Nine®	625	8.1a*	8.4*	4.3
	1250	8.2a*	8.6	4.5
	2500	8.3a*	8.6	4.3
Bonzi®	7	7.5a*	9.4	4.8
	25	6.8ab*	9.3	4.6
	50	6.4b*	8.7	4.7
Cycocel®	250	7.1a*	9.0	4.6
	500	7.0a*	9.2	5.0
	1000	6.7a*	8.5*	4.2*
Florel®	125	6.9a*	8.3*	4.3a*
	250	6.9a*	8.0*	4.3a*
	500	7.2a*	8.3*	4.2a*

* Indicates significant difference between growth regulator treated plants and control plant tested by Tukey's Studentized Range at $P < 0.05$; -,

Different letters within a column represent significant difference among treatments tested by Tukey's honest significant difference (HSD) at the 5% level.

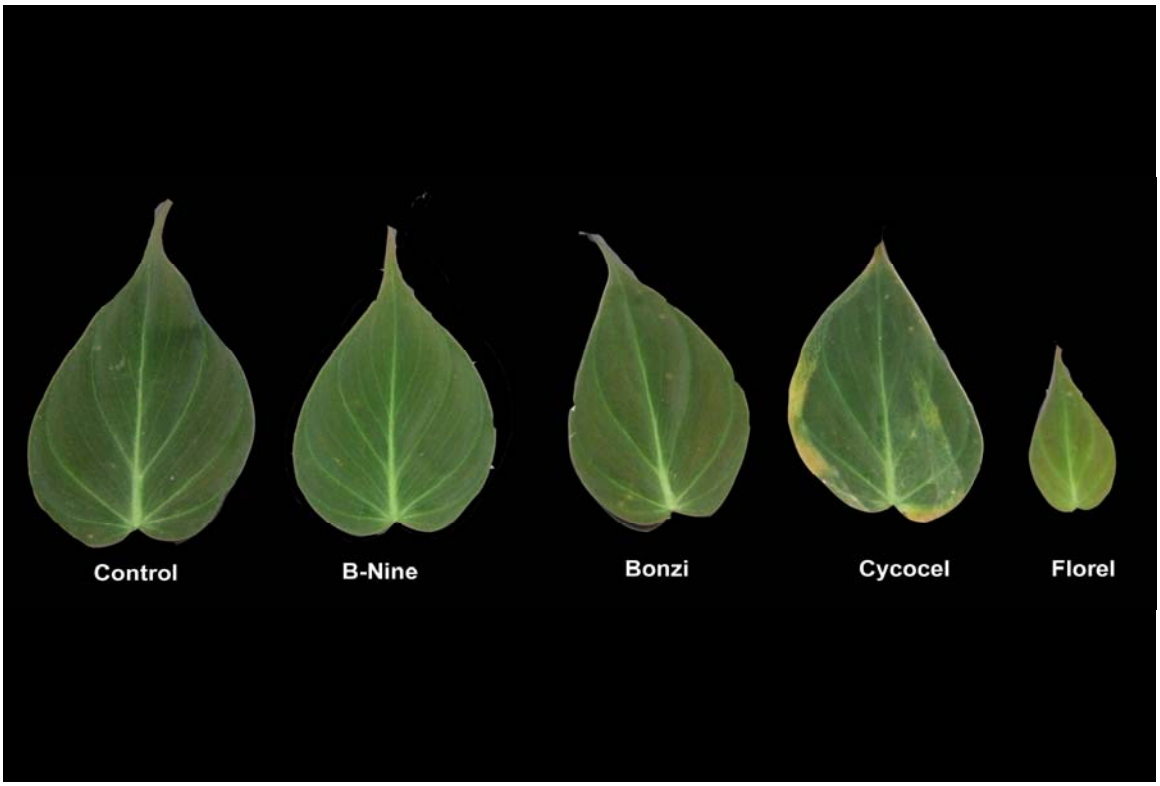

Figure 5-1. The largest leaf size for *P. micans* one month after one-time spraying with water, B-Nine® at 5000 mg L^{-1}, Bonzi® at 100 mg L^{-1}, Cycocel® at 2000 mg L^{-1}, and Florel® at 1000 mg L^{-1}.

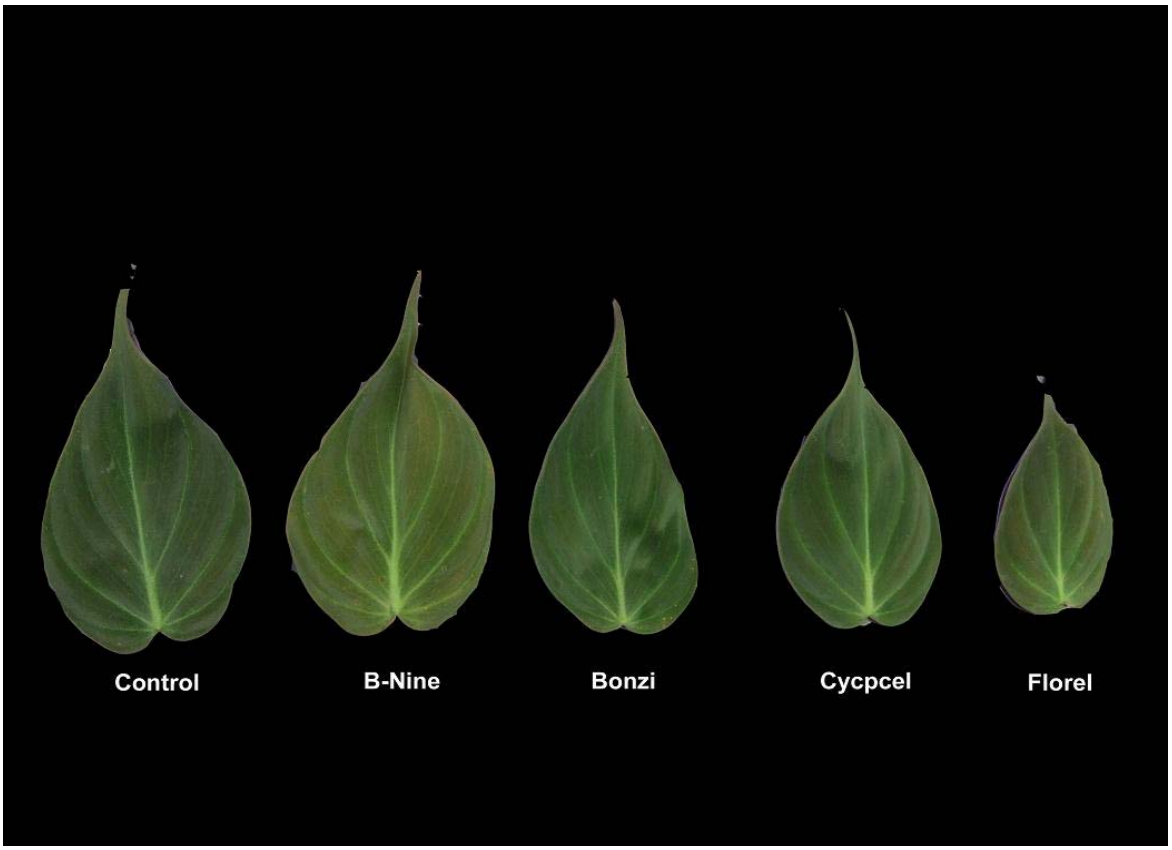

Figure 5-2. The largest leaf size for *P. micans* one month after one-time drenching with water, B-Nine® at 2500 mg L^{-1}, Bonzi® at 50 mg L^{-1}, Cycocel® at 1000 mg L^{-1}, and Florel® at 500 mg L^{-1}.

Discussion

The results from both foliar spray and soil drenching experiments showed that B-Nine® and Cycocel® with the concentrations applied were not effective in control of internode length (Table 5-1 and Table 5-2). Foliar spray of Florel® significantly reduced internode length, but at the same time it caused leaf drop, indicating that Florel® cannot be used on *P. micans* as foliar spray. It is not surprising that foliar application of Florel® resulted in defoliation because Florel® breaks down into ethylene, a naturally occurring compound, which causes leaf drop and blossom abortion. However, Florel® used as a soil drench did not cause defoliation or reduce internode length. However Florel® did significantly reduce leaf size, and thus is not a suitable growth regulator to control internode length in *P. micans*.

Bonzi® appears to have potential to reduce internode length in *P. micans*. The internode of plants sprayed at 100 mg L^{-1} or drenched at 50 mg L^{-1} with Bonzi® was 6.4 cm compared to 8.8 cm for the control. Bonzi® applications did not significantly reduce leaf size. Bonzi® is an effective inhibitor that blocks gibberellin biosysnthesis by inhibiting kaurene oxidase, an enzyme converting kaurene to kaurenoic acid (Wang et al. 1986). When gibberellin biosysnthesis is blocked, cell division still occurs, but the new cells do not elongate, which results in shoots with the same numbers of leaves but compressed internodes (Chaney 2003). Successful use of Bonzi® application to reduce internode length has been reported in *Plectranthus australis* R. Br., *Zebrina pendula* Schnizl., and *Ficus*. *benjamina* (Davis 1987) as well as *Gynura aurantiaca* (Blume) DC (Chen et al. 2002) and other floriculture crops (Barrett et al. 1994).

Although internode length was reduced by application of Bonzi®, it appears that further tests with increased concentration in both foliar spray and soil drenching is

needed to determine the optimum concentration rates for effective control of internode length and improve the growth form of *P. micans*.

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BIOGRAPHICAL SKETCH

Zhujun Xiong was born in Jiangxi province, P.R. China. She obtained her Bachelor of Science degree in biology from Fudan University in July 2007, Shanghai. At the same time, she was admitted to the graduate program in the Department of Environmental Horticulture, University of Florida. Her study was sponsored by Dr. Jianjun Chen with a research assistantship.

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