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Improving *in vitro* propagation of *Protea cynaroides* L. (King Protea) and the roles of starch and phenolic compounds in the rooting of cuttings

by

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Protea cynaroides (Photograph: G. Bredenkamp)

DECLARATION

I hereby certify that this thesis is my own work, except where duly acknowledged. I declare that this thesis that I hereby submit at the University of Pretoria has not previously been submitted by me for degree purposes at any other university.

Signature:……

(How-Chiun Wu)

CONTENTS

ACKNOWLEDGEMENTS v

LIST OF ABBREVIATIONS vi

LIST OF FIGURES vii

LIST OF TABLES x

ABSTRACT xi

INTRODUCTION 1

CHAPTER 1 LITERATURE REVIEW 3

1.1 Introduction 3

1.1.1 Carbohydrates 4

1.1.2 Etiolation 5

1.1.3 Phenolic compounds 6

1.2 *In vitro* propagation 7

1.2.1 Embryo culture 10

1.2.2 Micrografting 11

1.2.3 Somatic embryogenesis 13

1.2.4 Organogenesis 18

1.3 References 20

CHAPTER 2 *IN VITRO* CULTURE OF ZYGOTIC EMBRYOS OF *PROTEA CYNAROIDES* AND ITS ESTABLISHMENT IN *EX VITRO* ENVIRONMENT 38

2.1 Abstract 38

2.2 Introduction 38

2.3 Preliminary trials 41

2.3.1 Materials and methods 41

2.3.2 Results 41

2.4 Main trial 42
2.4.1 Materials and methods 42
2.4.2 Results 44
2.4.3 Discussion 46
2.4.4 Conclusion 48
2.4.5 References 50

CHAPTER 3 MICROGRAFTING OF *PROTEA CYNAROIDES* 61
3.1 Abstract 61
3.2 Introduction 61
3.3 Materials and methods 62
3.4 Results 64
3.5 Discussion 64
3.6 Conclusion 66
3.7 References 67

CHAPTER 4 PLANTLET REGENERATION OF *PROTEA CYNAROIDES* THROUGH DIRECT SOMATIC EMBRYOGENESIS AND MULTIPLE SHOOT DEVELOPMENT 72
4.1 Abstract 72
4.2 Introduction 72
4.3 Materials and methods 74
4.3.1 Plant materials 74
4.3.2 Culture media and growth conditions 74
4.3.3 Histology 75
4.3.4 Statistical analysis 75
4.4 Results 76
4.4.1 Induction of somatic embryos on zygotic embryos 76
4.4.2 Induction of somatic embryos on cotyledon explants 77
4.4.3 Germination of somatic embryos 78
4.4.4 Immature unfertilized ovules 79
4.4.5 Multiple shoot-bud development 80

4.5 Discussion 80
4.6 Conclusion 84
4.7 References 85

CHAPTER 5 ROLES OF STARCH AND PHENOLIC COMPOUNDS IN VEGETATIVE PROPAGATION OF *PROTEA CYNAROIDES* STEM CUTTINGS 101
5.1 Abstract 101
5.2 Introduction 101
5.3 Materials and methods 103
5.3.1 Plant material 103
5.3.2 Experiment 1 104
5.3.3 Experiment 2 106
5.3.4 Statistical analysis 110
5.4 Results 110
5.4.1 Experiment 1 110
5.4.2 Experiment 2 112
5.5 Discussion 114
5.6 Conclusion 119
5.7 References 120

CHAPTER 6 ALLELOPATHIC ACTIVITY OF PHENOLICS FROM *PROTEA CYNAROIDES* STEMS, AND THEIR ROLE AS ENDOGENOUS ROOTING REGULATORS *IN VIVO* 132
6.1 Abstract 132
6.2 Introduction 132
6.3 Materials and methods 134
6.3.1 Collection of plant material 134
6.3.2 Lettuce seed bioassay of stem extract 134
6.3.3 Osmotic interference 135
6.3.4 Extraction, separation, isolation and characterization procedures 135
6.3.5 Lettuce seed bioassay of 3,4-dihydroxybenzoic acid 136

6.3.6 *In vitro* bioassay of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants 136
6.3.7 Analysis of phenolic compounds by HPLC 137
6.3.8 Statistical analysis 137
6.4 Results 138
6.4.1 Osmotic potential of stem extracts 138
6.4.2 Bioassay of stem extract 138
6.4.3 Extraction, separation, isolation and characterization 138
6.4.4 Bioassay of 3,4-dihydroxybenzoic acid 139
6.4.5 Effects of 3,4-dihydroxybenzoic acid on *P. cynaroides in vitro* 139
6.4.6 Analysis of phenolic compounds by HPLC 140
6.5 Discussion 141
6.6 Conclusion 144
6.7 References 145

CHAPTER 7 GENERAL DISCUSSION AND CONCLUSION 155
7.1 References 160

SUMMARY 162

APPENDIX A Murashige and Skoog medium 165

APPENDIX B Starch analysis reagent 166

APPENDIX C Abbreviated analysis of variance (ANOVA) tables 168

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LIST OF ABBREVIATIONS

2,4-D	:	2,4-Dichlorophenoxyacetic acid
2iP	:	N^6-(2-isopentyl)adenine
ABA	:	Abscisic acid
AND	:	Anderson (1975) medium
BA	:	Benzyladenine
BAP	:	6-Benzylaminopurine
GA_3	:	Gibberellic acid
IAA	:	3-indolyl-acetic acid
IBA	:	3-indolebutyric acid
MS medium	:	Murashige and Skoog (1962) medium
NAA	:	1-Naphthalene acetic acid
NOA	:	2-Naphthyloxyacetic acid
TDZ	:	Thidiazuron
WPM	:	Woody plant medium (Loyd and McCown, 1981)
IEDC	:	Induced embryogenic determined cells
PEDC	:	Pre-embryogenic determined cells
CARD	:	Curve-fitting to allelochemical response data
PAR	:	Photosynthetic active radiation
PEG	:	Polyethylene glycol
$mOsm.kg^{-1}$	:	$MilliOsmol\ kilogram^{-1}$
ACN	:	Acetonitrile
HPFC	:	High performance flash chromatography
HPLC	:	High performance liquid chromatography
NMR	:	Nuclear magnetic resonance
MS	:	Mass spectrophotometer
TLC	:	Thin layer chromatography
UV	:	Ultraviolet

LIST OF FIGURES

Figure 2.1 The effects of two temperature regimes and different media on the germination percentage of *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. 53

Figure 2.2 The effects of two temperature regimes and different media on the growth of cotyledons in *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. 54

Figure 2.3 The effects of two temperature regimes and different media on root growth of *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. 55

Figure 2.4 **(A)** *P. cynaroides* seed (=achene); **(B)** *P. cynaroides* seed with hairs removed; **(C)** Excised *P. cynaroides* embryo. 56

Figure 2.5 *In vitro* germination of excised *P. cynaroides* embryos cultured on MS medium grown under light (12-hour photoperiod), after **(A)** 9 days; **(B)** 14 days; **(C)** 30 days, and in total darkness, after **(X)** 9 days; **(Y)** 14 days; **(Z)** 30 days. 57

Figure 2.6 Growth of long and twining cotyledons on *P. cynaroides* seedlings germinated in media containing GA_3. 58

Figure 2.7 **(A)** Two weeks after transferring to the mistbed, *in* vitro-formed roots died, and the emergence of new roots formed *ex vitro* is visible; **(B)** A seedling with healthy roots after 60 days in the mist bed. 59

Figure 2.8 Establishment of *in vitro*-germinated *P. cynaroides* seedlings in the mistbed. **(A)** After 30 days; **(B)** Transferred to black bag after 60 days. 60

Figure 3.1. **(A)** Thirty-day old sprouted axillary bud on established nodal explant (Wu, 2001) was used as microscion; **(B)** Roots of 30-day old *in vitro*-germinated *P. cynaroides* seedling were used as rootstock; **(C)** diagrammatic representation of the micrografting method used in this study; **(D)** Growth of new axillary buds on microscion and new roots on rootstock after 8 weeks. Bar ≈ 5 mm. 70

Figure 3.2 **Top row:** Formation of the graft union after 2 weeks in culture.
Bottom row: Sprouting of terminal and axillary buds on microscion after 8 weeks. 71

Figure 4.1 Direct formation of somatic embryos: (A) globular-stage embryos formed on the edges of a zygotic embryo; (B) Advanced torpedo-stage embryo formed on the abaxial side of a zygotic embryo; (C) Torpedo-stage embryo formed on the adaxial side of a zygotic embryo (arrow); (D) Cluster of somatic embryos from zygotic embryo in various stages of development. 91

Figure 4.2 Direct somatic embryo formation on excised cotyledons cultured on full-strength MS medium with no growth regulators in (A) the dark, and (B) under a 12-hour photoperiod. The development of somatic embryo into the (C) heart stage, and (D) torpedo stage, directly on excised cotyledons in the dark. 92

Figure 4.3 Growth of non-embryogenic, watery, fluffy callus on (A) zygote explant cultured on medium containing 2.5 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ, and on (B) excised cotyledon cultured on medium containing 1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP. 93

Figure 4.4 Development of isolated somatic embryo cultured on germination medium containing MS medium supplemented with 0.1 mg l^{-1} GA_3. (A) Embryo in advanced heart stage; (B) Embryo in cotyledonary stage, with accessory embryos attached (arrow). 94

Figure 4.5 Germination of normal embryos in MS medium containing GA_3. Emergence of first leaves from the germinated embryo (bottom right). 95

Figure 4.6 Germination of malformed embryos with (A) a single cotyledon; (B) Multiple cotyledons; (C) Two fused embryos attached. 96

Figure 4.7 Longitudinal section through (A) Embryo at globular-stage, with cell differentiation at root pole (Bar ≈ 500 µm); (B) Embryo at heart stage, with development of cotyledons (Bar ≈ 500 µm); (C) Embryo at cotyledonary stage showing vascular connections (Bar ≈ 500 µm); (D) Starch grains stained with safranin, enlarged from the cotyledon area of (C) (Bar ≈ 50 µm). 97

Figure 4.8 Immature unfertilized ovules, cultured on half-strength MS medium with 1 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ after (A) 0 days; (B) 5 days; (C) 4 weeks; (D) 8 weeks. 98

Figure 4.9 Clusters of shoot buds on cotyledons cultured on basal MS medium. 99

Figure 4.10 (A) Root initiation of excised shoot-bud placed on growth regulator-free MS medium after 4 weeks; (B) Root elongation after 6 weeks; (C) Callus formation on shoot-bud on 1 mg l^{-1} NAA. 100

Figure 5.1 **(A)** Rooting of *P. cynaroides* cuttings in seedling trays placed in a mistbed; **(B)** Rooting of untreated and **(C)** blanched *P. cynaroides* cuttings after 90 days. 126

Figure 5.2 The effects of pre-treatments on the rooting % of *P. cynaroides* cuttings after 90 days. 127

Figure 5.3 The effects of pre-treatments on the root dry mass of *P. cynaroides* cuttings after 90 days. 127

Figure 5.4 Dry root mass of cuttings during rooting period (60, 90 and 120 days after planting). 129

Figure 5.5 Changes in the total soluble phenolic content in different parts of *P. cynaroides* cuttings, during rooting. 130

Figure 5.6 Changes in the starch content in different parts of *P. cynaroides* cuttings, during rooting. 131

Figure 6.1 Column chromatography of 70% aqueous acetone extract from *P. cynaroides*. 150

Figure 6.2 Dose-response of stem extract on lettuce root growth. 150

Figure 6.3 NMR spectral analysis of Compound 1 showing pure 3,4-dihydroxybenzoic acid. 151

Figure 6.4 Chemical structure of 3,4-dihydroxybenzoic acid. 151

Figure 6.5 Dose-response of 3,4-dihydroxybenzoic acid on the root growth of lettuce seedlings. 152

Figure 6.6 Effect of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants. From left to right: 0, 1, 25, 50, 100, 500 mg l^{-1} (After 3 weeks in culture). 153

Figure 6.7 HPLC analysis of the basal end of stems. **(A)** Stems taken from motherplant; **(B)** After 90 days in mist bed when rooting was observed; **(C)** After 120 days when root growth was high. 154

LIST OF TABLES

Table 3.1 Effects of pre-treatments on grafting success, indicated by the formation of graft union and the growth of buds. 70

Table 4.1 Effects of light conditions on the induction of somatic embryos from zygotic embryos and cotyledons excised from *in vitro*-germinated seedlings, cultured on full-strength growth regulator-free MS medium. Data were taken after 8 weeks in culture. 89

Table 4.2 Effects of growth regulators on the germination of somatic embryos cultured in the dark. Half-strength MS medium was used as the basal medium in all treatments. Results were taken after 4 weeks in culture. 89

Table 4.3 Effects of growth regulators and light conditions on the growth of immature ovules after 8 weeks. 90

Table 5.1 Mean number of roots on the cuttings of the various treatments after 90 days in mistbed. 128

Table 5.2 Loss of nutrients from *P. cynaroides* cuttings by leaching with distilled water for 6, 12 and 24 hours. 129

Table 6.1 Response of *P. cynaroides* explants to a series of 3,4-dihydroxybenzoic acid concentrations in MS medium cultured *in vitro* (After 3 weeks in culture). 153

Improving *in vitro* propagation of *Protea cynaroides* L. (King Protea) and the roles of starch and phenolic compounds in the rooting of cuttings

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Abstract

Protea cynaroides L. (King Protea) is a well known cutflower. Seeds and stem cuttings are commonly used to propagate *P. cynaroides.* However, the success rate and rooting rate of seeds and cuttings, are inconsistent and slow. The potential of *in vitro* propagation as an alternative method to produce *P. cynaroides* plantlets was investigated. *In vitro* studies consisted of *in vitro* germination of mature zygotic embryos, micrografting and direct somatic embryogenesis of zygotic embryos and excised cotyledons. In the germination study, temperature was the most important factor in obtaining a high germination percentage. Alternating temperatures of 21±2ºC/12±2ºC (light/dark) was suitable for germination and over 90% of embryos germinated, while the germination percentage of embryos at 25±2ºC was poor. Plantlets were successfully established in *ex vitro* conditions when planted in a peat/coir/sand mixture. Micrografting of *P. cynaroides* was done by grafting microshoots (microscion), which was taken from *in-vitro*-established nodal explants, onto roots of decapitated *in-vitro*-germinated seedlings. After the graft union formed, buds on the microscion sprouted. A protocol to induce direct somatic embryogenesis was developed. Direct somatic embryogenesis was achieved on both *P. cynaroides* mature zygotic embryos and excised cotyledons. The addition of auxins such as NAA and 2,4-D singly or in combination with TDZ, BAP or kinetin suppressed the formation of somatic embryos. Formation of somatic embryos was observed in medium lacking growth regulators. Germination of somatic embryos was highest in medium containing GA_3. The roles of starch and phenolic compounds in the rooting of *P. cynaroides* cuttings were also studied. Starch and total soluble phenol analyses

results revealed a positive correlation between high root formation and increased starch and phenolic content. NMR and MS analyses identified high amounts of 3,4-dihydroxybenzoic acid in stems of *P. cynaroides*. *In vitro* bioassay showed that 3,4-dihydroxybenzoic acid stimulated and inhibited root growth of *P. cynaroides* explants, depending on the concentration. A link was made between the endogenous concentration levels of 3,4-dihydroxybenzoic acid and rooting of *P. cynaroides* stem cuttings. Findings of this study contribute towards a better understanding of the roles starch and phenolic compounds play in the rooting of *P. cynaroides*.

Keywords: *Protea cynaroides*, *in vitro* germination, micrografting, somatic embryogenesis, phenolic compounds, starch

INTRODUCTION

Protea cynaroides (King Protea) is a multi-stemmed, upright shrub that grows to between 0.3 and 2 m tall. It has sparse branches, with hairless stems (Rebelo, 2000). The leaves are round, oval or narrowly elliptic, ranging from 50 to 120 mm in length and 50 to 75 mm in width. The flowerhead sizes range from 120 mm to 300 mm in diameter and the colour of the bracts, which are either hairy or hairless, range from pink to creamy-white (Patterson-Jones, 2000).

The King Protea is the national flower of South Africa. They are widely spread throughout the south-western and southern parts of South Africa. Its magnificent inflorescence is a well known cutflower in many parts of the world. The most common methods of propagation are by seeds and stem cuttings. However, propagation by both seeds and stem cuttings have limitations in large-scale commercial production. Seed germination is usually inconsistent, even when the seed is treated with seed primers. However, the main problem with plants derived from seeds is genetic variation. This is particularly problematic when uniform blooms of a specific cultivar are highly sought after in the market place. Vegetative propagation of protea cuttings has become more common, nevertheless, this has its own limitations. The difficulty in inducing quick and consistent rooting of stem cuttings has not been overcome. At the moment, *P. cynaroides* cuttings take four to six months to root in the mistbed, prior to being transplanted to the field. After transplanting, it takes several years for the first high quality flower to be produced, which makes it an expensive flower to produce. Currently, some flowers are still picked from the wild, which firstly, do not always adhere to international standards, and secondly, cannot maintain a consistent flow of quality floral products to the floriculture industry (Coetzee, 2000).

Even with the abovementioned problems in the propagation of *P. cynaroides*, the cultivation of proteas in general has gradually increased over the years, mainly through the increase in area being planted. Although the majority is grown in the southern hemisphere, cultivation areas in the northern hemisphere have increased from 800 ha in 2000 to 900 ha in 2004 (Anonymous, 2005). South Africa is the world

leader regarding the total area of Proteaceae grown, which in 2004 was 3,853 ha, of which 2795 ha was broadcast sown. This is followed by Australia with an estimated 1,230 ha, while in the northern hemisphere, California leads the way with approximately 405 ha, followed by Israel with 270 ha. The amount of fresh Proteaceae flowers exported by South Africa has steadily increased from approximately 2,100 tons in the early nineties to over 4,200 tons in 2004 (Anonymous, 2005).

Very few studies on the *in vitro* propagation of *P. cynaroides* have been reported. Nevertheless, the establishment (Ben-Jaacov and Jacobs, 1986) and multiplication (Wu, 2001) of *P. cynaroides* explants have been investigated, where explants were successfully cultured *in vitro*. *In vitro* rooting of these explants were however, not successful. Furthermore, the use of other *in vitro* propagation methods, such as somatic embryogenesis and zygote culture to obtain rooted plantlets has not been reported.

In numerous studies, reviewed in Chapter 1, it has been shown that micropropagation techniques, whether through *in vitro* embryo culture, somatic embryogenesis, organogenesis or micrografting, can overcome common problems such as slow germination, low regeneration rate and poor rooting capacity of various plant species. The main objective of the research reported in this thesis was to introduce alternative propagation methods for *P. cynaroides*, which included *in vitro* culture of zygotic embryos, micrografting and somatic embryogenesis. These *in vitro* propagation practices could ultimately be used in practice to breed and mass propagate one of the most valuable *Protea* species. Furthermore, the aim of this study was to contribute new knowledge towards the understanding of the roles of starch and phenolic compounds in the root formation of *P. cynaroides*, through allelopathy and biochemical studies.