

**STUDIES ON THE *IN VITRO* CULTURE AND HEIGHT CONTROL OF  
ORNAMENTAL *PLECTRANTHUS***

**A Thesis**

**Presented to the Faculty of the Graduate School  
of Cornell University**

**In Partial Fulfillment of the Requirements for the Degree of  
Master of Science**

**by**

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**August 2009**

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## ABSTRACT

*Plectranthus* belongs to the *Lamiaceae* and consists of approximately 350 species. New *Plectranthus* cultivars that have gained popularity in the horticultural industry are usually sterile hybrids. Tissue culture techniques can be used as a tool to aid *Plectranthus* breeding programs. The number of works published on tissue culture of *Plectranthus* is limited, thus creating opportunity for further investigation. The objective of this study was to develop a complete micropropagation strategy making use of both direct and indirect organogenesis for *Plectranthus* 'Cape Angels Dark Pink' (CADP) and *P. zuluensis* (PZ). Half-strength Murashige and Skoog nutrients and vitamins formed the base medium for the tissue culture research. The chosen *Plectranthus* cultivars appeared to be sensitive to high cytokinin levels and both CADP and PZ produced the greatest number of shoots in the presence of 0.1 mg/L BA. The presence of auxin during multiplication did not increase the number of shoots produced from nodal sections. CADP shoot tips did not display a change in the average number of shoots produced on continuous subculture, while the average number of shoots produced by PZ increased over time. The greatest increase over time was seen at 0.1 mg/L BA. Different *Plectranthus* accessions display genotypic variance in the average number of shoots produced from shoot tips when cultured in the presence of 0.1 mg/L BA, with CADP and *P. purpurea* producing the greatest number of shoots from a single shoot tip. Acclimated microcuttings of CADP and PZ rooted with ease under *ex vitro* conditions. Near 100% establishment of *in vitro* cultured *Plectranthus* plantlets to the greenhouse was achieved.

The presence of 2,4-D was required for callus production in both CADP and PZ. Irradiance was important in callus production, with dark conditions favoring callus induction in PZ and light favoring callus production in CADP. White friable CADP

callus could be maintained on medium with 0.5 mg/L 2,4-D and kinetin, although it was not possible to induce shoot formation from either CAPD or PZ callus.

Ornamental horticulture has placed a major emphasis on the production of well-branched, compact-growing, and proportionate-looking plants. Commercial *Plectranthus* hybrids such as 'Mona Lavender' and the 'Cape Angels' series display vigorous growth that requires greenhouse growers to use several cultural techniques to produce plants with the characteristics that are desired by the commercial industry. Plant growth retardants (PGRs) are commercially available to use in producing more compact crops. The objective of this study was to identify appropriate PGRs and concentrations for use as foliar sprays on three commercial hybrid *Plectranthus* cultivars, 'Mona Lavender', 'Cape Angels Dark Pink', and 'Cape Angels White'. Daminozide (2500 – 5000 mg/L) and paclobutrazol (5 – 15 mg/L) that were applied as foliar sprays were not effective in growth retardation in the hybrid *Plectranthus*. Flurprimidol foliar sprays in the range of 10 – 20 mg/L proved to be effective in growth retardation without other visible negative effects on the plants. Flurprimidol treatments higher than 20 mg/L were excessive. Optimal rates of flurprimidol application on ornamental hybrid *Plectranthus* are currently being investigated.

This study provides a foundation for future research on tissue culture and micropropagation of ornamental *Plectranthus*. Results from the growth retardation study provide a guideline for effective growth regulator levels for height control of hybrid *Plectranthus* cultivars under northeastern greenhouse growing conditions in the United States.

## **BIOGRAPHICAL SKETCH**

Ockert F.C. Greyvenstein was born on 20 December 1983 in the small Highveld town of Carolina in South Africa. Ockert grew up on a small farm roughly 50 km east of the city of Pretoria. Apart from his parents John and Marina, Ockert has two siblings, an older sister Rina and a younger brother John. Growing up on a small farm in South Africa provided Ockert with the unique opportunity to interact with various plants and animals. Ockert's father, John Greyvenstein, is trained as a geneticist, and part of growing up for Ockert was observing his father manage a dog breeding program. Ockert has always had a great interest in breeding of ornamental plants and the box full of seeds, from native plants, under his bed in South Africa is proof of this. While still in high school, Ockert started a small scale sheep breeding program with the Damara breed and selected for multi horn qualities in his flock.

After completion of his high school training, from Hoërskool Erasmus in Bronkhorstspuit, Ockert achieved a B.Sc. with specialization in Biotechnology and B.Sc. (Hons) with specialization in genetics from the University of Pretoria. During summer holidays Ockert worked with Mr. Johan Potgieter at PANNAR seed company, located in Delmas South Africa, as research assistant on the sunflower breeding program. After completion of his undergraduate studies, Ockert went to Ireland to play and coach cricket for the 2006 season at Clontarf Cricket Club in Dublin, Ireland.

Upon his return to South Africa Ockert worked as a grower for Sunkloof wholesale nursery in South Africa. During this time he got in contact with Dr. Mark Bridgen from Cornell University's Horticulture department. With the advice and help from Dr. Bridgen, Ockert was accepted into the masters program in the Horticulture department at Cornell University, where he conducted his Masters research on southern African *Plectranthus*.

Upon completion of his masters degree from Cornell University, Ockert plans on furthering his scientific education and continue on to pursue a Ph.D.

This thesis is dedicated to my late grandfather:

Ockert Johannes Albertus Greyvenstein, 1919 – 2002

“All we have to decide is what to do with the time that is given us.” – Gandalf,

Lord of the Rings

## ACKNOWLEDGMENTS

First I would like to thank my major advisor Dr. Mark Bridgen for taking the risk of investing his time and energy and accepting me as a student. Dr. Bridgen's help and advice extended far beyond the normal duties of an advisor, and was the closest to a father figure I could have outside of South Africa. I would also like to thank Dr. Elizabeth Earle for serving on my committee. It was a great honor for me to use her laboratory and be able to stop into her office at any time and ask for advice, which she gave freely.

Thanks must be given to Françoise Vermeulen at the Cornell University statistical consulting unit. She was always patient with me and without her help doing the statistical analyses correctly would have been a nightmare.

Thanks also to Dr. Gert Brits from Brits Nursery in South Africa. His unconditional advice and knowledge of *Plectranthus* was a great help. Ernst van Jaarsveld, at Kirstenbosch botanical gardens in South Africa, helped me with the identification of several plants in the collection for which I am very grateful. Thank you to Mr. Johan Potgieter from PANNAR seed in South Africa who always gave me advice, and has served as a mentor to me for many years.

I must give thanks to the greenhouse staff at the Kenneth Post greenhouse complex at Cornell University's Ithaca campus for managing my plants in the greenhouse. Special thanks goes to Missy Bidwell and Jenny Rothenberg who were personally in charge of my plants.

Spending two summers at Cornell University's Long Island Horticultural Research and Extension Center was a great experience. Thank you to all the staff who kept my summers interesting. Everyone was always friendly and treated me with respect. I must give special thanks to Lynn Hyatt, Dr. Bridgen's laboratory technician, for always being willing to help me.

To all my friends inside and outside of the Horticulture department, who kept me sane. I appreciate it greatly that I could always find somebody to talk to and take a break from science. I must give special thanks to Jillian Ferdman and Kendra Hutchins for helping with my thesis; the two of you were always willing to read over my drafts and help control for the second language effect. Jillian, you were there for me throughout, thank you for that. Burtie van Zyl must get special mention; I never expected to be able to speak Afrikaans to anybody here at Horticulture department. Sonder ons gesselsies, lang middag tee en koffie sessies sou die lewe hier aansienlik moeiliker gewees het.

Finally I would like to thank my entire family back in South Africa for their continuous support. Special thanks to my immediate family; Pa, Ma, Rina, and John Jr.; your love and support provided me with a structure I could fall back on in tough times. Special mention must be given to my father, John M. Greyvenstein, who throughout my life has coached me in the natural sciences. Pa was altyd meer as gewillig om na my studies te luister en raad te gee waar Pa kon, ek wardeer dit baie.

## TABLE OF CONTENTS

<b>BIOGRAPHICAL SKETCH.....</b>	<b>III</b>
<b>DEDICATION.....</b>	<b>V</b>
<b>ACKNOWLEDGMENTS.....</b>	<b>VI</b>
<b>TABLE OF CONTENTS .....</b>	<b>VIII</b>
<b>LIST OF FIGURES .....</b>	<b>XI</b>
<b>LIST OF TABLES .....</b>	<b>XII</b>
<b>CHAPTER 1: LITERATURE REVIEW .....</b>	<b>1</b>
<b>1.1 INTRODUCTION .....</b>	<b>1</b>
<b>1.2 PLANT TISSUE CULTURE .....</b>	<b>2</b>
1.2.1 Tissue culture media .....	2
1.2.2 Organogenesis .....	4
1.2.3 Stages of micropropagation.....	4
<b>1.3 Tissue culture of <i>Plectranthus</i> and related plants.....</b>	<b>6</b>
1.3.1 Micropropagation of <i>Plectranthus</i> and related plants .....	6
1.3.2 Callus production in <i>Plectranthus</i> and related plants .....	12
<b>1.4 Plant growth regulators in the greenhouse.....</b>	<b>14</b>
1.4.1 Use of PGRs on <i>Plectranthus</i> and related plants.....	16
<b>CHAPTER 2: TISSUE CULTURE AND MICROPROPAGATION OF ORNAMENTAL <i>PLECTRANTHUS</i> .....</b>	<b>20</b>
<b>2.1 ABSTRACT .....</b>	<b>20</b>
<b>2.2 INTRODUCTION .....</b>	<b>21</b>
<b>2.3 MATERIALS AND METHODS.....</b>	<b>24</b>
2.3.1 <i>Plectranthus</i> collection and initiation of <i>in vitro</i> cultures.....	24
2.3.2 Direct organogenesis.....	25
2.3.2.1 Preliminary micropropagation studies .....	26
2.3.2.2 Major micropropagation study.....	26
Experiment 1 – Shoot production.....	26

Experiment 2 – Effect of IBA on shoot production.....	27
Experiment 3 - Shift in multiplication medium strategy .....	27
Experiment 4 – Explant effect on micropropagation.....	28
Experiment 5 - Comparison of micropropagation potential for several <i>Plectranthus</i> accessions .....	29
Experiment 6 - <i>In vitro</i> rooting of <i>Plectranthus</i> .....	30
Experiment 7 - <i>Ex vitro</i> rooting of <i>Plectranthus</i> .....	30
Experiment 8 – Cold storage of <i>in vitro Plectranthus</i> .....	31
Acclimation of <i>in vitro</i> cultured plantlets .....	31
2.3.3 Indirect organogenesis .....	32
2.3.3.1 Preliminary studies.....	32
Experiment 9 - Effect of cytokinins and auxins on leaf explants.....	32
Experiment 10 - Adventitious shoot and root formation .....	33
Experiment 11 - Callus Production .....	33
2.3.3.2 Major Study .....	34
Experiment 12 - Callus production, kinetin and 2,4-D factorial .....	34
Experiment 13 – Comparison of different cytokinins in callus production.....	34
Experiment 14 - Organogenesis from callus .....	35
2.3.4 Statistical analysis.....	35
<b>2.4 RESULTS .....</b>	<b>36</b>
2.4.1 Direct organogenesis.....	36
2.4.1.1 Preliminary micropropagation studies .....	36
2.4.1.2 Major micropropagation study.....	39
Experiment 1 – Shoot production.....	39
Experiment 2 – Effect of IBA on shoot production.....	42
Experiment 3 – Shift in multiplication medium strategy.....	42
Experiment 4 – Explant effect on micropropagation.....	43
Experiment 5 – Comparison of micropropagation potential for several <i>Plectranthus</i> accessions .....	44
Experiment 6 - <i>In vitro</i> rooting of <i>Plectranthus</i> .....	44
Experiment 7 - <i>Ex vitro</i> rooting of <i>Plectranthus</i> .....	46
Experiment 8 - Cold storage of <i>in vitro Plectranthus</i> .....	47
Acclimation of <i>in vitro</i> cultured plantlets .....	47
2.4.2 Indirect organogenesis .....	48
2.4.2.1 Preliminary studies.....	48
Experiment 9 - Effect of cytokinins and auxins on leaf explants.....	48
Experiment 10 – Adventitious shoot and root formation.....	48
Experiment 11 - Callus Production .....	50
2.4.2.2 Major studies .....	52
Experiment 12 - Callus production, kinetin and 2,4-D factorial .....	52
Experiment 13 – Comparison of cytokinins for callus production.....	54
Experiment 14 - Organogenesis from callus .....	55
<b>2.5 DISCUSSION .....</b>	<b>57</b>
2.5.1 Direct organogenesis.....	57

2.5.1.1	Preliminary micropropagation studies .....	57
2.5.1.2	Major micropropagation study.....	57
	Experiment 1 – Shoot production.....	57
	Experiment 2 – Effect of IBA on number of shoots produced .....	58
	Experiment 3 – Shift in multiplication medium strategy.....	58
	Experiment 4 – Explant effect on micropropagation.....	59
	Experiment 5 - Comparison of micropropagation potential for several <i>Plectranthus</i> accessions .....	60
	Experiments 6 and 7 – Rooting of <i>Plectranthus</i> .....	61
	Experiment 8 - Cold storage of <i>in vitro Plectranthus</i> .....	61
	Proposed protocol for direct organogenesis .....	62
2.5.2	Indirect organogenesis .....	65
2.5.2.1	Experiments 9 and 10 - Adventitious shoot formation .....	65
2.5.2.2	Experiments 11, 12, and 13 - Callus culture.....	66
2.5.2.3	Experiment 14 - Organogenesis from Callus.....	68
<b>2.6</b>	<b>CONCLUSIONS.....</b>	<b>68</b>
<b>CHAPTER 3: USE OF PLANT GROWTH REGULATORS FOR HEIGHT CONTROL OF ORNAMENTAL HYBRID <i>PLECTRANTHUS</i> .....</b>		<b>70</b>
<b>3.1</b>	<b>ABSTRACT .....</b>	<b>70</b>
<b>3.2</b>	<b>INTRODUCTION .....</b>	<b>70</b>
<b>3.3</b>	<b>MATERIALS AND METHODS.....</b>	<b>72</b>
	3.3.1 2008 Plant Growth Regulation Trial.....	73
	3.3.2 2009 Plant Growth Regulation Trial.....	75
	3.3.3 Statistical analysis.....	75
<b>3.4</b>	<b>RESULTS .....</b>	<b>76</b>
	3.4.1 2008 Plant Growth Regulation Trial.....	76
	3.4.2 2009 Plant Growth Regulation Trial.....	76
<b>3.5</b>	<b>DISCUSSION .....</b>	<b>81</b>
<b>3.6</b>	<b>CONCLUSIONS.....</b>	<b>83</b>
<b>CHAPTER 4: SUMMARY .....</b>		<b>84</b>
 <b>APPENDIX.....</b>		<b>87</b>
 <b>REFERENCES.....</b>		<b>89</b>

## LIST OF FIGURES

Figure 2.1 <i>Plectranthus</i> ‘Cape Angels Dark Pink’ (CADP) and <i>P. zuluensis</i> (PZ) shoot tips after eight weeks of culture in different combinations of BA and IBA. ...	37
Figure 2.2 Mean number of shoots produced from <i>Plectranthus</i> ‘Cape Angels Dark Pink’ (CADP) and <i>P. zuluensis</i> (PZ) shoot tips after eight weeks of culture. .	38
Figure 2.3 Number of shoots produced from <i>Plectranthus</i> ‘Cape Angels Dark Pink’ (CADP) and <i>P. zuluensis</i> (PZ) shoot tips after four subcultures. ....	40
Figure 2.4 Comparison of the mean number of shoots produced, from single nodal sections, after nine weeks in the absence and presence of IBA. ....	42
Figure 2.5 Tukey's HSD comparison across <i>Plectranthus</i> accessions for the mean number of both total and culturable number of shoots produced from shoot tips.....	45
Figure 2.6 Comparison of mean days to root for <i>Plectranthus</i> ‘Cape Angels Dark Pink’ shoot tips from two prior BA treatments, 0.05 and 0.1mg/L.....	46
Figure 2.7 Roots of <i>Plectranthus</i> ‘Cape Angels Dark Pink’ shoots after 15 days of growth in 1 peat: 1 perlite growing medium. ....	47
Figure 2.8 <i>Plectranthus</i> plantlets in the greenhouse after being taken out of tissue culture conditions. ....	49
Figure 2.9 <i>P.zuluensis</i> petiole showing adventitious shoot formation after eight weeks of growth on BA 1.0mg/L: IBA 0.1 mg/L. ....	51
Figure 2.10 <i>Plectranthus</i> ‘Cape Angels Dark Pink’ (CADP) and <i>P. zuluensis</i> (PZ) leaf cultures that were grown in both light and dark treatments at two-and four weeks of culture.....	51
Figure 2.11 Nodal sections of <i>Plectranthus</i> ‘Cape Angels Dark Pink’ (CADP) and <i>P. zuluensis</i> (PZ) from the, kinetin and 2,4-D factorial after four weeks of growth. ....	53
Figure 2.12 Proposed micropropagation strategy derived for <i>Plectranthus</i> 'Cape Angels - Dark Pink'.....	63
Figure 3.1 <i>Plectranthus</i> cuttings 21 days after taking the cuttings.....	74
Figure 3.2 Average plant growth of the three <i>Plectranthus</i> cultivars at all the treatment levels. ....	77
Figure 3.3 Total average plant growth of the <i>Plectranthus</i> cultivars used in the 2008 PGR study. ....	78
Figure 3.4 Mean growth of all three <i>Plectranthus</i> cultivars, at five flurprimidol levels at time of harvest. ....	78
Figure 3.5 Representative samples <i>Plectranthus</i> 'Mona Lavender' treated with different concentrations of flurprimidol.....	79
Figure 3.6 Effect of high concentrations (40 and 80 mg/L) of flurprimidol on <i>Plectranthus</i> 'Mona Lavender' compared to the control treatment.....	80

## LIST OF TABLES

Table 1.1 Summary of tissue culture conditions published for <i>Plectranthus</i> and selected studies on related plants.....	9
Table 1.2 Suggested plant growth regulator levels obtained on <i>Coleus</i> and <i>Salvia</i> cultivars.....	17
Table 2.1 Size classes used for grouping shoots.....	27
Table 2.2 Description of root growth scale .....	29
Table 2.3 Comparison of the mean number of shoots produced per shoot tip at each treatment over a period of 32 weeks, using Tukey's Honestly Significant Difference (HSD) test. ....	41
Table 2.4 Dunnett's comparison of <i>P. zuluensis</i> total shoots and culturable shoots, produced from single nodal sections on the shift in multiplication media compared to continuous culture at 0.1 mg/L BA. ....	43
Table 2.5 Tukey's HSD for culturable shoots produced by <i>Plectranthus</i> 'Cape Angels Dark Pink' (CADP) and <i>P. zuluensis</i> (PZ) shoot tip and nodal explants. ....	44
Table 2.6 Percentage of explants that showed callus formation for each of the four <i>Plectranthus</i> accessions. ....	50
Table 2.7 Tukey's HSD on the Least Square Mean of final explant width produced per species at each level of 2,4-D.....	52
Table 2.8 Percentage of light cultured <i>Plectranthus</i> 'Cape Angels Dark Pink' callus grouped in classes 1 – 5, at 0.1 mg/L 2,4-D by all of the cytokinin treatments. ....	54
Table 2.9 Percentage of dark cultured <i>Plectranthus zuluensis</i> explants, at 0.1 mg/L 2,4-D, scored in different callus growth classes.....	55
Table 2.10 Tukey HSD on the least square means of growth by callus explants for <i>Plectranthus</i> 'Cape Angels Dark Pink' (CADP) and <i>P. zuluensis</i> (PZ) by the four treatment combinations used for callus organogenesis. ....	56
Table 2.11 Comparison of conventional greenhouse cutting propagation of CADP to micropropagation using both culturable and total number of shoots produced. ....	64
Table 3.1 PGR treatments used in summer 2008 trial.....	73
Table 3.2 Tukey's Honestly Significant difference comparison of the mean growth for all three <i>Plectranthus</i> cultivars, 'Mona Lavender', 'Cape Angels Dark Pink' and, 'Cape Angels White' at time of harvest. ....	79

## CHAPTER 1: LITERATURE REVIEW

### 1.1 INTRODUCTION

*Plectranthus* is an old-world genus that belongs to the *Lamiaceae* and consists of approximately 350 known species distributed throughout summer rainfall areas of Africa, Madagascar, India, Australia, and a few of the Pacific islands (Van Jaarsveld, 2006). *Plectranthus* species are mostly soft, low-growing, semi-succulent, to succulent herbs or shrubs.

The name *Plectranthus* literally means spurflower, from the Greek words *plectron*, meaning spur, and *anthos*, meaning flower. *Plectron* refers to the spur at the base of the corolla tube of *Plectranthus fruticosus*, the first plant in this genus to be described (Codd, 1975). The name *Plectranthus* is confusing because only a couple of the species in the genus have this spur. *Plectranthus* is a very diverse genus with some species converging into other genera such as *Coleus*. There has been quite some taxonomic confusion around this genus, and DNA studies are needed to clarify some of the taxonomic confusion (Codd, 1975; Van Jaarsveld and Edwards, 1997; Van Jaarsveld, 2006).

Being such a widespread genus, *Plectranthus* has many ethnobotanical uses. Members of *Plectranthus* have been, and still are, used as medicine, food, fodder, herbs, building materials, and in horticulture (Lukhoba, *et al.*, 2006).

*Plectranthus* has been referred to as a “horticulturally neglected” genus (Van Jaarsveld and Edwards, 1987). *Plectranthus* is most commonly grown as foliage plants in pots or hanging baskets. Some of the southern African species produce surprisingly beautiful flowers (Brits, *et al.*, 2001), and there is great potential for further development of plants within *Plectranthus* for the horticultural market. Traditional breeding methods and *in vitro* techniques can be utilized in further development of ornamental *Plectranthus*. Several species have been horticulturally used with the best

known plants being the ‘Swedish Ivy’ (Van Jaarsveld, 2006). The name ‘Swedish Ivy’ can be confusing because it is actually the common name given to at least three species, *P. oertendahlii*, *P. verticillatus* (Van Jaarsveld, 2006) and *P. argentatus* (AboutGardenPlants, 2004). Other *Plectranthus* being cultivated for ornamental horticulture include: *P. argentatus*, *P. amboinicus*, *P. barbatus*, *P. ciliatus*, *P. ecklonii*, *P. fruticosus*, *P. hadiensis*, *P. madagascariensis*, *P. saccatus* and *P. zuluensis* (Koopowitz, 1998; AboutGardenPlants, 2004; Schoellhorn, 2004; Van Jaarsveld, 2006; Brits and Li, 2008).

Plants within *Plectranthus* are normally easy to propagate by taking stem cuttings (Van Jaarsveld and Edwards, 1987; Joffe, 1993; Ball-Horticultural-Company, 2009); the majority of the commercial varieties from Ball Horticultural Company are propagated asexually via cuttings (Ball-Horticultural-Company, 2009). The plants grown as species are usually fertile and a maximum of four small seeds per flower can be produced. Seeds are used to propagate species such as *P. argentatus* (Ball-Horticultural-Company, 2009). Seeds are not the preferred method for production because the crop time is usually longer and a less uniform crop is expected. Seed propagation, however, plays a very important part in the breeding of potentially new cultivars (Van Jaarsveld, 2006; Brits and Li, 2008).

## **1.2 PLANT TISSUE CULTURE**

Plant tissue culture can be defined as the culture of protoplasts, cells, tissues, organs, embryos, or seed on nutrient medium *in vitro* under controlled environmental conditions (Pierik, 1987).

### **1.2.1 Tissue culture media**

Tissue culture plants are grown on an artificial medium, which supplies all the necessary nutrients for healthy, vigorous growth. The medium provides all 17 essential

elements as described by Epstein (1972). These essential elements consist of both macro- and micro-plant nutrients that make up the mineral composition of the growth medium.

Plant growth regulators, or growth substances, such as cytokinins, auxins, gibberellins, abscisic acid and their analogues and inhibitors are added to tissue culture media to regulate and manipulate the growth of tissue culture plants or organs.

Sucrose is most commonly added to the medium as a carbon source to replace the carbon that the plants usually fix via photosynthesis. Other organic compounds that are added include vitamins and sometimes amino acids. Coconut water (CW) is sometimes added to provide nutrients and plant growth substances (George, *et al.*, 2008). The use of CW is limited in research because the precise composition is unknown; CW composition will vary largely and can therefore have an effect on the repeatability of experiments.

The most commonly used medium in tissue culture is the formulation by Murashige and Skoog (1962). Commonly referred to as MS medium, it was developed for optimal growth of tobacco callus. The elementary composition of plants varies and can be used to adapt the growing medium. Adaptations to growing medium can result in improved growth. For example Gonçalves, *et al.* (2005) used *Ceratonia siliqua* leaf macronutrient composition to adapt the tissue culture medium which resulted in improved growth.

Plant tissue cultures can grow in either liquid media or semi-solid media with a solidifying agent. Agar and gellan gum are the most commonly used gelling agents (George, *et al.*, 2008). There are, however, various alternatives to agar such as, synthetic polymers, alginate, and gelrite (Pierik, 1987).

### **1.2.2 Organogenesis**

There are several strategies that can be followed in a micropropagation program. Direct organogenesis, as the name implies, is the process whereby organs are formed from an explant without an intermediate callus phase. During direct organogenesis, organs can either form from existing meristematic tissues or *de novo* on the explant. Propagation via axillary branching is most acceptable for the production of plants identical to the starting material, mutations are more likely to occur when adventitious shoots are used for propagation.

Indirect organogenesis involves an intermediate callus phase. Callus is actively growing non-organized cells and is usually produced by the manipulation of growth regulators in the media. The growth regulator requirements for callus induction differ between plant species and even between genotypes within species. Callus induction is usually associated with a relatively high level of auxin (George and Sherrington, 1984). A change in growth regulators, usually to a higher cytokinin level, is associated with shoot differentiation from callus. The use of 2,4-D and NAA has been reported to increase the number of mutations (Pierik, 1987), and for this reason callus is not the preferred method of propagation if the goal is to produce genetically identical plants.

### **1.2.3 Stages of micropropagation**

There are five stages (0 – IV) of micropropagation that are recognized internationally (Debergh and Maene, 1981).

Stage 0 includes the selection and preparation of the mother plants that will be used as starting material for cultures. Using healthy, disease-free plants as starting material will increase the success rate of sterilization and establishment of cultures at stage I. In some cases stock plants are pre-treated with systemic pesticides and

screened for the presence of known viruses (Debergh and Maene, 1981; George, *et al.*, 2008).

Stage I includes the establishment of aseptic cultures *in vitro*. In order to obtain sterile cultures, the starting material is washed under running water followed by sterilization, usually with a sodium hypochlorite bleach treatment such as commercial Clorox® bleach. The most common explants at stage I are leaf pieces, nodal sections, and shoot tips. After sterilization the explants are cultured on tissue culture medium for a couple of days to observe for any obvious contamination. If no obvious contamination is present the explants can be moved to stage II (George, *et al.*, 2008).

Stage II is considered the proliferation stage, and thus the objective of stage II is to produce propagules which will be capable of growing into new plants. The most common method of proliferation is through the formation of axillary shoots. During stage II, cultures are typically grown with relatively higher cytokinin levels (Trigiano and Gray, 2005).

Stage III is the pre-transplant or rooting stage. The objective of stage III is to produce plants capable of photosynthesis and growth without the use of an external carbon source. Rooting of *in vitro* plantlets is usually required for successful transfer to the natural environment. Rooting takes place during stage III, and cultures are usually grown with a higher auxin level to promote rooting. Some plants root with ease and rooting is not always required during stage III; micropropagated blueberries for example, can be rooted under *ex vitro* conditions (Isutsa, *et al.*, 1994).

Stage IV is the phase where *in vitro* grown plants are transferred to the greenhouse. *In vitro* grown plants are produced in high humidity, which can result in less epicuticular wax on the leaves. The lack of epicuticular wax can result in a rapid loss of water for tissue culture plants when placed in a lower humidity environment (Sutter and Langhans, 1979). Stage IV normally consists of an acclimatization step in

which the plantlets are acclimated to a lower humidity environment. If stage III plants have been grown on a medium containing agar, the agar is usually washed away from the roots before transplanting to an appropriate rooting medium. After the plants are transplanted, they are usually kept in high humidity environment (e.g. a mist tunnel) for several days and from there they are moved to the greenhouse (George, *et al.*, 2008).

### **1.3 Tissue culture of *Plectranthus* and related plants**

#### **1.3.1 Micropropagation of *Plectranthus* and related plants**

Very limited work has been conducted on the micropropagation of plants within *Plectranthus*. Some work has been completed on micropropagation of *P. vetiveroides* (Sivasubramanian, *et al.*, 2002), a species that is used as a medicinal plant in India. Tissue culture protocols have been developed for *P. esculentus*, the edible Livingstone Potato (Allemann, 2002). Only one paper has been produced on the micropropagation of ornamental *Plectranthus*. The study conducted by Hu, *et al.* (2008) focused on *P. madagascariensis* and *Plectranthus x hilliardiae* ‘Edelblau’ and showed positive results for micropropagation via direct organogenesis of the two varieties studied. None of the papers produced on micropropagation of *Plectranthus* made use of an indirect organogenesis procedure.

In all of the above-mentioned studies, the method of propagation was via axillary branching. Standard or half-strength MS (Murashige and Skoog, 1962) media were used as the culture medium. In the case of *P. esculentus*, Alleman (2002) found that the addition of 2  $\mu\text{M}$   $\text{CuSO}_4$  to the culture medium resulted in better overall growth of plants. Hu *et al* (2008) found that half-strength MS nutrients and vitamins was optimal for both shoot and root growth. For *P. vetiveroides*, standard MS medium

was optimal for shoot production whereas half-strength MS medium produced better results in root production.

Numerous papers have been published on the micropropagation of other members of the *Lamiaceae*, including *Coleus forskohlii* (Bhattacharyya and Bhattacharya, 2001; Reddy, *et al.*, 2001). With the taxonomic confusion surrounding *Plectranthus*, *C. forskohlii* was renamed as *P. barbatus* during a revision of the genus by Codd in 1975. The name *C. forskohlii* will be used in this literature to refer to *P. barbatus*. Both direct and indirect organogenesis procedures have been produced for *C. forskohlii*. *C. forskohlii* is an important plant in Indian medicine, and produces forskolin in tuberous roots. A mass propagation protocol has been developed for *C. forskohlii*, making use of an indirect organogenesis pathway in which shoots are produced from leaf-derived callus (Reddy, *et al.*, 2001). Protocols for shoot multiplication via a combination of axillary branching and direct shoot morphogenesis have been developed (Bhattacharyya and Bhattacharya, 2001).

In the case of *C. forskohlii*, callus was produced on a standard MS medium supplemented with 0.5 mg/L kinetin. For the production of shoots from the leaf-derived callus, the medium was supplemented with a combination of 1.0 mg/L kinetin and 0.1 mg/L NAA (1-naphthaleneacetic acid) (Reddy, *et al.*, 2001). Similar to the other tissue culture studies of members in *Plectranthus*, Reddy *et al.* found that *C. forskohlii* produced better roots on half-strength MS medium devoid of growth regulators.

In another study with *C. forskohlii*, nodal sections and shoot tips were used as explants to develop a micropropagation protocol via direct organogenesis (Bhattacharyya and Bhattacharya, 2001). Basal MS medium was used, and kinetin in combination with IAA both at 0.1 mg/L gave the best shoot multiplication rate.

Table 1.1 provides a summary of media, growing conditions, and growth regulator combinations used in the above mentioned studies. Table 1.1 also provides the same information on selected tissue culture and micropropagation studies performed on plants related to *Plectranthus*.

In all of the studies that are referenced in Table 1.1, the methods used during Stage I of culture were very similar. In most cases plant material was obtained from greenhouse grown plants. These explants were washed under running tap water for five to ten minutes, followed by a sterilization procedure. The sterilization procedure usually involved a short dip in 70 % ethanol, followed by a bleach treatment of sodium hypochlorite in the ranges of 0.525 - 1.05 %. Sterilization in the sodium hypochlorite lasted between 10 and 20 minutes.

The preferred nutrient medium used for the culture of *Plectranthus* and related plants is basal MS medium or half-strength MS medium (Table 1.1). The carbon source in all the studies summarized was sucrose. Sucrose was used at either 3% or 2%. In a majority of the studies, half-strength MS medium performed better when root formation was required, although in some cases full strength MS medium performed well.

During Stage II, the micropropagation stage, BA and kinetin proved to be the most used cytokinins for shoot multiplication. The level of cytokinin to be used differed between the studies. Relatively high cytokinin levels were used in some studies while low levels were used in others. *Coleus forskohlii* shoot tips achieved a multiplication of 12.5 shoots per tip in the presence of 0.1 mg/L Kin (Bhattacharyya and Bhattacharya, 2001). Multiple shoots were produced from *Coleus blumei* leaf disks using a combination of BA at 0.1 mg/L with NAA at 0.01 mg/L (Hervey and Robbins, 1978). In the case of orange mint, a high BA level of 10 mg/L was used to promote multiple shoot formation on leaf disks (Van Eck and Kitto, 1992).

**Table 1.1 Summary of tissue culture conditions published for *Plectranthus* and selected studies on related plants.**

Species	Starting Explant	Nutrient Media	Light conditions	Growth phase	Growth regulator and other additions	Reference
<i>Plectranthus esculentus</i>	Shoot tip	MS	16 hr light	Multiplication	CuSO <sub>4</sub> 2.0 $\mu$ M	Allemann, 2002
<i>Plectranthus vetiveroides</i>	Nodal	MS	14 hr light	Multiplication	BA 1.0 mg/L	Sivasubramanian, <i>et al.</i> , 2002
		1/2 MS	14 hr light	Rooting	IBA 2.0 mg/L	
<i>Plectranthus madagascariensis</i>	Shoot tip	1/2 MS	16 hr light	Multiplication	BA 1.0 mg/L	Hu, <i>et al.</i> , 2008
		1/2 MS	16 hr light	Rooting	IBA or NAA at 0.5 mg/L	
<i>Coleus forskohlii</i> ( <i>P. barbatus</i> )	Leaf	MS	16 hr light	Callus	Kin 0.5 mg/L	Reddy, <i>et al.</i> , 2001
		MS	16 hr light	Shoot differentiation	Kin 1.0 mg/L + NAA 0.1 mg/L	
		1/2 MS	16 hr light	Rooting	None	
<i>Coleus forskohlii</i> ( <i>P. barbatus</i> )	Shoot tip	MS	18 hr light	Multiplication	Kin 0.1 mg/L + IAA 0.1 mg/L	Bhattacharyya and Bhattacharyya, 2001
		MS	16 hr light	Rooting	IBA 2.0 mg/L	
<i>Coleus blumei</i>	Leaf	BRVS <sub>2</sub>	Natural day	Shoot differentiation	BA 0.1 mg/L + NAA 0.01 mg/L	Hervey and Robbins, 1978
<i>Coleus blumei</i>	Leaf	MS	16 hr light	Shoot differentiation	BA 2.0 mg/L + IAA 1.0mg/L	Zagrajski, <i>et al.</i> , 1997
	Inter nodal	MS	16 hr light	Shoot differentiation	BA 1.0 mg/L + IAA 0.5 mg/L	
	Inter nodal	MS	16 hr light	Callus	BA 0.05 - 2.0 mg/L + IAA 0.5 - 2.0 mg/L	
<i>Coleus blumei</i>	Inter nodal	Gamborg B5	24 hr light	Callus	Kin 0.1 mg/L + 2,4-D 1.0 mg/L	Razzaque and Ellis, 1977
<i>Salvia nemrosa</i>	Shoot tip	MS	24 hr light	Multiplication	BA 0.5 - 2.0 mg/L + IAA 0.5 mg/L	Skala and Wysokinska, 2004
	Leaf	MS	24 hr light	Shoot differentiation	BA 0.2-1.0mg/L + IAA 0.5 mg/L or IBA 0.5 mg/L	
		MS	24 hr light	Rooting	NAA 0.5 mg/L	

**Table 1.1** continued

<i>Mentha spicata</i>	Shoot tip	MS	Dark	Callus	TDZ 4.0 mg/L + CW 250 ml/L	Li, <i>et al.</i> , 1999
		MS	Dark	Shoot differentiation	None	
<i>Mentha citrata</i>	Leaf	MS	Dark	Callus	BA 10 mg/L + CW 250ml/L	Van Eck and Kitto, 1992
		MS	16 hr light	Shoot differentiation	BA 10 mg/L + CW 250 ml/L	

- MS – Murashige and Skoog's (1962) medium

- BRVS<sub>2</sub> – Nutrient and vitamin solution (Robbins and Hervey, 1969)

- CW – Coconut water

- Kin – Kinetin; BA – 6-Benzyladenine; TDZ – thidiazuron; IAA - indole-3-acetic acid; IBA – indole-3-butyric acid; NAA –  $\alpha$ -naphthaleneacetic acid

Orange mint leaf explants underwent a callus phase before shoot differentiation began. From the literature studied, shoot multiplication proved to be most effective at cytokinin levels of 0.1 – 2.0 mg/L (Table 1.1).

With, *C. forskohlii* (Reddy, *et al.*, 2001), *C. blumei* (Razzaque and Ellis, 1977; Hervey and Robbins, 1978; Zagrajski, *et al.*, 1997), and *Salvia nemrosa* (Skala and Wysokinska, 2004), the addition of auxin in combination with cytokinin during the multiplication phase proved best whereas in others studies cytokinin alone was preferred. In micropropagation protocols developed for *P. vetiveroides* (Sivasubramanian, *et al.*, 2002) and *P. madagascariensis* (Hu, *et al.*, 2008) no auxin was used during shoot multiplication. In the case of orange mint, BA in combination with coconut water (CW) produced more shoots than when the auxins IBA and NAA were used (Van Eck and Kitto, 1992), although it was not reported whether the addition of an auxin was required for shoot induction. The composition of CW shows a lot of variation and it is difficult to repeat experiments where CW is used. The higher number of shoots produced from orange mint when CW was present could be due to some other shoot initiation compound present in the CW.

*Plectranthus* and related plants usually root with ease under *ex vitro* conditions. In all the studies mentioned in Table 1.1, plantlets easily produced roots under *in vitro* conditions. In some studies, the nutrients in basal MS were at high enough levels to inhibit rhizogenesis, and half-strength MS media promoted better root formation (Reddy, *et al.*, 2001; Sivasubramanian, *et al.*, 2002; Hu, *et al.*, 2008). The addition of auxin to the growth media is usually associated with promotion of rhizogenesis, and this proved to be the case with most of the tissue culture protocols studied on *Plectranthus* and related plants (Table 1.1). In the case of *C. forskohlii*, Reddy *et al.* found that no auxin was required to promote root formation.

During Stage IV in all the above-mentioned studies, well rooted *in vitro* plantlets were acclimatized in laboratory conditions for a period ranging from a couple of days to a month. In all cases, acclimated plants resulted in good establishment rates. For *P. vetiveroides* 82% of plantlets survived and for *P. esculentus* between 75 and 94% survival rates were achieved (Allemann, 2002; Sivasubramanian, *et al.*, 2002).

An extensive procedure was developed to acclimatize and establish *in vitro* produced *Coleus forskohlii* plantlets. Well rooted plantlets were first cultured in autoclaved tap water for a week and then moved to a vermiculite mixture where the plantlets were kept under high humidity in polythene bags for a month before the plants were moved to the greenhouse (Reddy, *et al.*, 2001).

### **1.3.2 Callus production in *Plectranthus* and related plants**

Apart from leaf-derived callus in *C. forskohlii* (Reddy, *et al.*, 2001), no other callus production protocols for *Plectranthus* have been produced. However, callus have been produced in other closely related members such as *Coleus* (Razzaque and Ellis, 1977; Reuff, *et al.*, 1988; Reddy, *et al.*, 2001) and *Mentha* (Van Eck and Kitto, 1992; Li, *et al.*, 1999).

*C. forskohlii* leaf pieces produced optimal callus for shoot production with the addition of kinetin at 0.5 mg/L (Reddy, *et al.*, 2001). Although 2,4-D is usually associated with *in vitro* callus production, the addition of 2,4-D was found to be inhibitory to callus formation on *C. forskohlii*. Callus from *C. forskohlii* was produced in 16 hour day light conditions.

The addition of an auxin in combination with a cytokinin was required for callus formation with *C. blumei*. Razzaque and Ellis (1977) used 2,4-D at 1.0 mg/L in combination with 0.1 mg/L kinetin in suspension culture for callus production. The callus cultures were maintained in continuous light. In a cryopreservation study

performed on *C. blumei*, callus suspension cultures were maintained with a growth regulator combination of 2.0 mg/L 2,4-D, 0.5 mg/L NAA, and 0.2 mg/L kinetin (Reuff, *et al.*, 1988). In both of the above mentioned studies, *C. blumei* cultures were used for forskolin production and no report was given on the callus's regeneration potential.

Callus was induced from leaf explants of native spearmint, *Mentha spicata* L., with a growth regulator combination of 4.0 mg/L thidiazuron (TDZ) and 250 ml/L CW in dark growing conditions (Li, *et al.*, 1999). Callus that was cultured on initiation medium for 14 days, followed by culturing on medium devoid of growth regulators in the dark, resulted in an average of 8.5 shoots produced per leaf explant (Li, *et al.*, 1999).

Orange mint leaf discs cultured in the dark for 60 days on a medium containing BA ranging from 4 – 10 mg/L and 250 ml/L CW produced callus that, when moved to low light conditions, produced shoots (Van Eck and Kitto, 1992). Orange mint also produced some organogenic callus when CW was substituted for NAA or 2,3,5-triiodobenzoic acid (TIBA), however a much larger regeneration ability was observed when CW was used instead of the two synthetic auxins. The higher organogenesis observed with the use of CW instead of NAA or TIBA was probably due to the interaction of BA with some of the other components in CW (Van Eck and Kitto, 1992).

From the mentioned studies it is clear that plants related to *Plectranthus* react very differently to tissue culture conditions. In some cases the addition of a low level cytokinin will be sufficient to produce regenerable callus, whereas in other cases a combination of auxins and cytokinins are required for callus production. From the literature there is no clear trend in callus production protocols for plants related to *Plectranthus*.

Irradiance has been known to affect callus formation. From the literature reviewed, the effect of light on plants related to *Plectranthus* is dependant on the species (Table 1.1). In some cases light may be promotive (Razzaque and Ellis, 1977; Zagrajski, *et al.*, 1997; Reddy, *et al.*, 2001) but in other cases darkness promotes callus formation and regeneration (Li, *et al.*, 1999).

#### **1.4 Plant growth regulators in the greenhouse**

In the commercial horticulture industry there has been a major emphasis on the production of compact, well-branched and proportionate looking potted plants (Wilkins, *et al.*, 1979; Larson, 1985). A large number of potted crops species have not been very well domesticated, and thus the genetics for short well-branched growing types have not been developed (Andersen and Andersen, 1999). In many cases growth retardation in vigorous growing plants is required.

Several techniques are practiced to produce potted crops with the characteristics desired by the horticulture industry. Growth control via manipulation of the plant's growing environment can be implemented. Plant height can be controlled through the use of temperature regulation, light intensity, controlled drought stress, nutrient stress, and mechanical stress (Andersen and Andersen, 1999; Schnelle, *et al.*, 1999). Controlling plant height in a commercial setup via manipulation of the plant's growing environment requires a high level of involvement from the grower, whereas the application of a chemical to the crop can be much more convenient. It is difficult to imagine any segment of agriculture that has made more extensive use of plant growth regulators (PGRs) in commercial production than ornamental horticulture. This could be because most ornamental plants are not consumed and are therefore less regulated.

Gibberellins are a large group of compounds that are usually associated with the promotion of stem growth (Taiz and Zeiger, 2002). Gibberellins have been used to promote stem elongation in floriculture crops. The best known use of the exogenous application of gibberellins in floriculture is their use as a substitute for cold requirement to induce flowering in azalea (Larson, 1985).

Most PGRs used for plant height restriction inhibit some step in the complex gibberellin synthesis pathway. There is a distinction between different PGRs used as growth retardants. The distinction is made between compounds that interfere with the biosynthesis of gibberellins and sterols and compounds whose effects are connected with ethylene production (Sauerbrey, *et al.*, 1987). Products such as ethephon and aminocyclopropane-carboxylic acid (ACC) are connected with cellular ethylene biosynthesis. Ethephon is directly converted to ethylene and ACC is a precursor in the ethylene synthesis pathway (Sauerbrey, *et al.*, 1987)

Retardants such as the triazoles, pyrimidines, and quaternary ammonium compounds interfere with the biosynthesis of gibberellins (Sauerbrey, *et al.*, 1987; George, *et al.*, 2008). Common names for some of the most used PGRs in floriculture are ancymidol ( $\alpha$ -cyclopropyl- $\alpha$ -(4-methoxyphenyl)-5-pyrimidinemethanol), chlormequat chloride (2-chloroethyl trimethylammonium chloride), daminozide (butanedioic acid mono (2,2-dimethyl hydrazide)), paclobutrazol (2R,3R+2S,3S)-1-(4-chlorophenyl-4,4-dimethyl-2-(1,2,4-triazol-1-yl))), and flurprimidol (( $\alpha$ -1-methylethyl)- $\alpha$ -(4-(trifluoromethoxy) phenyl)-5-pyrimidinemethanol). These growth retardants are often referred to as anti-gibberellins.

The use of PGRs can have beneficial effects other than those visibly observed. Some of these effects can be in the form of protection from pollutants such as ozone, drought, pests, and other physiological disorders (Larson, 1985). PGRs such as

paclobutrazol have also been used to promote adventitious root formation on *Plectranthus verticillatus* cuttings in the greenhouse (Davis, *et al.*, 1985).

#### **1.4.1 Use of PGRs on *Plectranthus* and related plants**

The effect of paclobutrazol on adventitious root formation on cuttings of *P. verticillatus* was investigated by placing cuttings in solutions of different concentrations of paclobutrazol ranging between 3.0 and 6.0 mg/L for 24 – 40 hours. It was found that paclobutrazol increased the number of roots formed on cuttings. The paclobutrazol treatments also had a residual height reduction effect on the plants (Davis, *et al.*, 1985).

In guidelines for warm climate production of *Plectranthus*, Rick Schoellhorn (2004) proposes the use of paclobutrazol as a drench at low concentrations ranging from 0.25 – 1.0 mg/L at two weeks after pinch. Schoellhorn also notes that daminozide and chlormequat used as sprays in low concentrations are also effective under Florida conditions. Culture information for *Plectranthus* ‘Mona Lavender’ recommends the use of paclobutrazol as a foliar spray in the ranges of 5.0 – 20.0 mg/L (Ball-Horticultural-Company, 2008). Ball Horticulture Company also recommends a tank mix of chlormequat chloride (750 – 1500 mg/L) and daminozide (2500 – 3500 mg/L) applied as a foliar spray.

Apart from the above-mentioned guidelines there are no other reports on the suggested use of PGRs on *Plectranthus* under greenhouse growing conditions. Several reports are available on the results of plant growth regulators applied to Coleus (*Solenostemon scutellarioides*) and ornamental members of *Salvia*. Table 1.2 provides a summary of the suggested PGRs and their rate of application on Coleus and *Salvia splendens* cultivars.

**Table 1.2 Suggested plant growth regulator levels obtained on Coleus and *Salvia* cultivars.**

<b>Species</b>	<b>Plant material</b>	<b>PGR</b>	<b>Concentration (mg/L)</b>	<b>Application</b>	<b>Applications</b>	<b>Reference</b>
<i>Solenostemon scutellarioides</i>	Seedling	Uniconizole	20 - 80	Spray	1x	Barrett and Nell, 1988
<i>Salvia splendens</i> 'Red Hot Sally'	Seedling	Uniconizole	20 - 80	Spray	1x	
Coleus	Flats and Pots	Ancymidol	33 - 66	Spray		Schnelle, <i>et al.</i> , 1999
<i>Salvia</i>	Flats and Pots	Paclobutrazol	50 - 63	Spray		
		Uniconizole	10 - 20	Spray		
		Ancymidol	33 - 66	Spray		
		Daminozide	5000	Spray	1x - 2x	
<i>Solenostemon scutellarioides</i>	Cuttings	Uniconizole	10 - 15	Spray		
		Uniconizole	16 - 32	Spray	1x or 2x	Cavins, <i>et al.</i> , 2002
<i>Coleus x hybridus</i> 'Wizard Velvet'	Cuttings	Flurprimidol	40	Spray	1x	Whipker, <i>et al.</i> , 2003
<i>Salvia splendens</i> 'Prestige Scarlet'	Cuttings	Flurprimidol	40 - 80	Spray	1x	
<i>Salvia splendens</i> 'Flamex 2000'	Seedling	Flurprimidol	0.5	Spray	1x	Vernieri, <i>et al.</i> , 2003

Different species and even cultivars within a species display genotypic variance in their response to the PGRs used (Seeley, 1979). Triazole compounds such as paclobutrazol and uniconazole acts as effective growth retardants on a wide range of plants (Barrett and Nell, 1988). Within the *Coleus* cultivars that have been studied, paclobutrazol applied as a spray was not very effective in retarding growth in both seedlings (Barrett and Nell, 1988) and cuttings (Cavins, *et al.*, 2002). Uniconazole applied as a spray in the range of 20 – 40 mg/L were more effective in retarding growth than paclobutrazol. Cavins, *et al.* (2002) observed a significant difference in the effectiveness of paclobutrazol and uniconazole between the two cultivars *Solenostemon scutellariodes* ‘Burgandy sun’ and ‘Solar storm’. Paclobutrazol had almost no effect when applied to ‘Solar storm’; whereas when paclobutrazol was applied to ‘Burgandy Sun’, sufficient growth retardation was observed. Uniconazole proved to be more effective than paclobutrazol for both cultivars (Cavins, *et al.*, 2002).

Paclobutrazol is not recommended for growth retardation on ornamental sages such as *Salvia splendens* (Schnelle, *et al.*, 1999). Comparison of the effectiveness of uniconazole, paclobutrazol, and daminozide on height control of bedding plants showed that uniconazole was more effective in height control in *Salvia* than paclobutrazol (Barrett and Nell, 1988).

Flurprimidol, commercially applied as Topflor®, proved to be effective in height control of both *Salvia splendens* and *Solenostemon scutellariodes* (Vernieri, *et al.*, 2003; Whipker, *et al.*, 2003). *Salvia splendens* ‘Vista Burgandy’ showed a linear relationship between plant height and increasing concentration of flurprimidol applied as a spray. Flurprimidol applied in the range of 40 – 80 mg/L produced good results in height restriction on *Salvia splendens* ‘Vista Burgandy’, although in the same study it was found that both paclobutrazol and uniconazole also yielded good results (Whipker, *et al.*, 2003).

The rates and results for different PGR applications in the literature vary. Apart from different species and cultivars having differences in their response to PGR treatments, there are other factors to consider when conducting PGR research. The environmental conditions, life stage, and age of the plants can influence the effectiveness of the PGR application. Schoellhorn (2004) mentions that *Plectranthus* should be treated with PGRs before the onset of flowering to have a good effect on height restriction.

Environmental factors such as growth medium, growing medium moisture, temperature, watering and fertilizer schedules, pests, and the use of surfactants can influence the effectiveness of the PGR application (Seeley, 1979; Larson, 1985). The equipment used in the application of PGR treatments can also influence the effectiveness of the retardant. The effectiveness of daminozide foliar sprays increased up to 12 times when the average droplet size decreased from 6000 to 25 microns (Seeley, 1979).

The mode of uptake for different growth retardants differ and also differ between species and cultivars. Some growth regulators such as chlormequat are effective when applied either as a drench or spray, depending on the species. Compounds such as daminozide are only effective when used as a foliar spray (Larson, 1985). In poinsettia a chlormequat drench application has a better activity than a foliar spray application (Larson, 1985). Drench applications typically use more of the growth retardant than a spray application, and one has to consider the costs involved with each treatment.

Selecting an appropriate growth regulator can be a challenging process for growers. In addition to selecting from a wide range of available growth regulators, growers must also consider the possible environmentally-dependent effects of each PGR, as well as the characteristics of each crop.

## CHAPTER 2: TISSUE CULTURE AND MICROPROPAGATION OF ORNAMENTAL *PLECTRANTHUS*

### 2.1 ABSTRACT

*Plectranthus* belongs to the *Lamiaceae* and consists of approximately 350 species. Some members of *Plectranthus* have beautiful flowers and display potential for further improvement in the horticulture industry. New *Plectranthus* cultivars that have gained popularity in the horticultural industry are sterile hybrids that require vegetative propagation. Tissue culture techniques can be very useful tools to aid in future breeding programs and creates an opportunity for further investigation. The objective of this study was to develop a complete micropropagation strategy developing both direct and indirect organogenesis protocols for *Plectranthus* ‘Cape Angels Dark Pink’ (CADP) and *P. zuluensis* (PZ). Half-strength Murashige and Skoog nutrients and vitamins were used as the base medium in all studies. The cultivars chosen for this study appeared to be sensitive to high cytokinin levels and both CADP and PZ produced the greatest number of shoots in the presence of 0.1 mg/L BA. CADP shoot tips did not display a change in the average number of shoots that were produced by continuous subculture. The average number of shoots produced by PZ increased over time, with the greatest increase over time at 0.1 mg/L BA. The presence of auxin during multiplication did not increase the number of shoots produced by CADP and PZ nodal sections. Different *Plectranthus* accessions display genotypic variance in the average number of shoots produced from shoot tips when cultured in the presence of 0.1 mg/L BA. CADP and PZ shoots rooted with ease *in vitro* and acclimated microcuttings of CADP and PZ rooted without the use of auxins under *ex vitro* conditions. The presence of 2,4-D was required for callus production in both CADP and PZ. Irradiance was important in callus production, with dark

conditions favoring callus induction in PZ while light conditions favored callus production in CADP. White friable CADP callus could be maintained on nutrient medium with 0.5 mg/L 2,4-D and kinetin, although it was not possible to induce shoot formation from either CAPD or PZ callus. Near 100% establishment rates of *in vitro* cultured *Plectranthus* plantlets to the greenhouse was achieved.

## 2.2 INTRODUCTION

*Plectranthus* belongs to the *Lamiaceae* and consists of approximately 350 known species distributed throughout summer rainfall areas of Africa, Madagascar, India, Australia, and a few of the Pacific islands (Van Jaarsveld, 2006). *Plectranthus* species are mostly soft low-growing, semi-succulent, to succulent herbs or shrubs.

*Plectranthus* has been referred to as a “horticulturally neglected” genus (Van Jaarsveld and Edwards, 1987). Several species have been used horticulturally, with the best known plants being the ‘Swedish Ivy’ (Van Jaarsveld, 2006). This common name causes confusion because it is the name given to at least three species, *P. oertendahlii*, *P. verticillatus* (Van Jaarsveld, 2006) and *P. argentatus* (AboutGardenPlants, 2004). As an ornamental, *Plectranthus* is most commonly grown as foliage plants in pots or hanging baskets. Some of the southern African species produce beautiful flowers (Brits, *et al.*, 2001), and there is great potential for further development of plants within *Plectranthus* for the horticultural market. Traditional breeding and *in vitro* techniques can be utilized to further the development of ornamental *Plectranthus*.

Tissue culture techniques have been widely used in plant breeding programs. *Plectranthus* crosses are difficult to make and hybrids are usually sterile (Brits and Li, 2008). *In vitro* techniques can be applied in a *Plectranthus* breeding program in several ways. First, there can be a relative fast increase in the numbers of plants with commercial potential, and help speed up the process of widely releasing a new

cultivar. Second, tissue culture techniques such as regeneration from callus can be used to create somaclonal variation where plants are sterile. Third, *in vitro* techniques can be used to create fertile tetraploids from sterile hybrids. Fourth, *in vitro* techniques can be used to produce disease free plants to serve as stock plants. Very limited work has been conducted on the tissue culture and micropropagation of plants within the genus *Plectranthus*. This creates an opportunity for further investigation and the creation of tissue culture protocols that can be used in a breeding program.

Numerous papers have been produced on other *Lamiaceae* that are closely related to *Plectranthus*. Micropropagation strategies have been developed for, *P.vetiveroides* (Sivasubramanian, *et al.*, 2002), *P.esculentus* (Allemann, 2002), and two ornamental members *P.madagascariensis* and *Plectranthus x hilliardiae* ‘Edelblau’ (Hu, *et al.*, 2008). None of the studies on *Plectranthus* micropropagation made use of an indirect organogenesis procedure; micropropagation via axillary branching was the method of choice in the studies above.

Callus and direct organogenesis protocols have been described for *Coleus forskohlii* (Bhattacharyya and Bhattacharya, 2001; Reddy, *et al.*, 2001). A mass propagation protocol has been developed for *Coleus forskohlii* making use of an indirect organogenesis pathway where shoots are produced from leaf derived callus (Reddy, *et al.*, 2001). Protocols for shoot multiplication via a combination of axillary branching and direct shoot morphogenesis have been developed for *C.forskohlii* (Bhattacharyya and Bhattacharya, 2001). Tissue culture and micropropagation protocols via axillary branching, direct shoot morphogenesis, and a callus phase have been described for *Coleus blumei* (Razzaque and Ellis, 1977; Hervey and Robbins, 1978; Zagrajski, *et al.*, 1997).

Work published on *Plectranthus* (Allemann, 2002; Sivasubramanian, *et al.*, 2002; Hu, *et al.*, 2008), *Coleus* (Razzaque and Ellis, 1977; Hervey and Robbins, 1978;

Reuff, *et al.*, 1988; Zagrajski, *et al.*, 1997; Bhattacharyya and Bhattacharya, 2001; Reddy, *et al.*, 2001; Rani, *et al.*, 2006), *Salvia* (Skala and Wysokinska, 2004), and *Mentha* (Van Eck and Kitto, 1992; Li, *et al.*, 1999) does not provide a clear guideline as to the growth regulators and concentrations to use for tissue culture of *Plectranthus*.

In these studies, Murashige and Skoog (1962) medium, also known as MS medium, is the most preferred nutrient medium used for *Plectranthus* and related plants. The cytokinins 6-benzylaminopurine (BA) and kinetin proved to be the most commonly used cytokinins during shoot multiplication. In some cases a relatively low level of cytokinin was required for shoot multiplication (Hervey and Robbins, 1978; Bhattacharyya and Bhattacharya, 2001), whereas in other studies a BA level as high as 10 mg/L (Van Eck and Kitto, 1992) gave the best results.

*Plectranthus* root with ease in *ex vitro* conditions, and the same proves to be true for plants related to *Plectranthus*. In some cases half-strength MS media was preferred for rhizogenesis (Reddy, *et al.*, 2001; Sivasubramanian, *et al.*, 2002; Hu, *et al.*, 2008) and in the majority of studies full-strength MS media was used for rhizogenesis. The addition of an auxin proved in the majority of cases studied to enhance rhizogenesis (Bhattacharyya and Bhattacharya, 2001; Sivasubramanian, *et al.*, 2002; Skala and Wysokinska, 2004; Hu, *et al.*, 2008) while in other cases no growth regulator was required for root formation (Reddy, *et al.*, 2001).

The objectives of this study were to develop a complete micropropagation strategy (stages 0 – IV) making use of both direct and callus organogenesis for the *Plectranthus* ‘Cape Angels Dark Pink’ (CADP) and *P.zuluensis* (PZ) accessions and compare the micropropagation strategy developed for CADP to other *Plectranthus* accessions in the collection.

## 2.3 MATERIALS AND METHODS

### 2.3.1 *Plectranthus* collection and initiation of *in vitro* cultures

Several accessions of *Plectranthus* were obtained (Appendix A). Once plants were established, they were grown and maintained under greenhouse conditions at the Kenneth Post Greenhouse (KPL) complex on Cornell University's Ithaca campus. The collection was also maintained at Cornell University's Long Island Horticultural Research and Extension Center in Riverhead, New York.

The plants were grown under natural day light conditions without the use of artificial lights. Growing temperatures were maintained at  $25^{\circ}\text{C} \pm 3^{\circ}\text{C}$ . Liquid fertilizer was applied with every watering at 150 ppm nitrogen from a 20-5-21 fertilizer with added Epsom salts.

The greenhouse-grown plant material was used to provide explants for *in vitro* cultures. Starting material was taken from greenhouse plants and cut into single node and shoot tip explants. The explants were washed under running tap water with a drop of Ultra Dawn® for 5 minutes. In order to determine the optimal sterilization strategy, three concentrations of sodium hypochlorite were used; 0.53, 0.78 and 1.05 % each with 0.1 % Tween 20. The sterilization treatments were applied for 15 minutes. The bleach treatments were preceded by a 30 second dip in 70% ethanol (EtOH), and followed by three rinses in sterile distilled water. Success of the sterilization procedure was scored five days after the cultures were initiated. The treatments were replicated over three dates and 16 replications per bleach treatment were used.

The nutrient medium that was used for all experiments was half-strength basal MS nutrients and vitamins (*Phyto* technology laboratories®, [www.phytotechlab.com](http://www.phytotechlab.com), product number M519). The carbon source was sucrose at 20 g/L and agar was the gelling agent at 7 g/L (*Phyto* technology laboratories® product number A111). All media used in this study were autoclaved, at  $121^{\circ}\text{C}$  and 103 kPa, after the pH was

adjusted to  $5.75 \pm 0.05$ . The above described media will be referred to as MS1 medium.

After sterilization, explants, single nodal sections and shoot tips 10 – 15 mm, were placed in culture tubes with 15 mL of MS1. All *in vitro* cultures, unless otherwise stated, were cultured in the growth room in the Earle Laboratory in Bradfield at Cornell University's Ithaca campus. Cultures were maintained at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , with a 16 hour photoperiod. The average photosynthetic photon flux on the benches in the culture room was  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ . These culture conditions will be referred to as standard culture conditions.

*Plectranthus* 'Cape Angels Dark Pink' (CADP) and *P. zuluensis* (PZ) was chosen as the two accessions to perform the majority of the research on. CADP was chosen because it is an interspecific hybrid that is currently grown in the industry. PZ is a species that displays horticultural potential due to its beautiful flowers and ability to grow in very shaded areas. Successful initiation of *in vitro* cultures of CADP, PZ, *P. ecklonii*, and *P. oertendahlii* were maintained by subculturing every four to six weeks. *In vitro* grown cultures were used as the starting material for all experiments to follow.

### **2.3.2 Direct organogenesis**

Unless otherwise stated, normal culture conditions were used for all experiments, all experiments were arranged in a completely randomized design with random numbers generated from [www.random.org](http://www.random.org), MS1 was used as the base medium, and control treatments consisted of plants cultured on MS1 devoid of growth regulators.

### 2.3.2.1 Preliminary micropropagation studies

Initial studies were conducted with CADP and PZ to determine a growth hormone concentration that produced near optimal multiplication. A factorial of 6-benzylaminopurine (BA), at 0.1 and 1.0 mg/L and Indole-3-butyric acid (IBA) at the same levels was used. Explants were cultured using Falcon™ 35 60 x 15mm pre-sterilized petri dishes. Two shoot tips were used per petri dish, for both CADP and PZ. The experiment was repeated twice with general observations made at four and eight weeks of culture.

A second preliminary experiment evaluated three cytokinins, BA, 6-( $\gamma,\gamma$ -dimethylallylamino) purine (2iP), kinetin (Kin), each added at concentrations of 0, 0.05, 0.1, 0.5, and 1.0 mg/L. *In vitro* grown shoot tips between 10 – 13 mm were used as explants with four replications per treatment for both CADP and PZ. After eight weeks, the total number of shoots that were produced was recorded and shoot height was measured. Shoots were grouped in different size classes. A shoot was counted if the first two leaves were visible with the naked eye.

The best treatments from the first two preliminary experiments were selected for further testing.

### 2.3.2.2 Major micropropagation study

#### *Experiment 1 – Shoot production*

Results from the preliminary studies determined that two concentrations of BA (0.05 and 0.1 mg/L) would be tested for both CADP and PZ, and two concentrations of 2iP (0.05 and 0.5mg/L) would be tested on PZ. *In vitro* grown shoot tips cut between 10 – 13 mm were used as explants. Magenta GA7 vessels (*Phyto* technology laboratories®, product number C907) with 30 mL of media were used to culture plants. Four shoot tips were placed in each culture vessel and the vessels were

replicated four times per treatment, resulting in a total of 16 explants per treatment. After eight weeks of culture the number of shoots that were produced was counted and shoot tips 10 – 13 mm in length were excised and subcultured on the same medium. This experiment was maintained for a period of four subcultures and terminated after the last subculture. At termination, shoots were grouped into one of four size classes (Table 2.1).

**Table 2.1 Size classes used for grouping shoots.**

<b>Class</b>	<b>Size (mm)</b>
1	< 5
2	5 - 10
3	11 - 20
4	> 20

*Experiment 2 – Effect of IBA on shoot production*

The objective of this experiment was to determine if the addition of auxin would have an effect on the number of shoots produced. The addition of IBA at 0.01 and 0.1 mg/L in combination with 0.1 mg/L BA was compared to a control treatment of only BA 0.1mg/L. Single nodal explants from *in vitro* grown CADP and PZ were used as explants. GA7 vessels with 30 mL media were used as to culture plants. Four nodal sections per magenta were used and the GA7 vessels were replicated four times per treatment. After nine weeks of growth, the number of shoots that were produced per explant was counted.

*Experiment 3 - Shift in multiplication medium strategy*

The objective of this study was to determine if a shift in growing medium would affect shoot production from CADP and PZ nodal sections. Single nodal sections were placed on MS1 containing BA at 0.1, 0.5, and 1.0 mg/L for four weeks.

After four weeks, nine nodal explants per 0.5 and 1.0 mg/L BA treatments were subcultured to GA7 vessels with 30 mL MS1 devoid of growth regulators. Explants cultured on 0.1 mg/L were not subcultured, because explants grown in the presence of 0.1 mg/L BA for eight weeks was the control protocol used to compare the shift in growing medium strategy. After a further four weeks of growth, the number of shoots that were produced was scored and grouped into one four size classes (Table 2.1). The number of “culturable” shoots was calculated as:

**Equation 2-1 Culturable shoots (CS):**

$$CS = 0.5(\text{Class2}) + \text{Class3} + 2.5(\text{Class4})$$

Culturable shoots are described as shoots that would easily be subcultured or moved to the next stage of micropropagation in a commercial scenario. Culturable shoots are the number of shoots that are expected to have the same multiplication potential as the initial explant. Shoots grouped in the first class are too small to easily be moved to the next phase of micropropagation and are thus left out of Equation 2-1. Roughly half of the shoots grouped in class two are expected to have the same production potential as shoots in the third size class. The third size class was taken as the optimal size, and thus every class three shoot is only counted once. Shoots grouped in class four can be large and shoots in excess of 20 mm can at least be cut into two or more explants, of 10 mm, to be used in a micropropagation protocol.

*Experiment 4 – Explant effect on micropropagation*

The following experiment was conducted to determine if there is a difference in the number of shoots that are produced from either a shoot tip or nodal explant. MS1 with 0.1 mg/L BA was used as the multiplication medium. Single nodal sections

and shoot tips 10 mm in length were used as explants and cultured in GA7 vessels with 30 mL of medium. Four explants were placed per culture vessel and replicated four times for nodes and shoot tips for both CADP and PZ. After eight weeks of culture the number of shoots that were produced was recorded. Shoots were grouped in four categories, Table 2.1. Root growth was classified on a one to five scale with one being no roots and five being the most (Table 2.2). Culturable shoots were calculated (Equation 2-1).

**Table 2.2 Description of root growth scale**

<b>Ranking</b>	<b>Root class description</b>
1	No roots visible
2	1 or 2 small roots
3	2 or more medium length roots
4	3 or more long roots
5	Many vigorous growing roots

*Experiment 5 - Comparison of micropropagation potential for several  
Plectranthus accessions*

This study was designed to compare the multiplication of several *Plectranthus* accessions using MS1 with BA at 0.1 mg/L. The *Plectranthus* accessions used in this study was selected in order to both represent plants that are commercially available and represent plants with very different growth characteristics. The number of shoots that were produced by CADP, PZ, *Plectranthus* ‘Mona Lavender’, *P. ecklonii* ‘Erma’, *P. oertendahlii* ‘Uvongo’ and *P. purpurea* were compared in this experiment. GA7 culture vessels with 30 mL medium were used as culture vessels. Four shoot tips between 10 – 13 mm were used per vessel, and were replicated five times per *Plectranthus* accession. Observations were made after a growth period of eight weeks.

The number of shoots produced were counted and grouped in to size classes (Table 2.1). Root growth was scored (Table 2.2).

#### *Experiment 6 - In vitro rooting of Plectranthus*

It was observed in previous studies that both CADP and PZ explants root with ease under *in vitro* conditions on MS1 media devoid of growth regulators. This experiment was conducted to determine the effect of two levels of IBA on *in vitro* rooting of plants following Stage II. Single nodal sections of CADP plants that were maintained for 11 weeks on MS1 with either 0.05 or 0.1 mg/L BA were used as explants. GA7 vessels with 30 mL of media were used to culture plants. Four explants were placed in each culture vessel and each vessel was replicated three times. The explants were checked at the same time each day to see if roots have formed. The number of days that it took for the first visible root to form was recorded. After 21 days of growth the roots were scored on a one to five scale (Table 2.2).

#### *Experiment 7 - Ex vitro rooting of Plectranthus*

The possibility of rooting shoots from culture directly into a greenhouse growing media was investigated. *In vitro* grown CADP and PZ plantlets were acclimatized by opening the culture vessel half way for 24 hours. CADP and PZ shoots ranging from 16 – 62 mm were used as explants. GA7 culture vessels were filled to 30 mm with a 1peat: 1perlite medium. Before adding the medium to the vessels, the growing mixture was moistened with distilled water. All of the leaves except the two uppermost leaves were removed from the shoots before being placed in the rooting vessel. Five shoots were placed per vessel and was replicated four times

for both CAPD and PZ. After 15 days of growth the roots were scored on a one to five scale (Table 2.2).

#### *Experiment 8 – Cold storage of in vitro Plectranthus*

CADP and PZ plants that were grown in culture tubes and GA7 vessels were stored under low temperature conditions to determine if *in vitro Plectranthus* can be cold stored. CADP and PZ plants were subcultured onto fresh MS1 medium and left under standard culture conditions for five days. After five days the plants were moved into a Percival growth chamber at 15°C constant with 16 hour photoperiod at an average photosynthetic photon flux of  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The same procedure was followed with CADP and PZ plants in both GA7 vessels and culture tubes. There were 36 CADP and 31 PZ tubes used with six CADP and seven PZ GA7 culture vessels. The total number of viable shoots from GA7 vessels was recorded 21 weeks after moving them into the cold. The total number of viable plants in culture tubes was recorded 30 weeks after moving the plants into the cold. Shoots were scored as viable when they were subcultured, to fresh MS1 and maintained under standard conditions, and within two weeks new growth was observed.

#### *Acclimation of in vitro cultured plantlets*

This section describes a protocol that was developed for the acclimation of *in vitro Plectranthus*. Well rooted *in vitro* grown *Plectranthus* plants were acclimatized by removing the caps of culture tubes for 24 hours. The plants were taken to Cornell University's greenhouses where all the agar was washed from the roots with lukewarm tap water. Plants were then planted in a 1peat: 1perlite mixture, and were placed in the

mist tunnel located in the propagation greenhouse kept at a constant  $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . After two weeks of growth in the mist tunnel the plants were moved to the greenhouse.

Sixteen plants each of CADP, PZ, *P. ecklonii* 'Erma', and *P. oertendahlii* 'Uvongo' were randomly selected (out of 36 culture tubes) after going through a cycle of micropropagation to compare plant uniformity. Plant uniformity was visually compared to the mother plants. Comparisons were based on growth habit, plant size, leaf size and color, and flower size and color of tissue culture plants versus the stock plants.

### **2.3.3 Indirect organogenesis**

Experiments where explants other than shoot tips and nodal sections were used will be described in this section. Unless otherwise stated, standard culture conditions were used in all studies, experiments were arranged in a completely randomized design with random numbers generated from [www.random.org](http://www.random.org), and MS1 was used as the base medium for all experiments. Dark grown conditions took place at  $25^{\circ}\text{C}$ . Falcon® 35 60 x 15 mm petri dishes ([www.vwr.com](http://www.vwr.com) product number 25384-324) will be referred to as petri dishes.

#### *2.3.3.1 Preliminary studies*

##### *Experiment 9 - Effect of cytokinins and auxins on leaf explants*

The response of *in vitro* grown leaf pieces to different cytokinins and auxins was investigated. BA and 2iP were the cytokinins and 1-naphthalneacetic acid (NAA) and IBA were the auxins. Full-strength MS nutrients and vitamins with 30 g/L sucrose (MS2) was used as the base medium for all the treatments with 6.5 g/L agar and was poured into petri dishes. All growth regulators were used in concentrations ranging

from 0.05 – 5.0  $\mu$ M (Appendix B). The basal ends of *in vitro* leaves were cut into 5 x 5 mm sections with each explant containing part of the leaf midrib. Three leaf explants, abaxial side on the medium, were used per petri dish. Explants from CADP, PZ, *P. oertendahlii*, and *P. ecklonii* ‘Erma’ were used. Explants cultured on MS2 media devoid of growth regulators served as control treatments. The experiment was repeated twice. Observations were made after five weeks of culture.

#### *Experiment 10 - Adventitious shoot and root formation*

The objective of this study was to determine the ability of *in vitro* leaf explants to directly produce roots and shoots. Experiment 10 was separated in two phases.

In the first phase, a factorial experiment with BA at 0.5, 1.0, and 5.0 mg/L and IBA at 0.1, 0.2, and 0.5 mg/L was designed. Two leaf pieces cut the same as in experiment 9, and petioles were used as explants per petri dish. Explants from CADP, PZ, *P. oertendahlii*, and *P. ecklonii* ‘Erma’ were used. The experiment was replicated twice and observations were made after three weeks of culture.

In the second phase, BA at 1.0 mg/L in combination with IBA or NAA at 0.01, 0.1, and 0.5 mg/L were tested to determine if adventitious shoots would form from leaf explants. The second phase consisted of three replications with one replication cultured in dark conditions. The cultures were examined weekly.

#### *Experiment 11 - Callus Production*

The following experiment was conducted to determine the most effective auxin and concentration to use for callus production. MS1 with BA 0.01 mg/L in combination with IBA, NAA, or 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.1, 0.5, and 1.0 mg/L were used. Two leaf pieces, one petiole, and a shoot tip were used as

explants per petri dish. Explants from CADP, PZ, and *P.ecklonii* 'Erma' were used in this experiment. The experiment consisted of three replications with one replication cultured in dark conditions. Observations were made at four and eight weeks of culture.

#### 2.3.3.2 *Major Study*

##### *Experiment 12 - Callus production, kinetin and 2,4-D factorial*

A factorial design with kinetin at, 0.1, 0.5, and 1.0 mg/L and 2,4-D at 0, 0.1, 0.5, and 1.0 mg/L was designed to determine optimal concentrations for callus production in CADP and PZ. Two single nodal sections were used as explants per petri dish. The experiment was repeated twice. All explants were subcultured after four weeks and final observations were made after eight weeks of culture. The presence or absence of roots, shoots, and callus were recorded, and the final width of the explant was measured in mm.

##### *Experiment 13 – Comparison of different cytokinins in callus production*

This experiment was designed to determine the efficacy of callus production for different cytokinins in combination with 2,4-D. Kinetin, BA, and zeatin at concentrations of 0, 0.1, and 0.5mg/L were used in all combinations with 2,4-D at 0.1 and 0.5mg/L. The effect of explant source was also investigated by using both nodal explants (two per petri dish) and leaf explants (three per petri dish). Three replicates of CADP leaf explants and two replicates of nodal explants were examined. One of the leaf explant replicates were cultured under dark grown conditions. Three replicates of PZ nodal explants and two replicates of leaf explants were studied. One PZ replicate of both leaf and nodal explants was cultured under standard culture conditions while

the remaining three replicates were cultured under dark conditions. Observations were made three weeks after the experiment was initiated. The browning of explants and presence or absence of roots and shoots were recorded. The amount of callus that was produced was scored on a one to five scale, with one being no callus and five being the most.

#### *Experiment 14 - Organogenesis from callus*

The objective of this study was to investigate the organogenesis from white friable CADP callus maintained on MS1 with 0.5 mg/L 2,4-D and 0.5 mg/L kinetin (CAC1 medium), and four week old PZ callus produced from dark cultured leaf pieces on MS1 medium with 0.1 mg/L 2,4-D and 0.5 mg/L kinetin (PZC1 medium). MS1 devoid of growth regulators, 0.1 mg/L BA alone, and 0.1 mg/L BA in combination with IBA at 0.01 and 0.1 mg/L were the media that were used. In CADP treatments, three callus clumps roughly 5 mm<sup>3</sup> per petri dish were used as the explants whereas for PZ, three four-week old leaf pieces (10 x 10 mm) with callus growth were the explants. The experiment was repeated three times. Final observations were made six weeks after the initiation of the experiment. The presence of shoots were recorded and root development was classified on a one to five scale, with one being no root growth and five being the most root growth. Growth of callus was scored on a one to five scale, with one being no callus growth and five being the most callus growth.

#### **2.3.4 Statistical analysis**

For all experiments, statistical analysis was performed using JMP® 7.0 (2007 SAS institute inc., Cary, North Carolina). Analysis of variance was performed by fitting standard least squares (SLS) models. The effect of random location for culture

vessels was taken into account in SLS models. All model assumptions were met. In cases where data did not fit the model assumptions, the count data was transformed either by taking the natural logarithm or by taking the square root of the count data. Tukey's honestly significant difference (HSD) was performed for multiple mean comparisons. Dunnett's method for comparing means to a control treatment was performed where means were compared to a control treatment. Where required ordinal logistic regression was performed on results grouped in different classes.

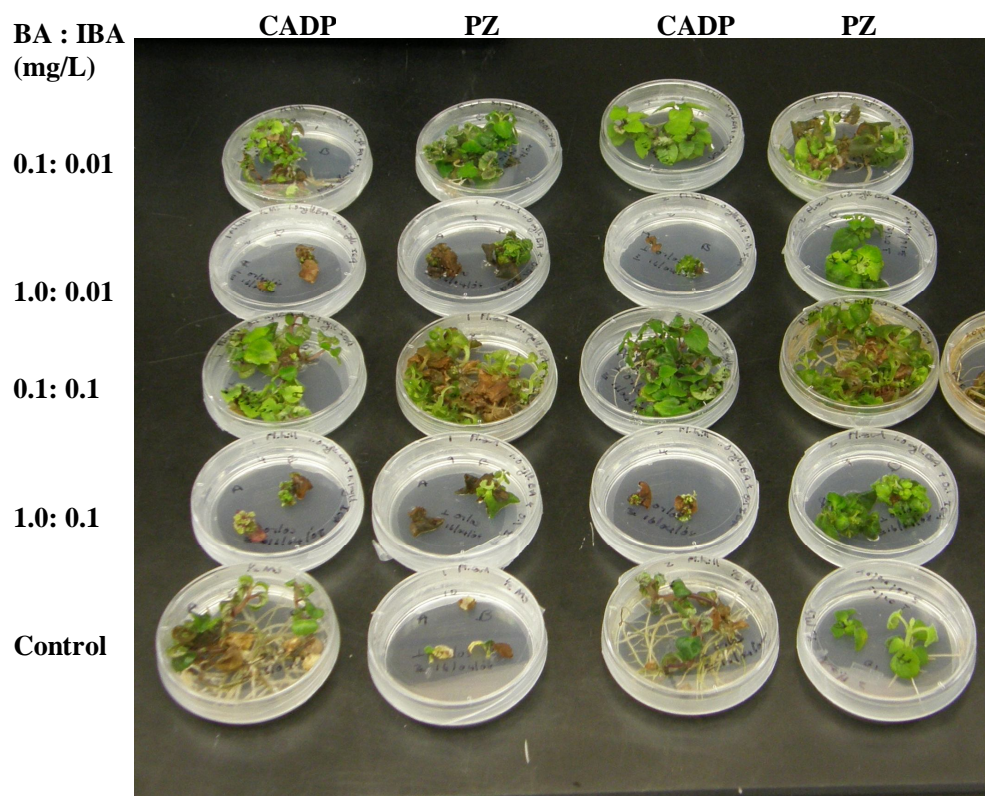
## **2.4 RESULTS**

### **2.4.1 Direct organogenesis**

#### *2.4.1.1 Preliminary micropropagation studies*

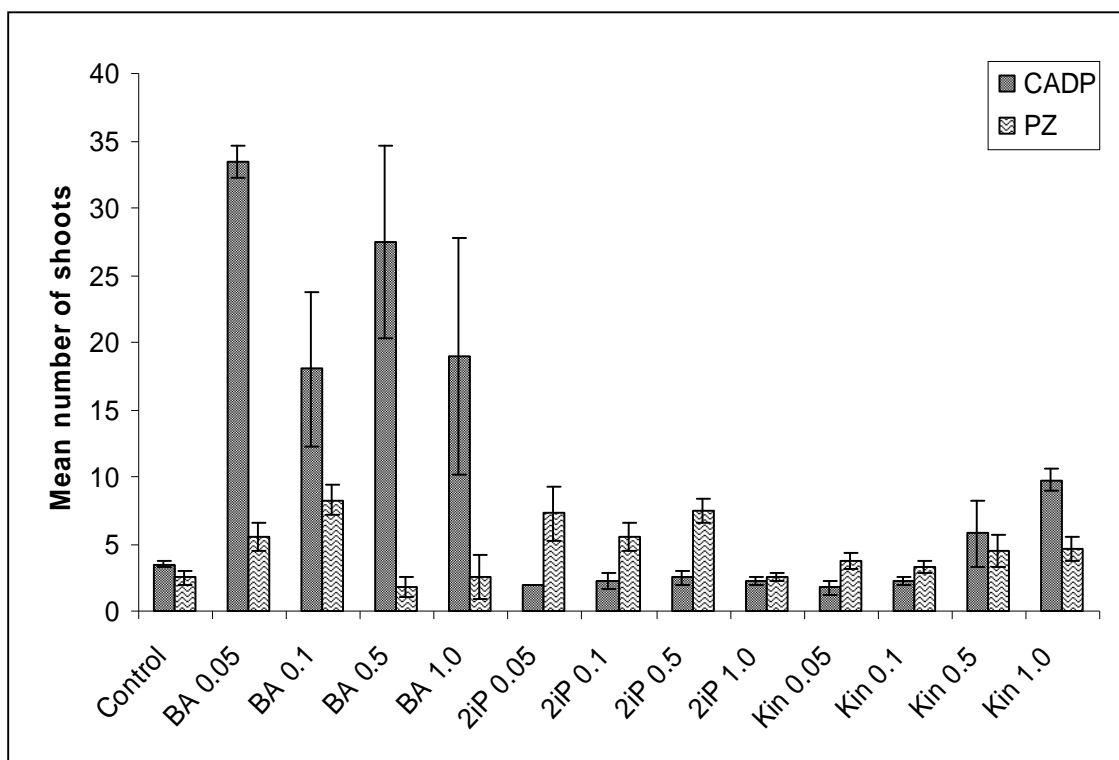
Preliminary micropropagation results showed that shoot tips from both CADP and PZ had better growth in the presence of lower BA levels. All of the explants had more vigorous growth when cultured with BA at 0.1mg/L (Figure 2.1). There was no noticeable effect of the auxin IBA on growth. All of the hormone combinations showed much less root growth than the control treatments (Figure 2.1).

Results from comparing the number of shoots produced per shoot tip from both CADP and PZ using BA, 2iP, and kinetin revealed that BA was the best at inducing axillary shoots from shoot tips (Figure 2.2). Comparisons of the different treatment means to the control treatments using Dunnett's method revealed two BA concentrations, 0.05 and 0.1 mg/L, for both CADP and PZ and the addition of 0.05 and 0.5 mg/L 2iP for PZ, to be used in future studies.



**Figure 2.1** *Plectranthus* ‘Cape Angels Dark Pink’ (CADP) and *P. zuluensis* (PZ) shoot tips after eight weeks of culture in different combinations of BA and IBA.

- BA, 6-benzylaminopurine
- IBA, Indole-3-butyric acid



**Figure 2.2 Mean number of shoots produced from *Plectranthus* ‘Cape Angels Dark Pink’ (CADP) and *P. zuluensis* (PZ) shoot tips after eight weeks of culture.**

- Error bars represent 95% confidence interval of the mean number of shoots produced
- All treatments in mg/L
- Control treatments are half-strength MS devoid of growth regulators
- BA, 6-Benzylaminopurine
- 2iP, 6-( $\gamma,\gamma$ -dimethylallylamino) purine
- Kin, Kinetin

### 2.4.1.2 Major micropropagation study

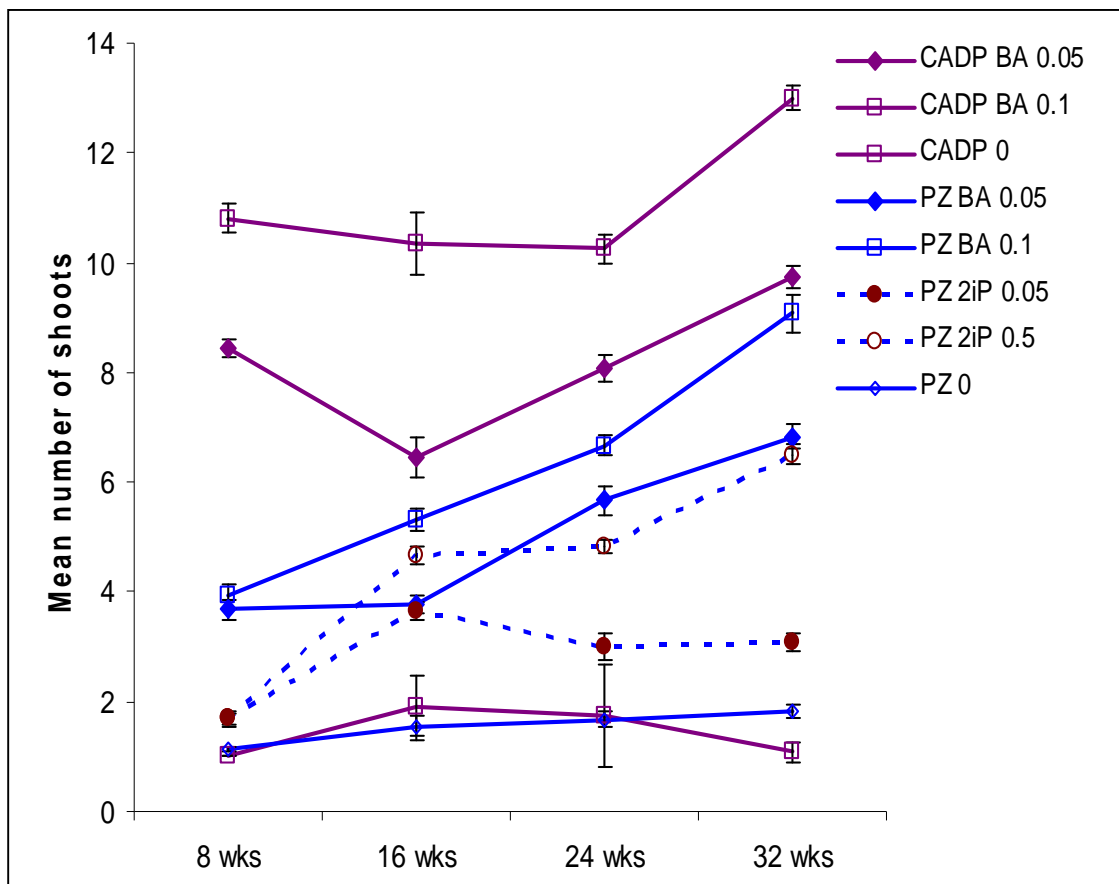
#### Experiment 1 – Shoot production

CADP shoot tips constantly produced a greater number of shoots than any of the PZ treatments. CADP cultured with BA 0.1 mg/L produced the greatest number of shoots (Figure 2.3). CADP explants did not show a significant increase in the number of shoots produced over time. PZ showed an increase in the number of shoots produced over time.

The control treatments were left out of the ANOVA process in order to meet all the assumptions for fitting an SLS model. In the case of CADP, both the main effects of treatment and weeks were highly significant with p values < 0.0001 and 0.006. The interaction term of treatment by week was not significant. In the case of PZ, both the main effects and the interaction term proved to be highly significant with p values < 0.0001. A prediction equation for the amount of shoots produced for PZ shoot tips over time per treatment can be written as in Equation 2-2. The change over time for PZ at BA 0.05 mg/L did not prove to be significantly different than the average change over time for all of the PZ treatments. The greatest increase for PZ over time was at 0.1 mg/L BA (Figure 2.3). The mean number of shoots produced over time by PZ increased significantly at treatment levels, 0.1mg/L and 2iP 0.5 mg/L (Table 2.3).

#### Equation 2-2 Expected number of shoots (ES) produced by *P. zuluensis* over time per cytokinin treatment.

$$\text{ES of } \begin{bmatrix} 2iP & 0.05 \\ 2iP & 0.5 \\ BA & 0.05 \\ BA & 0.1 \end{bmatrix} = \begin{bmatrix} -1.78 \\ -0.29 \\ 0.45 \\ 1.63 \end{bmatrix} + (0.147 \text{ weeks}) + (\text{weeks} - 19.43) \times \begin{bmatrix} -0.094 \\ 0.043 \\ -0.012 \\ 0.063 \end{bmatrix}$$



**Figure 2.3** Number of shoots produced from *Plectranthus* ‘Cape Angels Dark Pink’ (CADP) and *P. zuluensis* (PZ) shoot tips after four subcultures.

- Error bars represent standard error of the mean number of shoots produced at each subculture
- All treatments in mg/L
- Control treatments are half-strength MS devoid of growth regulators
- BA, 6-Benzylaminopurine
- 2iP, 6-( $\gamma,\gamma$ -dimethylallylamino) purine

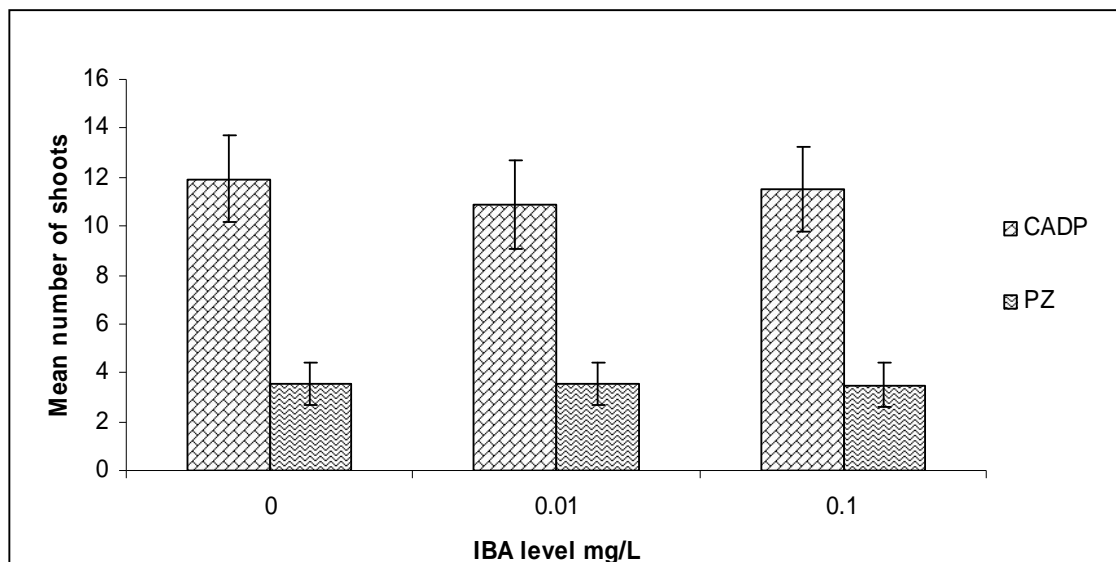
**Table 2.3 Comparison of the mean number of shoots produced per shoot tip at each treatment over a period of 32 weeks, using Tukey's Honestly Significant Difference (HSD) test.**

<b>Week</b>	<b>Treatment</b>	<b>Class</b>	<b>Mean</b>
Wk8	CADP BA 0.1	A	10.81
Wk8	CADP BA 0.05	B	8.44
Wk8	PZ BA 0.1	C	3.92
Wk8	PZ BA 0.05	C	3.69
Wk8	PZ 2iP 0.05	D	1.69
Wk8	PZ 2iP 0.5	D	1.69
Wk8	PZ 0	D	1.13
Wk8	CADP 0	D	1.00
Wk16	CADP BA 0.1	A	10.33
Wk16	CADP BA 0.05	B	6.44
Wk16	PZ BA 0.1	B C	5.33
Wk16	PZ 2iP 0.5	B C	4.67
Wk16	PZ BA 0.05	C D	3.78
Wk16	PZ 2iP 0.05	C D	3.67
Wk16	CADP 0	D	1.89
Wk16	PZ 0	D	1.56
Wk24	CADP BA 0.1	A	10.25
Wk24	CADP BA 0.05	B	8.08
Wk24	PZ BA 0.1	B C	6.67
Wk24	PZ BA 0.05	C	5.56
Wk24	PZ 2iP 0.5	C D	4.83
Wk24	PZ 2iP 0.05	D E	3.00
Wk24	CADP 0	E	1.75
Wk24	PZ 0	E	1.67
Wk32	CADP BA 0.1	A	13.00
Wk32	CADP BA 0.05	B	9.75
Wk32	PZ BA 0.1	B	9.08
Wk32	PZ BA 0.05	C	6.83
Wk32	PZ 2iP 0.5	C	6.50
Wk32	PZ 2iP 0.05	D	3.08
Wk32	PZ 0	D E	1.83
Wk32	CADP 0	E	1.08

- Treatments not connected by the same levels are significantly different,  $\alpha = 0.05$
- CADP, *Plectranthus* 'Cape Angels Dark Pink'
- PZ, *P.zuluensis*
- All treatments in mg/L
- Control treatments are half-strength MS devoid of growth regulators
- BA, 6-Benzylaminopurine
- 2iP, 6-( $\gamma,\gamma$ -dimethylallylamino) purine

### Experiment 2 – Effect of IBA on shoot production

The addition of a low level auxin, IBA (at 0.01 and 0.1mg/L), to a medium with 0.1 mg/L BA did not have a significant effect on the number of shoots produced by single nodal explants from CADP and PZ (Figure 2.4). The main effect of species was highly significant with, p value < 0.0001, whereas the presence or absence of IBA did not have a significant effect on the mean number of shoots produced per nodal section



**Figure 2.4 Comparison of the mean number of shoots produced, from single nodal sections, after nine weeks in the absence and presence of IBA.**

- Error bars represent 95% confidence interval of mean number of shoots produced
- All treatments contained 0.1 mg/L 6-benzylaminopurine
- CADP, *Plectranthus* 'Cape Angels Dark Pink'
- PZ, *P. zuluensis*
- IBA, Indole-3-butyric acid

### Experiment 3 – Shift in multiplication medium strategy

For this experiment the total number of shoots and culturable shoots were used as response variables, and ANOVA was performed by fitting a SLS model. For CADP, no significant differences were detected when the shift of medium treatments

were compared to the control treatment. In the case of PZ both, total number of shoots and culturable shoots increased significantly with the shift in growing medium strategy when it was compared to continuous culture on 0.1 mg/L BA (Table 2.4).

**Table 2.4 Dunnett’s comparison of *P. zuluensis* total shoots and culturable shoots, produced from single nodal sections on the shift in multiplication media compared to continuous culture at 0.1 mg/L BA.**

	Treatment	d  - LSD	p - Value
<b>Total shoots</b>	0.5 mg/L	1.908	< 0.0001
	1.0 mg/L	1.019	0.0003
	0.1 mg/L	-1	1
<b>Culturable shoots</b>	0.5 mg/L	1.583	0.0005
	1.0 mg/L	0.472	0.0139
	0.1 mg/L	-1.71	1

- |d|, Absolute difference = 2.33 shoots
- LSD, Least significant difference
- $\alpha = 0.05$
- Positive values show pairs of means that are significantly different
- BA. 6-benzylaminourine

#### *Experiment 4 – Explant effect on micropropagation*

No significant difference in the total number of shoots that were produced was detected between the different explants for both CADP and PZ. The only significant variable in the model was the species with a p value < 0.0001. When looking at the number of culturable shoots that were produced, the explant was a significant factor in the model with p value 0.0016. The explant effect was only significant in CADP and not PZ. Table 2.5 shows the difference in number of culturable shoots produced. The greatest number of culturable shoots was produced by CADP tip explants (Table 2.5).

**Table 2.5 Tukey's HSD for culturable shoots produced by *Plectranthus* 'Cape Angels Dark Pink' (CADP) and *P. zuluensis* (PZ) shoot tip and nodal explants.**

Treatment	Class	Least square Mean
CADP, Tip	A	4.56
CADP, Node	B	3.20
PZ, Tip	C	1.59
PZ, Node	C	1.34

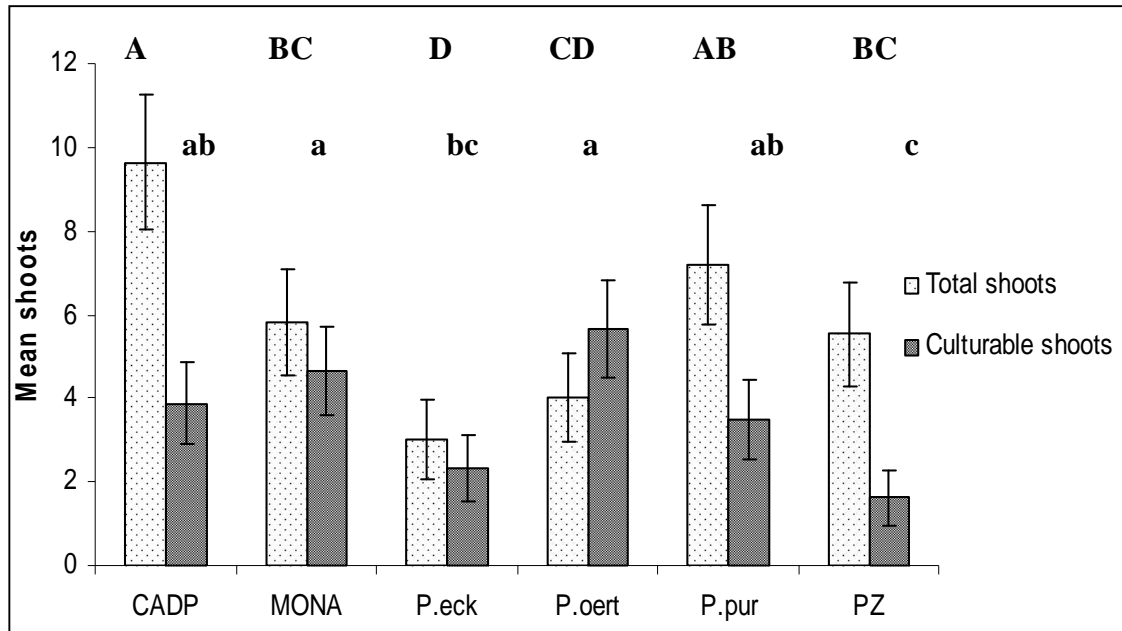
- Treatments not connected by the same letter are significantly different,  $\alpha = 0.05$

*Experiment 5 – Comparison of micropropagation potential for several *Plectranthus* accessions*

The effect of different *Plectranthus* accessions was highly significant with p value < 0.0001 for both the total number of shoots produced and the number of culturable shoots. For the total number of shoots, the accessions were separated into four groups whereas with culturable number of shoots the accessions were divided into three groups. The rankings of different *Plectranthus* accessions changed between the total number of shoots produced and culturable number of shoots (Figure 2.5).

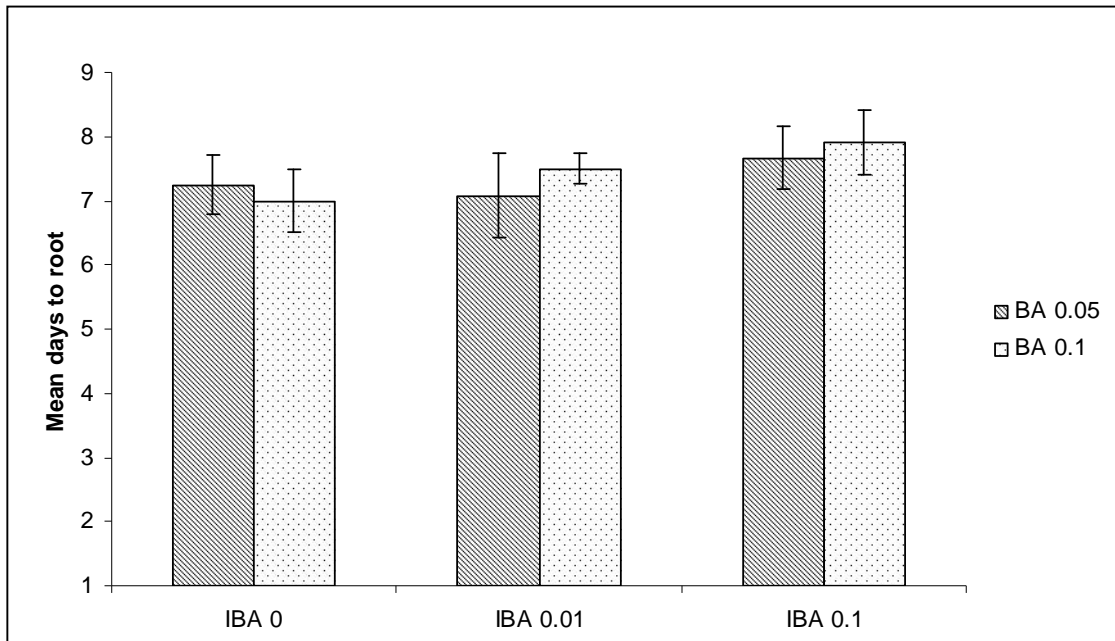
*Experiment 6 - In vitro rooting of *Plectranthus**

No significant differences on the number of days to root were detected among the treatments with the average time to form roots being 7.2 days (Figure 2.6). Ordinal logistic regression was performed on the classification of roots after 21 days of growth. None of the treatments had a significant effect on the classification of roots after 21 days of growth. Only one explant in this study was classified as class two, whereas all the rest were classified as class three and greater.



**Figure 2.5 Tukey's HSD comparison across *Plectranthus* accessions for the mean number of both total and culturable number of shoots produced from shoot tips.**

- *Plectranthus* accessions not connected by the same letter are significantly different,  $\alpha$  0.05
- Error bars indicate 95% confidence interval for mean number of total and culturable shoots produced respectively
- Capital letter indicate groups for total number of shoots
- Lowercase letters indicate groups for culturable number of shoots
- CADP, *Plectranthus* 'Cape Angels Dark Pink'
- MONA, *Plectranthus* 'Mona Lavender'
- P.eck, *P. ecklonii* 'Erma'
- P.oert, *P. oertendahlii* 'Uvongo'
- P.pur, *P. purpurea*
- PZ, *P. zuluensis*



**Figure 2.6 Comparison of mean days to root for *Plectranthus* ‘Cape Angels Dark Pink’ shoot tips from two prior BA treatments, 0.05 and 0.1mg/L.**

- Error bars represent 95% confidence intervals for mean number of days to root
- IBA levels in mg/L
- IBA, Indole-3-butyric acid

*Experiment 7 - Ex vitro rooting of Plectranthus*

Ordinal logistic regression was performed on the classification of the roots after 15 days of growth. Species and initial explant length was used as main effects in the model. None of the main effects proved to be significant, and 100% rhizogenesis was observed (Figure 2.7).



**Figure 2.7** Roots of *Plectranthus* ‘Cape Angels Dark Pink’ shoots after 15 days of growth in 1 peat: 1 perlite growing medium.

#### *Experiment 8 - Cold storage of in vitro Plectranthus*

CADP plants that were cultured in GA7 vessels for 21 weeks at 15°C had 100% viable shoots when they were removed from refrigeration. PZ plants that were cultured in GA7 vessels had 88% viable shoots after 21 weeks at 15°C. After 30 weeks of culture at 15°C, 97% of CADP plantlets in culture tubes were scored as viable whereas 100% of PZ plantlets were scored as viable.

#### *Acclimation of in vitro cultured plantlets*

The procedure that was described for the acclimation of *in vitro* cultured plantlets under the Materials and Methods section proved to be very successful to

establish tissue culture plant under greenhouse conditions. A 100% establishment rate can be achieved using the described methods. Figure 2.8 shows some plants that were successfully transferred from tissue culture to greenhouse conditions. Sixteen plantlets of CADP, PZ, *P. ecklonii* 'Erma', and *P. oertendahlii* 'Uvongo' were assessed for uniformity and all the plants displayed good uniformity based on visual the assessments described (Figure 2.8).

## **2.4.2 Indirect organogenesis**

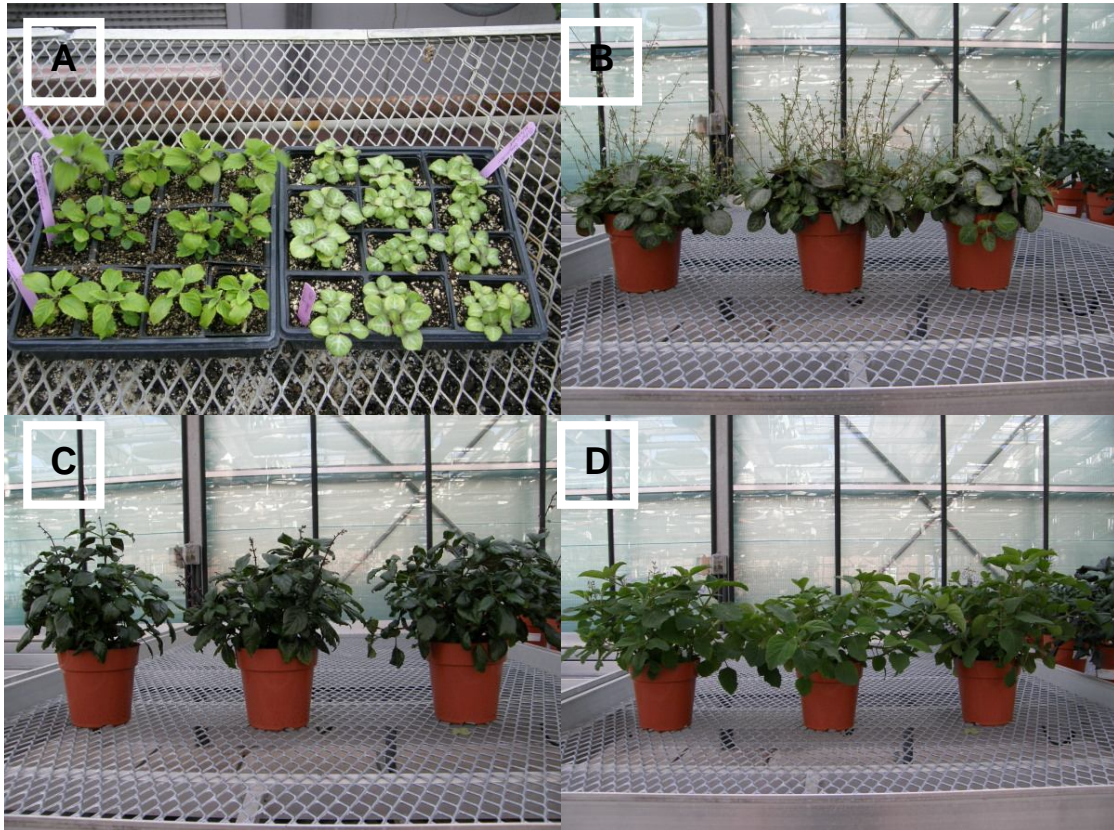
### *2.4.2.1 Preliminary studies*

#### *Experiment 9 - Effect of cytokinins and auxins on leaf explants*

The accessions, CADP, PZ, *P. ecklonii* 'Erma', and *P. oertendahlii* did not yield any promising results when cultured on media with auxin or cytokinin. After five weeks, almost all of the explants turned brown and did not show any signs of growth. One leaf explant of *P. ecklonii* 'Erma' cultured on media containing 0.93 mg/L NAA produced a single root from the midrib area. This was not observed on either of the other two explants in the same petri dish nor on explants in the second replication.

#### *Experiment 10 – Adventitious shoot and root formation*

The factorial experiment with BA at 0.5, 1.0, and 5.0 mg/L and IBA, 0.1, 0.2, and 0.5 mg/L did not produce any adventitious shoots. After three weeks of growth, the majority of the explants turned brown. Some of the CADP and PZ leaf and petiole explants showed a small amount of callus formation around the cut edges of the explants. The majority of callus was produced at 1.0 mg/L BA, and the level of IBA



**Figure 2.8** *Plectranthus* plantlets in the greenhouse after being taken out of tissue culture conditions.

- A – *Plectranthus* plantlets moved to greenhouse after 14 days in misting tunnel
- B – *P. oertendahlii* mature plants moved out of tissue culture. Plants display good uniformity
- C – *Plectranthus* ‘Cape Angels Dark Pink’ mature plants moved out of tissue culture. Plants display good uniformity
- D – *P. zuluensis* mature plants moved out of tissue culture. Plants out display good uniformity

did not seem to affect the growth. One PZ petiole cultured in the presence of BA at 1.0 mg/L and IBA at 0.1mg/L produced an adventitious shoot after eight weeks of culture (Figure 2.9).

BA at 1.0 mg/L in combination with IBA or NAA at 0.01, 0.1, and 0.5 mg/L did not produce any adventitious shoots. Both leaf and petiole explants eventually turned brown regardless of the *Plectranthus* accession. Differences between light-and dark-grown cultures were visible; dark grown cultures stayed green and healthy looking for longer periods of time (Figure 2.10).

#### *Experiment 11 - Callus Production*

Leaf pieces and petiole explants turned brown within four weeks of culture and callus was only produced on the shoot tips. Table 2.6 indicates the treatments that successfully produced callus. The auxin 2,4-D was superior for callus induction and promoted callus formation in three of the four *Plectranthus* accessions.

**Table 2.6 Percentage of explants that showed callus formation for each of the four *Plectranthus* accessions.**

	<b>IBA 0.1</b>	<b>IBA 0.5</b>	<b>IBA 1.0</b>	<b>NAA 0.1</b>	<b>NAA 0.5</b>	<b>NAA 1.0</b>	<b>2,4-D 0.1</b>	<b>2,4-D 0.5</b>	<b>2,4-D 1.0</b>
<b>CADP</b>	0	0	0	0	0	0	100	100	100
<b>PZ</b>	0	0	0	50	50	100	100	0	0
<b><i>P. oertendahlii</i></b>	0	0	0	0	0	0	0	100	100
<b><i>P. ecklonii</i></b>	0	0	100	0	0	0	0	0	0

- Treatment levels given in mg/L
- CADP, *Plectranthus* ‘Cape Angels Dark Pink’
- PZ, *P. zuluensis*
- Data only given for the two shoot tip replicates



Figure 2.9 *P.zuluensis* petiole showing adventitious shoot formation after eight weeks of growth on BA 1.0mg/L: IBA 0.1 mg/L.

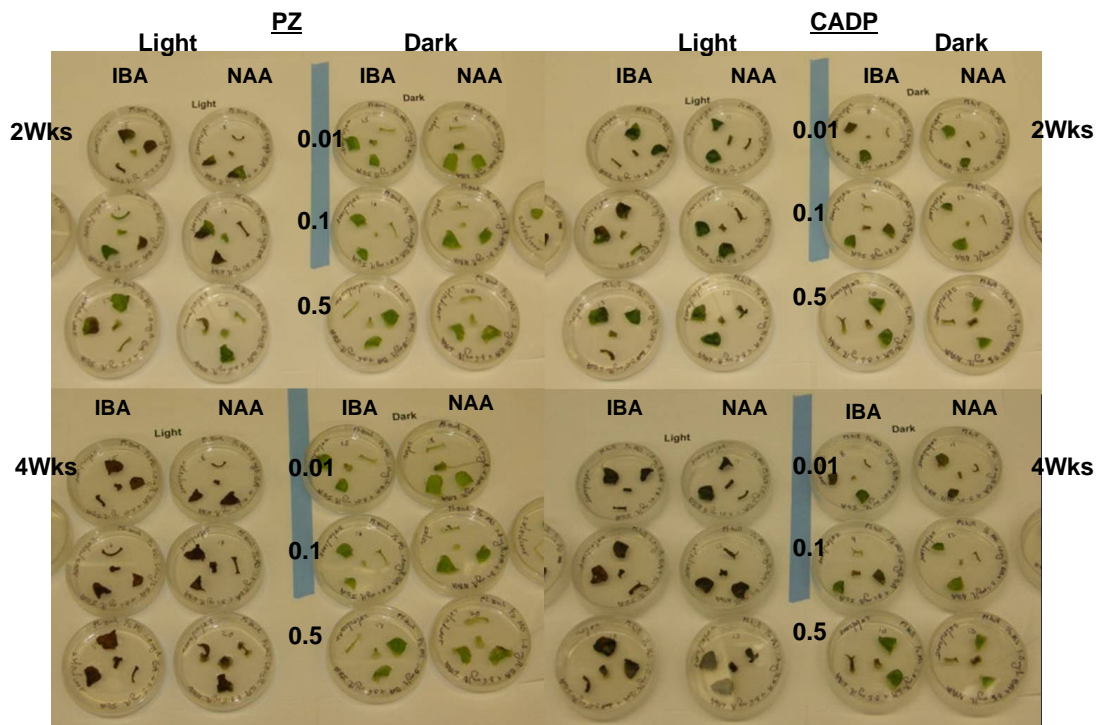


Figure 2.10 *Plectranthus* 'Cape Angels Dark Pink' (CADP) and *P. zuluensis* (PZ) leaf cultures that were grown in both light and dark treatments at two-and four weeks of culture.

- Treatments in mg/L

#### 2.4.2.2 Major studies

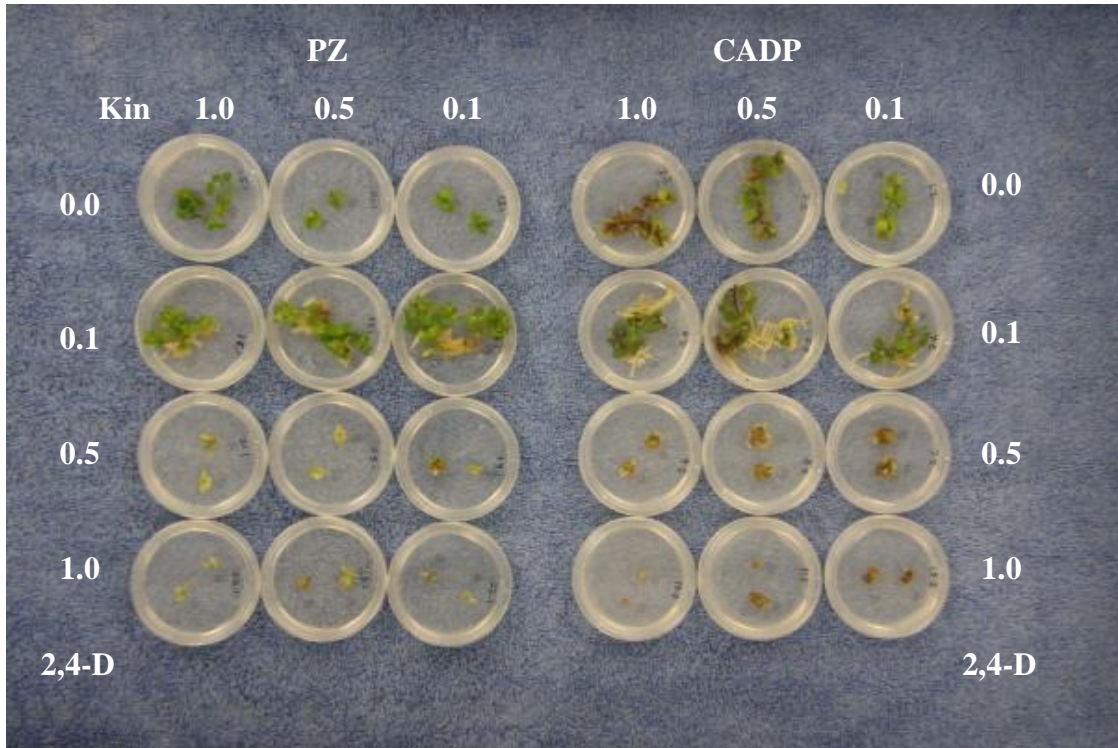
##### *Experiment 12 - Callus production, kinetin and 2,4-D factorial*

As a method to quantify the amount of callus, the final explant width was measured and analysis of variance was performed on the final width of the explants. In order for the model assumptions to hold, 2,4-D at 0.0 mg/L were left out of the analysis. Species and the level of 2,4-D were highly significant with p values < 0.0001. The level of kinetin was reported not to be significant with p value 0.30. CADP in the presence of 0.5 mg/L 2,4-D produced the most callus whereas the majority of the PZ explants did not produce substantial callus (Table 2.7). The presence of 2,4-D was required for callus to be produced in *Plectranthus* (Figure 2.11).

**Table 2.7 Tukey's HSD on the Least Square Mean of final explant width produced per species at each level of 2,4-D**

<b>Treatment</b>	<b>Class</b>	<b>LS Mean Width</b>
CADP, 0.5 mg/L	A	13.17
CADP, 1.0 mg/L	B	8.08
CADP, 0.1 mg/L	B	8.00
PZ, 0.5 mg/L	B C	6.08
PZ, 1.0 mg/L	C	4.58
PZ, 0.1 mg/L	C	4.46

- Treatments not connected by the same letter are significantly different,  $\alpha = 0.05$
- CADP – *Plectranthus* ‘Cape Angels Dark Pink’
- PZ – *P. zuluensis*



**Figure 2.11** Nodal sections of *Plectranthus* ‘Cape Angels Dark Pink’ (CADP) and *P. zuluensis* (PZ) from the, kinetin and 2,4-D factorial after four weeks of growth.

- Treatment levels given in mg/L

*Experiment 13 – Comparison of cytokinins for callus production*

The analysis for the amount of callus that was produced was separated into cultures that were maintained under light and dark conditions. CADP produced better callus in this study at 0.1 mg/L 2,4-D. No CADP explants at 0.5 mg/L 2,4-D produced callus classified better than class three. Table 2.8 shows the percentage for each CADP light treatment scored at 0.1 mg/L 2,4-D. Zeatin (0.5 mg/L) produced the most callus in class five (Table 2.8).

**Table 2.8 Percentage of light cultured *Plectranthus* ‘Cape Angels Dark Pink’ callus grouped in classes 1 – 5, at 0.1 mg/L 2,4-D by all of the cytokinin treatments.**

	Callus Class				
	1	2	3	4	5
<b>Control</b>	80.0	20.0	0.0	0.0	0.0
<b>BA 0.1</b>	10.0	30.0	60.0	0.0	0.0
<b>BA 0.5</b>	10.0	40.0	50.0	0.0	0.0
<b>Kin 0.1</b>	50.0	40.0	10.0	0.0	0.0
<b>Kin 0.5</b>	10.0	50.0	30.0	10.0	0.0
<b>Zeatin 0.1</b>	0.0	30.0	60.0	10.0	0.0
<b>Zeatin 0.5</b>	0.0	30.0	40.0	10.0	20.0

CADP dark-grown cultures did not produce good callus in comparison with explants that were cultured under light conditions. Only 16.7% of the dark cultured CADP explants were grouped in class three, whereas the rest were grouped either in classes one or two.

PZ did not produce significant amounts of callus under light conditions and none of the light-grown PZ cultures produced callus classified greater than class two. An ordinal logistic regression model was fit on the dark grown PZ cultures. The main effects of the cytokinin treatment, level of 2,4-D, and the interaction term was found to be highly significant with p value < 0.0001. The type of explant, internodal or leaf,

was also included in the analysis and it was found that the type of explant was not significant with a p value 0.316. PZ explants that were cultured in the dark did not produce substantial callus in the presence of 0.5 mg/L 2,4-D. Only 6.1% of all the explants cultured on 0.5 mg/L 2,4-D were scored as class three, whereas the rest were all scored as classes one and two. Dark cultured PZ explants in the presence of 0.1 mg/L 2,4-D produced more callus than when cultured with 0.5 mg/L 2,4-D. Dark cultured PZ explants on 0.1 mg/L 2,4-D resulted in 51% explants scored in class three or greater. Zeatin at 0.5 mg/L and kinetin at 0.5 mg/L produced the most callus scored in classes three and higher (Table 2.9).

**Table 2.9 Percentage of dark cultured *Plectranthus zuluensis* explants, at 0.1 mg/L 2,4-D, scored in different callus growth classes.**

	Callus Class				
	1	2	3	4	5
<b>Control</b>	83.3	11.1	0.0	0.0	0.0
<b>BA 0.1</b>	0.0	71.4	28.6	0.0	0.0
<b>BA 0.5</b>	0.0	85.7	14.3	0.0	0.0
<b>Kin 0.1</b>	14.3	42.9	14.3	28.6	0.0
<b>Kin 0.5</b>	0.0	14.3	28.6	42.9	14.3
<b>Zeatin 0.1</b>	0.0	14.3	57.1	28.6	0.0
<b>Zeatin 0.5</b>	0.0	0.0	28.6	42.9	28.4

#### *Experiment 14 - Organogenesis from callus*

No explant and treatment combination formed shoots from callus. Callus growth class was used as a continuous variable in fitting an SLS model. The main effect of species was highly significant with a p value < 0.0001. The absence or presence of BA on callus growth was found to be significant with p value 0.0007. The main effect of the level of IBA was not significant in the model with a p value 0.0864. Callus growth in the presence of 0.01 mg/L IBA was statistically significantly more

than callus growth at IBA levels, 0 and 0.1 mg/L. The interaction term of species by IBA level showed to be significant with p value 0.0081, resulting in CADP having more callus growth than PZ (Table 2.10). The best callus growth results were produced by CADP at 0.1 mg/L IBA (Table 2.10).

**Table 2.10 Tukey HSD on the least square means of growth by callus explants for *Plectranthus* ‘Cape Angels Dark Pink’ (CADP) and *P. zuluensis* (PZ) by the four treatment combinations used for callus organogenesis.**

Treatment	Class	LS mean
CADP,4	A	4.22
CADP,2	A B	4.00
CADP,3	B C	2.89
CADP,1	B C D	2.78
PZ,2	C D E	2.33
PZ,3	C D E	2.11
PZ,4	D E	1.56
PZ,1	E	1.44

- Treatments not connected by the same letter are significantly different,  $\alpha$  0.05
- Treatment 1 – MS1 devoid of growth regulators
- Treatment 2 – 0.1 mg/L BA
- Treatment 3 – 0.1 mg/L BA + 0.01 mg/L IBA
- Treatment 4 – 0.1 mg/L BA + 0.1 mg/L IBA

Ordinal logistic regression on root growth by the callus cultures revealed that species was the only significant factor in the model, with a p value 0.0005, whereas the treatment or the species by treatment interaction terms were found not to be significant.

More roots were produced by PZ cultures than CADP with 11.1% of PZ explants producing roots in class five, whereas none the CADP explants produced any roots scored as a class five. In total, 30.5% of PZ explants produced roots classed three or greater, whereas only 2.8% of CADP explants produced roots classified as class three or greater.

## 2.5 DISCUSSION

### 2.5.1 Direct organogenesis

#### 2.5.1.1 Preliminary micropropagation studies

As indicated in Figure 2.1, there was no noticeable effect of IBA on growth of CADP and PZ shoot tips. In this study, both PZ and CAPD had better growth on the lower level of BA. When all of the hormone combinations were visually compared to the control treatment, there was more root growth in the control treatments. This study indicated that the two *Plectranthus* accessions, CAPD and PZ, are probably sensitive to high levels of growth regulators.

#### 2.5.1.2 Major micropropagation study

##### *Experiment 1 – Shoot production*

The results of this study, summarized in Figure 2.3, show that CADP produces more shoots in the presence of 0.1 mg/L BA than with 0.05 mg/L. For CADP there was no significant change in the number of shoots produced over time, whereas the number of shoots produced by PZ increased over time (Figure 2.3 and Table 2.3). The greatest increase in the number of shoots produced over time for PZ occurred at the two BA treatments and 0.5 mg/L 2iP. It appears that the continuous subculture of PZ on a cytokinin medium will increase the total number of shoots produced per explant; Equation 2-2 provides a linear estimate for the increase over time. However it is expected that the observed increase over time will reach a plateau and possibly start to decrease over time. A similar trend was seen with *C. forskohlii* callus that was cultured on multiplication medium for six subcultures; the initial increase in the number of shoots that were produced stabilized for some time and then started to decrease (Reddy, *et al.*, 2001). This experiment was terminated at the end of the fourth

subculture. A future study, possibly using shorter subculture times of four or six week intervals, will provide more information regarding the number of shoots produced over time for periods longer than four subcultures.

#### *Experiment 2 – Effect of IBA on number of shoots produced*

The result of adding IBA to multiplication medium summarized in Figure 2.4 show that the addition of auxin in combination with BA does not increase the number of shoots produced. The results were clear, and no further investigation regarding the addition to auxin during the micropropagation stage was conducted. In micropropagation studies that were performed on *S. nemrosa* (Skala and Wysokinska, 2004) and *C. forskohlii* (Bhattacharyya and Bhattacharya, 2001), a combination of cytokinin and auxin was used during the multiplication phase. The study by Skala and Wysokinska (2004) did not report results for micropropagation when a cytokinin without auxin during multiplication was used. The results obtained in this study are similar to the hormones used in the multiplication of *P. vetiveroides* (Sivasubramanian, *et al.*, 2002) and *P. madagascariensis* (Hu, *et al.*, 2008). In both studies 1.0 mg/L BA was reported for use during the multiplication stage. In neither of the two studies was the result of combining cytokinin and auxin during the multiplication stage reported. The result from this study indicates that the addition of a low level of auxin during the multiplication stage is not necessary for CADP and PZ.

#### *Experiment 3 – Shift in multiplication medium strategy*

The CADP explants showed great variation in the number of shoots that were produced during the shift in growing medium strategy. The number of shoots produced by CADP shifted from 1.0 mg/L BA to MS1 devoid of growth regulators

ranged from zero up to 19 shoots. The average number of shoots that were produced however, did not differ from continuous culture on 0.1 mg/L BA. Continuous culture on 0.1 mg/L produced much less variation. The two phase multiplication procedure does not seem to be a better strategy in the multiplication of CADP, but gave promising results with PZ. The technique of exposing plant material to more than one medium to enhance organogenesis has been successfully applied to other plants such as maize callus (Duncan and Widholm, 1988) and apple leaf explants (Yancheva, *et al.*, 2003). In both studies, the initial exposure times were much shorter, between three and six days, than the four week exposure used in this study. The results indicate that a strategy that makes use of two types of medium to enhance shoot multiplication, especially on PZ, should be investigated as a method for increasing the number of shoots produced from a single explant.

#### *Experiment 4 – Explant effect on micropropagation*

When looking at the total number of shoots that were produced from both CADP and PZ, there was no significant difference in the numbers produced from either nodal or shoot tip explants. Shoot tips produced a statistically significant higher number of culturable shoots in CADP. It is debatable whether this difference would be significant to the commercial industry. The equation for culturable shoots gives an indication of the average size of shoots produced, and the fact that only a slight difference was seen in the culturable number of shoots from CAPD suggests that both shoot tip and nodal explants can be used equally well in a commercial laboratory. The advantage of being able to use both nodes and tips as explants is obvious; it will enable more explants to be harvested during the multiplication process.

*Experiment 5 - Comparison of micropropagation potential for several  
Plectranthus accessions*

Plants display variation within and between species in how they react to tissue culture conditions. As was expected, there were differences in the total number of shoots and culturable number of shoots that were produced by the different *Plectranthus* accessions. The mode of multiplication tested was developed using CADP and it was thus not surprising to see CADP produce the greatest number of shoots (Figure 2.5). The results indicate that the use a low level of BA will increase the number of shoots that will be produced in a range of *Plectranthus* species. The use of 0.1 mg/L BA, however, is not the optimal level for all *Plectranthus*. Studies on PZ and CADP using shoot tips as explants on a medium with no growth regulator did not show multiplication. This study did not make use of control treatments; using a control treatment devoid of growth regulators will give a truer indication of the relative increase in the number of shoots produced per accession by using 0.1 mg/L BA in the medium.

The number of culturable shoots is an indication of shoot elongation. It was interesting that *P. oertendahlii* had a higher average number of culturable shoots than total number of shoots produced, thus *P. oertendahlii* produced fewer but longer shoots. The high number of culturable shoots is because Equation 2-1 adds two and a half times the shoots in the largest size class. High cytokinin levels have been known to inhibit shoot elongation. It would be expected that in cases where no inhibition of shoot elongation is observed, that higher levels of cytokinins for multiplication can be investigated.

### *Experiments 6 and 7 – Rooting of Plectranthus*

Both CADP and PZ explants rooted with ease throughout this investigation. Because both accessions rooted with ease, both *in vitro* and *ex vitro*, simplifies the process of micropropagation. Rhizogenesis by CADP and PZ took place with greater ease than in the case of *P. vetiveroides* (Sivasubramanian, *et al.*, 2002) where no rooting was reported without the use of auxin. In the study done on *P. madagascariensis* (Hu, *et al.*, 2008) auxin was used in the rooting phase. There are reports on *C. forskohlii* where no auxin was required for rhizogenesis (Reddy, *et al.*, 2001) and in another study IAA was used to aid the rooting process (Bhattacharyya and Bhattacharya, 2001).

The ease with which the microcuttings rooted after 15 days of growth shown in Figure 2.7 will reduce cost and time in a commercial propagation program. In situations where microcuttings root with ease, the propagation laboratory can acclimatize, harvest, and ship the cuttings directly to the client, thus saving on time and production costs. In this study 100% rooting was obtained with cuttings ranging in sizes of 16 – 62 mm. The initial size of microcuttings did not influence the rooting; it seems possible to root a large number of microcuttings that are at least 15 mm or larger with good establishment rates. An investigation into what the smallest cutting for rooting under *ex vitro* conditions could be of interest for future commercial operations.

### *Experiment 8 - Cold storage of in vitro Plectranthus*

The ability to slow culture growth can be very useful in commercial programs either for storing stock plants or storing finished plants that are ready for shipping. By lowering the temperature of *Plectranthus* cultures it was possible to efficiently store

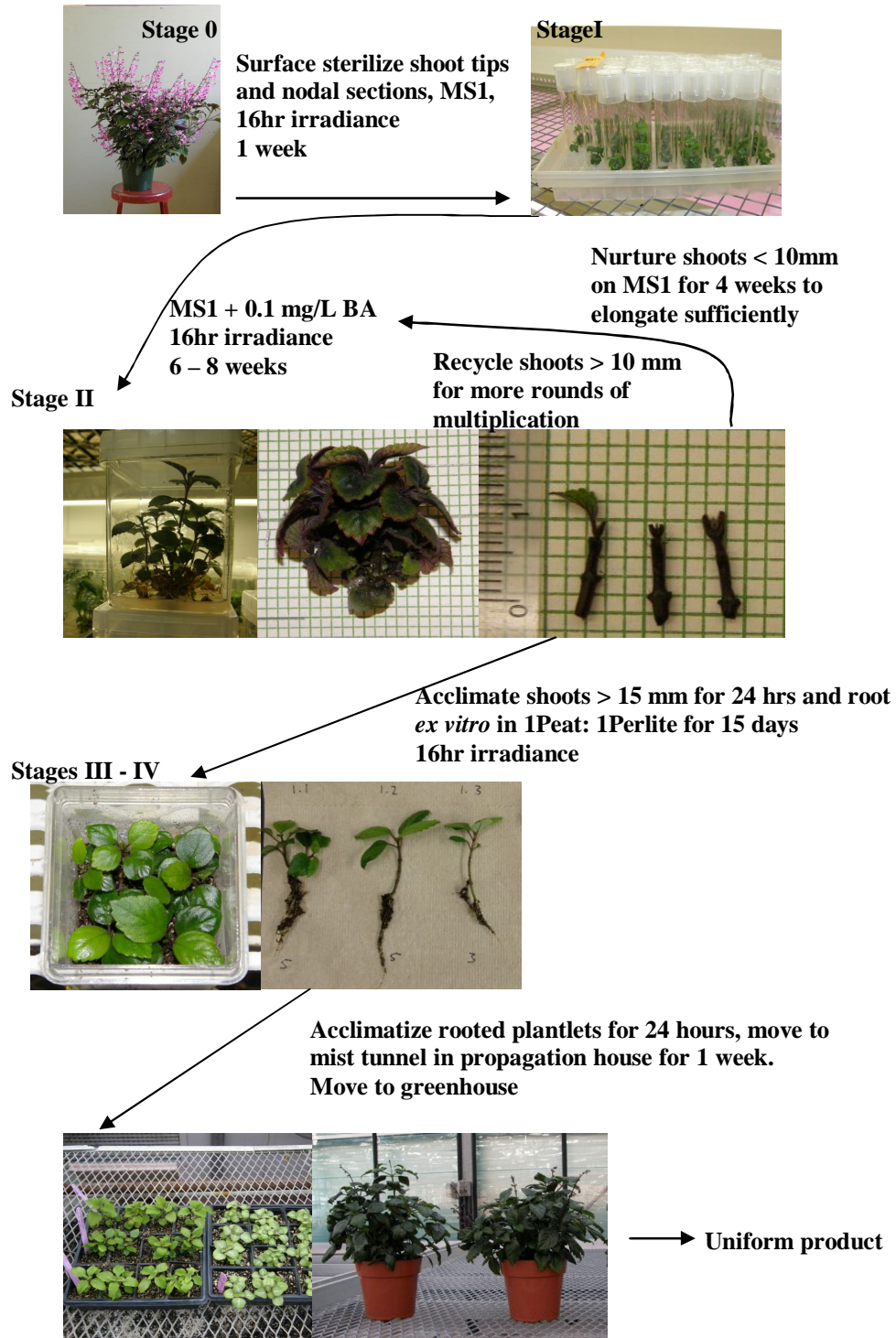
plants for up to 30 weeks without any subculturing. Storage at 15°C was sufficient to slow down growth of the plants, although when cultures were stored at 10°C (data not presented), both CADP and PZ plantlets had high mortality rates. The problem observed in this study was that the medium started to dehydrate, especially in the GA7 vessels. By using Parafilm® to seal culture vessels better storage of *Plectranthus* cultures at 15°C may be possible for longer periods of time.

#### *Proposed protocol for direct organogenesis*

Using the micropropagation protocol shown in Figure 2.12, it is possible to produce more plantlets after a year of micropropagation than what is possible when using conventional cutting propagation in a greenhouse (Table 2.11).

Approximately 28 cuttings can be harvested from a mature CADP greenhouse plant grown in a standard 6” container. CADP cuttings are expected to take an average of 16 weeks to produce a plant in a 6” container from where 28 cuttings can again be harvested. The calculations in Table 2.11 make use of a 95% success rate of cuttings and the same for initiation of *in vitro* cultures. Cuttings used in conventional propagation consist of two nodes and plants are expected to yield at least double the amount of starting material (nodal sections and shoot tips) to use for micropropagation.

The calculations in Table 2.11 make use of an average of four culturable shoots produced from a single shoot tip after an eight week multiplication phase (data from experiment 5). The number of plants that can be produced using culturable shoots is very conservative and an underestimation of the total amount of plants that can be produced. The estimation of plants produced by using culturable shoots does not take into account the shoots that are not used in the calculation of culturable shoots



**Figure 2.12 Proposed micropropagation strategy derived for *Plectranthus* 'Cape Angels - Dark Pink'**

- Stages 0 – IV as described by Debergh and Maene (1981)
- MS1, Half-strength Murashige and Skoog (1962) nutrients and vitamins, with 20 g/L sucrose solidified with 7.0 g/L agar
- 16 hr irradiance at average photosynthetic photon flux of  $70 \mu\text{mol m}^{-2} \text{s}^{-1}$
- BA, 6-benzylaminopurine

**Table 2.11 Comparison of conventional greenhouse cutting propagation of CADP to micropropagation using both culturable and total number of shoots produced.**

<b>Using conventional propagation:</b>		
<b>Week</b>	<b>Calculation</b>	<b>Number of plants</b>
16	$28 \times 0.95 =$	26.6
32	$26.6 \times 28 \times 0.95 =$	707.6
48	$707.6 \times 28 \times 0.95 =$	18821.1
64	$18821.1 \times 28 \times 0.95 =$	500641.2
<b>Using culturable shoots:</b>		
<b>Week</b>	<b>Calculation</b>	<b>Number of plants</b>
1	$56 \times 0.95 =$	53.2
8	$53.2 \times 4 =$	212.8
16	$212.8 \times 4 =$	851.2
24	$851.2 \times 4 =$	3404.8
32	$3404.8 \times 4 =$	13619.2
40	$13619.2 \times 4 =$	54476.8
48	$54476.8 \times 4 =$	217907.2
56	$217907.2 \times 4 =$	871628.8
64	$871628.8 \times 4 =$	3486515.2

- Average number of cuttings harvested from a mature plant grown in 6" standard pot = 28
- Expect 95% success with cuttings in conventional propagation system
- Expect 95% success initiation of *in vitro* cultures
- Average of 16 weeks is required to produce a plant in 6" pot from where an average of 28 cuttings can be harvested
- Average number of culturable shoots used in calculation = 4 (data from experiment 5)

(Equation 2-1). The smallest shoots can be nurtured on a nutrient medium to elongate and then be used in the multiplication stage. Using the total number of shoots to estimate the number of plants that can be produced will result in an over estimation of the total number of plants that can be produced. This overestimation is because not all the shoots produced after an eight week culture cycle can be expected to have the same multiplication potential due to their differing sizes.

Using the culturable number of shoots in the prediction calculations result in more plants than what can be produced via conventional greenhouse production. Apart from the faster multiplication of plants there are other advantages to using

micropropagation. Some of the advantages include the fact that micropropagation will take up much less space than conventional propagation. In addition, plants require very little attention between subcultures and can also be efficiently stored for periods of at least 30 weeks. The comparison of costs involved with micropropagation and conventional propagation should be investigated before final conclusions can be drawn about which method is best to use.

## **2.5.2 Indirect organogenesis**

### *2.5.2.1 Experiments 9 and 10 - Adventitious shoot formation*

The use of *Plectranthus* explants other than existing meristems was not promising for the production of adventitious shoots. Leaf explants proved to be successful as explants for shoot production in other *Lamiaceae* related to *Plectranthus*. Successful shoot formation from leaf explants of *Coleus blumei* (Hervey and Robbins, 1978; Zagrajski, *et al.*, 1997), *Coleus forskohlii* (Reddy, *et al.*, 2001), *Salvia nemrosa* (Skala and Wysokinska, 2004), and *Mentha citrata* (Van Eck and Kitto, 1992) has been reported.

Rhizogenesis occurred readily on leaf explants although it was not possible to initiate shoots. It was, however, noted that irradiance could be important in the case of leaf explants, especially in PZ. Leaf explants cultured in the dark stayed green for longer periods (Figure 2.10). In the case of *M. citrata* (Van Eck and Kitto, 1992), the leaf explants were first cultured in the dark for a period of up to 60 days before they were moved to the light where shoots formed. *C. blumei* leaf disks produced shoots within five weeks (Hervey and Robbins, 1978), whereas in the case of *C. forskohlii* (Reddy, *et al.*, 2001) organogenesis was preceded by a callus phase. There is no clear trend in shoot initiation from leaf explants in plants related to *Plectranthus*.

The genotype used can greatly affect the outcome of tissue culture experiments. It is possible that the accessions used in this study, CADP and PZ, are resistant to adventitious shoot formation. This study only made use of adenine derived cytokinins. The use of phenylurea derivatives such as thidiazuron to aid in shoot initiation was not investigated. Combinations of more plant growth regulators in combination with different irradiance strategy could possibly yield positive results in adventitious shoot formation from explants other than existing meristems.

#### 2.5.2.2 *Experiments 11, 12, and 13 - Callus culture*

From the preliminary results it appeared that leaves were not good sources of explants as starting material for callus. The best callus was obtained using 2,4-D in the ranges of 0.1 – 1.0 mg/L. The results indicate that CADP produces more callus at a higher concentration of 2,4-D than PZ. After the preliminary experiments it was decided to only use 2,4-D as the auxin to promote callus growth. Visually it appeared that 0.5 mg/L 2,4-D (Figure 2.11) was the best for callus production with no observed effect of kinetin; this observation was supported by the statistical analysis. After eight weeks of culture PZ explants did not produce good callus. When a replicate of the experiment was placed under dark conditions it was clear that PZ produced significantly more callus (data not shown).

Irradiance has been known to affect callus formation. The effect of light on plants related to *Plectranthus* is dependant on the species; in some cases light may be promotive (Razzaque and Ellis, 1977; Zagrajski, *et al.*, 1997; Reddy, *et al.*, 2001) and in other cases darkness promotes callus formation and regeneration (Li, *et al.*, 1999). The observation that PZ produced more callus under dark conditions was again observed in the study where different cytokinins in combination with 2,4-D were

investigated. From these results it can be concluded that dark conditions favor callus induction in PZ.

The result that PZ explant source, nodal or leaf, cultured in the dark did not have a significant effect on the amount of callus, shows that PZ leaf explants can be used in future tissue culture studies. This result contradicts the preliminary results that indicated that leaf explants are not a good source of explants in callus production.

Zeatin and kinetin at 0.5 mg/L produced the best callus growth for both PZ and CADP. PZ callus that was produced in the presence of both zeatin and kinetin, was white and friable. Callus that was produced from CADP in the presence of zeatin and kinetin was green, hard and compact in the presence of zeatin and white and friable in the presence of kinetin. Although it is not apparent from the results presented in Table 2.8, CADP produced the healthiest looking callus at 0.5 mg/L kinetin and 0.5 mg/L 2,4-D. Kinetin at 0.5 mg/L was chosen for callus induction in both CADP and PZ.

It was possible to maintain CADP callus cultures for several subcultures by using the same medium used in callus induction, MS1 medium with 0.5 mg/L 2,4-D and kinetin. Throughout this study it was very difficult to maintain PZ callus. Regardless of subculturing to fresh medium, PZ callus turned brown after four weeks of culture. The medium used to initiate PZ callus was MS1 with 0.5 mg/L kinetin and 0.1 mg/L 2,4-D. The use of activated charcoal and anti-oxidants such as ascorbic acid in the culture medium to avoid browning was not investigated. It is possible that PZ callus required higher levels of nutrients to maintain callus. MS1 medium makes use of half-strength MS nutrients and vitamins, thus the PZ callus could be starved of nutrients before the subculture. Shorter subculture intervals or full-strength MS nutrient medium could be a possible solution to the browning of PZ callus cultures after four weeks.

### 2.5.2.3 Experiment 14 - Organogenesis from Callus

The main objective of the study was to produce shoots from callus pieces. At no point during this investigation were shoots ever produced from callus growth. However, rhizogenesis did occur, and this gives hope that the callus that is produced from the described protocol can differentiate and produce shoots. Explants from PZ produced more roots than CADP; this could possibly be explained by the presence of the original PZ explants from which the callus was formed.

It would be interesting to determine if callus with different morphological properties, such as green hard callus that can be produced with Zeatin from CADP, will produce shoots.

The use of more growth regulators in more combinations would give a better indication as to the possibility of organogenesis from the callus that was produced in this study. Treatment protocols where callus are subjected to two types of media can possibly result in shoot formation. An example is where maize callus cultures were placed on a medium containing 3.5 mg/L BA for six days and then moved to a medium devoid of growth regulators produced large numbers of shoots (Duncan and Widholm, 1988).

## 2.6 CONCLUSIONS

It was possible to obtain and maintain sterile *in vitro* cultures of several *Plectranthus* accessions. This study provides a foundation for those who are interested in the tissue culture and micropropagation of ornamental *Plectranthus*. At this point it is possible to produce and store large numbers of genetically identical CADP and PZ plants in shorter time than can be done via taking cuttings in the greenhouse. Having a reliable micropropagation protocol can be very useful in breeding programs where, as in *Plectranthus*, the majority of the plants produced are sterile and crosses are

produced with difficulty. Tissue culture techniques can be used to build up plant numbers from these crosses relatively fast, and help speed up the process for release of new cultivars

The objective to establish a protocol for all five stages of micropropagation for ornamental *Plectranthus* was met. Successful protocols for stages 0 – IV are described for both PZ and CADP and provide a basis for micropropagation of other *Plectranthus*.

It was not possible to produce an indirect organogenesis protocol for the accessions that were used in this study. It is known that some genotypes do not produce regenerable callus, and this could be the case with CADP and PZ. Members of *Plectranthus* are excellent candidates for mutation breeding. Callus cultures are useful in mutation studies and the creation of somaclonal variation. An indirect-organogenesis protocol will be of great use in future *Plectranthus* breeding programs and more work is required to produce such a protocol.

## **CHAPTER 3: USE OF PLANT GROWTH REGULATORS FOR HEIGHT CONTROL OF ORNAMENTAL HYBRID *PLECTRANTHUS***

### **3.1 ABSTRACT**

Ornamental horticulture has placed a major emphasis on the production of well-branched, compact-growing potted plants. Commercial *Plectranthus* hybrids such as ‘Mona Lavender’ and the ‘Cape Angels’ series display vigorous growth that requires greenhouse growers to use several cultural techniques to produce plants with the characteristics that are desired by the commercial industry. Plant growth regulators (PGRs) that function as anti-gibberellins are commercially available to use in producing more compact crops. The objective of this study was to identify appropriate PGRs and concentrations for use as foliar sprays on three commercial hybrid *Plectranthus* cultivars. Daminozide (2500 – 5000 mg/L) and paclobutrazol (5 – 15 mg/L) were not effective in growth retardation in hybrid *Plectranthus*. However, flurprimidol in the range of 10 – 20 mg/L proved to be effective in growth retardation without other visible negative effects on the plants. Flurprimidol treatments higher than 40 mg/L are excessive. Current research is continuing to determine optimal rates of flurprimidol application on ornamental hybrid *Plectranthus*.

### **3.2 INTRODUCTION**

The ornamental horticulture industry has placed a major emphasis on the production of well-branched, compact, and proportionate-looking potted plants (Wilkins, *et al.*, 1979; Larson, 1985). Commercial *Plectranthus* such as ‘Mona Lavender’ and the ‘Cape Angels’ series are interspecific hybrids (Brits and Li, 2008) that display vigorous growth and have tremendous potential to be used as ornamental

plants. Although these plants have beautiful flowers, their aggressive growth habit sometimes produces plants that are very large and not suitable for standard, commercial greenhouse culture.

Several techniques can be used to produce potted crops with the compact growth characteristics desired by the horticulture industry. These include growth control via manipulation of the crop's growing environment. Plant height can be controlled through the use of temperature regulation, light intensity, controlled drought stress, nutrient stress, and mechanical stimulation (Andersen and Andersen, 1999; Schnelle, *et al.*, 1999). However these techniques are not very precise and require a high level of involvement from the grower. Alternatively, various plant growth regulators (PGRs) can be applied to help control plant height; this can often be much more convenient than trying to manipulate multiple factors in the growing environment.

Gibberellins are a large group of compounds that are usually associated with the promotion of stem growth and have been used to promote stem elongation in floriculture crops (Larson, 1985). Most PGRs commercially used for growth retardation inhibit some step in the gibberellin synthesis pathway to reduce stem elongation. Retardants such as triazoles, pyrimidines, and quaternary ammonium compounds interfere with the biosynthesis of gibberellins (Sauerbrey, *et al.*, 1987; George, *et al.*, 2008). Common names for some of the most used PGRs in floriculture are ancymidol ( $\alpha$ -cyclopropyl- $\alpha$ -(4-methoxyphenyl)-5-pyrimidinemethanol), chlormequat chloride (2-chloroethyl trimethylammonium chloride), daminozide (butanedioic acid mono (2,2-dimethylhydrazide)), paclobutrazol (2R,3R+2S,3S)-1-(4-chlorophenyl-4,4-dimethyl-2-(1,2,4-triazol-1-yl))), and flurprimidol (( $\alpha$ -1-methylethyl)- $\alpha$ -(4-(trifluoromethoxy)phenyl)-5-pyrimidinemethanol). These growth regulators are often referred to as anti-gibberellins.

There are limited studies that are published on the use of growth retardation of *Plectranthus*. The effect of paclobutrazol on adventitious root formation of *P. verticillatus* has been investigated (Davis, *et al.*, 1985), where it was found that *P. verticillatus* cuttings treated with paclobutrazol concentrations ranging between 3.0 and 6.0 mg/L increased the number of roots formed on cuttings and also had a residual height restriction effect. Rick Schoelhorn (2004) has provided guidelines for warm weather *Plectranthus* production and proposes the use of paclobutrazol as a drench at concentrations of 0.25 – 1.0 mg/L two weeks after pinch. Culture information provided by Ball Horticulture company (2008) for *Plectranthus* ‘Mona Lavender’ suggests paclobutrazol as a foliar spray, 5.0 – 20.0 mg/L, and also recommends a tank mix of chlormequat chloride, 750 – 1500 mg/L, and daminozide, 2500 – 3500 mg/L, applied as a foliar spray.

Growth regulators have been successfully used with species closely related to *Plectranthus*. According to Cavins *et al.* (2002), uniconazole applied as a foliar spray at 20 – 40 mg/L on cuttings of *Coleus* cultivars proved to be effective in retarding growth, while paclobutrazol produced varying results on growth retardation. The use of flurprimidol has been reported to be effective in height control of *Salvia splendens* and *Solenostemon scutellarioides* (Vernieri, *et al.*, 2003; Whipker, *et al.*, 2003)

The objective of this study was to identify PGRs and concentrations for use as foliar sprays on three commercial hybrid *Plectranthus* accessions.

### **3.3 MATERIALS AND METHODS**

All growth regulator studies were conducted at the Cornell University Long Island Horticultural Research and Extension Center (LIHREC) in Riverhead, New York.

### 3.3.1 2008 Plant Growth Regulation Trial

Cuttings from *Plectranthus* ‘Mona Lavender’ (MONA), *Plectranthus* ‘Cape Angels – Dark Pink’ (CADP), and *Plectranthus* ‘Cape Angels – White’ (CAW) were rooted in mid May 2008. After three weeks, well-rooted cuttings (Figure 3.1) were planted in 6” standard plastic pots, using Sunshine Mix # 8 ([www.sunagro.com](http://www.sunagro.com)). The plants were grown under natural daylight for two weeks. After the two week period all the cuttings were pinched and subjected to short day conditions (10 hour daylight), at ambient air temperatures approximately 21 – 25°C nights and 25 – 30°C days.

Plant growth regulators were applied three weeks after the cuttings were planted (Table 3.1). Tap water served as solvent for all the PGR treatments. The treatments were applied as a foliar spray, and were “sprayed to run-off” (Seeley, 1979). Treatments that consisted of two applications received a second application two weeks after the first. After a six week growing period under short days, the plants were grown under natural day length conditions. Plants were fertilized at each watering with a 20-5-21 fertilizer with added Epsom salts at 150 ppm nitrogen.

**Table 3.1 PGR treatments used in summer 2008 trial**

<b>Growth Regulator</b>	<b>Concentration (mg/L)</b>	<b>Application</b>
Paclobutrazol	5	1x
Paclobutrazol	5	2x
Paclobutrazol	10	1x
Paclobutrazol	10	2x
Paclobutrazol	15	1x
Paclobutrazol	15	2x
Daminozide	2500	1x
Daminozide	2500	2x
Daminozide	5000	1x
Daminozide	5000	2x

- Plants receiving only water were used as control treatments
- Paclobutrazol administered as Bonzi®, EPA 100-996
- Daminozide administered as B-Nine®, EPA 400-69



**Figure 3.1** *Plectranthus* cuttings 21 days after taking the cuttings

- A – Cutting not well rooted; cuttings with such poor roots were not used in this study
- B – Better root formation; no cuttings with poorer root formation was used
- C – Very well rooted cutting

Plant height at time of application of PGR and height at harvest were measured for all plants. Plant growth was calculated as the height at harvest minus the initial plant height. The number of days to harvest was also recorded. Plants were scored as ready for harvest when at least three racemes were in full flower. Three replicates per treatment were used, and the plants were arranged in a completely randomized block design.

### **3.3.2 2009 Plant Growth Regulation Trial**

Cuttings were rooted and potted in the same manner as described in the 2008 trial; however, the cuttings were taken at the end of January 2009. Flurprimidol (Topflor® Ornamental Plant Growth Regulator, EPA 67690-20) added to tap water at concentrations of 10, 20, 40, and 80 mg/L were applied as a foliar spray six weeks after the cuttings were planted. Pots receiving only water served as control treatments. The plants were grown under natural day length conditions.

Plant height at the time of application of PGR and at the time of flowering was recorded. Plant growth was calculated as plant height at harvest minus initial plant height. Six replications per treatment for each of the three *Plectranthus* accessions were used. All the plants were arranged in a completely randomized block design.

### **3.3.3 Statistical analysis**

All statistical analysis was performed using JMP® 7.0 (2007 SAS institute inc., Cary, NC.). Plant growth was calculated by subtracting plant height at the time of application from plant height at the time of harvest. Tukey's Honestly Significant Difference test was performed for multiple mean comparisons. Analysis of variance was performed by fitting a standard least squares model (SLS). All the model assumptions were satisfied.

### **3.4 RESULTS**

#### **3.4.1 2008 Plant Growth Regulation Trial**

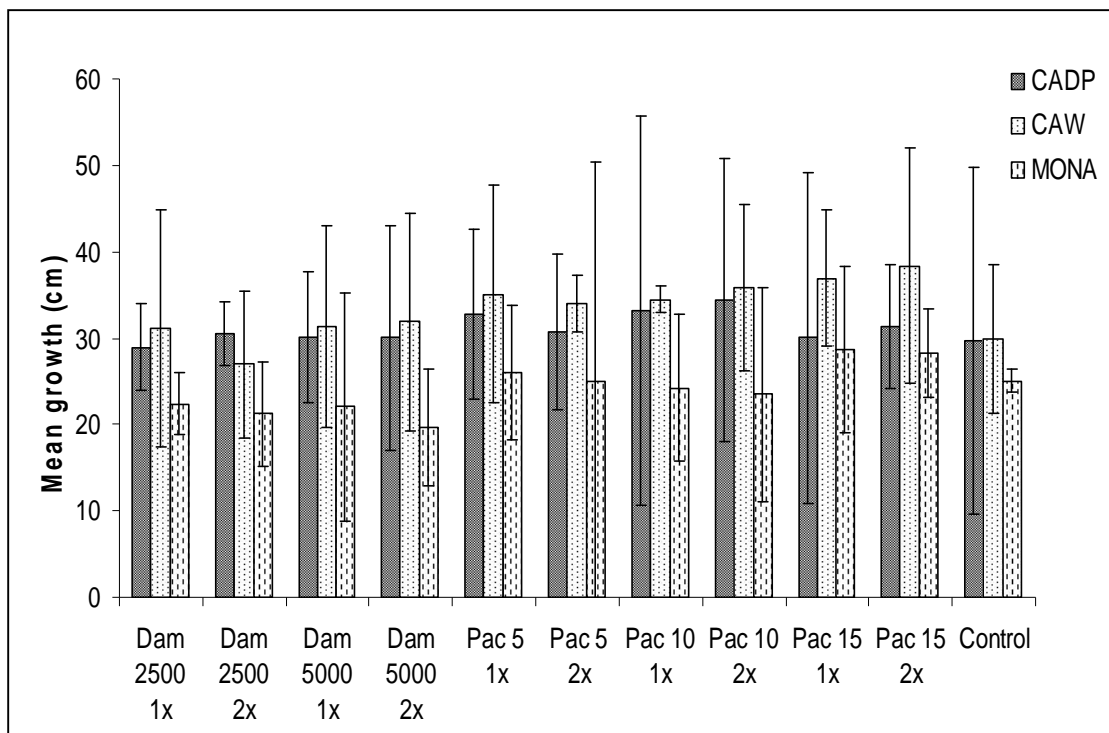
No difference in plant growth was detected between the different treatments and Dunnett's comparison of the different PGR treatments to the control did not reveal any significant differences between the treatments for all three of the cultivars used (Figure 3.2). The cultivar variable was the only significant effect on plant growth,  $p$  value  $< 0.0001$ . *Plectranthus* 'Mona Lavender', showed the least amount of growth among the three cultivars (Figure 3.3). 'Mona Lavender' matured earlier than the 'Cape Angels' cultivars. On average 'Mona Lavender' was scored ready to harvest after nine weeks of growth whereas the 'Cape Angels' cultivars were harvested after 12 weeks of growth

#### **3.4.2 2009 Plant Growth Regulation Trial**

Analysis of variance on plant growth revealed that the concentration of flurprimidol was the only significant effect on plant growth with  $p$  value  $< 0.0001$ . Table 3.2 shows the differences of the mean combined growth for all three *Plectranthus* accessions at each flurprimidol treatment. Neither the cultivar nor the interaction term between cultivar and the level of flurprimidol were significant in this study (Figure 3.4). During the statistical analysis there were some outlier values in the 'Cape Angels – Dark Pink' at 10 mg/L. Removal of the outliers did not change the significance of any of the effects in the model. Removal of the outliers resulted in a better fit of the model used.

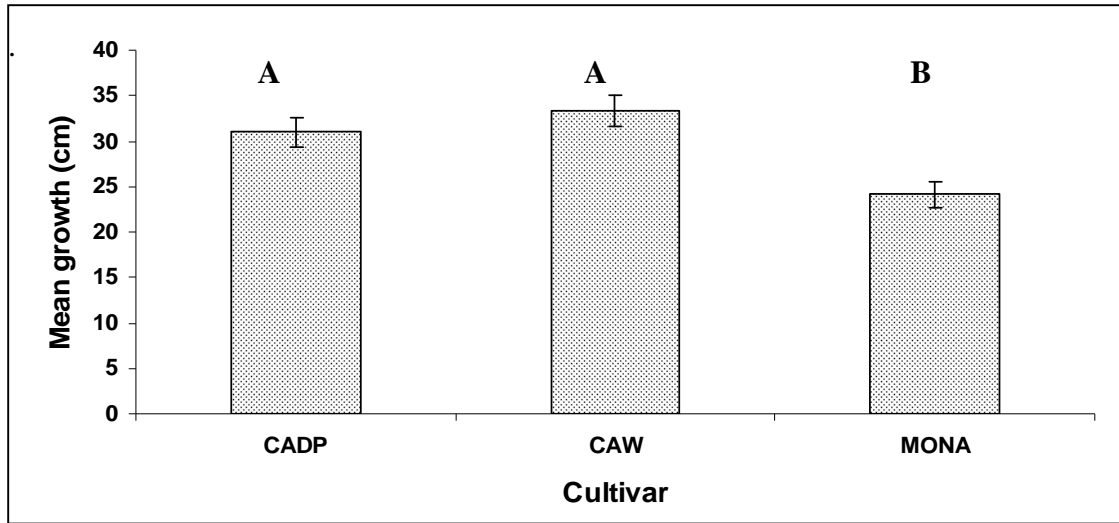
There was no significant difference in the number of days to flower between any treatments or cultivars. The overall mean number of days to flowering for the trial was 43 days after the application of flurprimidol. Plants treated with 10 and 20 mg/L

flurprimidol did not show morphological differences other than having reduced height (Figure 3.5), whereas treatments higher than 20 mg/L resulted in distorted growth with flower spikes bending and falling over (Figure 3.6).



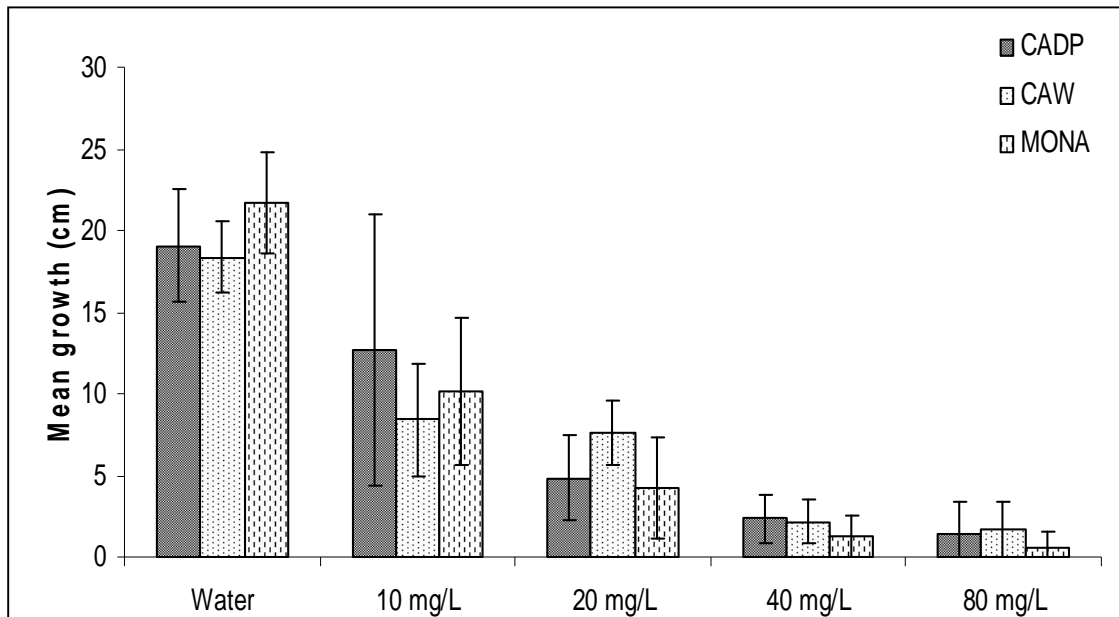
**Figure 3.2 Average plant growth of the three *Plectranthus* cultivars at all the treatment levels.**

- Error bars represent 95% confidence interval of mean growth
- CADP, 'Cape Angels Dark Pink', CAW, 'Cape Angels White', MONA, 'Mona Lavender'
- Dam, daminozide, Pac, paclobutrazol
- Treatments in mg/L



**Figure 3.3 Total average plant growth of the *Plectranthus* cultivars used in the 2008 PGR study.**

- Average growth includes the averages of all the treatments for each cultivar
- Cultivars not connected by the same letter are significantly different,  $\alpha$  0.05
- Error bars represent 95% confidence interval of mean growth
- CADP, 'Cape Angels Dark Pink', CAW, 'Cape Angels White', MONA, 'Mona Lavender'
- 'Mona Lavender' were harvested after nine weeks of growth, whereas the 'Cape Angels' cultivars were on average harvested after 12 weeks of growth



**Figure 3.4 Mean growth of all three *Plectranthus* cultivars, at five flurprimidol levels at time of harvest.**

- Error bars represent the 95% confidence interval of the mean growth
- CADP, 'Cape Angels Dark Pink', CAW, 'Cape Angels White', MONA, 'Mona Lavender'

**Table 3.2 Tukey's Honestly Significant difference comparison of the mean growth for all three *Plectranthus* cultivars, 'Mona Lavender', 'Cape Angels Dark Pink' and, 'Cape Angels White' at time of harvest.**

<b>Treatment</b>	<b>Level</b>	<b>Mean Growth</b>
Water	A	19.69
10 mg/L	B	10.42
20 mg/L	C	5.31
40 mg/L	D	1.93
80 mg/L	D	1.17

- Treatments not connected by the same letter are significantly different,  $\alpha$  0.05



**Figure 3.5 Representative samples *Plectranthus* 'Mona Lavender' treated with different concentrations of flurprimidol.**



**Figure 3.6** Effect of high concentrations (40 and 80 mg/L) of flurprimidol on *Plectranthus* 'Mona Lavender' compared to the control treatment.

### 3.5 DISCUSSION

In the 2008 study no differences in plant growth were detected between any of the treatments. This study only made use of three replications per treatment, and in some cases a plant was lost. Such low numbers make for unreliable statistics and can explain the large 95% confidence intervals seen in Figure 3.2. ‘Mona Lavender’ has a more compact and branched growth habit than the two ‘Cape Angels’ cultivars. The differences in height between the cultivars can possibly be attributed to the genetics and the fact that ‘Mona Lavender’ was on average ready to harvest three weeks earlier than the ‘Cape Angels’.

Different cultivars display genotypic variance in their response to PGR treatments, and it was expected that the cultivar effect would be significant on the plant growth. PGR treatments that delay flowering are unwanted, and it was observed that a two-time application of daminozide at 2500 mg/L resulted in two out of three plants having delayed flowering, whereas only one out of three control plants had delayed flowering.

The result that cultivar differences did not affect plant growth between treatment time and harvest in the 2009 trial is contradictory to the 2008 results, where the cultivar effect was the only significant effect on plant growth. Plant age at treatment application and growing conditions varied over the two trials which could have had an effect on the results. The 2008 trial was conducted during the summer whereas the 2009 trial was started in mid winter. The plants in the 2008 trial received PGR treatments three weeks after being planted into pots whereas in 2009 plants only received the PGR treatments six weeks after being potted.

The objective of this study was to identify common commercial PGRs for use in spray applications. Commercial growers prefer spray applications due to the relative ease of application compared to a drench application. Drench applications require

much larger volumes of chemicals. The use of a spray application for paclobutrazol was based on the *Plectranthus* 'Mona Lavender' culture guide provided by Ball Horticulture company (2008). Studies using paclobutrazol applied as a spray on *Coleus* (*Solenostemon scutellariodes*) cultivars yielded variable results (Barrett and Nell, 1988), and was influenced by the genotype used (Cavins, *et al.*, 2002). Uniconazole has proven to be more successful than paclobutrazol in height control of plants closely related to *Plectranthus* (Barrett and Nell 1988; Schnelle *et al.* 1999; Cavins *et al.* 2002).

Rick Schoellhorn (2004) suggests the use of paclobutrazol (0.52 – 1.0 mg/L) as a drench for *Plectranthus*. It is possible that a paclobutrazol drench could produce better results than a foliar spray treatment for *Plectranthus*.

Flurprimidol applied as a foliar spray in the range of 40 – 80 mg/L has been effective in height control of *Salvia splendens* and *Solenostemon scutellariodes* (Vernieri, *et al.*, 2003; Whipker, *et al.*, 2003). From the 2009 PGR trial, flurprimidol appears to be highly effective in height control in all three the *Plectranthus* cultivars used. Flurprimidol treatments between 5 and 20 mg/L should be sufficient for height control of hybrid *Plectranthus* cultivars such as those used in this study. The observation that no morphological differences apart from a more compact growth habit, were detected among plants at the 10 and 20 mg/L treatments are very promising for future use of flurprimidol as a growth retardant on *Plectranthus* cultivars.

No measurements were made on plant diameter during the course of this study. Restriction of plant diameter is also an important factor to keep in mind during growth retardation studies; usually a 30% retardation in growth is desired by the floriculture industry (Cavins, *et al.*, 2002). Plant diameter should be taken into account in future

*Plectranthus* PGR studies. Future studies making use of paclobutrazol drenches and mixtures of growth regulators such as daminozide and chlormequat are required.

The costs involved in using growth regulators for production of potted *Plectranthus* must be investigated. Studies comparing the use of chemical growth regulators for height control versus cultural practices for height control would be valuable for commercial producers of *Plectranthus* hybrid cultivars.

### **3.6 CONCLUSIONS**

The results from this study indicate that the efficacy of different PGRs differ among *Plectranthus* cultivars and provide a guideline for further experimentation on the use of plant growth regulators with commercial *Plectranthus* cultivars.

Paclobutrazol and daminozide foliar spray applications do not appear to be effective in height restriction of commercial *Plectranthus* hybrid cultivars. Flurprimidol gave very promising results for use on *Plectranthus* hybrid cultivars. The results presented merit further studies to determine PGRs and rates required for hybrid *Plectranthus* cultivars grown as potted crops. Current research is continuing to determine optimal application rates of flurprimidol.

## CHAPTER 4: SUMMARY

The two *Plectranthus* accessions chosen for the tissue culture study displayed variation in their response to *in vitro* treatments. *Plectranthus* ‘Cape Angels Dark Pink’ (CADP) constantly produced more shoots on culture medium containing 0.1 mg/L BA. The number of shoots produced by CADP did not change over time with continuous culture on the same cytokinin medium. *P.zuluensis* (PZ) produced fewer shoots per explant than CADP, however the number of shoots produced by PZ explants increased with continuous sub-culture on a cytokinin medium. Both accessions used in this study also responded in similar fashion to some treatments. The addition of auxin to the multiplication medium did not have an effect on the number of shoots produced from either CADP or PZ. Both CADP and PZ rooted with ease under *in vitro* conditions, and acclimated microcuttings from both accessions achieved 100% rooting in 15 days under *ex vitro* conditions. When the multiplication potential of several *Plectranthus* accessions was tested, the different accessions displayed variation in the number of shoots produced. CADP and *P.purpureus* produced the most shoots from a single shoot tip after eight weeks of culture on half-strength MS medium with 0.1 mg/L BA.

The objective of developing a complete direct-organogenesis micropropagation protocol was achieved, and multiplication by axillary branching was the method of choice. *In vitro* cultured *Plectranthus* plantlets can be acclimatized to greenhouse conditions with near 100% establishment rates.

The objective of developing an indirect-organogenesis protocol was not met. The presence of 2,4-D was required for callus induction in both accessions, although PZ responded better to lower levels of 2,4-D and CADP produced better callus at 0.5 mg/L. Irradiance is important in callus formation in *Plectranthus*. Dark conditions favored callus production in PZ whereas light conditions were favorable for callus in

CADP. It was possible to produce callus from both CADP and PZ. It was possible to maintain CADP callus, but PZ callus could not be maintained for more than four to five weeks. Rhizogenesis from callus explants could be achieved although it was at no point possible to produce shoots from callus of both CADP and PZ.

The fact that it was not possible to produce shoots from callus should not discourage future studies to achieve this goal. Plants in the *Lamiaceae* that are closely related to *Plectranthus* readily produce shoots from callus and explants other than existing meristems, and it is expected that the same can be achieved with ornamental *Plectranthus*. Members of *Plectranthus* are excellent candidates for mutation breeding. Callus cultures are useful in creation of somaclonal variation. An indirect-organogenesis protocol will be of great use in future *Plectranthus* breeding programs.

The results from this study provide positive results for micropropagation and tissue culture of *Plectranthus*. Large numbers of *Plectranthus* plants can be produced via axillary branching in a relatively short time period. Although multiplication via indirect-organogenesis was not achieved, this study will aid future studies on *Plectranthus* tissue culture. This work will not only be applicable to the horticultural industry. Some members of *Plectranthus* produce secondary metabolites that are being investigated for their medicinal properties, and these results should be very useful to future tissue culture research on non-ornamental *Plectranthus*.

Hybrid ornamental *Plectranthus* such as ‘Mona Lavender’ and the ‘Cape Angels’ series display vigorous tall growth under greenhouse conditions. Foliar applications of daminozide and paclobutrazol did not seem to effectively control plant height. Foliar sprays of flurprimidol in the 10 – 20 mg/L range proved to effectively restrict plant height in the hybrids investigated in this study. Flurprimidol applications higher than 40 mg/L were excessive and resulted in deformed plants with flower

spikes falling over. Current research is ongoing to determine optimal rates of flurprimidol application on ornamental hybrid *Plectranthus*.

## APPENDIX

Appendix A. *Plectranthus* collection maintained at Cornell University.

<b>Species / Cultivar</b>	<b>Source / Origin</b>
<b>Southern African</b>	
<i>P. ambiguus</i>	Ernst van Jaarsveld
<i>P. ambonicus</i>	Logees
<i>P. ambonicus</i> - 'Variegated'	Logees
<i>P. ambonicus</i> - 'Ochre Flame'	Glasshouse Works
<i>P. ecklonii</i> - 'Erma'	Glasshouse Works
<i>P. ecklonii</i> - Pink	Rosanna Freyre
<i>P. ecklonii</i> - Purple	Rosanna Freyre
<i>P. ernstii</i>	Glasshouse Works
<i>P. fruticosus</i>	Logees
<i>P. grandidentatus</i>	Glasshouse Works
<i>P. hereroensis</i>	Rosanna Freyre
<i>P. hilliardiae</i>	Glasshouse Works
<i>P. madagascariensis</i>	Rosanna Freyre
<i>P. madagascariensis</i> - 'Lothlorien'	Glasshouse Works
<i>P. madagascariensis</i> - 'Marginatus'	Glasshouse Works
<i>P. oertendahlii</i>	Logees
<i>P. oertendahlii</i> - 'Uvongo'	Glasshouse Works
<i>P. saccatus</i> - 1	Ernst van Jaarsveld
<i>P. saccatus</i> - 2	Ernst van Jaarsveld
<i>P. saccatus</i> - 3	Ernst van Jaarsveld
<i>P. strigosus</i> - 'Mbabane'	Glasshouse Works
<i>P. venterii</i>	Rosanna Freyre
<i>P. venterii</i> - 'Snowflake'	Glasshouse Works
<i>P. verticillatus</i>	Glasshouse Works
<i>P. verticillatus</i> - 'Variegatus'	Glasshouse Works
<i>P. zuluensis</i> 1	Glasshouse Works
<i>P. zuluensis</i> 2	Rosanna Freyre
<b>Non Southern African</b>	
<i>P. argentatus</i>	Glasshouse Works
<i>P. discolor</i>	Glasshouse Works
<i>P. fosterii</i> - 'Green + Gold'	Glasshouse Works
<i>P. fosterii</i> - 'Green on Green'	Glasshouse Works
<i>Plectranthus</i> - 'Menthol Eucalyptus'	Glasshouse Works
<i>P. prostratus</i>	Glasshouse Works
<i>P. purpureus</i>	Glasshouse Works
<i>P. purpureus</i>	Rosanna Freyre
<i>Plectranthus</i> - 'Turpentine'	Glasshouse Works
<i>P. tomentosus</i>	Rosanna Freyre
<i>Plectranthus</i> - 'Cerveza and Lime'	Rosanna Freyre

Appendix A. continued

**Hybrid accessions**

<i>Plectranthus</i> - 'Cape Angels White'	Rosanna Freyre
<i>Plectranthus</i> - 'Cape Angels Light Pink'	Rosanna Freyre
<i>Plectranthus</i> - 'Cape Angels Dark Pink'	Logees
<i>Plectranthus</i> - 'Cape Angels Purple'	Rosanna Freyre
<i>Plectranthus</i> - 'Mona Lavender'	Glasshouse Works
<i>Plectranthus fruticosus x ciliatus</i>	Rosanna Freyre

Logee's Tropical Plants (2009) – www.logees.com

Glasshouse Works (2009) – www.glasshouseworks.com

Appendix B. Concentrations of growth regulators used in experiment 9.

<b>μM</b>	<b>BA (mg/L)</b>	<b>2iP (mg/L)</b>	<b>IBA (mg/L)</b>	<b>NAA (mg/L)</b>
0.05	0.011	0.011	0.011	0.009
0.5	0.113	0.102	0.102	0.093
5.0	1.126	1.102	1.102	0.931

- BA, 6-benzylamino purine
- 2iP, 6-( $\gamma$ -dimethylallylamino) purine
- IBA, Indole-3-butyric acid
- NAA, 1-naphthaleneacetic acid

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