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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)

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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

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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

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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.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Vegetative propagation is the reproduction of plants identical in genotype to the source plant (Hartmann, Kester, Davies and Geneve, 1997). Hence, the objective is the formation of adventitious roots since it is the main regenerative process and the prerequisite for successful vegetative propagation. The main advantage of vegetative propagation is that growers are able to achieve genetic uniformity among the newly propagated plants, which is of utmost importance for cutflower producers. However, the difficulties in achieving root formation in certain plants, as well as the high costs of various propagation facilities such as mistbeds, fogging systems, temperature and light manipulation, are the disadvantages of vegetative propagation.

There are two types of adventitious roots: preformed and wound-induced roots (Hartmann, Kester, Davies and Geneve, 1997). Preformed root initials are normally dormant until the stems are made into cuttings and placed under environmental conditions which are conducive to emergence of the primordia as adventitious roots (Lovell and White, 1986). Wound-induced roots develop only after the cutting is made and is therefore formed *de novo* (Davies, Lazarte and Joiner, 1982). When a cutting is made, the stem responds to the wounding to protect the cut surface from desiccation and infections. These responses include: 1) the formation of a necrotic plate, a corky substance (suberin) seals the wound, and the xylem is plugged with gum; 2) cell division takes place and a layer of parenchyma cells (callus) is formed; 3) cells close to the vascular cambium and phloem start to divide to initiate *de novo* adventitious roots (Cline and Neely, 1983). The stages of development of *de novo* adventitious roots are: 1) dedifferentiation, 2) cell initiation to form root initials, 3) development of root initials into root primordia, and 4) the growth and elongation of the root primordia (Girouard, 1967).

A special adaptation of proteas to nutrient-deficient soils is the growth of unique roots known as proteoid roots (Vogts, 1982). Proteoid roots are dense clusters of fine rootlets that are produced by all cultivated species of protea (Lamont, 1986). Individual proteoid roots vary in length from a few millimetres to over 10 cm in length and can consist of up to thousands of hairy rootlets. The rootlets readily form a 2 – 5 cm thick mat at the soil surface in localized wet pockets of soil (Lamont, 1986). The primary function of proteoid roots is to enhance nutrient uptake, particularly in poor soils. It has been shown that proteoid root formation is suppressed in clayey soils, and the production of proteoid roots decreases markedly when soil nutrient availability is increased (Gardner, Parbery and Barber, 1982), indicating that the formation of proteoid roots is temporary, which takes place only when insufficient nutrients and moisture are present. Proteoid roots were not observed during the study of adventitious root formation in *P. cynaroides* (Chapter 5).

The first part of the following literature review deals with relationships between vegetative reproduction and carbohydrate, etiolation and phenolics. The second part describes alternative propagation methods that could be applied to *P. cynaroides*.

1.1.1 Carbohydrates

Reducing sugars such as glucose and fructose, non-reducing sugar such as sucrose, and starches (storage carbohydrates) are the most important carbohydrates for the rooting process (Haissig, 986). Carbohydrates are important in root formation because they are the basic building blocks of structural elements and are used as energy sources in the plant (Struve, 1981). The amount of carbohydrates required to fulfill this function has not been defined. Carbohydrates may regulate the number of roots to be supported and their subsequent growth, however, it is not necessarily a controlling mechanism (Veierskov, 1988). This may explain numerous apparent contradictory findings of positive correlations between carbohydrates and rooting in certain plant species, and negative correlations in others. Veierskov, Stummann and Henningsen (1982) reported a positive correlation in *Pisum sativum* plants, where a high carbohydrate content in the cuttings increased the number of roots formed. However, it has been proposed that a positive correlation between starch and rooting may be due to the supply of photosynthate being insufficient to support rooting (Veierskov, 1988).

Conversely, a negative correlation was observed in *Pinus sylvestris* by Hansen, Stromquist and Ericsson (1978), where an increase in carbohydrate content reduced the number of roots formed. Similarly, Nanda and Anand (1970) and Okoro and Grace (1976) showed that starch content was not related to rooting. Therefore, in this thesis (Chapter 5), the role of starch in the rooting of *P. cynaroides* was investigated to provide explanations to the low rooting rate of *P. cynaroides*.

1.1.2 Etiolation

Etiolation is defined as growing plants in total darkness (Bassuk and Maynard, 1987). Other practices related to etiolation are banding and blanching of the stem, which are both localized light exclusion techniques. Banding involves etiolating the entire plant until the new shoots have grown to a suitable length. Subsequently, shading is gradually reduced and the shoot is allowed to turn green, while an adhesive band is wrapped around the portion of the shoot that will become the cutting base. Blanching involves the plant being grown under the usual light conditions and once the normal development of new shoots has been completed, the future cutting base is banded with adhesive tape for several weeks (Bassuk and Maynard, 1987). Over the years, all the various light exclusion treatments, whether etiolation, shading, banding or blanching, have in most cases improved rooting of numerous plants. Of these species, many were difficult-to-root woody plants (Maynard and Bassuk, 1987).

For example, Reid (1923) successfully rooted *Camphora* spp. cuttings, which is one of the earliest reports on the promotion of rooting by etiolation. Subsequently, the success of rooting of various plant genera through etiolation has been increased. These include *Acer* spp., *Betula papyrifera, Carpinus* spp., *Corylus americana*, *Pinus* spp. and *Quercus* spp. (Maynard and Bassuk, 1985), *Persea americana* (Frolich, 1961), *Hibiscus rosa-sinensis* and *Phaseolus vulgaris* (Herman and Hess, 1963), *Syringa vulgaris* (Patience and Alderson, 1985) and *Malus* spp. (Gardner, 1936; Delargy and Wright, 1978; Delargy and Wright, 1979; Sun and Bassuk, 1991).

Although the various etiolation techniques have resulted in numerous successes in promoting rooting of difficult-to-root species, the mechanisms of etiolation is still not fully understood. However, several anatomical and biochemical studies have led to

some elucidation. Less lignification was found in etiolated stem tissues than in light-grown tissues (Reid, 1923). A decrease in cell wall thickness and an increase in protoplasmic cell contents were observed which led to the idea that the ease of rooting in etiolated cuttings was due to a change in the mechanical properties of the stem. In addition, Gardner (1936) reported that blanched shoots had more undifferentiated tissues that may have led to easier root initiation. Furthermore, Frolich (1961) showed a negative relationship between total light duration and rooting of mung beans, where the longer the mung beans were exposed to light, the poorer was the rooting.

Plant growth regulators such as auxin and rooting co-factors may also play a role in the rooting of *P. cynaroides* cuttings. However, the focus of this study was on the changes of endogenous concentrations of starch and phenolic compounds during rooting of cuttings. No research on etiolation of *P. cynaroides* has been done, therefore blanching, which could be used as an etiolation technique for proteas, was investigated (Chapter 5).

1.1.3 Phenolic compounds

When cuttings are prepared for rooting, they are wounded when removed from the motherplant. This wounding leads to the release and oxidation of phenolics which were formerly contained in cell compartments. The roles of these phenolics could include inhibition of the growth of microbes and assisting in the formation of lignin around the wound to act as a physical barrier against diseases (Salin and Bridges, 1981).

Numerous research papers have suggested that endogenous phenolic compounds may play a role in the rooting ability of stems. For example, before the seasonal increase in the rooting ability of apple ‘M26’ shoots, the amount of phloridzin, which is a phenolic found in apple, increased in the xylem of those shoots (Roy, Roychoudhury, Bose and Basu, 1972). Phloroglucinol, which is also a well-known phenolic, has been shown to promote rooting in apple shoots (Jones and Hatfield, 1976; James and Thurbon, 1981; James, 1983) and in *Prunus* species (Jones and Hopgood, 1979). However, one should keep in mind that a compound could either be a promoter or inhibitor of root formation depending on its concentration. In addition, it is generally

agreed that a number of different factors and compounds work together during rooting, rather than a certain compound alone, whether phenolics, auxins or other endogenous promoters/inhibitors.

Interestingly, Spiegel (1954) found that the rooting of easy-to-root *Vitis* sp. was inhibited when supplied with leachates taken from the bases of difficult-to-root cuttings. Bioassay investigations carried out on extracts taken from various vines also showed that difficult-to-root species have relatively higher amounts of inhibitors than easy-to-root species. On the other hand, in *Hibiscus* extract bioassay results led Hess (1964) to conclude that a higher concentration of promotory substances are found in easy-to-root *Hibiscus* materials than in difficult-to-root species. The effects of phenolic compounds on the rooting of *P. cynaroides* have not been investigated. Therefore, phenolic compounds from *P. cynaroides* cuttings were isolated and identified. Allelopathic potential of these compounds and their effects on root formation were studied (Chapter 6).

1.2 *In vitro* propagation

The potential of *in vitro* propagation using tissue and organ culture for rapid mass propagation of Proteaceae has been researched relatively extensively (George, 1996). Most of the methods used involved using stem or leaf segments as explants, which are established, multiplied, rooted, acclimatized and planted out into *ex vitro* conditions. However, in terms of obtaining a propagation protocol that is relatively simple and reliable, which can be used in a commercial environment, it has still not been successful. This is evident in the fact that Proteaceae plants are still not being extensively mass-propagated using tissue culture techniques, which is often the standard procedure in numerous economically important cutflowers.

It is surprising that even though *P. cynaroides* is probably the most popular and recognizable species in the Proteaceae family, very little research has apparently been done on its *in vitro* propagation. As a result, rooted *P. cynaroides* plantlets derived from tissue culture have yet to be achieved. Early work on *in vitro* establishment of *P. cynaroides* explants were reported by Ben-Jaacov and Jacobs (1986). Gibberellic acid

(GA_3) was found to be an essential growth regulator to start *P. cynaroides* in culture. More specifically, they concluded that the addition of 10 mg l^{-1} GA_3 and 2 mg l^{-1} BA (Benzyladenine) into Anderson medium (AND) (Anderson, 1975) was the most suitable combination for establishment. Following this, an alternative establishment medium was reported, where antioxidants were used as a pretreatment to reduce oxidative browning, resulting in the promotion of bud sprout (Wu and du Toit, 2004). These established explants were subsequently multiplied in a multiplication medium. However, rooting of these explants was not successful.

Research on *in vitro* propagation of other Proteaceae have also been published, such as *Protea obtusifolia* (Watad, Ben-Jaacov, Cohen, Tal and Solomon, 1992), where etiolated, multinodal shoot segments were established through bud sprouting and elongation, which were promoted by the addition of 2 mg l^{-1} GA_3 and 1 mg l^{-1} BAP (6-Benzylaminopurine). Similarly, Rugge (1995) also used multinodal explants to establish *Protea repens* on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) medium supplemented with 1 mg l^{-1} BA. Bud break was increased with the addition of 6 mg l^{-1} GA_3. Axillary shoot multiplication was achieved in *Leucadendron* when a mixture of 0.89 µM BA and 0.89 µM kinetin was added into the medium (Pérez-Francés, Expósito and Rodríguez, 1995).

In *Leucospermum*, shoots of the cultivar 'Red Sunset' were successfully established on AND liquid medium containing 2 mg l^{-1} BA (Ben-Jaacov and Jacobs, 1986), as well as using multinodal stem segments cultured on full strength MS medium with 2 mg l^{-1} kinetin (Rugge, Jacobs and Theron, 1989). Axillary shoots of *Leucospermum* 'Hawaii Gold' were induced to proliferate on modified MS medium supplemented with 0.2 mg l^{-1}, and rooted *in vitro* by dipping in 50 or 100 mg l^{-1} IBA (3-indolebutyric acid) (Kunisaki, 1989). Dwarf clones of *Leucospermum cordifolium* were also micropropagated using 1 mg l^{-1} GA_3, 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA (1-Naphthalene acetic acid). Best *in vitro* rooting was achieved when 1 mg l^{-1} IBA was added to the medium (Tal, Solomon, Ben-Jaacov and Watad, 1992). Tal, Ben-Jaacov and Watad (1992) further reported that high light intensities and a low relative humidity are best suited for *in vivo* rooting of micropropagated *Leucospermum.*

In *Grevillea* spp., cultivars 'Robyn Gordon' and 'Crosbie Morrison' were propagated via tissue culture by adding 1 µM NOA (2-Naphthyloxyacetic acid) and 0.1 µM 2iP (N^6-(2-isopentyl)adenine) into the growth medium, and rooted *in vitro* with 10 µM IBA in the rooting medium. (Gorst, Bourne, Hardaker, Richards, Dircks and de Fossard, 1978). Ben-Jaacov and Dax (1981) proliferated shoot segments of *Grevillea rosmarinifolia* on half-strength MS medium containing 0.5 mg l^{-1} BA, which were rooted on paper bridges placed in liquid MS medium containing 0.1 mg l^{-1} NAA. Six other *Grevillea* species were established *in vitro* when cultured on half-strength MS medium with 1 mg l^{-1} BAP (Watad, Ben-Jaacov, Tal and Solomon, 1992). Highest rooting percentage was recorded with 1 mg l^{-1} NAA added into the rooting medium. The endangered *Grevillea scapigera* was micropropagated using leaf sections to obtain adventitious shoot growth (Bunn and Dixon, 1992). Shoot tips were initiated on filter paper placed in liquid Woody Plant Medium (WPM) (Loyd and McCown, 1981), supplemented with 20 µM zeatin riboside and 2 µM GA_3.

Single-node explants of *Telopea speciosissima* were established in culture on modified MS medium (Seelye, Butcher and Dennis, 1986). Shoots then proliferated when grown on medium with 0.05 mg l^{-1} IBA, 0.3 mg l^{-1} BA and 2 mg l^{-1} GA_3. Similarly, Offord, Campbell and Mullins (1992) established *Telopea speciosissima* on a modified MS medium, with microshoot proliferation achieved with 1.25 µM BA and 1 µM GA_3 added to the medium. Microshoots were then subsequently rooted *in vitro* on agar, filter paper bridges and crushed quartz–sand (Offord and Campbell, 1992). Best rooting was obtained on the crushed quartz-sand containing 50 µM IBA.

Other *Protea* species that have been micropropagated include *Serruria florida*, where shoot proliferation was achieved on AND, MS and WPM liquid media (Ben-Jaacov and Jacobs 1986). Furthermore, numerous side shoots formed when the explants were subcultured onto MS medium containing 5 mg l^{-1} BA. Shoot tips of *Stirlingia latifolia* were successfully multiplied on half-strength MS medium supplemented with 5 µM 2iP or 0.5 µM BA (Bunn and Dixon, 1992). These microshoots were subsequently rooted *in vitro* in rooting medium containing IBA. *Alloxylon flammeum* was best grown via tissue culture on 0.6 µM BA, which produced the most number of usable shoots, whilst 3 – 25 µM 2iP gave the longest shoots (Donovan, Offord and Tyler, 1999).

1.2.1 Embryo culture

Embryo culture is defined as the *in vitro* isolation and growth of an immature or mature embryo to obtain a viable plant (Pierik, 1987). In the culture of immature embryos, the embryos are excised and cultured to avoid abortion. This procedure is commonly referred to as 'embryo rescue'. However, failure in culturing these immature embryos is generally high. Success rates depend largely on the development stage of the immature embryo. In addition, the nutritional requirements of immature embryos are complex, thus, composing a suitable nutrient medium is difficult (Pierik, 1987). Immature embryo cultures are often used by plant breeders for biochemical studies and interspecific hybridization, where a particular useful character from wild species, such as disease resistance, is transferred into cultivated species (Hadley and Openshaw, 1980). Embryo culture is also useful for determining seed viability and studying nutritional and physical requirements for embryonic development (Razdan, 1993). Mature (zygotic) embryos are easier to culture, where the use of simple medium is generally sufficient, although low temperatures, absence of light, and gibberellic acid, which is known to promote germination, are commonly applied during embryo culture. Mature embryo culture is mainly used to overcome seed dormancy, improve germination rates and shorten the breeding cycle, since chemical inhibitors are not present after the removal of the seed coat. This is particularly advantageous in plant species where germination is extremely slow, or does not occur at all, when using conventional germination techniques. From a commercial point of view, for highly-sought after seedlings that do not germinate easily under conventional propagation methods, embryo culture may be used as an alternative method for mass production.

Embryo culture was first described in the early 20th century, and subsequently, numerous studies have been reported over the years. The first publication on embryo culture was by Hannig (1904), who isolated *Cochleria* and *Raphanus* embryos, which were subsequently grown into transplantable seedlings. Following Hannig's work, Brown (1906) studied the nutritional requirements of excised barley embryos. *Linum* embryos were also one of the first to be successfully cultured (Laibach, 1929), where hybrid plants were raised from an interspecific cross, which in nature, failed to germinate. Other early embryo culture research were reported in *Prunus* spp. (Tukey,

1933; Tukey, 1934; Tukey, 1938), *Datura stramonium* (Van Overbeek, Conklin and Blakeslee, 1942), *Iris* spp. (Randolph and Cox, 1943), *Hordeum vulgare* (Kent and Brink, 1947), *Phaseolus* spp. (Honma, 1955), *Musa balbisiana* (Cox, Stotzky and Goos, 1960), and *Cocos* sp. (De Guzman, del Rosario and Eusebio, 1971). Zygotic papaya embryos were first cultured by Phadnis, Budrakker and Kaulgud (1970). This was followed by the successful embryo rescue of the interspecific cross between the incompatible *C. papaya* and *C. cauliflora* (Khuspe Hendre, Mascarenhas and Jagannathan, 1980). Similarly, Chung and Kim (1990) produced interspecific hybrids between *Glycine max* and *G. tomentella* through embryo culture. Self-sterile *Litchi chinensis* embryos, which were excised and cultured successfully, produced up to four axillary shoots each when the cotyledons and shoot tips were cultured separately (Amin, Razzaque and Anisuzzaman, 1996). Embryos of cross-bred seeds of peach (*Prunus persica*), which did not germinate under conventional methods, germinated *in vitro* after excision and stratification (Chopra, Kanwar, Gosal, Dhaliwal and Chanana, 1996).

In vitro germination of zygotic embryos has been studied in several Proteaceae species. Excised embryos of *Protea compacta*, *Leucadendron daphnoides L. cordifolium* (Van Staden and Brown, 1973), *Protea magnifica* (Deall and Brown, 1981) and *Leucadendron tinctum* (Brown and Dix, 1985),) were germinated in Petri dishes. In other studies, whole seeds of *P. compacta, P. barbigera*, *L. cordifolium* and *L. daphnoides* (Brown and van Staden, 1971) were used. *In vitro* germination of *P. cynaroides* excised embryos has not been reported in literature. Factors affecting *in vitro* germination of excised embryos of *P. cynaroides* and the transfer to the *ex vitro* environment are reported in Chapter 2.

1.2.2 Micrografting

Micrografting or *in vitro* grafting was first successfully carried out by Murashige, Bitters, Rangan, Roistacher and Holliday (1972). Newly-germinated seedlings are often used as the rootstock, although *in vitro*-rooted microshoots can also be used. The top of the rootstock seedling is cut off and the desired scion is grafted on. Depending on scion, different micrografting techniques are used. In the case where relatively large shoots are used, their bases are cut into a wedge (‘V’) shape, and

inserted into a vertical cut on the rootstock. If meristem tips are used as the scion, it is usually placed directly onto the cambium or cortex on the cut surface of the rootstock (George, 1996).

Problems encountered during micrografting include tissue blackening and death of the scion caused by oxidation of the cut surface, incompatibility between scion and rootstock, and desiccation of the graft area. Procedures to remedy tissue blackening have been developed. These include soaking the scion in a growth regulator or antioxidant solution such as ascorbic acid, thiourea, cysteine, chlorhydrate, sodium diethyl-dithiocarbamate (DIECA), or placing a drop of the solution onto the severed rootstock before inserting the scion (George, 1996). In *Citrus*, where micrografting has been used extensively to eliminate viruses, Edriss and Burger (1984) placed the shoot tips into a solution containing 10 mg l^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid), which doubled the number of successful micrografts. Similarly, Starrantino and Caruso (1988) soaked both the microscion and the tip of the rootstock in 0.5 mg l^{-1} BAP for 20 minutes before micrografting. Alternatively, an agar block, which contained mineral salts with or without hormones, was placed at the graft area between the scion and rootstock, helped in preventing dehydration of the scion (Pliego-Alfaro and Murashige, 1987). Furthermore, in order to improve the success rates, scions have been pre-cultured for a short period of time before being micrografted. Jonard, Hugard, Macheix, Matinez, Mosella-Chancel, Poessel and Villemur (1983) reported that pre-culturing the scion on medium containing 0.1 mg l^{-1} zeatin, encouraged rapid shoot formation after the micrograft has been successful.

Micrografting has been reported in numerous plant species. These include: *Persea americana* (Pliego-Alfaro and Murashige, 1987), *Anacardium occidentale* (Ramanayake and Kovoor, 1999; Mneney and Mantell, 2001; Thimmappaiah, Puthra and Anil, 2002), *Citrus* spp. (Murashige *et al*., 1972; Navarro, Roistacher and Murashige, 1975; Jonard *et al*., 1983), *Opuntia* spp. (Estrada-Luna, López-Peralta and Cárdenas-Soriano, 2002), *Picea* spp. (Ponsonby and Mantell, 1993), *Pistacia vera* (Abousalim and Mantel, 1992), *Prunus* spp. (Deogratis, Lutz and Dosba, 1986) and *Sequoia sempervirens* (Huang, Luis, Huang, Murashige, Mahdi and van Gundy, 1992).

Micrografting has mainly been used in fruit species to eliminate viruses or to overcome incompatibility between the microscion and rootstock. Micrografting has not been studied in *P. cynaroides*. Investigation into the use of micrografting as a technique to obtain rooted plantlets is reported in Chapter 3.

1.2.3 Somatic embryogenesis

Somatic embryogenesis is a process whereby a single cell or a group of cells initiate a developmental pathway that leads to reproducible regeneration of embryos ('embryoids'), which are capable of germinating and growing into complete plants (Razdan, 1993). The earliest work on plant regeneration was reported by Levine (1950), who after removing indole-acetic acid (IAA) from the growth medium, obtained roots and shoots from carrot callus. Wiggans (1954) also observed carrot plantlet regeneration when tissue was transferred from a medium containing adenine sulphate to a medium lacking it. However, it was Steward, Mapes and Mears (1958) who first described proembryo-like stages in carrot plantlet regeneration. This was followed by Reinert (1959) who proposed that carrot plantlets grew from bipolar embryos that were derived from single cells. Kato and Takeuchi (1963), Nakajima (1963) and Wetherell and Halperin (1963) also demonstrated that embryos formed in cultures were derived from mature organs of carrot plants.

According to Sharp, Sondahl, Caldas and Maraffa (1980), Evans, Sharp and Flick (1981) and Sharp, Evans and Sondahl (1982), somatic embryogenesis is initiated by either 'pre-embryogenic determined cells' (PEDC) or by 'induced embryogenic determined cells' (IEDC). In the PEDC pathway, direct embryogenesis occurs, without an intervening callus phase, from cells which are predetermined to become embryo-producing. Nutrient media and other *in vitro* conditions only serve to enhance the process. Physiologically, explants from which direct embryogenesis is most likely to occur, are juvenile. These include zygotic embryos, young seedlings, pollen microspores within the anther, tissues of all or part of the ovary, or ovules (George, 1993). On the other hand, in the IEDC pathway, indirect embryogenesis requires the differentiated cells of an explant to be induced to divide as undifferentiated callus, and then for certain cells to be re-determined to the embryogenic pathway, normally by exposure to growth hormones. Once the embryogenic state has been reached, plantlets

are produced. Direct somatic embryogenesis is less widely observed than indirect somatic embryogenesis. This is mainly due to the fact that conditions to obtain direct embryogenesis can be more critical than those required to produce embryogenic callus (George, 1996).

1.2.3.1 Stages in the development of somatic embryos

It is sometimes difficult to distinguish between plantlets that have been grown from embryos and adventitious shoots. However, detailed anatomical studies of somatic embryos will reveal a shoot and root pole (i.e. bipolar), a shoot axis and cotyledons (in dicotyledons). In addition, unlike axillary or adventitious buds, somatic embryos have no vascular connections with the underlying parental tissue (George, 1996). Somatic embryos and zygotic embryos are structurally similar, although somatic embryos do not have an orderly pattern of cell division, which is probably due to the different environmental conditions the cells are exposed to. Stages through which dicotyledonous somatic embryos develop are described as follows (George, 1996):

1) Pro-embryo stage: Small cluster of meristematic cells.
2) Globular stage: These are larger groups of cells, which are yet to have a definite embryo-like shape.
3) Heart stage: The cotyledonary initials are separated from the root pole.
4) Torpedo stage: An elongated form of the heart shape.
5) Cotyledon stage: Small seedling with cotyledons and root.

1.2.3.2 Selection of explant

Various parts of a plant can be used to induce somatic embryogenesis in culture. Direct embryogenesis has been induced on explants derived from seedlings or more mature organs, and zygotic embryos or their component parts. Examples of explants derived from young seedlings include: Petioles in *Apium graveolens* (Zee and Wu, 1980), cotyledonary node of *Corylus avellana* (Pérez, Fernandez and Rodriguez, 1983) and cotyledons of *Manihot esculenta* (Stamp, 1987). Embryogenesis induced on explants derived from mature organs include: leaf discs of *Amaranthus hypochondriacus* (Flores, Thier and Galston, 1981), mechanically isolated mesophyll

cells of *Asparagus officinalis* (Urigami, Sakai and Nagai, 1990), leaf midribs of *Dendranthema grandifolia* (May and Trigiano, 1991), and mesophyll protoplasts of *Medicago sativa* (Dijak and Simmonds, 1988). Examples of direct embryogenesis from zygotic embryos and their component parts include: Scutellum of *Sorghum bicolor* immature embryos (Thomas, King and Potrykus, 1977), immature zygotic embryo of *Brassica napus* (Pretova and Williams, 1987), cotyledons from *Camellia japonica* embryo (Kato, 1989), immature zygotic embryo of *Anacardium occidentale* (Gogate and Nadguada, 2003), and mature zygotic embryo of *Hyoscyamus niger* (Tu, Sangwan and Sangwan-Norreel, 2005).

Indirect embryogenesis has been successfully induced in numerous plant species such as: the immature zygotic embryos of *Mangifera indica* (Litz, Knight and Gazit, 1984) and *Glycine max* (Finer and Nagasawa, 1988), immature zygotic embryos of *Quercus* (Gingas and Lineberger, 1989), suspension cultures of *Pinus strobus* (Finer, Kriebel and Becwar, 1989), cell suspensions of *Ipomoea batatas* (Chée and Cantliffe, 1989), cell cultures of *Betula pendula* (Kurtén, Nuutila, Kauppinin and Rousi, 1990), shoot apices of *Pisum sativum* (Kysely and Jacobsen, 1990), and cell suspension cultures of *Prunus Persica* (Raj Bhansali, Driver, and Durzan, 1991).

Somatic embryos have also been obtained from haploid organs such as the pollen, anther and unfertilized ovules. Research has shown that pollen and anther culture can be induced to give rise to vegetative cells instead of pollen grains (George, 1996). The normal development pattern of the pollen is changed, and instead of the pollen producing gametes and a pollen tube, microspores are produced, which are capable of forming callus tissue and haploid pro-embryos. Formation of plantlets from pollen microspores is called androgenesis. Guha and Maheshwari (1964) regenerated haploid plants from pollen of *Datura innoxia* using intact anthers. Nitsch and Nitsch (1969) subsequently obtained pollen-derived embryos in *Nicotiana*. Batty and Dunwell (1989) reported that more pollen-derived embryos were produced when potato anthers were cultured in maltose than in sucrose.

Another method of obtaining haploid plants is to use unfertilized ovules, ovaries or flower buds. Such a method is referred to as gynogenesis (George, 1996). Gynogenesis was achieved in *Beta vulgaris* using unfertilized ovules (Doctrinal,

Sangwan and Sangwan-Norreel, 1989). Somatic embryos derived from unfertilized ovules were also obtained in onion (*Allium cepa*), where young developing embryos sprouted from split ovules (Campion and Alloni, 1990). Other examples of haploid plantlets produced include: ovules of *Gerbera jamesonii* (Sitbon, 1981; Meynet and Sibi, 1984), ovules of *Beta vulgaris* (Hosemans and Bossoutrot, 1983) and ovaries of maize (Truong-Andre and Demarly, 1984),

1.2.3.3 Growth media, hormone supplements and culture conditions

From extensive research carried out on somatic embryogenesis on a wide range of plants, a few general rules for the induction of somatic embryos have been written and are often applied. Nitrogen, usually in reduced form such as ammonium salts, is needed during embryo initiation and maturation (Razdan, 1993). Other nutritional additives, which contain various forms of nitrogen, such as coconut milk, casein hydrolysate and amino acid are also used in the growth medium. Potassium is also known to promote embryogenesis, particularly if nitrogen is restricted (Pierik, 1987). Although numerous types of basal media have been used to induce somatic embryogenesis, the MS medium or modified versions thereof, are the most commonly used media (George, 1996). The presence or the lack of certain macronutrients in the media is often essential for the induction and development of somatic embryos. For example, Reynolds (1990) reported that calcium was needed to induce somatic embryos in horsenettle pollen. Also, Walker and Sato (1981) showed that alfalfa embryos did not develop in the absence of ammonium or nitrate.

The most common type of auxin used to induce somatic embryogenesis is 2,4-D, although other auxins such as NAA, IBA and IAA are also used (George, 1996). The type of auxin and the concentrations used vary greatly, depending on the type of plant species. In direct embryogenesis, where explant tissues are embryogenically determined, it may not be necessary to add growth regulators to obtain embryos. In addition, in the case where spontaneous somatic embryos have been formed directly from *Citrus sinensis* nucellus tissues, it is thought that the nucellus tissue is auxin-habituated, since the addition of auxin depressed embryogenesis (Button, Kochba and Bornman, 1974). Furthermore, in certain cases the competence of cells, which become embryogenically determined, is increased with the addition of auxin.

Numerous papers have shown that direct embryogenesis is induced without any growth hormones: *Camellia reticulata* (Plata and Vieitez, 1990), *Citrus* (Rangan, Murashige and Bitters, 1968; Gmitter and Moore, 1986), *Daucus carota* (Smith and Krikorian, 1988), and *Mangifera indica* (Litz, Knight and Gazit, 1982). Many reports state that auxin is required for the induction of indirect somatic embryogenesis. Generally, auxin is required in the growth medium to induce embryogenic callus. In addition, it has also been established that continuous exposure of embryos to auxin can be detrimental to their development (Merkle, Parrott and Flinn, 1995). Halperin and Wetherell (1964) first observed that maintaining carrot embryos at the globular stage in 2,4-D inhibited their development and led to abnormal growth. Similar observations were made in *Cronilla varia* (Dusková, Opantrny, Sovová and Dusek, 1990).

Other common growth hormones used in somatic embryogenesis include cytokinin, ABA (abscisic acid) and GA_3. Cytokinins such as kinetin are often added with auxin to the media for the induction of somatic embryogenesis (George, 1996). In embryogenic-determined explants, the addition of cytokinins only is sometimes sufficient to induce somatic embryos. For example, only the addition of BAP was required to induce somatic embryos in *Pelargonium* (Marsolais, Wilson, Tsujita and Senaratna, 1991) and *Trifolium* (Maheswaran and Williams, 1985; Maheswaran and Williams, 1986). Abscisic acid is normally used in the later stages of indirect embryogenesis for maturation and germination of embryos. In most experiments, ABA has been reported to inhibit somatic embryo formation. However, Qureshi, Kartha, Abrams and Steinhauer (1989) reported that ABA promoted the production of embryogenic callus and suppressed precocious germination in zygotic embryos of wheat. Gibberellic acid has also, in most cases been found to suppress embryogenesis, although Shekhawat and Galston (1983) and Mehra and Sachdeva (1984) used gibberellic acid to induce embryogenesis in *Vigna aconitifolia* and *Malus domestica*, respectively.

The growth condition requirements of somatic embryos are dependent on the plant species (George, 1996). However, light is generally needed to promote embryogenesis, although various levels of irradiance ranging from low to total darkness have been reported to be critical in some species. Similarly, the temperature

requirements for the growth of somatic embryos are also species-dependent. Although high temperatures are normally favourable for embryogenesis, certain types of cultures, such as anther cultures, require a cold shock to initiate the formation of embryos (Pierik, 1987).

Somatic embryogenesis has not been reported in *P. cynaroides*. In other proteas, very few research papers have been published. An early study by Van Staden and Bornman (1976) obtained initiation and growth of *Leucospermum cordifolium* callus. In addition, callus and proteoid rootlet formation were obtained on cotyledonary explants of *Protea neriifolia*, but attempts to induce adventitious root and shoot development were unsuccessful (Van Staden, Choveaux, Gililand, McDonald and Davey, 1981). Somatic embryogenesis was, however, achieved in *P. repens*, where somatic embryos formed directly on the base of shootlets and callus, of which some developed into plantlets (Rugge, 1995). Chapter 4 reports the regeneration of *P. cynaroides* plantlets from cotyledon and zygotic explants through direct somatic embryogenesis.

1.2.4 Organogenesis

Various parts of intact plants grown *in vitro* on nutrient media can form new shoots, root and even flower initials without prior growth of callus tissue, i.e. direct organogenesis (George, 1996). Juvenile tissues of explants derived from the germination of zygotic embryos often form shoots readily. However, organogenesis is highly dependent on the morphogenetic potential of the plant species concerned, as well as the organ part from which organogenesis takes place. Adventitious shoot buds are often derived from cotyledons, leaves, roots and stems. Some explants have the capacity to regenerate shoots directly, without the addition of growth regulators, although the addition of these growth regulators usually increases the regeneration rate. In most cases, growth regulators are essential for the formation of adventitious shoots and roots. Examples of direct organogenesis where shoot formation were obtained include: *Glycine max* where cotyledonary nodes were used (Saka and Cheng, 1980), and leaf segments of *Petunia* sp. (Economou and Read, 1981). Adventitious shoots have also been reported to derive from root explants. For example, *Rubus* sp.

(Borgman and Mudge, 1986) and *Nicotiana* spp. (Zelcer, Soferman and Izhar, 1983) were induced to form shoots from root cultures.

Indirect organogenesis can occur from callus tissue that has been maintained in culture for a prolonged period of time. As in the case with direct organogenesis, the effective concentrations of growth regulators such as auxin and cytokinin vary from one plant species to another. There are two ways in which indirect organogenesis can arise (George, 1996): 1) On callus tissue which has been produced on the original explant, or 2) on callus tissue in which morphogenesis capacity has been induced, which forms plant organs once it is transferred to another medium.

Root initiation can occur simultaneously with shoot formation, although shoots are often excised from the original explant or callus, and rooted separately in rooting media. Generally, adventitious root formation is promoted when the ratio between auxin and cytokinin is greater than one. However, the capacity of tissues to produce endogenous hormones, or to metabolize exogenous growth regulators, differs. Therefore, certain callus tends to produce shoots and not roots, and *vice versa*. Indirect organogenesis have been reported in *Citrus grandis*, where seedling stem and leaf tissues were induced to form callus, which were subcultured to new media to form adventitious shoots (Chaturvedi and Mitra, 1974). Similarly, hypocotyls of young *Brassica* spp. were continuously subcultured to form callus tissue, on which adventitious shoots were initiated (Dietert, Barron and Yoder, 1982). Shoot apical meristems of *Chrysanthemum morifolium* (Sangwan and Harada, 1977), ovaries of *Freesia hybrida* (Bach, 1987) and stem segments of *Camellia* hybrids (Tosca, Pandolfi and Macchi, 1992), were induced to form callus and shoots.

Currently, methods to multiply *P. cynaroides* explants are limited. Preliminary studies of plantlet regeneration through direct multiple shoot-bud development of *P. cynaroides* explants are reported in Chapter 4, with the view to promote an alternative propagation method.

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CHAPTER 2

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

2.1 Abstract

Germination of *Protea cynaroides* seeds takes several weeks under conventional methods in soil. In addition, poor and inconsistent germination is also often observed. In this study, preliminary experiments confirmed the poor germination of *P. cynaroides* seeds under conventional methods. Furthermore, germination of seeds *in vitro* was also poor, which was mainly due to the inability of the radicle to protrude through the seed coat. The effects of GA_3, temperature and light on the *in vitro* germination of *Protea cynaroides* zygotic embryos were studied. Temperature was the most important factor in obtaining a high germination percentage. Alternating temperatures of 21±2ºC/12±2ºC (light/dark) was optimal for germination and over 90% of embryos germinated, while the germination percentage of embryos at 25±2ºC was poor. The incorporation of GA_3 into the growth medium had no effect on germination percentage, however, the cotyledons of seedlings germinated in this medium were long and abnormal, while the roots were stunted. The presence of light was not necessary since the embryos germinated similarly in a 12-hour photoperiod and in total darkness. The roots of the seedlings formed *in vitro* were incapable of functioning in *ex vitro* conditions. However, the plantlets were able to produce new roots in *ex vitro* conditions. A higher percentage of plantlets survived when transferred to the medium containing a peat/coir/sand mixture than those planted in silica sand.

2.2 Introduction

Propagation by seeds (=achenes) is widely used by *Protea* growers to obtain new plants. However, mass propagation of members of the Proteaceae, including *Protea cynaroides*, are known to be difficult. This is mainly because protea seeds germinate

poorly and erratically (Deall and Brown, 1981). In addition, germination usually takes place over a long period of time. Several research papers have shown that endogenous inhibitors contribute to poor germination of protea seeds: Brown and van Staden (1971) showed that inhibitors found in aqueous seed extracts of *Protea compacta, Protea barbigera, Leucospermum cordifolium* and *Leucadendron daphnoides* may be responsible for regulation of seed germination. It was subsequently reported that the primary inhibitor found in the seed extracts had coumarin-like properties (Van Staden and Brown, 1972). However, a later study showed that a lack of promoters rather than the presence of inhibitors was responsible for poor germination of *P. compacta* (Brown and van Staden, 1975a).

Numerous papers have described various methods to improve the germination percentages of different members of Proteaceae. Brown and van Staden (1973) showed that scarification, and to a lesser extent, stratification of the seeds, resulted in the promotion of *P. compacta* and *L. daphnoides* seed germination. It is postulated that chilling treatments lead to physical changes in the covering structures, which allow a greater penetration of oxygen, resulting in the enhancement of the metabolic activities of embryos (Wareing, 1969). Germination of *L. daphnoides* seeds increased by 50% through stratification and by 400% through incubation at high oxygen concentrations (Brown and van Staden, 1975b). Furthermore, stratification of *P. magnifica* seeds at 5ºC also led to high germination percentages (Deall and Brown, 1981).

Other methods to improve germination percentage include using chemicals or growth regulators, either as a pretreatment or as an additive in the growth medium. *Leucospermum* seeds soaked in hydrogen peroxide germinated significantly better than untreated seeds (Brits, 1986). Soaking *P. compacta* seeds in gibberellic acid (GA_3) and cytokinins also improved their germination (Brown and van Staden, 1973). Similarly, an improvement in the germination of *L. cordifolium* seeds was obtained by soaking the seeds in GA_3 and removing the outer seed coat before transferal to Petri dishes. Also, higher seed germination percentages of *P. eximia* and *P. neriifolia* were reported for GA_3-soaked seeds (Rodríguez-Pérez, 1995). However, conflicting results in the same experiment were observed for *P. cynaroides* when GA_3 did not significantly improve the germination percentage.

The role which the seed coat plays in inhibiting seed germination has been investigated. Brown and van Staden (1973) reported that the seed coat imposes dormancy and inhibit germination either by limiting water uptake or by acting as a physical barrier to germination of seeds. However, when they used excised embryos, in which the entire seed coat was removed, germination of *P. compacta* and *L. daphnoides* remained poor, but it was mentioned that a large number of embryos were exposed to microbial attack (Brown and van Staden, 1973). Germination of excised embryos of *L. cordifolium* (Van Staden and Brown, 1973), *P. magnifica* (Deall and Brown, 1981) and *Leucadendron tinctum* (Brown and Dix, 1985) were significantly improved by removal of seed coats.

The majority of the aforementioned experiments were carried out either in the field or in Petri dishes. Very few studies have been done *in vitro*. One such study was by Van Staden, Brown and Button (1972), who germinated excised embryos of *P. compacta* in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing various concentrations of growth regulators. Their findings showed that the addition of 1 mg l^{-1} GA_3 in the growth medium gave the best germination percentage of 43%, which is considered to be very low.

In vitro propagation methods are used worldwide on numerous plant species for rapid mass propagation. It is also used for growing seedlings from seeds that are difficult to germinate under conventional methods. The main objective of this study was to develop a method for rapid germination of *P. cynaroides* seeds in order to obtain large numbers of healthy plantlets. A preliminary trial was conducted to determine the germination percentage of *P. cynaroides* seeds and excised mature zygotic embryos. Thereafter, an *in vitro* germination study of excised mature zygotic embryos was conducted to investigate the effects of GA_3, temperature and light conditions on germination percentage, cotyledon and root mass.

2.3 Preliminary trial

2.3.1 Materials and methods

Mature *P. cynaroides* seeds were purchased from a commercial producer (Silverhill Seeds[1]). Firstly, seeds were screened by hand to select viable seeds for germination. Only plump, healthy-looking seeds were used. Thereafter, the seeds were treated with commercially available seed primers (Instant Smoke Plus Seed Primer, Kirstenbosch National Botanical Garden[2]), which entailed soaking them in 50 ml water containing the primer for 24 hours, as per instructions. One hundred seeds were sown during autumn, in a germination tray containing peat and sand (1:3 v:v). Trays were placed in a greenhouse at 24ºC±2. The growth medium was kept moist, while water logging was prevented by the presence of drainage holes at the bottom of the tray. Simultaneously, seeds were also germinated *in vitro*. One hundred seeds were sterilized in sodium hypochlorite (1%) for 5 minutes and placed upright on MS medium in a growth chamber at 25±2ºC. The temperature of 25±2ºC was used because *P. cynaroides* explants were successfully cultured *in vitro* at this temperature in previous studies (Wu, 2001). Cool, white fluorescent tubes provided 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR) to the explants. A 12-hour photoperiod was used. The germination percentage of the seeds in each trial was recorded. The seeds were considered germinated when the radicle emerged.

2.3.2 Results

Results from both experiments indicated extremely low germination percentages. After 8 weeks, 8% and 10% germination was observed in the germination tray and *in vitro* trials, respectively. The germination percentage increased to its highest level (35%) after 12 weeks in the germination tray, while the germination percentage of the *in vitro* trial remained at 10% after 12 weeks. Subsequent intervention involving removal of the seeds from the growth medium and visually inspecting them, revealed no signs of germination, and the seeds appeared to be dormant. The low germination percentage of seeds *in vitro* was mainly due to the inability of the radicle to emerge

[1] P O Box 53108, Kenilworth, 7745, South Africa
[2] Rhodes drive, Newlands, Cape Town, South Africa.

from the seed coat. Although the radicles of several seeds were able to protrude through the physical barrier, no plantlets were obtained since the cotyledons were unable to emerge. In the majority of the seeds, the seed coat seemed to impose dormancy, probably physically as well as through chemical inhibitors. Based on the results of these preliminary trials, it was decided that henceforth excised embryos would be used for germination under *in vitro* conditions.

2.4 Main trial

2.4.1 Materials and methods

2.4.1.1 Sterilization procedure

The same batch of seeds used in the preliminary trials was used. Firstly, the hairs on the seeds were removed by hand, in order to better expose the seed coat to sterilants during surface sterilization (Figure 2.4A, Figure 2.4B). Each seed was then carefully screened by hand, and only plump, healthy-looking seeds were used. The seeds were surface-sterilized in 0.35% sodium hypochlorite (NaOCl) for five minutes, followed by three minutes in concentrated sulphuric acid (H_2SO_4). Thereafter, the seeds were stirred in sterilized distilled water for 10 minutes. Each seed coat was then cut open using a scalpel and a pair of forceps, and the embryo removed (Figure 2.4C). The embryos were carefully placed into the medium, in an upright position.

2.4.1.2 Medium treatments

Three different types of media were used in this experiment, which all contained half-strength MS medium (Appendix A) as the basal medium. Forty explants were used in each treatment. The first medium contained no growth regulators, the second contained 1 mg l^{-1} GA_3, and the third medium contained 10 mg l^{-1} GA_3. All the media were also supplemented with sucrose (3%), and Gelrite® (3 g l^{-1}). The pH of all three media was adjusted to 5.7 before autoclaving. Ten ml of each medium were dispensed into 50 cm^3 glass test tubes, and capped. The media were sterilized in an autoclave (Hirayma Hiclave®, Model HA – 300D) at 104 KPa at 121ºC for 20 minutes.

2.4.1.3 Growth condition treatments

Excised zygotic embryos were germinated in either light or dark conditions. For the light treatment, a 12-hour photoperiod was used. Cool, white fluorescent tubes provided 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR) to the explants. In the other treatment, the explants were grown in total darkness for the entire germination period.

Two different temperature regimes were used to study the effects of temperature on the germination of the embryos exposed to the two light regimes. For the first temperature treatment the embryos were kept in a growth chamber at 25±2ºC for the entire germination period. In the other treatment, alternating temperatures of 21±2ºC/12±2ºC (light/dark) were used. The embryos were considered germinated when the growth of the radicle took place.

2.4.1.4 Transfer to *ex vitro* environments

Twenty *in vitro*-germinated embryos were planted out either into pasteurized silica sand or into a mixture of peat, coconut coir and sand (1:1:1 v:v:v), with no bottom-heating. After removing the plantlets from the test tubes, they were carefully washed in tap water to remove any medium attached to them. The plantlets were then transplanted to a fibre-glass-covered mistbed facility equipped with a fogging system, which kept the relative humidity above 95%, and a water sprinkler system that irrigated every 2 minutes for 30 seconds. The Photosynthetic Active Radiation (PAR) of the mistbed was 400 µmol m^{-2} s^{-1}, and the average temperature was 28±2ºC.

2.4.1.5 Statistical analysis

A completely randomized design was used in all the experiments. Significant differences in the germination percentage between treatments were tested using Chi-square analysis. Means of root and cotyledon fresh mass were separated using Tukey's studentised test at the 5% level of significance. All statistical analyses were performed using the SAS program (SAS Institute Inc, 1996). ANOVA is shown in Tables C1 and C2, Appendix C.

2.4.2 Results

2.4.2.1 Effects of treatments on germination percentage

The germination patterns of the zygotic embryos were the same in all the growth media under both light (12-hour photoperiod) and dark conditions, except that greening of the cotyledons did not occur in the dark. The first visible change noted was the separation of the cotyledons, which was followed by the growth of the radicle. Afterwards, further development of the seedlings continued until healthy plantlets were obtained (Figure 2.5).

The results showed that temperature had the most influence on the germination percentage of zygotic embryos. Irrespective of the light conditions and media, significant differences in the germination percentage were found between embryos grown at the alternating temperature regime of 21±2ºC/12±2ºC and the constant temperature of 25±2ºC (Figure 2.1). The largest difference in germination percentage was observed under lights on the basal MS medium without GA_3, where 90% of embryos germinated at 21±2ºC/12±2ºC compared to only 20% at 25±2ºC.

With regard to the medium treatments, in the 21±2ºC/12±2ºC temperature range, no significant differences were found in the germination percentage of zygotic embryos between the basal MS medium and those supplemented with GA_3, irrespective of whether it was under light or dark conditions (Figure 2.1). However, in the 25±2ºC temperature treatment, the germination percentage of explants on basal MS medium without GA_3 was significantly lower than those grown in medium containing 1 mg l^{-1} GA_3 in the light (Figure 2.1A), as well as both those media containing either 1 mg l^{-1} GA_3 or 10 mg l^{-1} GA_3 in the dark (Figure 2.1B).

Germination of the zygotic embryos was not influenced by the light conditions. Within the same temperature and growth media treatments, no significant differences were observed between the explants cultured under lights and those in the dark (Figure 2.1). This indicates that light does not play an important role, and is not essential during the germination process.

2.4.2.2 Effects of treatments on cotyledon and root growth of zygotic embryos

From the results of statistical analyses, no interaction effects were found between media, light and temperature treatments in both cotyledon and root fresh mass. However, significant interaction effects ($P \leq 0.05$) were found between media and light, and media and temperature in cotyledon fresh mass, whereas in the root fresh mass of the seedlings, interaction effects between media and light, media and temperature, and light and temperature, were significant ($P \leq 0.05$).

Under both light and dark conditions, cotyledons of seedlings grown on both 1 mg l^{-1} GA_3 and 10 mg l^{-1} GA_3 were long and twisted (Figure 2.6). No significant differences were found between cotyledon mass of seedlings grown at 1 mg l^{-1} and 10 mg l^{-1} GA_3, however, the cotyledon mass of seedlings germinated on the basal MS medium without GA_3 was significantly lower than for those exposed to GA_3 (Figure 2.2). In certain cases, the cotyledon mass of seedlings cultured in GA_3 were up to three times higher than those cultured in MS only. On the other hand, GA_3 had an opposite effect on root growth to that of cotyledon growth. The root growth of germinated embryos was significantly inhibited by GA_3. Fresh root mass of germinated seedlings grown on the MS medium without GA_3 was significantly higher than that of seedlings in media supplemented with either 1 mg l^{-1} or 10 mg l^{-1} GA_3, under both light and dark conditions (Figure 2.3).

Although temperature influenced the germination percentage of the embryos, cotyledon and root growth of the germinated seedlings were not significantly affected. Despite the poor germination percentages of embryos at 25±2ºC, the fresh mass of cotyledons and roots were similar to those cultured at 21±2ºC/12±2ºC (Figure 2.2; Figure 2.3). Significant differences were only observed in root mass of the different temperature treatments when they were cultured in 1 mg l^{-1} GA_3 or 10 mg l^{-1} GA_3 media treatment. For instance, the root mass of seedlings grown in the dark, under 21±2ºC/12±2ºC in 1 mg l^{-1} GA_3 and 10 mg l^{-1} GA_3 was significantly higher than those grown under 25±2ºC in the respective media (Figure 2.3B).

Cotyledons of etiolated seedlings, which germinated on the basal MS medium, were pale in colour due to the lack of chlorophyll formation (Figure 2.5Z). Even though the

etiolated cotyledons were longer, their mass was not significantly different to that of the light-grown cotyledons. This was mainly due to the etiolated cotyledons being narrower and more slender. In addition, root growth was also similar between light-germinated seedlings and etiolated seedlings. Within the same growth medium, no significant differences were observed in the root fresh mass between seedlings germinated under different light conditions (Figure 2.3). However, in appearance, besides the lack of colour, the roots of the etiolated seedlings were shorter and thicker (Figure 2.5X-Z).

2.4.2.3 Establishment in mistbed

In vitro-formed roots of the seedlings died soon after transferal to the mistbed. However, after 14 days newly formed roots were visible (Figure 2.7A) on plantlets growing in the peat/coir/sand mixture, while none was visible on plantlets in the silica sand. After 30 days, the number of plantlets (80%) growing in the peat/coir/sand mixture was significantly higher ($P \leq 0.05$) than those transplanted to silica sand (20%). After 60 days, plantlets with new leaves and healthy roots (Figure 2.7B) were transplanted to larger bags (Figure 2.8).

2.4.3 Discussion

Temperature played the most significant role in the germination of *P. cynaroides* zygotic embryos. Results clearly showed that the germination percentage of embryos at alternating temperatures of 21±2ºC/12±2ºC was significantly higher in comparison to those grown at 25±2ºC. Thus, the use of alternating temperatures of 21±2ºC/12±2ºC is an important factor in controlling germination of *P. cynaroides* embryos. This finding may also be applicable to other *Protea* species since the low germination percentage of these excised embryos at 25±2ºC cultured in 1 mg l^{-1} GA_3 (45% germination) was similar to that reported by Van Staden, Brown and Button (1972) for other *Protea* species. In their study, where *P. compacta* excised embryos were germinated *in vitro* at 25ºC with a 12-hour photoperiod, the highest germination percentage of 43% was obtained on an MS medium containing 1 mg l^{-1} GA_3.

The results of this study show that GA_3 plays a secondary role to temperature in the germination process, since at the less favourable temperature (25±2ºC), GA_3 was able to only improve the germination percentage from 20% to 45% in the light, and from 30% to 55% in the dark, while at the temperature range of 21±2ºC/12±2ºC, up to 90% germination was achieved in the absence of GA_3 (Figure 2.1). Furthermore, the different concentrations of applied GA_3 did not influence germination and seedling growth significantly, since germination percentage, root and cotyledon mass of seedlings cultured in media with 1 mg l^{-1} and 10 mg l^{-1} were similar.

The results show that excised embryos of *P. cynaroides* will germinate if the temperature is suitable. This finding contrasts, to a certain extent, with suggestions appearing in literature that endogenous inhibitors (Brown and van Staden, 1971) or the lack of promoters (Brown and van Staden, 1975a) may be partly responsible for poor seed germination of the related species *P. compacta* and *L. daphnoides*.

The importance of alternating temperatures cannot be underestimated. According to George (1993), adjusting the temperature of the growth chamber to that of its natural habitat can be advantageous in stimulating growth of explants of a particular species – keeping in mind that in the natural environment, seeds are exposed to temperatures that fluctuate widely, particularly between day and night. Therefore, in the case of *P. cynaroides*, the importance of using alternating temperatures lies in the fact that *Protea* seeds apparently germinate best during autumn where fluctuating diurnal temperatures is prevalent (Vogts, 1982). In addition, explants have been reported to root better *in vitro* under lower temperatures, for example, *Digitalis lanata* explants rooted best at 19ºC/14ºC (day/night) (Schöner and Reinhard, 1982).

The inhibitory effect of GA_3 on root growth was clear. The stunted growth of embryo roots in the media containing either 1 mg l^{-1} GA_3 or 10 mg l^{-1} GA_3 supports reports that GA_3 diminishes or prevents the formation of roots *in vitro* (George, 1993). Furthermore, it is often reported that addition of GA_3 to the medium results in the production of elongated and narrow leaves (De Fossard and de Fossard, 1988). In the present study, the effect of GA_3 in promoting the formation of abnormally long and twisty cotyledons was very distinctive in the seedlings (Figure 2.6). The fresh mass of the cotyledons cultured in either 1 mg l^{-1} or 10 mg l^{-1} GA_3 was at least twice as high

as that of seedlings grown in the basal medium without GA_3. Similar effects were found on pumpkin cotyledons where fresh mass cultured in 1 mg l^{-1} GA_3 were more than 40% higher than the control (Kursanov, Kulaeva and Mikulovich, 1969). Although GA_3 did not inhibit the growth of the seedlings in the present study, their stunted roots led to poor anchorage of the seedlings, and due to their over-developed cotyledons, the seedlings were top-heavy and abnormal (Figure 2.6). Besides the exhibition of pale colour and typical elongated vegetative growth seen in etiolated plants, the embryos grown in the same temperature regime germinated equally well, irrespective of light or dark conditions (Figure 2.1). Etiolated seedlings subsequently placed under light eventually turned green.

Results on the establishment of *in vitro* plantlets in *ex vitro* conditions showed that roots formed *in vitro* were rendered non-functional *ex vitro*. Roots which were formed *in vitro* were delicate and died soon after being transferred. Fourteen days after transferring to *ex vitro* conditions, the growth of new roots was visible (Figure 2.7A). This however, occurred more in plantlets grown in the peat/coir/sand mixture than in silica sand, which corresponded to the significantly higher survival rate of plantlets grown in the peat/coir/sand mixture in comparison to the plantlets grown in silica sand. High water-holding capacity and good aeration are important requirements in the soil to which plantlets are transplanted (George, 1996). The combination of peat, coir and sand used in the present study provided both these requirements adequately, while the inadequate water-holding capacity of silica sand was probably the main reason for the poor growth observed on it. In the propagation of *Leucospermum* hybrid 'Hawaii Gold' (Kunisaki, 1989) and *Grevillea scapigera* (Bunn and Dixon, 1992), the micropropagated plantlets were also successfully established in *ex* vitro conditions using medium containing peat.

2.4.4 Conclusion

A protocol for the *in vitro* germination of *P. cynaroides* embryos has been developed. In addition, because high germination percentages were obtained in a relatively short period of time, it may be a viable option in a commercial environment. Furthermore, the establishment of these plantlets in *ex vitro* conditions was unproblematic,

particularly in growth medium containing a peat/coir/sand mixture, therefore, the methods described in this study can be used as an alternative propagation method to obtain disease-free seedlings. *In vitro* germination of embryos could also be used in breeding programmes to propagate seeds of newly bred hybrids, or seeds of rare cultivars that are not easily available. The optimal cultural conditions identified in this study may be applicable to other *Protea* species, which may also be difficult to germinate with conventional methods.

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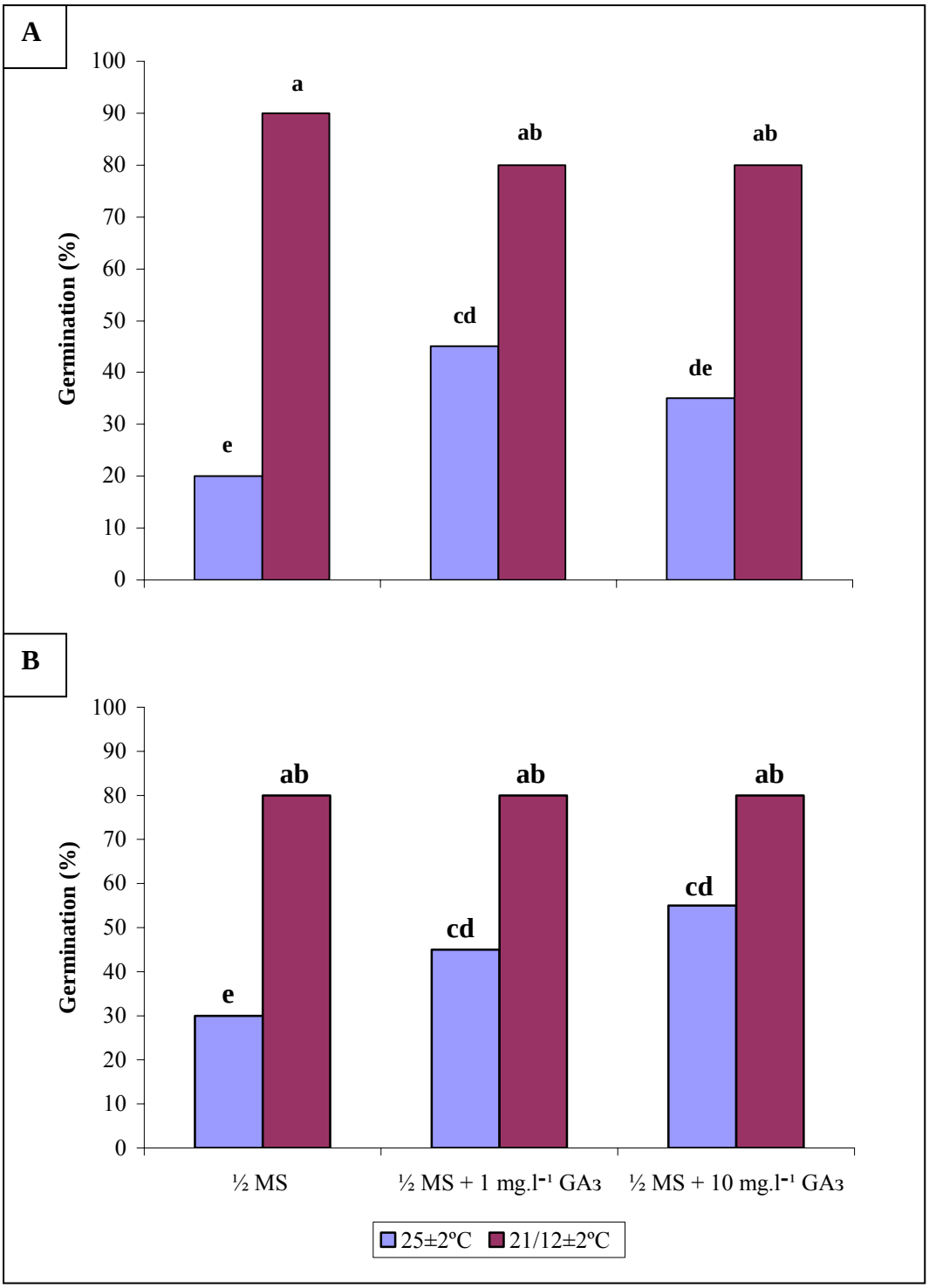

Figure 2.1. The effects of two temperature regimes and different media on the germination percentage of *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. Means with different letters differ significantly at according to Chi-square analysis ($P \leq 0.05$).

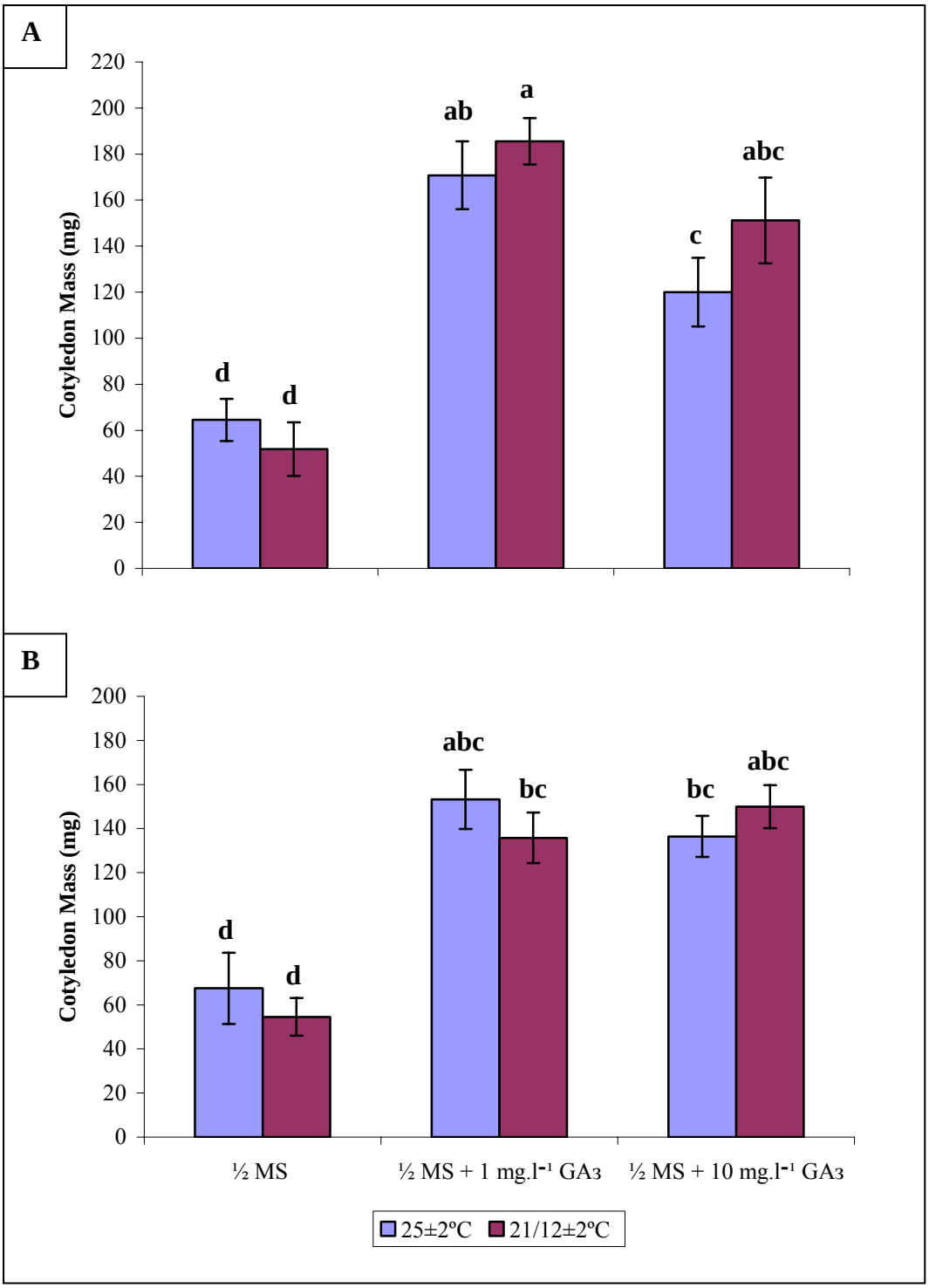

Figure 2.2. The effects of two temperature regimes and different media on the growth of cotyledons in *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. Means with different letters differ significantly according to Tukey's studentised test ($P \leq 0.05$). (LSD = 47.811)

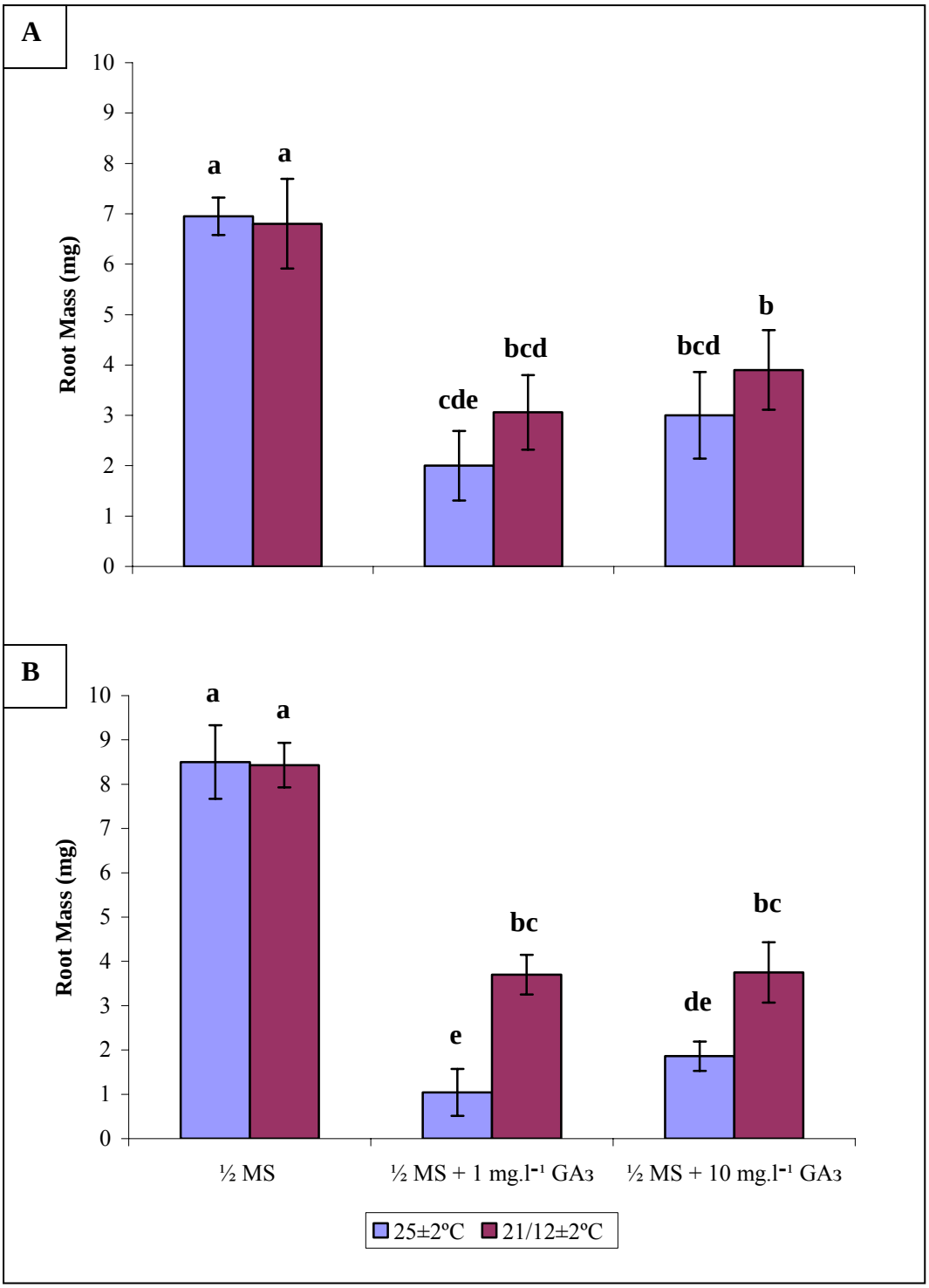

Figure 2.3. The effects of two temperature regimes and different media on root growth of *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. Means with different letters differ significantly according to Tukey's studentised test ($P \leq 0.05$). (LSD = 1.816)

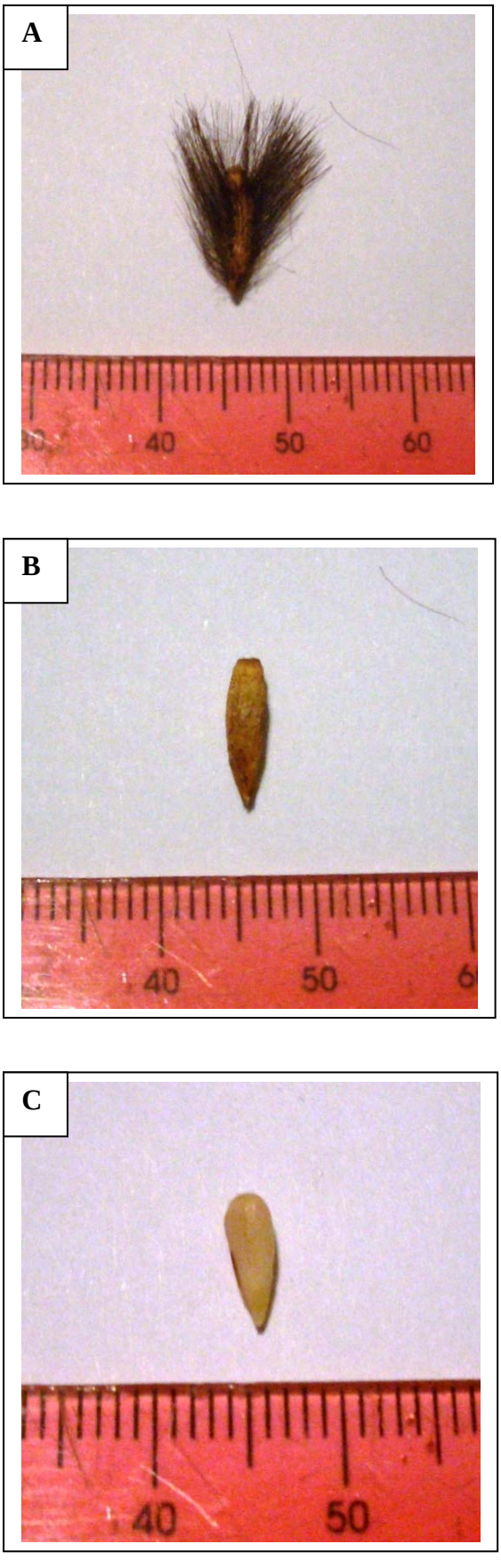

Figure 2.4. (A) *P. cynaroides* seed (=achene); **(B)** *P. cynaroides* seed with hairs removed; **(C)** Excised *P. cynaroides* embryo.

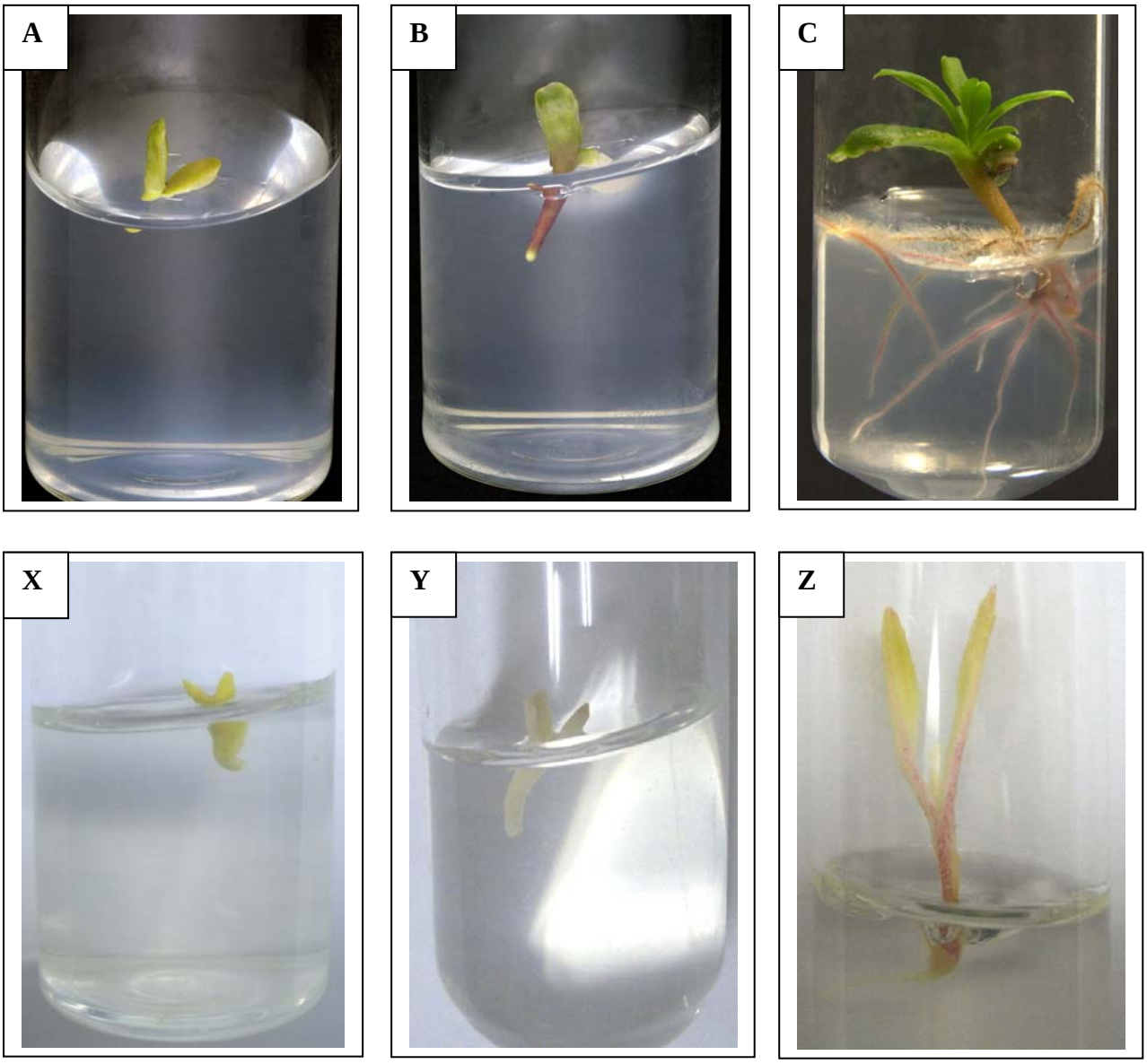

Figure 2.5. *In vitro* germination of excised *P. cynaroides* embryos cultured on MS medium grown under light (12-hour photoperiod), after **(A)** 9 days; **(B)** 14 days; **(C)** 30 days, and in total darkness, after **(X)** 9 days; **(Y)** 14 days; **(Z)** 30 days.

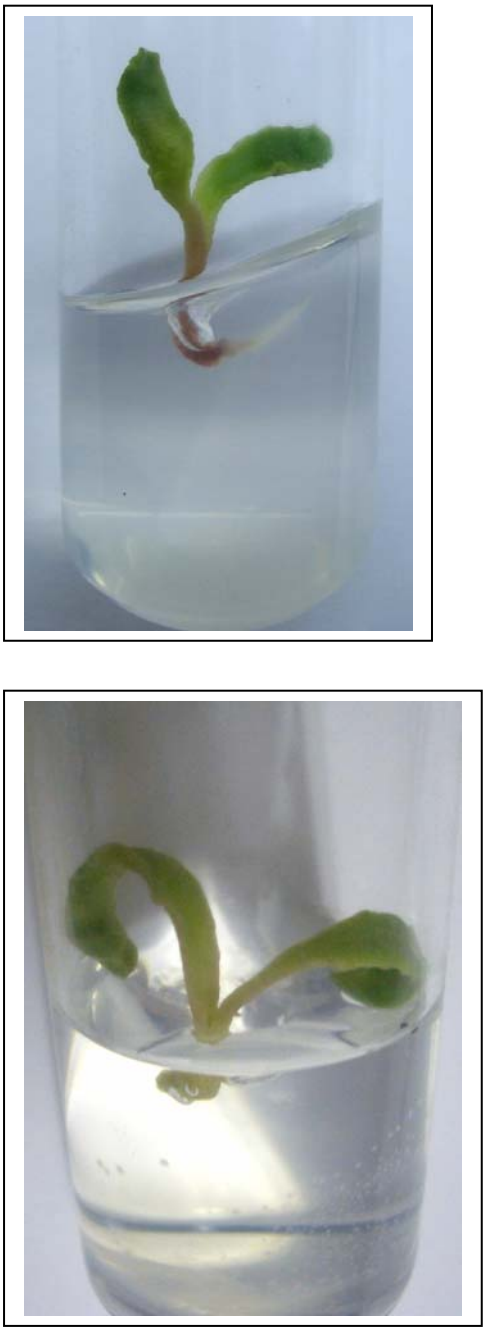

Figure 2.6. Growth of long and twining cotyledons on *P. cynaroides* seedlings germinated in media containing GA_3.

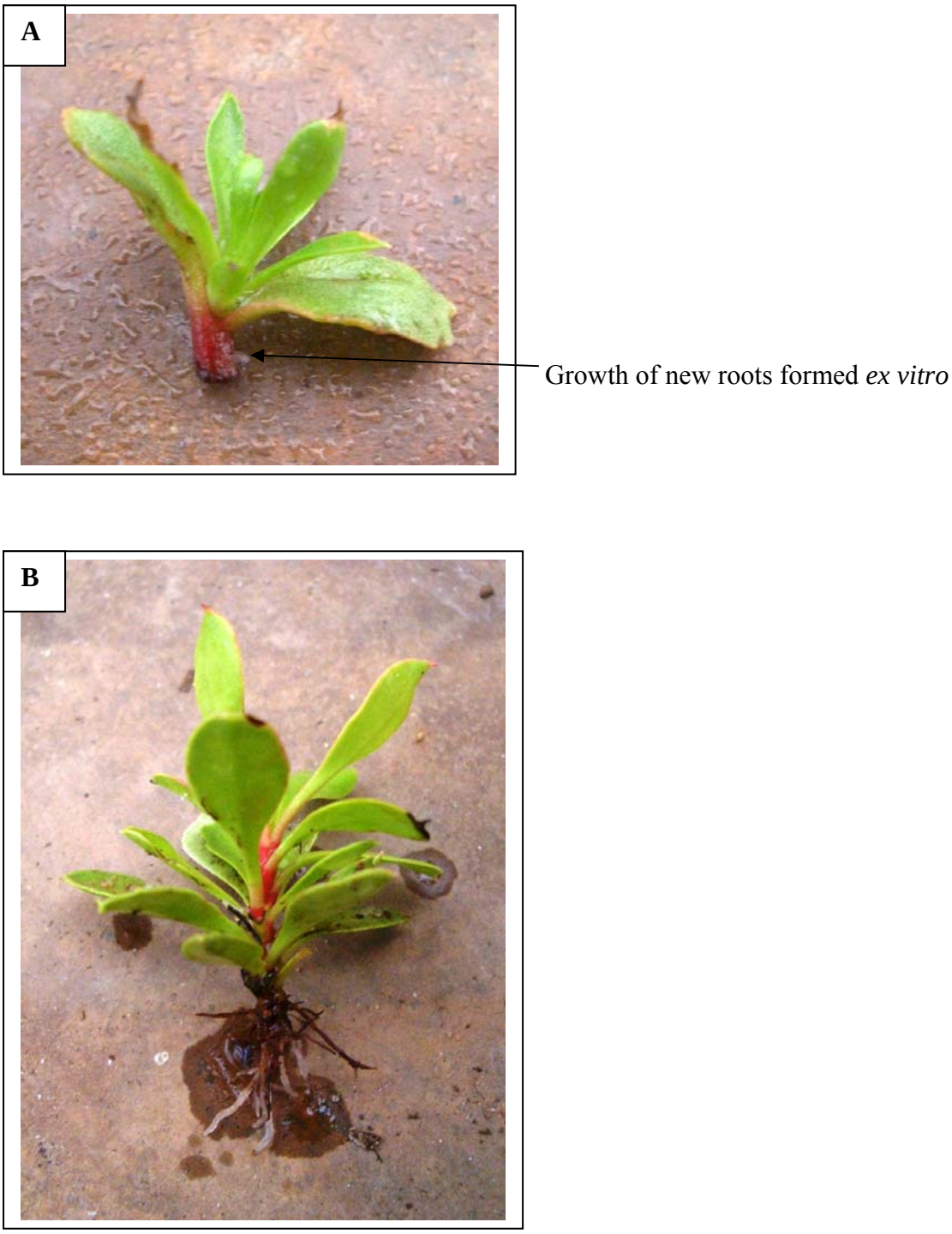

Figure 2.7. (A) Two weeks after transferring to the mistbed, *in* vitro-formed roots have died, and the emergence of new roots formed *ex vitro* is visible; **(B)** A seedling with healthy roots after 60 days in the mistbed.

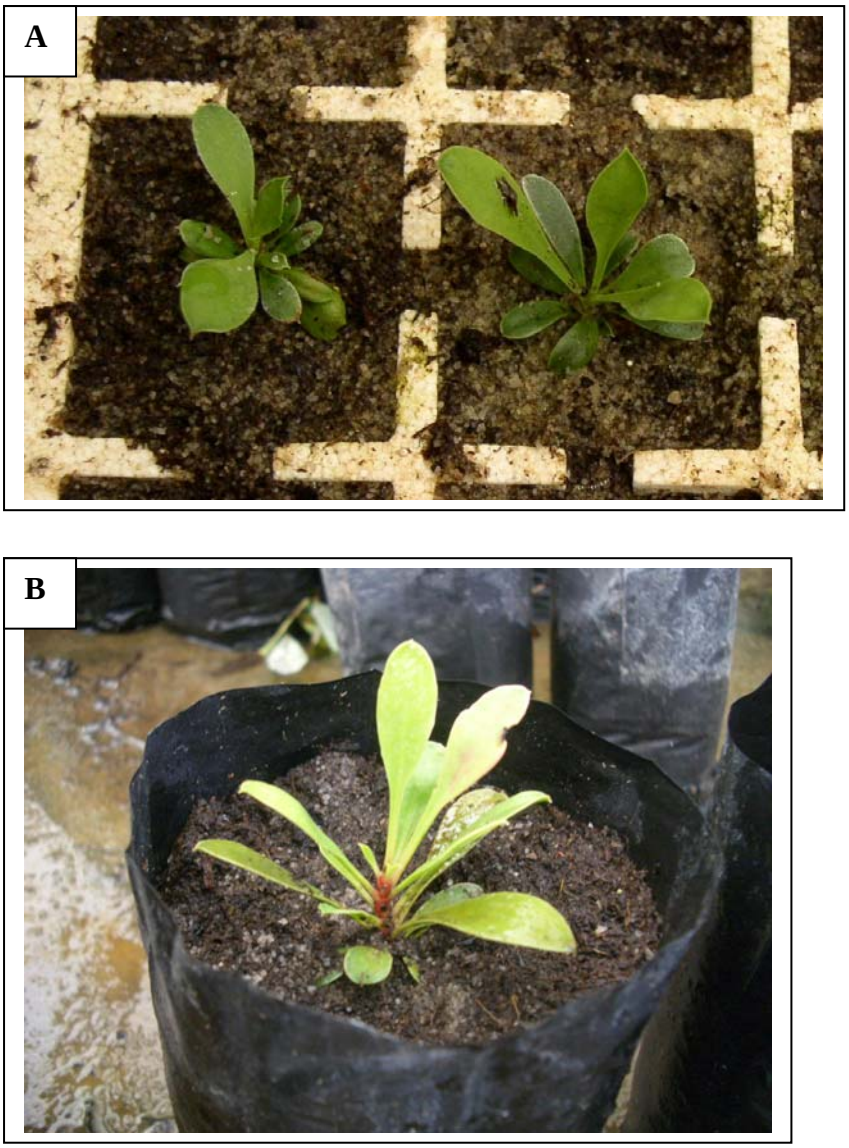

Figure 2.8. Establishment of *in vitro*-germinated *P. cynaroides* seedlings in the mistbed. **(A)** After 30 days; **(B)** Transferred to black bag after 60 days.

CHAPTER 3

MICROGRAFTING OF *PROTEA CYNAROIDES*

3.1 Abstract

The inability to induce rooting of *in vitro*-established *Protea cynaroides* microshoots has prevented the production of complete plantlets. A successful shoot-tip micrografting technique was developed using *in vitro*-germinated *P. cynaroides* seedlings as rootstocks and axenic microshoots established from pot plants as microscions. Thirty-day old seedlings, germinated on growth-regulator-free, half-strength Murashige and Skoog medium, were decapitated and a vertical incision made from the top end. The bottom ends of microshoots established on modified Murashige and Skoog medium were cut into a wedge ('V') shape, and placed into the incision. The micrografted explants were cultured in a growth chamber with the temperature adjusted to 25±2°C, with a 12-hour photoperiod. Best results were obtained by placing the microscions directly onto the rootstock without any pre-treatments. Dipping the explants in anti-oxidant solution or placing a layer of medium around the graft area led to the blackening of the microscion.

3.2 Introduction

In vitro propagation is commonly used to mass-produce disease-free plantlets that take a long time to grow under conventional vegetative propagation methods. *In vitro* propagation of *P. cynaroides* has had limited success. *In vitro* establishment (Ben-Jaacov and Jacobs, 1986; Wu and du Toit, 2004) and *in vitro* multiplication (Wu, 2001) have been reported. However, *in vitro* rooting of these microshoots has not been achieved. Conventional *in vitro* rooting methods, such as the addition of various rooting hormones into the growth media, have not been successful in inducing microshoots to form roots. Furthermore, phenolic exudates and the slow growth of microshoots further affect its ability to form roots (Thimmappaiah, Puthra and Anil, 2002).

Micrografting is mostly used for obtaining virus-free plants, separating viruses in infections, breeding specific genotypic combinations, and for studying graft incompatibility between scions and rootstocks (Burger, 1985; Navarro, 1988). Other uses that have also been investigated include: rejuvenation of adult avocados by grafting onto juvenile rootstocks (Pliego-Alfaro and Murashige, 1987) and germplasm exchange in cashew (Mantell, Boggetti, Bessa, Lemos, Abdelhadi and Mneney, 1997). In addition, microshoots that are difficult to root, or do not form roots at all *in vitro*, can be micrografted onto rootstocks to obtain rooted plantlets (Thimmappaiah *et al*., 2002). Several factors affect the success rate of the micrografts. These include the size of the scion, as well as the cultural conditions in which the scion was grown (Navarro, Roistacher and Murashige, 1975). They reported that using larger scions improved the grafting success rate. In addition, using etiolated rootstock seedlings increased the frequency of successful grafts.

Although *in vitro* micrografting has been reported in many plants such as cashew (Ramanayake and Kovoor, 1999; Mneney and Mantell, 2001, Thimmappaiah *et al*., 2002), citrus (Navarro *et al*., 1975; Navarro, 1981), pistachio (Abousalim and Mantel, 1992), prickly pear cactus (Estrada-Luna, López-Peralta and Cárdenas-Soriano, 2002) and *Prunus* spp. (Deogratias, Lutz and Dosba, 1986), micrografting of *Protea* spp. has yet to be achieved. The aim of this study was to develop a micrografting technique suitable for *P. cynaroides* through which rooted genetically uniform plantlets can be produced *in vitro.*

3.3 Materials and methods

In vitro-germinated seedlings were used as the rootstock. Half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Appendix A) was used as the growth medium. The seed preparation procedures and the growth conditions are described in Chapter 2. To obtain sterile microscions, microshoots, which sprouted from axillary nodes of *in vitro*-established nodal explants, were used. In order to induce the sprouting of the microshoot on the nodal explants, the *in vitro* establishment of *P. cynaroides* nodal explants was done according to Wu and du Toit (2004): Shoot segments were taken from 1-year old *P. cynaroides* plants grown in

black plastic bags in a greenhouse (Greenhouse temperature: 22ºC – 25ºC). After removing the leaves, the explants (± 1 cm, containing one to two nodes) were placed under running water for 2 hours. This was followed by dipping them into 70% ethanol for 10 seconds and in 0.35% sodium hypochlorite for 6 minutes. Afterwards, the explants were stirred in an antioxidant solution containing 100 mg l^{-1} ascorbic acid and 1500 mg l^{-1} citric acid for 1 hour. The explants were then placed onto the establishment medium, which contained half-strength MS medium. Sucrose (20 g l^{-1}), activated charcoal (3 g l^{-1}), ethylenediaminetetraacetate (EDTA) (50 mg l^{-1}) and Gelrite® (3 g l^{-1}) were also added. Additional growth regulators such as GA_3 (30 mg l^{-1}), BAP (2 mg l^{-1}) and myo-inositol (100 mg l^{-1}) were also added into the medium. The pH of the medium was adjusted to 5 before autoclaving. The explants were placed in a growth chamber with a 16-hour photoperiod and temperature of 25±2ºC. Cool, white fluorescent tubes provided 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR). After 30 days, the sprouted axillary bud was removed from the nodal explant, and prepared to be used as the microscion (Figure 3.1A).

Micrografting was done by grafting the microscion onto the *in vitro*-germinated seedlings. Ten 30-day old seedlings (Figure 3.1B) were decapitated, and a two millimetre vertical incision was made from the top of each rootstock. Ten microscions were prepared by cutting the microshoots (Figure 3.1A) evenly into 5 mm length, and each bottom end into a wedge (‘V’) shape (Figure 3.1C). The microscion was then quickly and firmly placed into the vertical incision of the rootstock. No wrapping was done at the graft area. The micrografted explants were cultured in a growth chamber with the temperature adjusted to 25±2°C, with a 12-hour photoperiod.

Due to the tendency of the newly-grafted explants turning black because of phenolic oxidation, three treatments were applied to the microscions, namely: 1) untreated, 2) soaking the microscions in 100 mg l^{-1} ascorbic acid and 150 mg l^{-1} citric acid for 10 minutes, and 3) placing a layer of solidified half MS medium around the graft area. Ten grafts were made in each treatment using almost uniform microscions and roostocks. Data were analyzed with the Chi-square analysis method in the SAS programme (SAS Institute Inc, 1996).

3.4 Results

Micrografting success was determined by whether the graft union formed or not, and the subsequent growth of the bud on the microscion. The ability of the graft unions to form successfully varied from 10% to 80% between the three treatments (Table 3.1). The success rate of the untreated (control) microscions was significantly higher ($P \leq 0.01$) than the other two treatments, with 80% of the graft unions forming after 2 weeks. After the formation of the graft union, terminal and axillary buds on the microscion began to swell and expand. After 8 weeks, sprouting of the buds was observed, which attained lengths of up to 5 mm with 3 leaves (Figure 3.2). Desiccation of microscions of the remaining control micrografts was the result of poor contact between microscion and rootstock.

In the two treated micrografts, wetness of microscion and the graft area was a possible cause to the failure of the graft union to form. Only one graft union of each of the other two treatments formed successfully. However, due to excessive wetness of the microscions from the pre-treatments and the medium applied around the graft area, the entire microscion soon turned brown and died.

3.5 Discussion

Jonard, Hugard and Macheix (1983), Edriss and Burger (1984) and Starrantino and Caruso (1988) increased the number of successful micrografts by pre-treating the microscion with hormonal solutions or anti-oxidants. In addition, Thimmappaiah *et al* (2002) reported that dipping the microscion in anti-browning solution (ascorbic acid and citric acid) reduced phenolic browning and drying of the cashew microscions. Pliego-Alfaro and Murashige (1987) applied a layer of moist nutrient agar at the graft area to prevent drying of the microscion, which improved the formation of the graft unions in avocados. In contrast, Ponsonby and Mantell (1993) reported that by applying antioxidants or medium solution to the graft area significantly reduced graft union successes. They proposed that this may have been due to excessive moisture created by the applied solutions, which caused the dilution of substances responsible for the establishment of a functional graft union.

In *P. cynaroides* micrografts, soaking the microscions in pre-treatment solutions, or applying a layer of medium to the graft area only aggravated the tissue blackening. Instead, best results were obtained when clean, dry, untreated microscions were used. This is in agreement with Navarro (1988) who reported that fast operation during micrografting was more effective in avoiding phenolic oxidation than using antioxidants. The wetness caused by the antioxidant and nutrient-agar treatments promoted the spread of phenolic oxidation from the graft area to the rest of the explant. A possible reason for the lack of inhibition of phenolic oxidation by the antioxidant solution may be the use of insufficient concentrations of ascorbic and citric acids. It is likely that the amount of phenolic compounds found in *P. cynaroides* is considerably higher than those found in cashew microscions, since the concentrations of ascorbic acid (100 mg l^{-1}) and citric acid (150 mg l^{-1}) used in this study was identical to that used by Thimmappaiah *et al* (2002) in micrografting cashew plantlets, with the soaking period twice as long.

Furthermore, the results of this study showed that the application of a layer of moist nutrient agar around the graft area to prevent dehydration of the microscion and rootstock was not necessary, since the rate at which the graft union formed was quick enough to heal the wounds and prevent moisture loss. This is despite the finding that a moisture film at the graft area was beneficial in conventional grafting (Hartmann, Kester, Davies and Geneve, 1997).

The firm placement of the microscion onto the rootstock to ensure good contact was essential for the formation of the graft union. Only one dislocation of the microscion occurred in the control treatment, while the majority of the microscions were able to establish vascular connection with the rootstock as indicated by wound callus formation in the 2nd week. It has been reported that the formation of callus is a good indication of grafting success, since the callus provides the initial pathway for water until vascular connections are formed between the rootstock and scion (Hartmann, Kester, Davies and Geneve, 1997). Dislocation of the micrograft resulted in drying-out of the microscion, in which no callus formation was observed. Dislocation of the microscion has also been reported as a cause of micrografting failures in cashew (Ramanayake and Kovoor, 1999).

The micrografting success rate of the untreated *P. cynaroides* microscions (80%) achieved in this study is relatively high, in comparison to 30 – 50% success rates in micrografting of *Citrus* explants (Navarro *et al*., 1975), and success rates of up to 85% were obtained when *Citrus* microscions were pre-treated in kinetin solution (Edriss and Burger, 1984). The growth-regulator-free half-strength MS medium was ideal for the growth of the micrografts. New roots formed simultaneously with the opening and growth of the bud after 8 weeks (Figure 3.1D; Figure 3.2).

3.6 Conclusion

Micrografting of *P. cynaroides* was successful by using dry microscions and rootstocks. In addition, ensuring good contact between the microscion and the rootstock was essential for the graft unions to form successfully. The micrografting method reported in this paper can be used to obtain well-rooted, disease-free microshoots within 60 days. Furthermore, this technique can be adopted in breeding of specific genotypes or other species that are difficult to root. For future studies, allowing more time for the treated microscions to dry will further test the effect of wetness as a cause for micrografting failure. In addition, the use of higher antioxidant concentrations is needed to determine the optimal concentrations for the prevention of phenolic oxidation.

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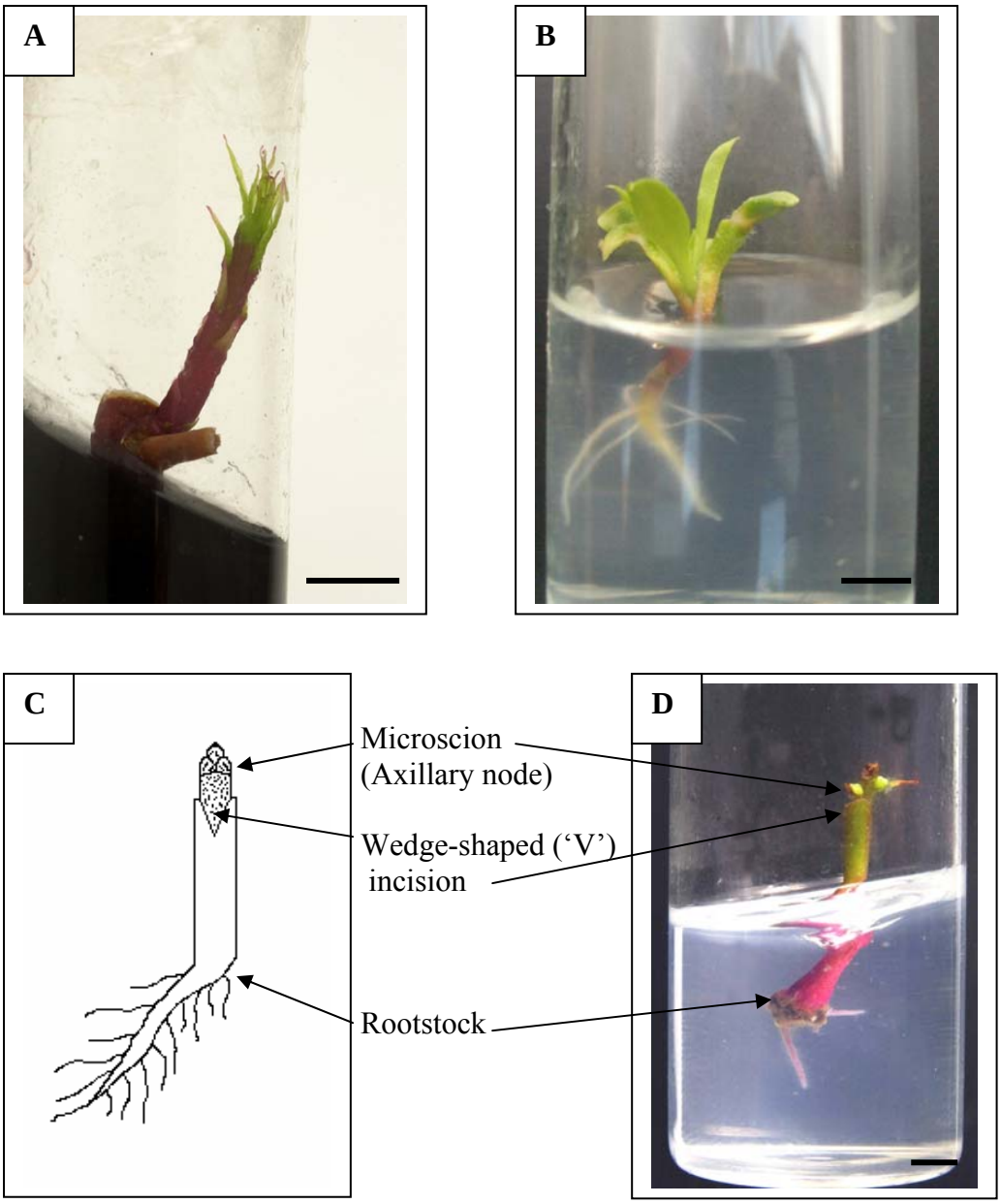

Figure 3.1. (A) Thirty-day old sprouted axillary bud on established nodal explant (Wu, 2001) was used as microscion; **(B)** Roots of 30-day old *in vitro*-germinated *P. cynaroides* seedling were used as rootstock; **(C)** A diagrammatic representation of the micrografting method used in this study;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

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

Treatment	Grafting success (%)
Control	80 a
Antioxidant Pre-treatment[1]	10 b
Medium application at graft area[2]	10 b

[1] Ascorbic acid (100 mg l^{-1}) and citric acid (150 mg l^{-1})
[2] Half-strength MS medium

Percentages followed by different letters are significant at $P \leq 0.01$ according to Chi-square.

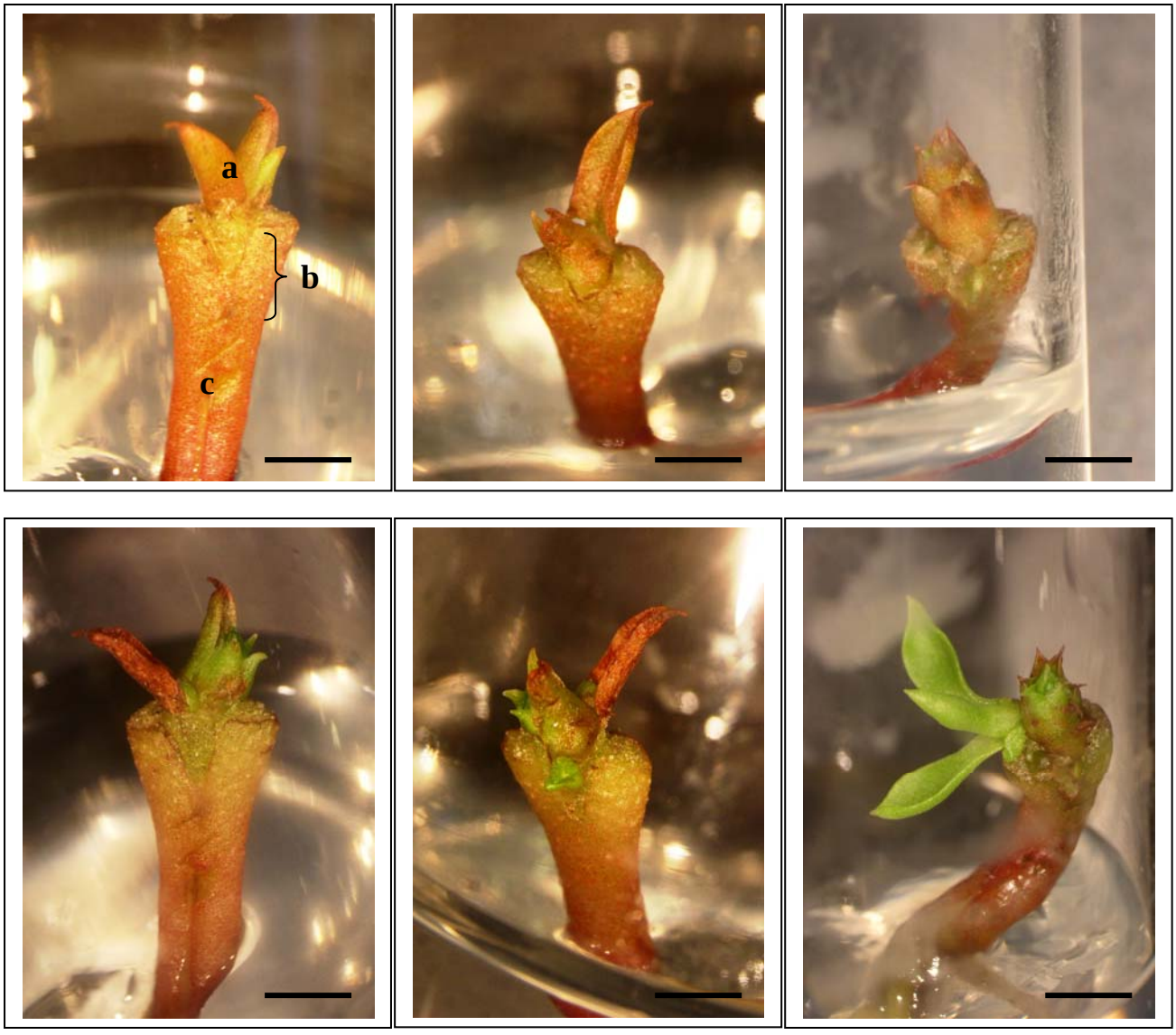

Figure 3.2. Top row: Formation of the graft union after 2 weeks in culture. **Bottom row:** Sprouting of terminal and axillary buds on microscion after 8 weeks. **a** = microscion; **b** = graft union of wedge-shaped ('V') incision; **c** = rootstock. Bar ≈ 5 mm.

CHAPTER 4

PLANTLET REGENERATION OF *PROTEA CYNAROIDES* THROUGH DIRECT SOMATIC EMBRYOGENESIS AND MULTIPLE SHOOT DEVELOPMENT

4.1 Abstract

Little research has been done on the *in vitro* propagation of *Protea cynaroides*. Somatic embryogenesis is often used to propagate a number of plant species and can potentially be used to rapidly propagate *P. cynaroides*. From the results of this study, somatic embryos formed directly on both *P. cynaroides* mature zygotic embryos and excised cotyledons cultured on MS medium without growth regulators. The addition of growth regulators such as NAA (1; 2.5 and 5 mg l^{-1}) and 2,4-D (1; 2.5 and 5 mg l^{-1}), in combination with TDZ (0.2 mg l^{-1}), BAP (0.2 mg l^{-1}) or kinetin (0.2 mg l^{-1}) suppressed the formation of somatic embryos. After eight weeks in culture, formation of somatic embryos was observed. Zygotic explants formed the most embryos when cultured in a 12-hour photoperiod in comparison to explants cultured in the dark. Up to 83% of these embryos germinated after transferal to the germination medium containing 0.1 mg l^{-1} GA_3. Significantly fewer embryos germinated in MS medium with no growth regulators, or supplemented with higher concentrations of GA_3, while low germination percentages were also observed in MS media containing casein hydrolysate and coconut water. The germination of normal embryos was observed only in medium containing either no growth regulators, 0.1 mg l^{-1} GA_3 or 0.5 mg l^{-1} GA_3. All embryos that germinated in high concentrations of GA_3 were malformed.

4.2 Introduction

Plant regeneration *via* somatic embryogenesis has the potential to produce a large number of plantlets in a relatively short period of time. Although numerous plant species are reportedly capable of forming somatic embryos, very few reports of somatic embryogenesis have been documented for members of the Proteaceae family. In the *Protea* genus only *Protea repens* have been reported to form somatic embryos

(Rugge, 1995). Van Staden, Choveaux, Gilliland, McDonald and Davey (1981) were able to induce callus and proteoid rootlet formation in *Protea neriifolia*, but attempts to initiate shoot and root development were unsuccessful. In another commercially-important Proteaceae species, *Serruria florida*, explants were induced to form somatic embryos (Rugge, van der Merwe, Jacobs and Theron, 1989).

The slow growths of established explants, as well as their inability to form roots, are stumbling blocks encountered during micropropagation of *P. cynaroides* (Wu and du Toit, 2004). This was shown by Ben-Jaacov and Jacobs (1986) and Wu and du Toit (2004), where *P. cynaroides* explants were established successfully *in vitro* by inducing axillary buds to sprout. However, *in vitro* rooting of those explants was unsuccessful. In addition, phenolic oxidation of the explants has been reported to be a problem, which resulted in their death. Thus, somatic embryogenesis can potentially be used as an alternative *in vitro* propagation method to produce *P. cynaroides* plantlets.

Histological studies have been used to investigate cell, tissue and organ development in somatic embryos (Stamp, 1987; Samaj, Bobak and Erdelsky, 1990; Puigderrajols, Celestino, Suils, Toribio and Molinas, 2000). Histological studies are also conducted to investigate the presence of starch grains in plant cells, which are revealed by various types of stains applied to the plant tissue. It is often reported that starch serves as an energy source for cell division and tissue formation in somatic embryos (Samaj *et al*., 1990). Thus, the identification of starch grains in a certain region of an embryo is indicative of active tissue development.

The main objective of this study was to investigate and determine the ideal explant, optimum growth conditions and suitable culture medium for the induction, development and germination of somatic embryos. This in turn would allow the development of a protocol for somatic embryogenesis of *P. cynaroides* from which plantlets could be produced rapidly.

4.3 Materials and methods

4.3.1 Plant materials

Excised, mature zygotic embryos, cotyledons from *in vitro*-germinated seedlings and immature unfertilized ovules were used as source material for the induction of somatic embryos. The methods of excision and sterilization of the mature zygotic embryos are described in Chapter 2. The cotyledons were obtained from newly *in vitro*-germinated seedlings and cut into similar sizes (5 mm x 5mm). All the cotyledons were placed on their adaxial side on the growth medium. The immature unfertilized ovules were collected from 30-day old inflorescences of 5-year old established *P. cynaroides* motherplants grown in a field situated near Cullinan (25º40'32S; 28º31'20E; Altitude 1482 metres) in the Highveld region (summer rainfall) of South Africa. After the ovary was carefully opened with a sterile needle, the immature unfertilized (haploid) ovule was removed and placed onto the culture medium (Figure 4.8A).

4.3.2 Culture media and growth conditions

For the induction of somatic embryos, forty explants were used in each medium treatment. Full-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Appendix A) and 3% (w/v) sucrose and Gelrite® (3 g l^{-1}) was used as the basal medium. The pH of all the media was adjusted to 5.7 before autoclaving. 2,4-Dichlorophenoxyacetic acid (2,4-D) (1, 2.5 and 5 mg l^{-1}) and 1-naphthalene acetic acid (NAA) (1, 2.5 and 5 mg l^{-1}) were either added singly or in combination with the following: thidiazuron (TDZ) (0.2 mg l^{-1}), 6-benzylaminopurine (BAP) (0.2 mg l^{-1}), or kinetin (0.2 mg l^{-1}). Other media supplements included coconut milk (5% and 10%), and casein hydrolysate (100 mg l^{-1} and 200 mg l^{-1}), which were added singly into the basal medium containing no growth regulators. In addition, full-strength basal MS medium with no growth regulators was also used as induction medium.

In the germination study, thirty embryos in the heart-shaped stage, which formed in the induction stage, were carefully removed from the source explants and transferred onto sterilized filter paper (Whatman No. 1) lining 9-mm Petri dishes containing

germination medium. Half-strength MS medium was used with the following growth regulators added singly: GA_3 (0.1, 0.5, 1, 2.5, 5, 10, 20 mg l^{-1}), casein hydrolysate (100, 200 mg l^{-1}) and coconut water (50, 100 mg l^{-1}). Half-strength basal MS medium with no growth regulators was also used as a germination medium.

For the induction of somatic embryos, the explants were either cultured under a 12-hour photoperiod or in total darkness. Illumination was supplied by cool, white fluorescence tubes providing 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR). For germination, somatic embryos were grown in the dark only. The temperature was adjusted to 25±2ºC for the induction stage, while 21±2/12±2ºC was used in the germination stage since this alternating temperature regime has shown to improve germination of zygotic embryos in the previous study (See Chapter 2).

4.3.3 Histology

Anatomical investigations were performed on the somatic embryos to observe the presence of starch grains and study the histodifferentiation of somatic embryos through the various stages. The methods used in the preparation of microscope slides were adapted from O'Brien and McCully (1981). Somatic embryos in the globular, heart and cotyledonary stages were first fixed in formalin: acetic acid: 50% ethanol (1: 1: 18), and then dehydrated in an ethanol: distilled-water series (30:70; 50:50; 70:30; 100:0). Finally, the sample was embedded in paraffin wax (melting point 60ºC). Samples were cut in a semi-thin rotary microtome (Reichert-Jung 2040®) at a thickness of 7 µm. Sections were attached to glass slides and stained with safranin and fast-green, which are used to reveal the presence of starch grains (Peacock, 1935; Plata and Vieitez, 1990). A Wild Leitz GMBH® (Model 020-057.010) light microscope was used to observe the slides at 50x and 500x magnification.

4.3.4 Statistical analysis

In the somatic embryo induction, the percentage of explants that formed embryos, and the number of embryos formed per explant was recorded. For the germination stage, the total number of embryos that germinated per treatment, as well as the number of normal-germinated embryos were recorded. A completely randomized design was

applied Where appropriate, Chi-Square analysis and Tukey's Studentised range test were used to compare treatment means. All statistical analyses were done in the SAS program (SAS Institute Inc, 1996). ANOVA is shown in Table C3, Appendix C.

4.4 Results

4.4.1 Induction of somatic embryos on zygotic embryos

4.4.1.1 MS medium without growth regulators

Six weeks after culturing the explants on the induction medium, the first somatic embryos began to appear on the zygotic embryos cultured on full-strength basal MS medium with no growth regulators (Figure 4.1). However, the majority of somatic embryos were formed after eight weeks in culture. All of these somatic embryos differentiated directly, without an intermediate callus phase. Somatic embryos formed on edges (Figure 4.1A), the abaxial side (Figure 4.1B) and the adaxial side (Figure 4.1C) of the zygotic embryo. Somatic embryos formed on the basal MS medium under both light (12-hour photoperiod) and dark conditions, although under light conditions, significantly more ($P \leq 0.001$) explants formed embryos (70%), compared to 37.5% of explants forming embryos in the dark (Table 4.1). Moreover, significantly more embryos were formed per explant under the light treatment (8.7 embryos) than in the dark (5.5 embryos) (Table 4.1). The somatic embryos that formed on these basal MS media were able to develop from the globular through to the heart stages, with some embryos developing further into the torpedo-shape stage (Figure 4.1D). In addition, a low number of embryos germinated when kept on MS medium with no growth regulators.

4.4.1.2 MS medium supplemented with growth regulators and other additives

After 8 weeks, somatic embryos also appeared on zygotic embryos cultured in the MS media containing coconut water or casein hydrolysate. However, the number of zygotic explants that formed embryos was less than 10%, and these results were considered insignificant. In addition, all the media containing various concentrations

of 2,4-D or NAA, singly or in combination with TDZ, BAP or kinetin, cultured under light or dark treatments did not induce formation of any somatic embryos, nor were there signs of embryogenic callus formation. One exception to this trend was found on MS medium containing 1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP in the dark, where explants showed some form of callus growth, however, no somatic embryos were produced. In general, zygotic embryos cultured on MS medium containing growth regulators or other additives formed fluffy, watery-looking callus (Figure 4.3A). Attempts to induce embryos on these calluses were unsuccessful. Eventually, these calluses turned brown and died.

4.4.2 Induction of somatic embryos on cotyledon explants

4.4.2.1 MS medium without growth regulators

After 8 weeks in culture, somatic embryos also formed directly on cotyledonary explants grown on full-strength MS medium lacking growth regulators, under both light and dark conditions (Table 4.1; Figure 4.2). These embryos developed through the globular to the heart and torpedo stages (Figure 4.2C; Figure 4.2D). The percentage of cotyledonary explants that produced embryos was significantly less ($P \leq 0.001$) than those produced by the zygotic embryo explants cultured on the same medium treatment and light conditions (Table 4.1). Twenty-five percent and 22.5% of cotyledons formed embryos in light and dark conditions, respectively. In addition, the number of embryos formed per explant was significantly less than the amount formed on zygotic explants (Table 4.1).

4.4.2.2 MS medium supplemented with growth regulators and other additives

Similar to the zygotic explants, the direct formation of somatic embryos was suppressed by the addition of growth regulators to the culture medium. Only a few watery calluses formed scantily, and no embryos were induced on the excised cotyledons cultured on any MS medium supplemented with growth regulators. Figure 4.3B illustrates the effect of growth regulators such as 1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP on the callusing of cotyledonary explants. These explants were covered in watery callus, and showed no ability to produce somatic embryos. Furthermore,

unlike the zygotic cultures, in light, no embryos were formed in the medium containing coconut milk or casein hydrolysate, while less than 10% of these explants formed embryos in the dark.

4.4.3 Germination of somatic embryos

Histodifferentiation of somatic embryos was rapid from the heart stage to the cotyledonary stage (Figure 4.4). Germination of somatic embryos was observed in all the germination media containing different concentrations of the various growth regulators. However, the germination percentage varied widely from 10% to 83% (Table 4.2). Although germination of the embryos was achieved in the medium lacking growth regulators, germination was favoured in medium supplemented with GA_3. After four weeks in culture, highest germination percentage was observed in the medium containing 0.1 mg l^{-1} GA_3 (83.3%), which was significantly higher ($P \leq 0.001$) than the medium with 0.5 mg l^{-1} GA_3 (60%) (Table 4.2). Significantly fewer embryos germinated in media supplemented with higher concentrations (1 - 20 mg l^{-1}) of GA_3, casein hydrolysate or coconut water. Furthermore, 70% of the embryos that germinated in 0.1 mg l^{-1} GA_3 were normal, i.e. they consisted of two separate cotyledons, and a single radicle (Figure 4.5). Somatic embryos which germinated in the medium containing either no growth regulators or 0.5 mg l^{-1} GA_3 yielded only 55.6% and 50% normal germination, respectively, while in all the other media treatments, the majority of embryos that germinated were malformed. The malformed embryos either had single, multiple or fused cotyledons, or fused embryos (Figure 4.6).

Somatic embryos at globular, heart and cotyledonary stages are illustrated in Figure 4.7. Undifferentiated epidermal cells occur around the entire globular embryo (Figure 4.7A), while the development of the radicle and shoot poles is revealed by the presence of smaller differentiated cells at each polar end (Figure 4.7A). In the heart-shaped embryos, accumulation of starch grains occurs in the vicinity of the developing cotyledons, indicating that cell division and tissue growth is taking place (Figure 4.7B). In the cotyledonary-stage embryo, the vascular tissues are visible along the centre of the two cotyledons, which connect up toward the upper centre of the embryo (Figure 4.7C). Starch deposits are also abundant in cells in the immediate

vicinity of the vascular tissues, particularly on the left side of the somatic embryo (Figure 4.7C). Fewer cells containing starch were found in the lower part of the embryo, especially towards the radicle pole (Figure 4.7C). Figure 4.7D illustrates a close-up of starch granules that are stored in slightly elongated parenchyma cells in a region close to the vascular tissue where cell activity are taking place.

4.4.4 Immature unfertilized ovules

Use of full-strength MS medium in all media treatments led to the browning and death of most immature unfertilized ovules. Half-strength MS medium was then used in place of full-strength MS medium, while growth regulator concentrations remained the same. Results showed that continuous darkness is required for immature unfertilized ovules to grow. The majority of explants cultured in the dark showed significant growth, while a low percentage of ovules cultured in light responded positively (Table 4.3).

Ovule explants cultured in the dark expanded rapidly, and tissue growth was observed after 5 days (Figure 4.8B). This rapid growth of the ovules continued after 4 weeks in culture (Figure 4.8C), and was similar in appearance at all medium treatments supplemented with various concentrations of auxins and cytokinins. However, the rapid growth of ovules decreased considerably after 8 weeks in culture, which is shown by their similarity in size to when they were 4-weeks old (Figure 4.8D). No differences in the appearance of the ovules could be observed between the various medium treatments after 8 weeks. Significant differences were, however, observed in the amount of ovules that showed tissue growth and expansion (Table 4.3). A high percentage of ovules (100%) cultured on MS medium without growth regulators showed tissue expansion and growth, while significantly fewer ovules showed growth on media supplemented with low concentrations (1 or 2.5 mg l^{-1}) of 2,4-D or NAA in combination with 0.2 mg l^{-1} TDZ (Table 4.3). Ovule growth was generally low in media containing BAP, which evidently resulted in a high percentage of ovule death. All ovules died in media containing kinetin. Subsequent transfers of healthy ovules to fresh media did not alter their growth patterns, and neither proembryonic callus nor somatic embryos were induced to form on ovules in any of the medium treatments.

4.4.5 Multiple shoot-bud development

Ten percent of zygotic embryos cultured under light (12-hour photoperiod) on MS medium lacking growth regulators germinated into seedlings (Figure 4.9). Multiple shoot-bud development was observed on these germinated seedlings, where approximately 8 shoot-buds were produced per seedling. These multiple-bud shoot developments were, however, not observed on any explants grown in the dark. Shoot-derived plantlets were obtained after removing each shoot-bud from the seedling and rooting them by placing them flat on MS medium in a Petri dish placed upright. Rooting was observed after 4 weeks on MS medium without growth regulators (Figure 4.10A, Figure 4.10B), while callusing of the explants occurred in media containing 1 mg l^{-1} NAA (Figure 4.10C).

4.5 Discussion

Results of this study showed the high competency of *P. cynaroides* zygotic explants to consistently form somatic embryos directly in medium lacking growth regulators, without an intervening callus phase. In addition, these results clearly showed that the addition of exogenous growth regulators, such as NAA, 2,4-D, TDZ, BAP and kinetin inhibited the induction of somatic embryos, while other additives such as casein hydrolysate and coconut water suppressed embryo induction. Inhibition or suppression of embryo induction by growth regulators is rare, since most research papers report the use of some sort of growth regulators (mostly auxins) for the induction of somatic embryos. These results strongly suggest that the source material (zygotic embryos and cotyledons) may be auxin-habituated and are predetermined to somatic embryogenesis (George, 1993).

Results from the direct formation of somatic embryos on zygotic embryos and cotyledons of *P. cynaroides* are in accordance with Sharp, Sondahl, Caldas and Maraffa (1980), where no auxin was required for somatic embryogenesis of the pre-embryogenic determined cells (PEDC). The PEDC were probably determined during a prior mitotic event *in situ*, before their transfer to a cell culture environment. Furthermore, in such cases, the process of embryogenesis is autonomous, and

therefore, once embryo initiation has started, these cells are then able to fulfill their commitment to the embryogenic pattern of development, even in the absence of exogenous growth regulators. These embryos can continue to develop and germinate on the same medium without transfer to fresh medium (Sharp *et al*., 1980).

As mentioned earlier, research papers showing direct somatic embryo formation on growth medium lacking growth regulators are limited. Nevertheless, Gingas and Lineberger (1989) reported that in *Quercus rubra*, the highest percentage of somatic embryos was obtained from immature zygotic embryo tissues cultured on media lacking growth regulators. Similarly, Plata and Viéitez (1990) obtained somatic embryos on MS medium with no growth regulators from cotyledon sections and embryonic axis of *Camellia reticulata*, which completed their development on the same medium.

Although significantly fewer somatic embryos were formed in the dark on explants cultured on MS medium lacking growth regulators (Table 4.1), the ability of the explants to form embryos in both light and dark is noteworthy. This is similar to reports in *Q. rubra*, where embryos formed in both light and dark conditions, however, the highest number of somatic embryos was formed in light (Gingas and Lineberger, 1989). In contrast, high irradiance inhibited embryogenesis in *Glycine max* (Lazzeri, Hildebrand and Collins, 1987). Thus, it is possible that a promotion in the induction of somatic embryos could be affected by a different photoperiod or light intensity. Further research is needed to determine the relationship between different levels of light and the induction of somatic embryos in *P. cynaroides*.

The accumulation of starch grains in parenchyma cells occurred in the vicinity of the developing cotyledons (Figure 4.7). Starch, which is synthesized in the plastids (Esau, 1960), is produced from sucrose supplied in the culture medium (Thorpe, Joy and Leung, 1986). The accumulation of starch grains is known to be a prerequisite for energy-demanding morphogenesis processes (Thorpe and Meier, 1974). Furthermore, starch also acts as a direct cellular reserve of the energy required for morphogenesis, since it disappears rapidly as meristemoids are formed. Therefore the location of the starch grains found in the somatic embryo is an indication that cell differentiation is

taking place, which is seen in the development of cotyledons from the heart stage to the cotyledonary stages (Figure 4.7B; Figure 4.7C).

Gibberellic acid is often used to stimulate the germination of somatic embryos in the post-initiation medium (George, 1993). In this study, the lowest concentration (0.1 mg l^{-1}) of GA_3 promoted the germination of somatic embryos, and as the concentration increased, the germination percentage decreased. The GA_3 concentration that promoted germination in this experiment is similar to that used in other studies. For instance, mostly in the range of 0.3 - 1 mg l^{-1} GA_3, germination of somatic embryos were successful in *Vitis* sp. (Mullins and Srinivasan, 1976), *Citrus* sp. (Kochba, Button, Spiegel-Roy, Bornman and Kochba, 1974) and *Panicum maximum* (Lu and Vasil, 1982). However, there are also numerous papers reporting the germination of somatic embryos in media with no growth regulators, such as in *Olea europaea* (Rugini, 1988), *Dendranthema grandiflora*. (May and Trigiano, 1991), and *Phragmites australis* (Lauzer, Dallaire and Vincent, 2000).

Incidences of malformed embryos were observed in all the medium treatments at the germination stage. The occurrence of malformed embryos seemed to be dependent on the concentration of growth regulators, particularly GA_3, since the lowest concentration of GA_3 (0.1 mg l^{-1}) produced the highest percentage of normal-growing embryos, while as the GA_3 concentration increased, the percentage of embryo malformation in *P. cynaroides* also increased. The malformed embryos were nevertheless able to develop into relatively normal plantlets since most of them possessed deformed cotyledons, while their radicles were intact, which allowed nutrient uptake and further development. A possible remedy to reduce the occurrence of malformed somatic embryos is to incorporate abscisic acid (ABA) into the medium. Manipulation of ABA concentrations has been shown to increase the frequency of embryos to reach maturity (Ammirato, 1988). Its presence in the growth media has been reported to be essential for the normal growth of somatic embryos, and credited with the elimination of abnormal forms of embryos (Ammirato, 1973; Ammirato, 1974). In the current study, we did not find it necessary to approach this method, since the correct concentration of GA_3 was identified to produce healthy, normal plantlets.

In this study, the unsuccessful induction of somatic embryos from unfertilized ovules showed the difference in the requirement of growth media and conditions between zygotes and ovules of *P. cynaroides*. Due to the requirement of complex nutrient media and the sensitivity of ovules to physical conditions, the induction of somatic embryos from unfertilized ovules has often failed (Campion and Alloni, 1990). Among woody plants, somatic embryogenesis has been one of the most thoroughly studied aspects in *Citrus* species (Litz, Moore and Srinivasan, 1985). However, attempts to induce somatic embryogenesis in unfertilized ovules in monoembryonic genotypes have failed (Button and Kochba, 1977; Litz *et al*., 1985). Furthermore, the use of haploid explants to obtain somatic embryos in other plant species has also been relatively unsuccessful. Even in cases where successful induction of somatic embryos from haploid ovules has been reported, the success rates were very low. For instance, for *Allium cepa* (Campion and Alloni, 1990), *Beta vulgaris* (Hosemans and Bossoutrot, 1983) and *Gerbera jamesonii* (Meynet and Sibi, 1984), the yield of somatic embryo induction was only 0.28%, 2.1% and 7%, respectively. These low success rates show that the induction of somatic embryos is very difficult when haploid explants are used. Nevertheless, from the high regenerative capacity shown by the zygotic explants of *P. cynaroides* in this study, it is likely that the regeneration of haploid plantlets from unfertilized ovules can be achieved. Further studies are required to determine their nutrient and environmental requirements such as light and temperature conditions, which are probably the most important factors in culturing these delicate explants.

In the present study, the majority of zygotic embryos formed somatic embryos, while a few germinated into seedlings. However, from those zygotic embryos that did germinate, multiple shoot-bud developments were observed at the cotyledonary node region. This indicates the existence of totipotent cells at this particular region. Although these multiple shoot-buds developed in MS medium without growth regulators, it is often reported that cytokinins such as BAP added singly into the growth medium is required to induce multiple shoot development. Multiple shoot development from embryos has been reported in soybeans (Cheng, Saka and Voqui-Dinh, 1980) and almonds (Hisajima, 1982). Further research is needed to establish the effects of cytokinins on the development of multiple shoot-buds in *P. cynaroides*.

4.6 Conclusion

This study has shown that zygotic explants possess a high competency to form somatic embryos, which can be germinated in medium containing low concentrations of GA_3 in the dark. The establishment of a protocol for direct somatic embryogenesis of *P. cynaroides* improves the prospects of producing *Protea* spp. quickly and efficiently. This is particularly important in a floriculture industry such as proteas where lengthy propagation time and inconsistent rooting is common, partly because seed and vegetative propagation, which have their limitations, are still the most commonly used methods for propagation. The relatively short period of time required to obtain germinated somatic embryos indicate that direct embryogenesis has great potential to be used in the mass production of *P. cynaroides*, as well as in plant breeding research. Further research regarding the establishment of the germinated somatic embryos in *ex vitro* conditions is needed. In addition, the development of multiple shoot-buds on the zygotic explant may be used as an alternative propagation method, but further research is needed.

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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.

Explant	Photoperiod	% of explants forming somatic embryos[x]	Mean number of embryos per explants[y]
Zygote	12-h	70.0 a	8.67 ±1.52 a
	24-h Dark	37.5 b	5.53 ±1.49 b
Cotyledon	12-h	25.0 b	4.10 ±1.20 b
	24-h Dark	22.5 b	4.44 ±1.13 b

[x] Data were subjected to Chi-Square analysis. Percentages of 40 explants within column followed by different letters are significantly different at $P \leq 0.001$.
[y] Means within the same column followed by a different letter are significantly different at $P \leq 0.05$ using Tukey's Studentized range test.

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.

GA_3 (mg l^{-1})	Casein hydrolysate (mg l^{-1})	Coconut water (ml l^{-1})	Total germination (%)[x]	Germination of normal embryos (%)
-	-	-	30.0 c	55.6 b
0.1	-	-	83.3 a	72.0 a
0.5	-	-	60.0 b	50.0 b
1	-	-	20.0 c	0 c
2.5	-	-	20.0 c	0 c
5	-	-	20.0 c	0 c
10	-	-	10.0 c	0 c
20	-	-	10.0 c	0 c
-	100	-	20.0 c	0 c
-	200	-	23.3 c	0 c
-	-	50	30.0 c	0 c
-	-	100	40.0 bc	0 c

[x] Percentages of 30 explants within the same column followed by different letters are significantly different at $P \leq 0.001$ according to Chi-square.

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

Growth medium				Ovule growth (%)	
Half-strength MS + Growth regulators (mg l^{-1})				12-Hour photoperiod	Total darkness
2,4-D	NAA	BAP	TDZ		
-	-	-	-	40.0 c	100 a
1	-	-	-	0.0 d	0.0 d
2.5	-	-	-	0.0 d	63.3 b
5	-	-	-	0.0 d	76.6 b
1	-	0.2	-	0.0 d	0.0 d
2.5	-	0.2	-	0.0 d	53.3 bc
5	-	0.2	-	0.0 d	0.0 d
1	-	-	0.2	0.0 d	73.3 b
2.5	-	-	0.2	0.0 d	60.0 b
5	-	-	0.2	0.0 d	0.0 d
-	1	-	-	0.0 d	30.0 c
-	2.5	-	-	0.0 d	63.3 b
-	5	-	-	0.0 d	53.3 bc
-	1	0.2	-	0.0 d	16.6 c
-	2.5	0.2	-	0.0 d	30.0 c
-	5	0.2	-	0.0 d	0.0 b
-	1	-	0.2	0.0 d	66.6 b
-	2.5	-	0.2	0.0 d	76.6 b
-	5	-	0.2	0.0 d	0.0 d

Percentages followed by different letters are significantly different at $P \leq 0.05$ according to Chi-square.

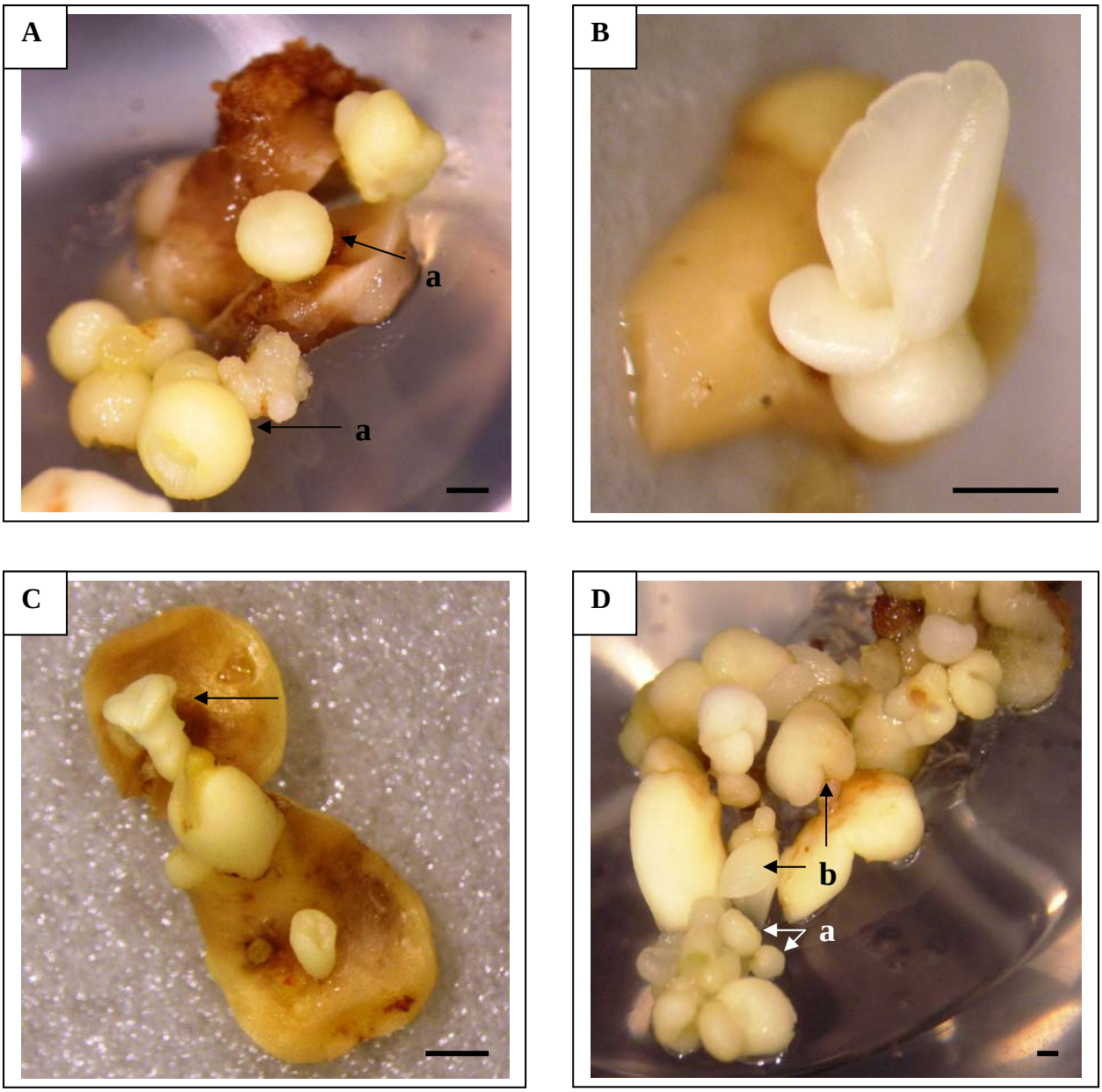

Figure 4.1. Direct formation of somatic embryos: (**A**) globular-stage embryos formed on the edges of a zygotic embryo; (**B**) Advanced torpedo-stage embryo formed on the abaxial side of a zygotic embryo; **(C)** Torpedo-stage embryo formed on the adaxial side of a zygotic embryo (arrow); **(D)** Cluster of somatic embryos from zygotic embryo in various stages of development. **a** = embryos in globular stage; **b** = embryos in heart stage. Bar ≈ 1 mm.

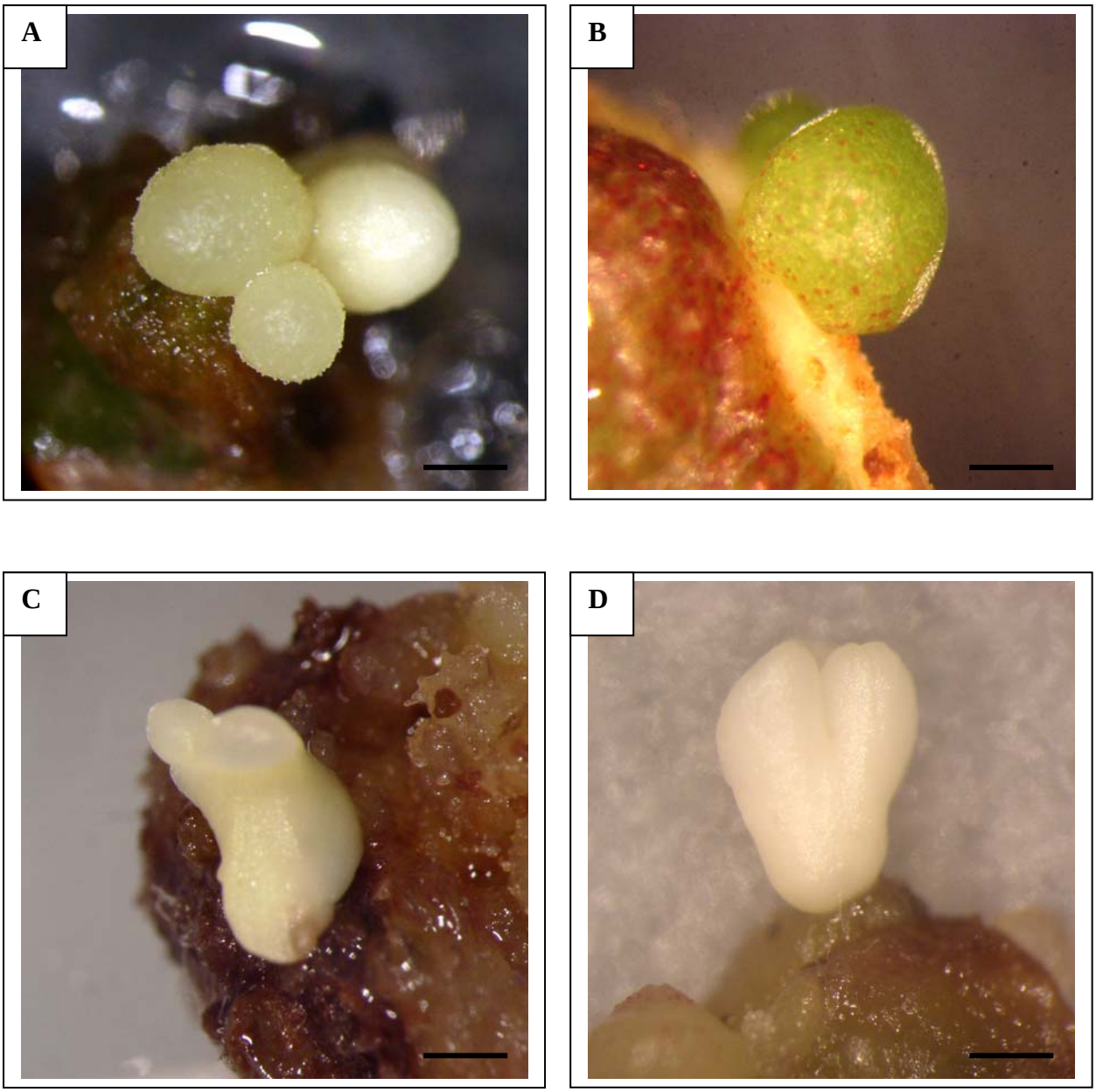

Figure 4.2. Direct somatic embryo formation on excised cotyledons cultured on full-strength MS medium with no growth regulators in **(A)** the dark, and **(B)** under a 12-hour photoperiod. The development of somatic embryo into the **(C)** heart stage, and **(D)** torpedo stage, directly on excised cotyledons in the dark. Bar ≈ 1 mm.

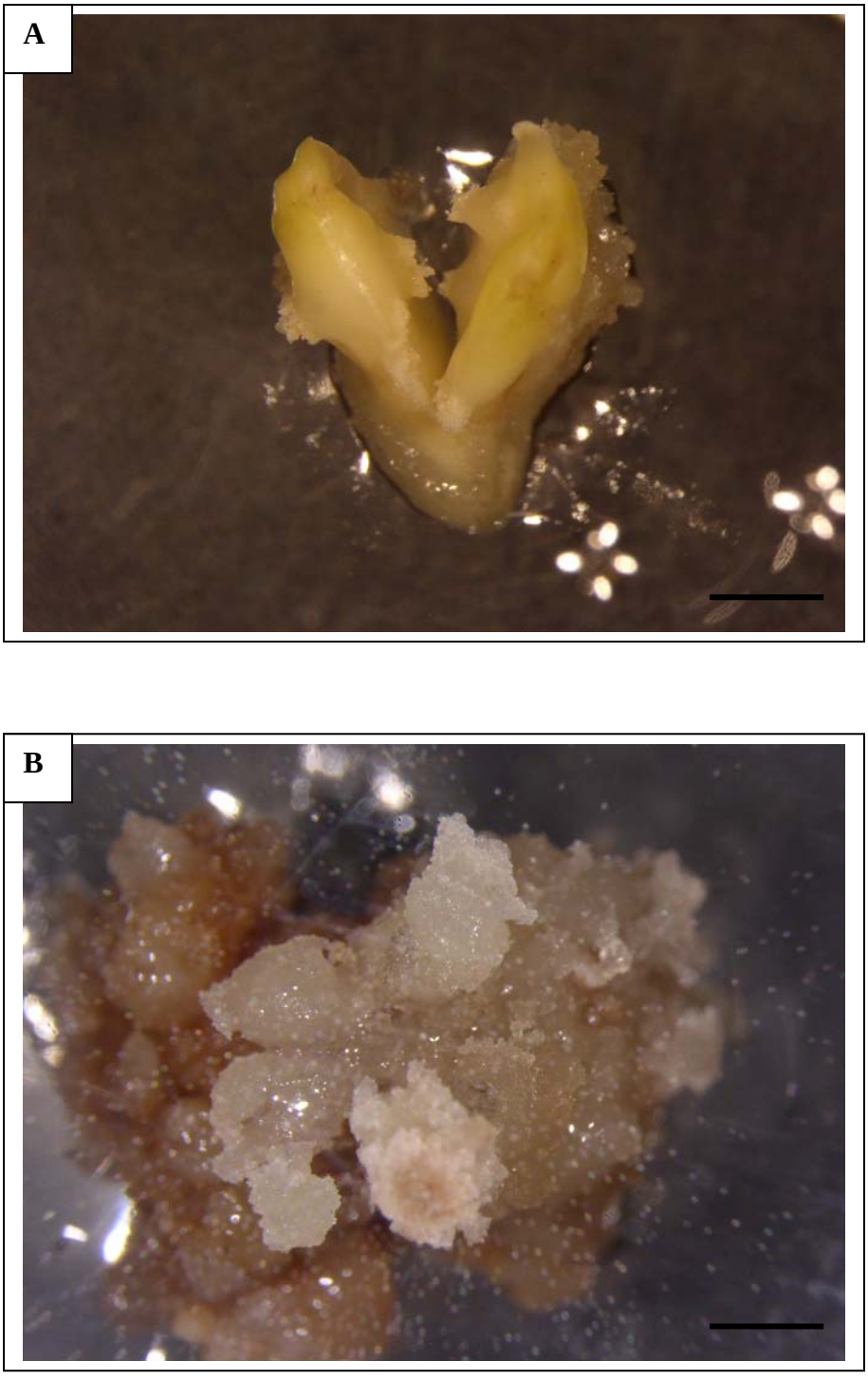

Figure 4.3. Growth of non-embryogenic, watery, fluffy callus on **(A)** zygote explant cultured on medium containing 2.5 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ, and on **(B)** excised cotyledon cultured on medium containing 1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP. Bar ≈ 5 mm.

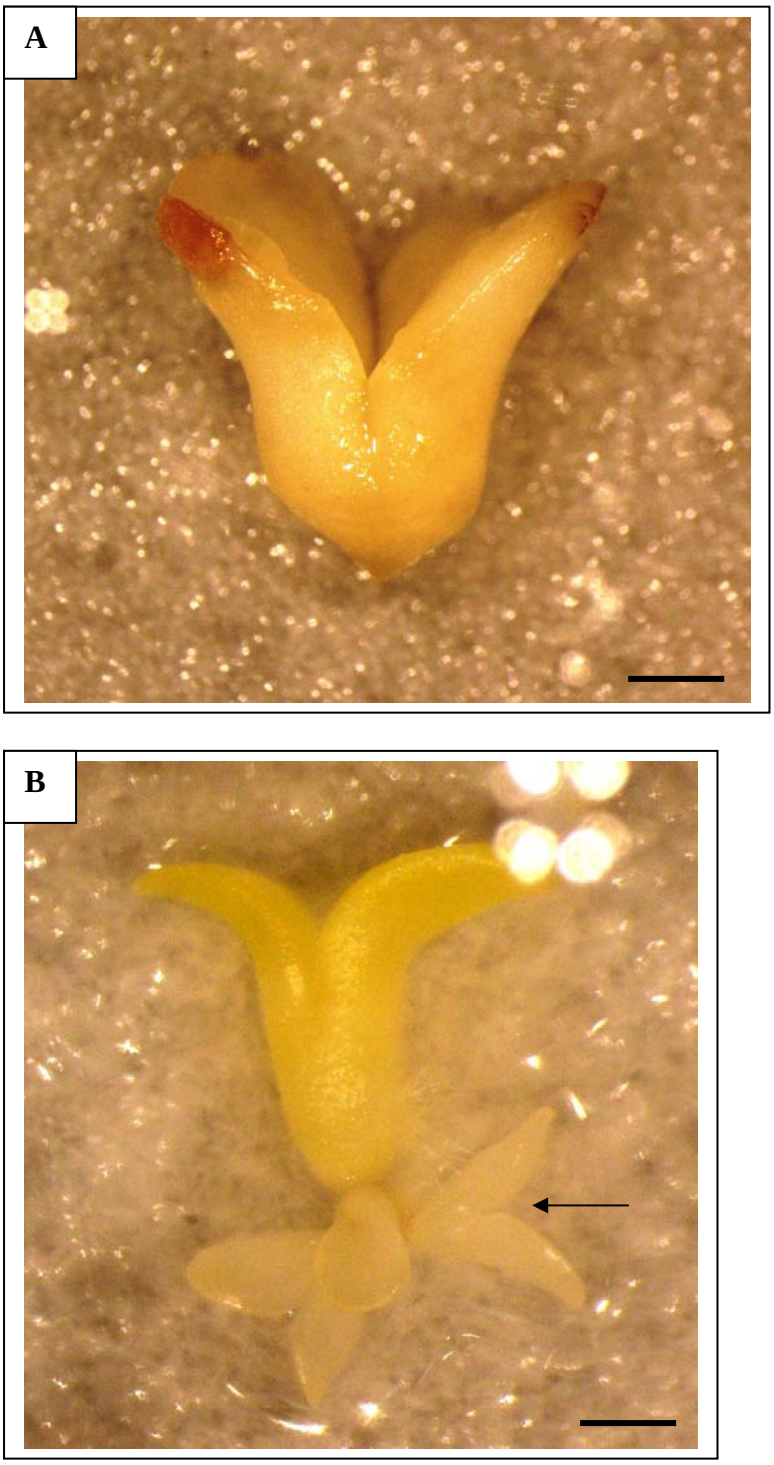

Figure 4.4. Development of isolated somatic embryo cultured on germination medium containing MS medium supplemented with 0.1 mg l^{-1} GA_3. **(A)** Embryo in advanced heart stage; **(B)** Embryo in cotyledonary stage, with accessory embryos attached (arrow). Bar ≈ 1 mm.

Figure 4.5. Germination of normal embryos on MS medium containing GA_3. Emergence of first leaves from the germinated embryo (bottom right). **a** = two separate cotyledons; **b** = radicle. Bar ≈ 5 mm.

Figure 4.6. Germination of malformed embryos with **(A)** single cotyledon; **(B)** multiple cotyledons; **(C)** two fused embryos attached. Bar ≈ 1 mm.

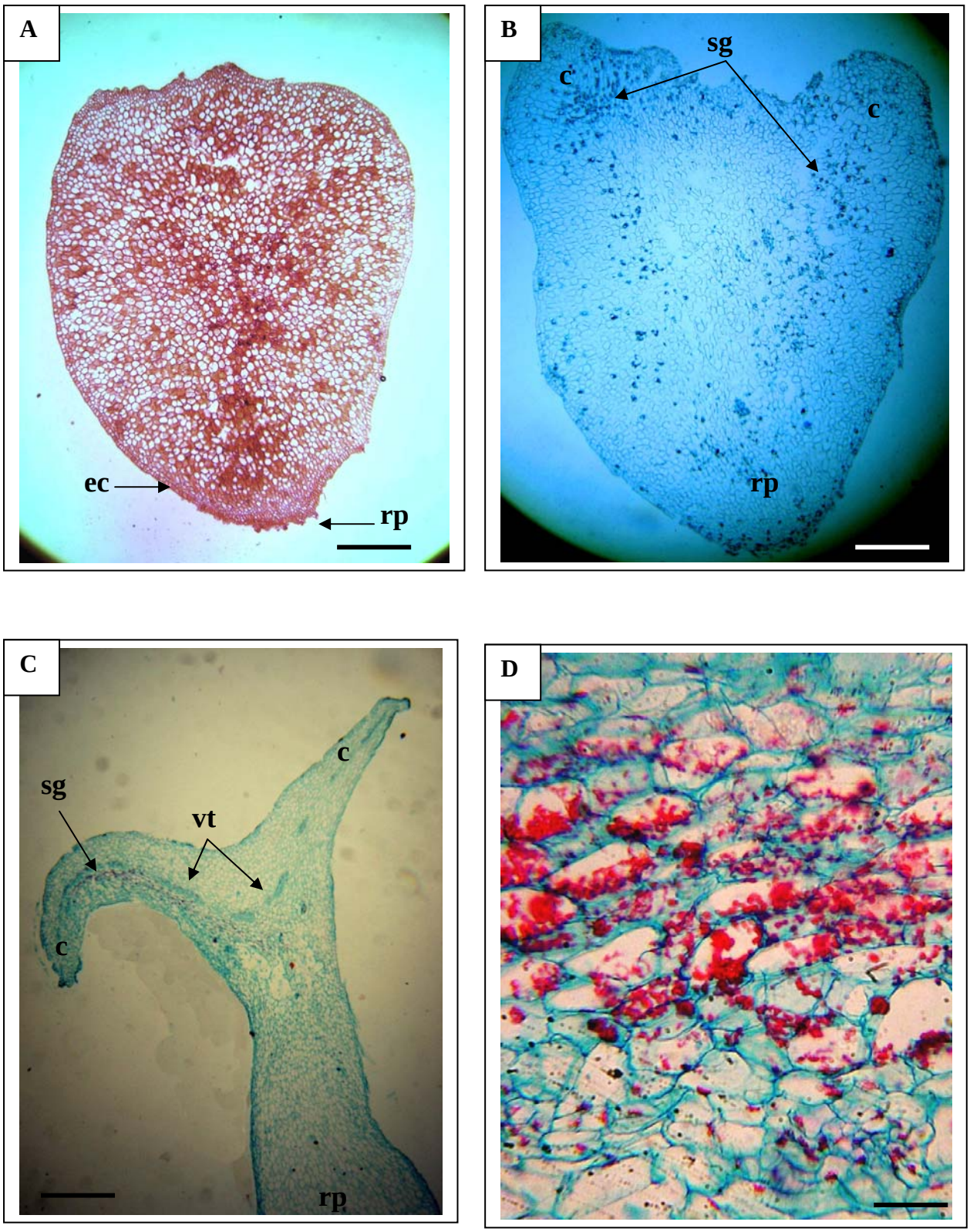

Figure 4.7. Longitudinal section through **(A)** Embryo at globular-stage, with cell differentiation at root pole (Bar ≈ 500 µm); **(B)** Embryo at heart stage, with development of cotyledons (Bar ≈ 500 µm); **(C)** Embryo at cotyledonary stage showing vascular connections (Bar ≈ 500 µm); **(D)** Starch grains stained with safranin, enlarged from the cotyledon area of **(C)** (Bar ≈ 50 µm). **c** = cotyledon; **ec** = epidermal cells; **rp** = radicle pole; **sg** = starch grains; **vt** = vascular tissue.

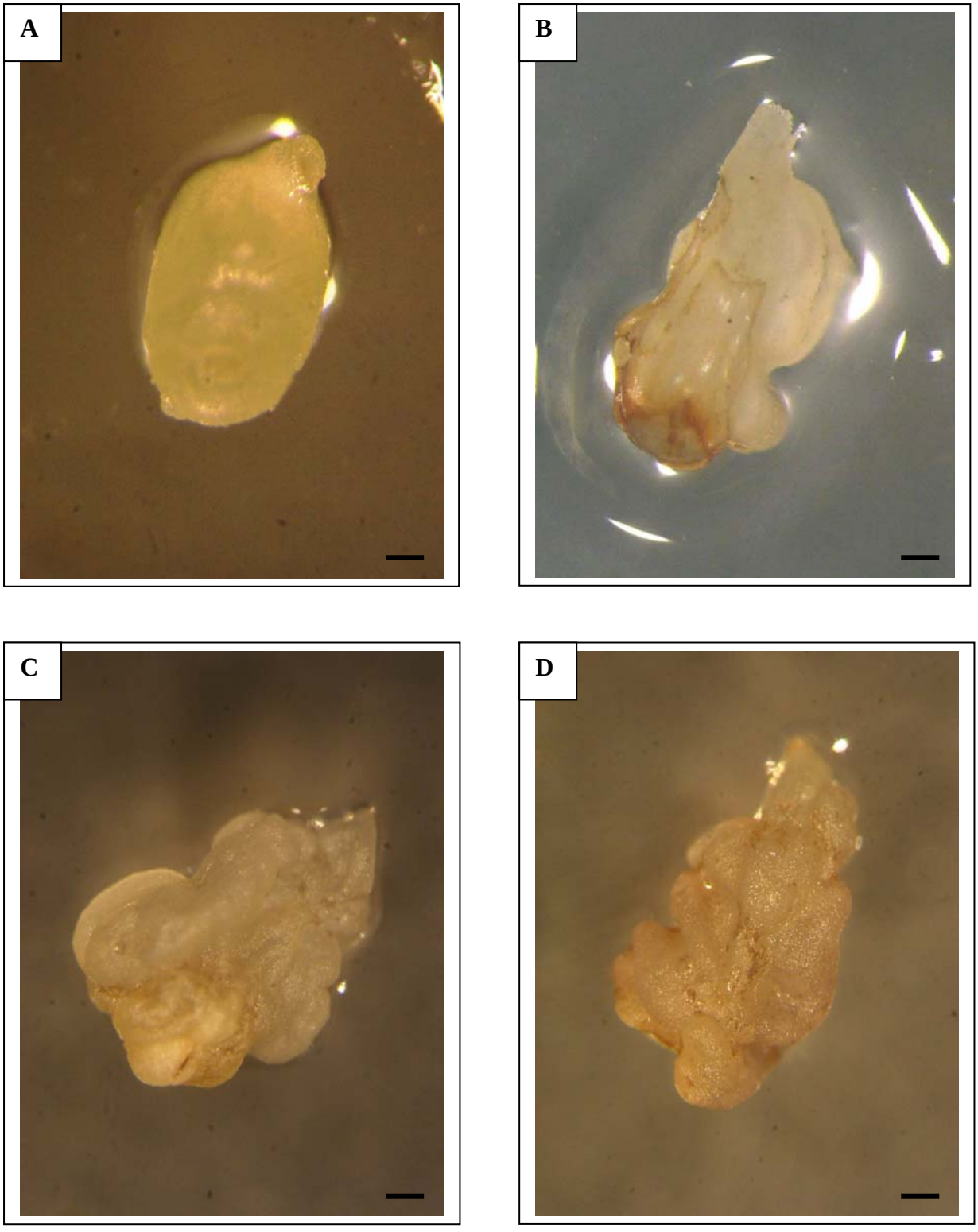

Figure 4.8. Immature unfertilized ovules, cultured on half-strength MS medium with 1 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ after **(A)** 0 days; (**B**) 5 days; **(C)** 4 weeks; **(D)** 8 weeks. Bar ≈ 1 mm.

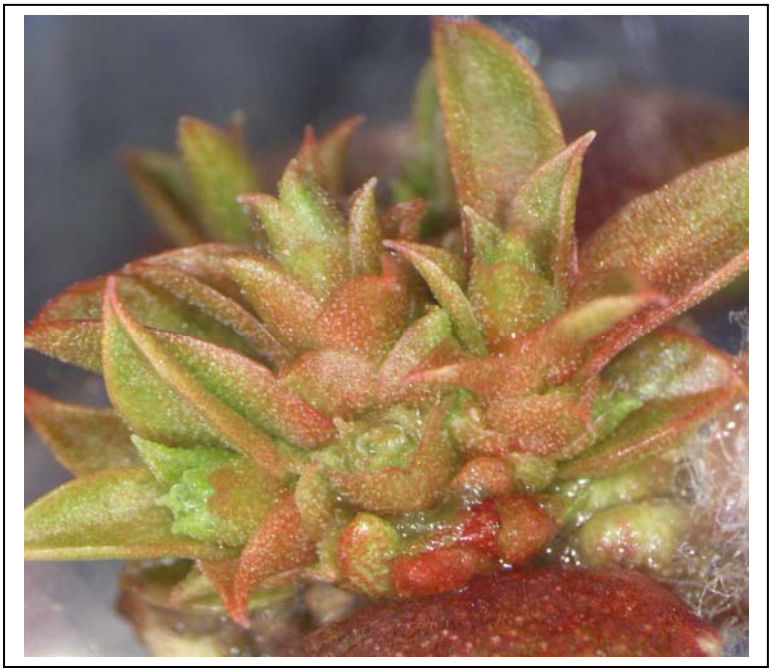

Figure 4.9. Clusters of shoot buds on cotyledons cultured on basal MS medium.

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 in 1 mg l^{-1} NAA. Bar ≈ 1 mm.

CHAPTER 5

ROLES OF STARCH AND PHENOLIC COMPOUNDS IN VEGETATIVE PROPAGATION OF *PROTEA CYNAROIDES* STEM CUTTINGS

5.1 Abstract

Vegetative propagation of *Protea cynaroides* stem cuttings is known to be slow and inconsistent. In this study, cuttings were treated either by blanching, leaching or rooting hormone before planting into the mistbed to improve the rooting rate and rooting percentage. The rooting percentage and the mean root dry mass of *P. cynaroides* stem cuttings were significantly improved by the blanching treatment. Starch and total phenol analyses results illustrated a positive correlation between high root formation and increased starch and phenolic content by the blanching treatment. Significantly higher amounts of starch and total phenols were found in the basal-end of blanched cuttings than the control, from planting time until the cuttings were well-rooted after 90 days. The blanched cuttings were ready to be transplanted after 90 days, compared to the control, which was only ready at day 120. Leaching of cuttings in water resulted in poor rooting percentage and low root dry mass, which may have been caused by the loss of macro- and micro-nutrients from the cuttings. Analyses of the leachate showed that significant amounts of N were leached from the cuttings, while a lesser amount of P, K Ca and Mg were also leached. Phenolic compounds, which may be responsible for stimulating root formation, may also have been leached from the cuttings. However, further studies in this regard are needed.

5.2 Introduction

P. cynaroides plants show great variation in nature with many different sizes, colours and flowering time (Vogts, 1982). Therefore, due to the genetic variability of seeds, vegetative propagation is the method used by growers in order to achieve and maintain genetic uniformity in the commercial production of *P. cynaroides* cut flowers. However, *P. cynaroides* is a woody plant, which typically has a poor

physiological capacity for adventitious root formation, and is notoriously known as a difficult-to-root ornamental plant. Because of this difficulty, growers have had to set up expensive propagation facilities, such as fogging systems and heating beds, to try and construct an environment conducive to rooting. This has resulted only in a slightly higher rooting percentage, and cuttings can still take up to six months (180 days) to root.

Vegetative propagation without the use of rooting hormones (auxin-types) has been used in other members of Proteaceae such as *Leucospermum reflexum, L. nutans, Mimetes lyrigera* and *Serruria florida* (Admiraal, 1966). However, rooting hormones are usually applied to improve rooting, particularly in difficult-to-root species of Proteaceae. Rousseau (1967), Jacobs and Steenkamp (1975) and Brits (1986) reported that the application of IBA resulted in high rooting percentages in *Mimetes* sp. and *Leucospermum* sp. Rousseau (1967) also recommended that applying IBA in powder form on *P. cynaroides* cuttings would provide an average of 50% rooting success. Similarly, Faruchi, Ackerman, Gilad, Ben-Jaacov and Riov (1997) obtained 66% rooting in *Protea obtusifolia* cuttings with IBA powder. Nevertheless, these rooting percentages are considered very low, particularly in a commercial environment.

For improving the rooting of cuttings, the beneficial effects of etiolation, whether it is done with the stockplant or parts of the stem, has been widely reported. Stockplant etiolation practices such as blanching, where the part of the stem that is to become the cutting base is etiolated while the remaining part of stem is allowed to continue developing in light, have often been used. For example, blanching has been used in the propagation of *Malus* spp. (Delargy and Wright, 1979; Harrison-Murray, 1981; Howard, Harrison-Murray and Arjal, 1985; Sun and Bassuk, 1991), *Dahlia* spp. (Biran and Halevy, 1973), and various other woody plants (Maynard and Bassuk, 1985; Maynard and Bassuk, 1987). In these studies, the rooting of plants that were known to be difficult-to-root was improved.

Carbohydrates are important basic building blocks of structural elements (Struve, 1981), and is known to play a role in rooting of cuttings. However, the relationship between carbohydrate content and rooting has been controversial. Both negative and positive correlations have been found between carbohydrate content and rooting:

Negative correlation was observed in *Pinus* cuttings by Hansen, Stromquist and Ericsson (1978), where an increase in carbohydrate content reduced the number of roots formed. Similar results were found in *Populus* cuttings by Nanda and Anand (1970) and Okoro and Grace (1976). Conversely, positive correlations were reported in *Humulus lupulus* (Howard and Sykes, 1966), *Populus* sp. (Nanda, Jain and Malhotra, 1971) and in *Pisum sativum* (Veierskov, Stummann and Henningsen, 1982).

Another factor that has been demonstrated to play an important role in the rooting of stem cuttings is phenolic compounds. Numerous reports have shown both stimulatory and inhibitory effects of phenols in the rooting of cuttings. For instance, Dhawan and Nanda (1982) illustrated stimulatory effects of coumarin in the rooting of *Impatiens* cuttings. In addition, phenols such as phloridzin and phloroglucinol have been shown to improve the rooting of apples (Gur, Gad and Haas, 1988). However, for *Chamaelaucium* hardwood cuttings, which contained high concentrations of cinnamic acid, it was found that root formation was inhibited (Curir, Sulis, Mariani, Sumere, Marchesini and Dolci, 1993). Furthermore, the leaching out of water-soluble inhibitors such as phenolics from cuttings has been shown to improve rooting, where improvements in the rooting of *Vitis* cuttings was obtained by placing cut canes in water (Spiegel, 1954).

Numerous factors are known to play a role in the rooting of cuttings. The main objective of this study was to determine the effects of leaching, blanching, and rooting hormones on the rooting rate and rooting percentage of *P. cynaroides* cuttings. In addition, the starch content and total soluble phenol levels in cuttings were analyzed throughout the rooting period to determine their influence on root formation.

5.3 Materials and methods

5.3.1 Plant material

P. cynaroides stem cuttings that were used in this study were collected during autumn

from motherplants grown in an open field situated near Cullinan (25º40'32S; 28º31'20E; Altitude 1482 metres) in the Highveld region (summer rainfall) of South Africa. The treatments applied to the stem cuttings included leaching, etiolating the cutting bases of the stem (blanching), and rooting hormone. In all the treatments, almost identical terminal semi-hardwood stems of the current year's growth, which were approximately 15 cm in length, were used.

5.3.2 Experiment 1

5.3.2.1 Rooting of stem cuttings, rooting medium and growth conditions

The rooting medium consisted of a peat moss and polystyrene ball (1:1 v:v) mixture in seedling trays (Figure 5.1A). The cuttings were rooted in a mistbed where they were irrigated with micro-jet sprinklers, which automatically irrigated every 20 minutes for 1 minute. No bottom heating was used during the propagation period. The air temperature of the mistbed was 27ºC±2.

In order to determine the effects of leaching on the rooting of *P. cynaroides* cuttings, semi-hardwood stems were soaked in distilled water for different periods, which consisted of 6, 12 or 24 hours. After the respective leaching periods, cuttings were removed from the water and planted into the rooting medium. Unleached cuttings were used as the control. The analyses of macro- and micro-nutrients in the stems and leachate solution were done and the percentage of nutrients leached from the cuttings was calculated (see 5.3.2.2).

For the blanching treatment, once suitable stems were identified on the motherplants, 20 mm of the portion of the stem that was to become the cutting base was covered with black insulation tape to prevent exposure to sunlight. The remaining top portion of each of the stem cuttings was exposed to sunlight to allow normal growth to continue. After 30 days, the blanched stems were removed from the mother plants by cutting just below the insulation tape. The tapes were then removed and the leaves of the bottom three-quarters of each cutting were stripped, prior to placement in the rooting medium. Untreated cuttings (control) were collected at the same time from the motherplants and planted directly into the rooting medium.

For the rooting hormone treatment, commercially available Seradix® No. 2 (Active ingredient: 4-(indol-3-yl)-butyric acid (IBA) (8 g kg^{-1})) was used. The cuttings were dipped into the rooting hormone powder and immediately placed into the rooting medium.

Weekly observations were made, and data, which included the rooting percentage, root dry mass, root length and the number of roots, were recorded after 90 days.

5.3.2.2 Analyses of macro- and micro-nutrients in stem cuttings and leachate solution

The analyses of macro- and micro-nutrients in the stem samples were carried out according to ALASA: Methods for soil and plant analysis (1998). Semi-hardwood stems, which were suitable to be used as cuttings, were freeze-dried (Edwards® Modulyo Freeze Drier), weighed and ground into powder using a pestle and mortar. For the analysis of N and P, 0.25 g was weighed and digested in a 5 ml mixture of H_2SO_4 (sulphuric acid), Se (Selenium metal grey powder) and H_2O_2 (Hydrogen peroxide) and incubated at 400ºC for approximately 30 minutes, or until the solution became clear. Afterwards, the mixture was made up to 75 ml using distilled water. Nitrogen and phosphate readings were taken using a Skalar® AutoAnalyzer II flow system.

For the analysis of K, Ca and Mg, 0.5 g was weighed and digested in a 5 ml mixture of HCLO (Perchloric acid) and HNO_3 (Nitric acid). The solution was then incubated at room temperature for 80 minutes. During the incubation period, the temperature was gradually increased from room temperature to 120ºC. The mixture was then made up to 75 ml using distilled water. Potassium, calcium and magnesium values were read in an AA (Atomic Absorption - Varian®, Model: SprectrAA – 200), according to ALASA: Methods for soil and plant analysis (1998).

The water that was used to leach the cuttings was also analyzed for N, P, K, Ca and Mg. The analysis procedure was conducted according to the Handbook of standard soil testing methods for advisory purposes (1990). For the quantification of nitrogen,

NH_4 and NO_3 were analyzed in a BUCHI® Kjeldahl Unit. Phosphate levels were determined using a Skalar® AutoAnalyzer II flow system, while the Atomic Absorption (Varian®, Model: SprectrAA – 200) was used to determine the amount of K, Ca and Mg in the leachate solution.

From the results of the analyses of macro- and micro-nutrients in the stem cuttings and the leachate solution, the percentage of macro- and micro-nutrients leached from the cuttings was calculated with the following equation:

$$\% \text{ Leached} = \frac{\text{Amount of nutrients in leachate solution (mg } 100\text{ml}^{-1})}{\text{Amount of nutrients in stem (mg g}^{-1}) \times \text{Dry mass of stem (g)}} \times 100$$

5.3.3 Experiment 2

5.3.3.1 Rooting response of cuttings after 60, 90 and 120 days

Experiment 1 was repeated using almost identical plant materials, rooting medium and growing conditions. However, only the control and the blanching treatments, which performed the best in Experiment 1 in terms of rooting percentage, root dry mass and number of roots formed, were repeated. Weekly observations were made, and the root dry mass was recorded after 60, 90 and 120 days. In addition, at each time interval, total soluble phenols (see 5.3.3.2), and total starch content (see 5.3.3.3) of the stem cuttings were analyzed.

5.3.3.2 Determination of total soluble phenols

a) Sample preparation

Untreated (control) and blanched cuttings of Experiment 2 (see 5.3.3.1) were collected after 0, 60, 90 and 120 days. At each time interval, the stems of the control and blanched treatments were cut into three parts: 1) cutting base (bottom 2 cm), 2)

middle section, and 3) top part of the stem. The latter two parts were divided into equal lengths. Together with the aforementioned three plant parts, the leaves were included in this analysis. The various parts of the stem and leaves were separately freeze-dried (Edwards® Modulyo Freeze Drier) and ground into fine powder using a pestle and mortar. Afterwards, of the 20 replications available in each treatment, six samples were randomly selected. Three groups of pairs were then randomly formed and pooled together. Thereafter, the samples were filtered through a 1 mm tea sieve and 0.05 g was weighed into 10 cm^3 test tubes.

b) Reagents

The Folin-Ciocalteu reagent was purchased from Sigma-Aldrich. All solutions were prepared with distilled water, and all the salts and solvents used were of analytical grade.

c) Extraction and quantification of total soluble phenols

The procedure for the extraction and quantification of total phenolic compounds was adapted from Fourie (2004). The solvent used was methanol:acetone:water (7:7:1). One millilitre of the solvent was added to 0.05 g powdered sample. It was then placed in an ultrasound waterbath for 3 minutes, and then centrifuged (Kubota® 2010 Centrifuge) for 30 seconds. The extraction procedure was repeated twice. The concentration of phenolic compounds was determined using the Folin-Ciocalteu reagent (Bray and Thorpe, 1954). A 96-well ELISA plate was used for the reaction mixture. A dilution series (10 – 1000 $\mu g.ml^{-1}$ methanol) were used to prepare standard curves for ferulic- and gallic acid for the quantification of phenolic content. The reaction mixture comprised of 175 µl distilled water + 5 µl standard or extract sample + 25 µl Folin-Ciocalteu reagent + 50 µl 20% (v/v) Na_2CO_3. The samples were then incubated at 40ºC for 30 minutes. Afterwards, the absorbance was read at 690 nm using an ELISA reader (Multiskan Ascent V1.24354 – 50973 (Version 1.3.1)). The phenolic concentration is expressed as gallic acid equivalents per gram dry sample material.

5.3.3.3 Analysis of starch content

a) Sample preparation

The preparation of the samples is described in the 'Determination of total soluble phenols' section above (5.3.3.2). The samples were weighed (0.05 g) and placed into test tubes.

b) Reagents for procedure

1) Ethanol (99.9%)
2) Termamyl®
3) Amyloglucosidase (Novo® 300L)
4) Acetate buffer (Appendix B)
5) Glucose solution: 1000 µl ml^{-1} (Prepared from AR glucose)
6) Glucose oxidase colour solution (Appendix B)

c) Procedure for extraction and starch determination

The enzymic-chlorometric procedure by Rasmussen and Henry (1990) and Kaiser and Wolstenholme (1994) was followed. To each test tube was added 5 ml of 80% ethanol, capped with a marble, and incubated in a waterbath (80°C) for 30 minutes. Thereafter, the test tubes were centrifuged (Kubota® 2010 Centrifuge) for 10 minutes at 3000 rpm. Once centrifuged, the supernatant was decanted and discarded. The abovementioned procedure was repeated twice to remove all free sugars.

Thereafter, acetate buffer (2.5 ml) and Termamyl® (50 µl) were added to the residue in the test tubes, which were then capped with marbles and incubated in a waterbath (90°C) for 30 minutes, and then allowed to cool to room temperature. Afterwards, 50 µl amyloglucosidase (Novo® 300L) was added and incubated in the waterbath (60°C) for 20 hours.

After removing the test tubes from the waterbath, they were again centrifuged for 10 minutes at 3000 rpm. Then, 25 µl aliquots of the resultant supernatant were pipetted to new test tubes and made up to 5 ml with glucose oxidase colour solution. Subsequently, test tubes were capped with marbles and incubated in a waterbath (40°C) for 15 minutes. Finally, the test tubes were left to stand at room temperature for 60 minutes.

The samples were poured into cuvettes and placed into the spectrophotometer (Pharmacia LKB Ultrospec III). Absorbance values were read at 505 ηm and compared to a glucose standard curve (Appendix B). Percentage starch was calculated from the following formula:

$$\% \text{ Starch} = \frac{C \times D \times K}{W} \times 100$$

where,

C	=	concentration of glucose sub-sampled for colour development (the spectrophotometer readout)
D	=	104.0 (Dilution Factor)
K	=	0.9 (Water of Hydrolysis Constant)
W	=	Total dry mass of sample (mg)

The calculation for the dilution factor depended on the expected starch concentration. The concentration of dry material was 0.05 g / (2.5 + (50 µl)) ml^{-1}.
25 µl was sub-sampled and analyzed for colour development. The dilution factor was calculated as follows:

$$2.5 + 2(0.05) / 0.025$$
$$= \mathbf{104.0}$$

The amount of glucose that was detected by the spectrophotometer was multiplied by the dilution factor to calculate the % starch on a mass glucose/mass dry mass basis.

5.3.4 Statistical analysis

In the rooting study of both Experiments 1 and 2, a completely randomized design was used, with 20 replications for each treatment. Three replications were used in the total soluble phenolic determination and starch content analyses. Where appropriate, Chi-Square analysis and Tukey's Studentised range test were applied to compare treatment means. All statistical analyses were done in the SAS program (SAS Institute Inc, 1996). ANOVA are shown in Tables C4 – Table C43, Appendix C.

5.4 Results

5.4.1 Experiment 1

5.4.1.1 Rooting response of cuttings to leaching, blanching, and rooting hormone

In all treatments, roots that formed on cuttings were of adventitious type. Proteoid root formation was not observed on any of the cuttings. This was probably because the rooting medium contained adequate nutrients. Proteoid roots generally form when soil nutrients are deficient (see Chapter 1). Compared to the leaching and rooting hormone treatments, the blanched cuttings were the only ones that were well-rooted and ready to be transplanted to the field after 90 days. In fact, when the cuttings were removed from the rooting medium, their roots had already penetrated the bottom of the container. This suggests that the cuttings were suitable to be transplanted even before 90 days. Figure 5.1B and Figure 5.1C clearly illustrates the difference in the amount of roots formed in the control and blanched cuttings.

The effects of all the treatments on the rooting percentage of the cuttings after 90 days are illustrated in Figure 5.2. The highest rooting percentage was obtained in blanched cuttings (100%), which was significantly higher than the control (60%), 6-hour leaching (40%), 12-hour leaching (30%), 24-hour leaching (40%), and rooting hormone (60%).

Furthermore, the effects of blanching in the improvement of the amount of roots formed is shown in Figure 5.3, where a significantly higher mean root dry mass of the blanched cuttings was obtained in comparison to the other treatments. The mean root dry mass between the control and the rooting hormone treatments were not significantly different, which indicates that rooting hormones did not improve the rooting of *P. cynaroides* cuttings. In addition, besides the poor rooting percentage of the leached cuttings, the number of roots formed was also very low. The amount of roots formed by the leached cuttings was significantly lower than the control, which is shown by the mean dry root mass of cuttings leached for 6 hours (28 mg), 12 hours (11 mg) and 24 hours (17 mg) (Figure 5.3).

The low rooting capacity of the leached cuttings could be explained by the amount of nutrients lost from the cuttings through leaching (Table 5.2). Nitrogen was leached in the largest quantity from the cuttings when placed in water for 6 hours (34.62%), 12 hours (35.53%) and 24 hours (20.86%). A possible explanation for the lower percentage of nitrogen leached (20.86%) from cuttings in the 24-hour leaching treatment, may be due to the re-uptake of the nutrient (N) after a prolonged period of soaking. In addition, smaller amounts of P, K, Ca and Mg were also leached from the cuttings in all the different leaching time periods (Table 5.2).

The poor rooting shown by the leached cuttings is further illustrated by the number of roots formed and their lengths (Table 5.1). In general, very few roots were formed by the leached cuttings, particularly by the cuttings that were leached for either 12 hours or 24 hours. In addition, a large number of the roots formed were less than 10 mm in length (Group 1, Table 5.1), indicating that root growth had just started, compared to the blanched cuttings, which had relatively higher number of roots in most of the root length categories. Comparisons between the untreated cuttings (control) and the cuttings treated with rooting hormone revealed that the number of roots formed in each root length category was similar, which further shows that the use of rooting hormones did not increase the overall rooting of *P. cynaroides* cuttings. However, compared to the blanched cuttings, the untreated cuttings formed significantly less roots in all the root length categories except in Group 4 (31 – 40 mm) where the number of roots formed was similar (Table 5.1).

5.4.2 Experiment 2

5.4.2.1 Rooting response of cuttings after 60, 90 and 120 days

Figure 5.4 illustrates the root growth of cuttings in the control and blanched treatments throughout the entire rooting period. Root initiation was first observed after 60 days in both the control and blanched cuttings. At this stage, although the mean root dry mass between the two treatments was not significant (Figure 5.4), visual observations indicated that more roots had formed on the blanched cuttings than on the control. In terms of the rooting rate, the response of the untreated (control) and the blanched cuttings in this experiment was very similar to the results of Experiment 1. As found in Experiment 1, after 90 days, the blanched cuttings had sufficiently rooted for transplanting, while rooting of the untreated cuttings was still poor. The results of this experiment showed that the untreated cuttings (control) were only suitable for transplanting after 120 days. Furthermore, the difference in the rate of root formation between the two treatments was clearly shown by the mean root dry mass recorded at the respective collection dates (Figure 5.4). The rooting progress of the cuttings in the control and blanched treatments in relation to the starch and total phenol content is discussed below.

5.4.2.2 Determination of total soluble phenols

Figure 5.5 illustrates the changes of total phenol content in the different parts of the control and the blanched cuttings during a rooting period of 120 days. At the basal end of the cuttings, where rooting took place, the total phenol content of both the control and blanched treatments increased steadily throughout the propagation period. However, the total phenol levels of the blanched treatment were significantly higher than the control from day 0 to day 90 (Figure 5.5). In particular, the total phenols of the blanched cuttings were already significantly higher at day 0 (when the banding tapes were removed), which clearly showed that blanching caused an increase in the accumulation of phenolics in stems. Furthermore, the increase in the total phenol content throughout the entire propagation period correlated with the progressive rooting of the cuttings shown by the increase of root dry mass in Figure 5.4. The phenolic content of the cutting base of blanched cuttings was at its highest level (84

mg g^{-1}) at day 90, which is at the same time that considerable rooting took place. This is illustrated in Figure 5.4, where the mean root dry mass of blanched cuttings was significantly higher than the control. High rooting of the untreated cuttings was only observed after 120 days when the phenol content reached its highest level (78 mg g^{-1}), which incidentally was similar to the levels obtained in blanched cuttings at 90 days. Thus, this demonstrates that blanching the cuttings lead to an earlier accumulation of phenolics in the cuttings, which resulted in quicker rooting of the cuttings by approximately 30 days.

In the middle part of the cutting, the total phenol content of the blanched cuttings was significantly higher than the control (Figure 5.5), which may be partly due to an increase in the accumulation of phenols in this area caused by the etiolation effect in the basal end below it. Although the total phenol levels of the blanched cuttings (middle part) remained significantly higher than the untreated cuttings throughout the entire rooting period, a sharp increase was observed in both treatments from day 0 to day 60, after which, the phenol levels remain relatively constant (Figure 5.5).

The amounts of total phenols found in the top part of the cuttings as well as in the leaves in the control and blanched treatments were very similar (Figure 5.5). At the top end of the cuttings in both treatments, steady increases in total phenol took place until day 90 when the highest level was obtained. The phenol content remained constant thereafter. However, in the leaves the total phenol content was the highest after 60 days, after which, a steady decrease occurred until day 120.

5.4.2.3 Analysis of starch content

A striking difference between the starch content of the blanched cuttings and the untreated cuttings was that while the starch content of the blanched cuttings remained relatively consistent throughout the entire rooting period (highest level reached after 60 days), the starch levels of the untreated cuttings fluctuated throughout the rooting period (Figure 5.6). In addition, from day 0 to day 90, a higher percentage of starch was found in the blanched cuttings than the untreated cuttings in all parts of the stem. Of particular interest, when the cuttings were taken from the motherplant (day 0 - when banding tape was removed in the blanched treatment), the amount of starch in

the basal end of the blanched stems (34.65%) was significantly higher than the control (26.44%), which strongly suggests that etiolation caused an increase in the accumulation of starch (Figure 5.6).

After 90 days, when high root growth took place in the blanched cuttings, starch content was found to be significantly higher than the control. After which, the starch levels in the blanched cuttings decreased in all the plant parts from day 90 to day 120 when root formation was not as vigorous as the control, and a reduction in the rooting rate was observed (Figure 5.4). On the other hand, in the control the starch percentage increased considerably from day 90 to day 120 (Figure 5.6) when high rooting took place, which is illustrated by the significant increase of root dry mass (Figure 5.4).

5.5 Discussion

The results of the rooting studies clearly showed a significant improvement in the rooting percentage and the amount of roots formed when the stems were blanched for 30 days before planting (Figure 5.2). Similar findings were reported by Doud and Carlson (1977) where striking differences between the rooting of etiolated and non-etiolated cuttings were obtained in both the percentage rooting and the number of roots per stem. In addition, Delargy and Wright (1979) and Sun and Bassuk (1991) also reported improved rooting percentages of apple rootstock cuttings by blanching. Similarly, Maynard and Bassuk (1987) showed that *Carpinus* and *Pinus* cuttings formed more roots per cutting when blanched than the control.

In addition to the improved rooting percentage, the rate of root formation in the blanched cuttings was quickened, which was shown by the fact that cuttings were ready for transplant at day 90, while rooting of the untreated cuttings was still relatively low (Figure 5.1). Davis and Potter (1983) also reported that localized etiolation of stock plants accelerated the development of adventitious roots and decreased the time to obtain well-rooted *Rhododendron* cuttings. It is likely that the increase in the rooting rate of blanched *P. cynaroides* cuttings was directly linked to the high accumulation of total phenol content in the basal end of the cuttings, since it is known that phenolics are important in the rooting process by protecting the

endogenous natural-occurring auxin – indole-3-acetic acid (IAA) – from destruction by the enzyme IAA oxidase (Donoho, Mitchell and Sell, 1962; Fadl, El-Deen and El-Mahady, 1979), or by acting as precursors to lignin formation for structural support (Haissig, 1986). However, it has been illustrated that etiolation causes a reduction in the production of lignin, i.e. thinner cell walls, which is often characteristic of dark-grown plants. Thus, instead of forming lignin, phenolics are channeled to enhance root initiation, which correlates with the ability of etiolated cuttings to have higher success of rooting for a longer period of time than light-grown cuttings (Englert, Maynard and Bassuk, 1991). This correlation is shown in reports where etiolation improves rooting by increasing the herbaceous nature of the rooting region (Biran and Halevy, 1973). Similarly, Herman and Hess (1963) reported that etiolated stems were relatively herbaceous because they had thinner cell walls and more undifferentiated tissues. In addition, Druart, Keevers, Boxus and Gaspar (1982) showed that etiolation treatments lead to an increase in the phenolic content of apple shoots, which resulted in the stimulation of rooting.

Histological investigation of the cuttings was not done in the present study. However, by visual observation, besides the loss of colour at the blanched section (basal end) of each cutting due to the lack of light, the etiolated section seemed to have lost its structural strength and became more herbaceous. It is likely that the changes in structural characteristics of the etiolated sections in *P. cynaroides* cuttings were caused by a reduction in the production of lignin, resulting in thinner cell wall, as discussed above.

Results for total phenols in the different parts of the stem and leaves (Figure 5.5) revealed a positive correlation between phenolic content and rooting of the cuttings. The highest amounts of phenolics were found in the leaves, confirming that phenolic compounds are synthesized in the chloroplasts and transported to the vacuole for storage (Mueller and Beckman, 1974). Overall, the total phenol content increased throughout the rooting period in all parts of the cutting, except the leaves, which decreased from day 60 onwards. It is likely that this was due to the distribution of stored phenols from the leaves to the cuttings, in particular to the basal end where rooting took place. Furthermore, results indicated that rooting of *P. cynaroides* cuttings took place when a specific concentration of endogenous phenolic compounds

was reached, since rooting occurred in both blanched and untreated cuttings when the total phenol levels reached relatively high levels, viz., 84.15 mg g^{-1} and 78.42 mg g^{-1}, respectively (Figure 5.5). Moreover, due to the etiolation effect, blanched cuttings had higher total phenol content when planted, which probably resulted in earlier rooting (after 90 days) than the untreated cuttings (after 120 days).

The mobilization and deposition of starch usually take places in the dark (Daie, 1985), which is probably the reason why a higher amount of starch is often found in etiolated stems. In this study, the starch percentage of the basal ends of blanched cuttings was determined immediately after the blanching process was complete (Day 0). The results of the starch analyses showed that localized etiolation (blanching) increased the accumulation of starch in the *P. cynaroides* stem, which was significantly higher than the control, while the starch percentage of the other parts of the cuttings remained similar. This agrees with the finding by Doud and Carlson (1977) that stem etiolation caused a significant increase in starch levels. They concluded that the increase of starch in the stem seemed to favour positional root initiation, where roots were produced almost exclusively in association with the starch-rich nodal gap of the stem. Similarly, Howard and Ridout (1992) reported that the accumulation of starch in etiolated cuttings improved the rooting of *Syringa vulgaris*.

As described in Chapter 1, different research papers reporting on the relationship between carbohydrates and rooting have shown both positive and negative correlations in different plant species. However, other literature has cautioned against concluding that carbohydrates (including starch) have a regulatory role in root formation (Veierskov, 1988; Hartmann, Kester, Davies and Geneve, 1997). The relationship that has been demonstrated with the present study suggests that starch serves as an energy source for the production of new tissues and organs, such as roots.

Although the optimum starch content for rooting initiation has not been critically defined in *P. cynaroides*, the comparison between the starch contents of untreated and blanched cuttings may have shed some light on the reason why untreated *P. cynaroides* cuttings generally take long to form roots. A possible explanation may be that the starch content in *P. cynaroides* motherplants are relatively low when cuttings are taken, while the amount of starch required to provide sufficient energy source for

rooting to take place in *P. cynaroides* cuttings is high. This leads to the need of a prolonged period of time for sufficient starch to accumulate so that root formation can be initiated. On the other hand, the blanching of stems on the motherplant seems to be able to increase the accumulation of starch, which results in sufficient energy sources for the rooting of cuttings to commence at an earlier time. Struve (1981) has stated that the ability of root formation to take place is dependent on the amount of stored carbohydrates (starch) that a cutting contains at the time of severance from the motherplant. If motherplants are depleted of carbohydrates, and stem cuttings are removed to be rooted, the energy charge will be too low to support rooting (Veierskov, 1988).

As described above, numerous studies have investigated the roles of phenolic compounds and starch in rooting of cuttings. However, in the majority of these investigations, the roles which phenolics and starch play during rooting are reported separately. No literature could be found that directly links phenolic compounds and starch (or other carbohydrates). This is possibly because in different hypotheses on root formation, such as those by Bouillenne and Bouillenne-Walrand (1955), Hess (1965), Haissig (1974) and Jarvis (1986), strong links between phenolic compounds and auxins (IAA), rooting-co-factors, or rhizocaline are made, which takes place in the early phase of root induction. On the other hand, the role of starch or other carbohydrates as an energy source in rooting is usually considered at the root initiation stage. It has been proposed that carbohydrates in general may also play a role in osmoregulation, cellular solvent capacity, and other physiochemical phenomena (Haissig, 1986). Thus, there is a possibility that there may be direct interactions between phenolics and carbohydrates during root formation, whether at the early induction phase, or during the late initiation phase. The present study focused on changes in the levels of total soluble phenol concentration and total starch content during rooting of *P. cynaroides*. This approach can serve as a starting point for future investigations on possible relationships between phenols and starch.

Root formation on leached *P. cynaroides* cuttings was very poor. This is in contrast to a report by Spiegel (1954) where the rooting of grapes was improved by leaching the cuttings in water. Similarly, Vieitez (1964) observed that the normally difficult-to-root chestnut cuttings rooted when placed in running water, which suggested that the

lack of rootability of chestnuts might be due to the presence of water-soluble inhibitors. However, with *P. cynaroides* cuttings, the loss of nutrients through leaching, particularly nitrogen, indicated that the cuttings were deficient in nutrients after the leaching process, which probably reduced their rooting capacity. This resulted in poor rooting percentages and low mean root dry mass shown in Figure 5.2 and Figure 5.3, respectively. Similarly, Sharp (1955) reported in their study that large amounts of nitrogen (16%) that were leached from peach, grape and blueberry cuttings reduced their rooting capacity. According to Tukey (1962), during the process of plant growth and development, loss of important nutrients will greatly influence the plant's behaviour. Furthermore, the severity of the loss of nutrients was also exacerbated by the fact that the cuttings were semi-hardwood, which is known to be more easily leached of its nutrients during rooting than herbaceous cuttings (Good and Tukey, 1966). This has been attributed to an increased proportion of nutrients being in exchangeable forms in hardwood cuttings, whereas in herbaceous cuttings, nutrients are quickly metabolized within cells which are difficult to leach (Good and Tukey, 1966).

Another factor that may have influenced the poor rootability of the leached cuttings was the possibility that water-soluble phenolics were also leached from the cuttings. It is possible that the endogenous concentration levels of phenolic compounds required to stimulate root formation were reduced from the leaching process, thus, resulting in poor root formation. In this study, we focused on the two treatments that performed well, i.e. control and blanched cuttings. Thus, for future studies, investigations into the amount of phenolics present in the leachate will uncover more information.

Rooting hormones are commonly used to improve the rooting of cuttings. However, the results obtained in this study indicated that there was no increase in rooting percentage or root dry mass in cuttings treated with rooting hormones. The results of the rooting hormone treatment relates to information obtained from a local protea grower that the addition of rooting hormones does not improve rooting of protea cuttings in general, and therefore are not used in their cuttings (G. Bredenkamp, personal communication, 2005). A possible reason may be that the concentration (8 g kg^{-1}) of the active ingredient (IBA) used in the commercial rooting hormone (Seradix® No. 2) was not high enough to stimulate rooting in *P. cynaroides*. It is also

likely that the application method of the rooting hormone on the cuttings was not effective. The use of a rooting hormone in aqueous solution instead of powder form may result in better uptake of the active ingredient, and hence, lead to better rooting.

5.6 Conclusion

A positive correlation was found between etiolation and starch, and etiolation and total phenol content. Blanching the cutting base of stems prior to planting improved rooting percentage, root dry mass, number of roots formed by the cuttings, and accelerated the rooting process, which was probably due to a higher accumulation of starch and an increase in the total phenol content in the etiolated area of the cuttings. Treating the cuttings with rooting hormones did not improve the rooting of the cuttings. Leaching of cuttings in water led to losses of macro- and micro-nutrients, which resulted in very poor rooting of the cuttings. Results from the analyses of the leachate and cuttings showed that a large percentage of N was leached from the cuttings, while smaller amounts of P, K, Ca and Mg were leached. In addition, the possibility of phenolic compounds being leached out, which may have influenced the rootability of the cuttings, cannot be ruled out. Further studies in this regard as well as the activity of endogenous IAA and IAA-oxidase are needed.

5.7 References

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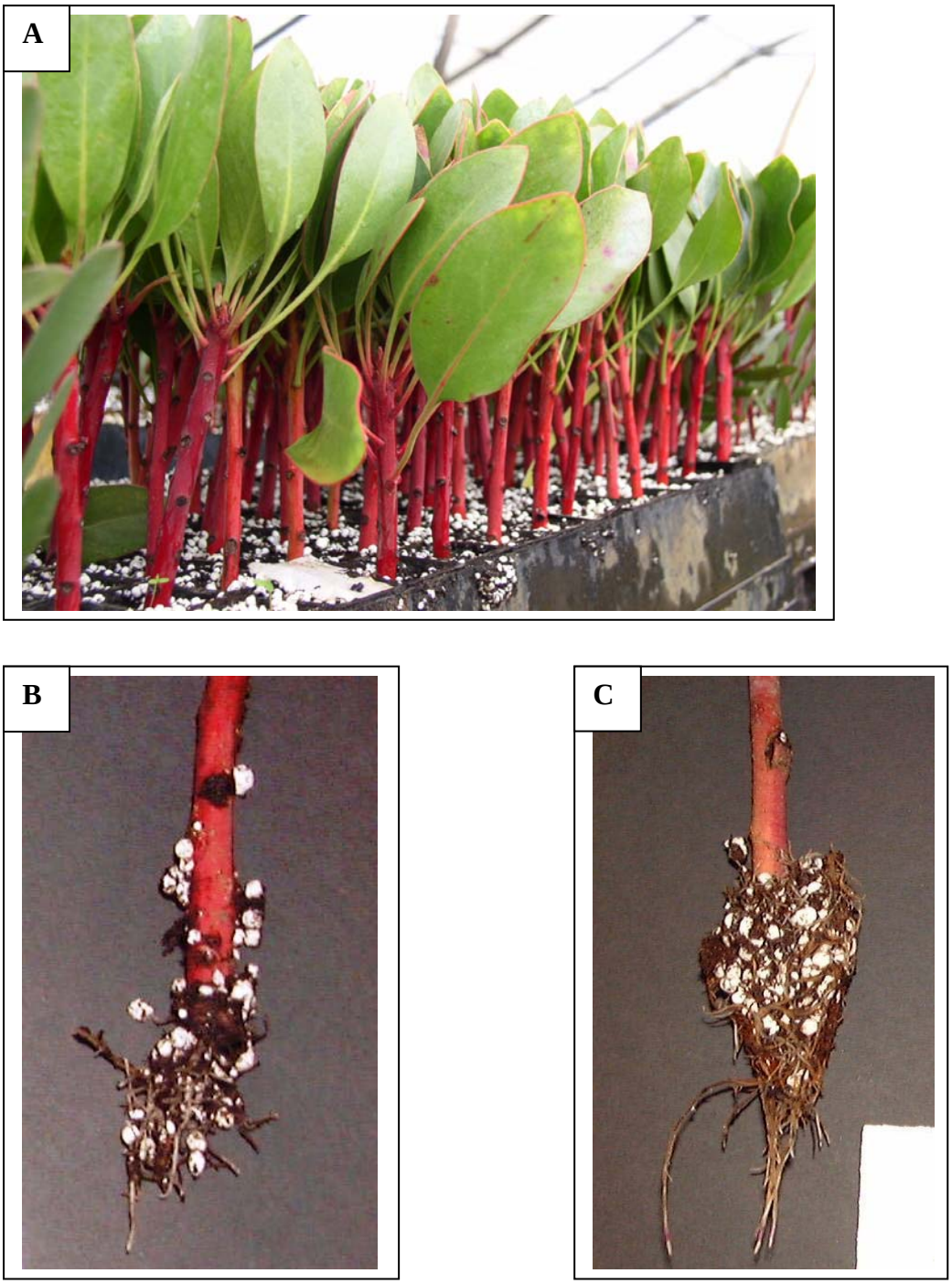

Figure 5.1. (A) Rooting of *P. cynaroides* cuttings in seedling trays placed in a mistbed; **(B)** Rooting of untreated and **(C)** blanched *P. cynaroides* cuttings after 90 days.

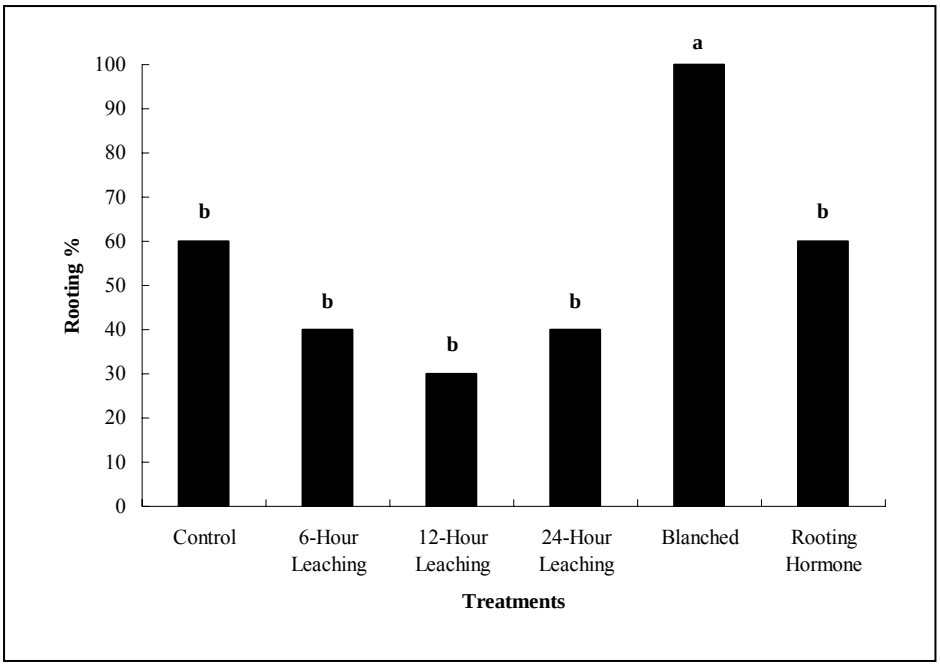

Figure 5.2. The effects of pre-treatments on the rooting % of *P. cynaroides* cuttings after 90 days. Different letters indicate significant differences at $P \leq 0.05$ according to Chi-square.

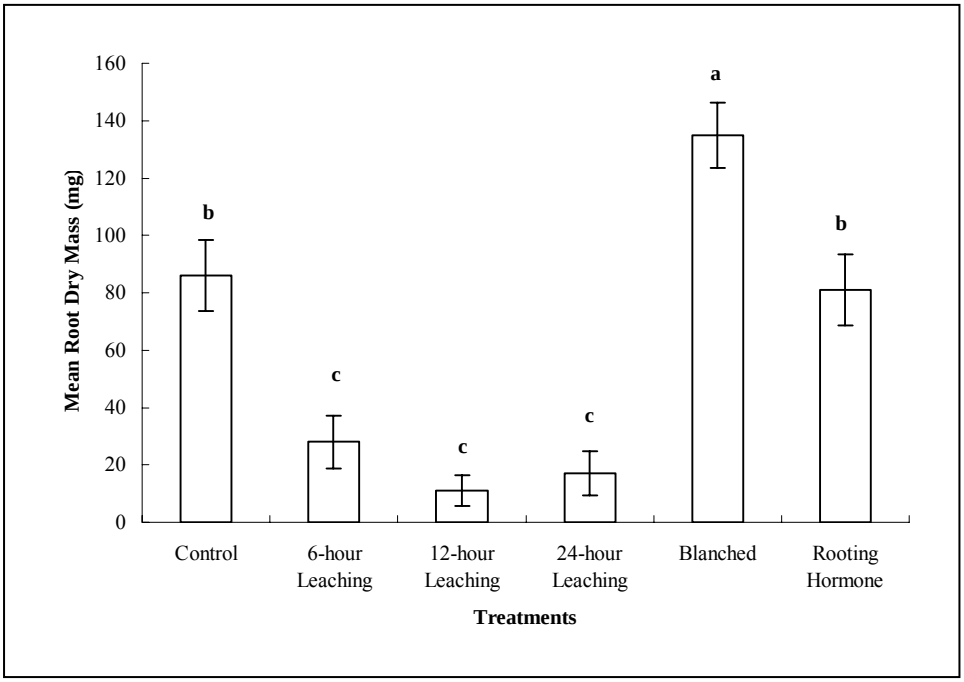

Figure 5.3. The effects of pre-treatments on the root dry mass of *P. cynaroides* cuttings after 90 days. Different letters indicate significant differences at $P \leq 0.001$ according to Tukey's studentised test. (LSD = 42.72).

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

Treatments	Mean number of roots categorized by root length					
	Group 1 (1 - 10 mm)	**Group 2 (11 - 20 mm)**	**Group 3 (21 - 30 mm)**	**Group 4 (31 - 40 mm)**	**Group 5 (41 - 50 mm)**	**Group 6 (>51 mm)**
Control	6.6 ±2.6 ab	4.8 ±2.2 b	5.8 ±3.6 b	4.0 ±0.7 a	4.4 ±2.3 b	3.0 ±1.4 b
6-hour leaching	5.6 ±1.1 b	3.6 ±1.3 bc	3.0 ±0.7 bc	2.0 ±1.2 bc	2.2 ±1.3 bc	1.6 ±0.5 bc
12-hour leaching	2.0 ±1 b	1.0 ± 0 c	2.0 ±1.2 c	1.2 ±0.4 c	0.0 ±0 c	1.0 ±0 bc
24-hour leaching	4.2 ±1.3 b	1.4 ±0.5 c	1.6 ±0.5 c	1.2 ±0.4 c	1.0 ±0 bc	0.0 ±0 c
Blanching	11.6 ±3.4 a	10.8 ±2.8 a	11.6 ±0.5 a	4.2 ±1.1 a	9.6 ±3.8 a	5.8 ±2.5 a
Rooting hormone	4.4 ±1.5 b	3.6 ± 0.9bc	4.8 ±1.1 bc	3.2 ±1.3 ab	4.4 ±0.5 b	0.0 ±0 c

Means within each column followed by different letters are significantly different at $P \leq 0.001$ according Tukey's studentised test.

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

Leaching time (Hours)	Nutrients				
	N	P	K	Ca	Mg
	% Leached				
6	34.62	1.57	1.93	2.33	1.64
12	35.53	1.57	1.26	2.33	1.91
24	20.86	3.14	0.50	2.33	1.64

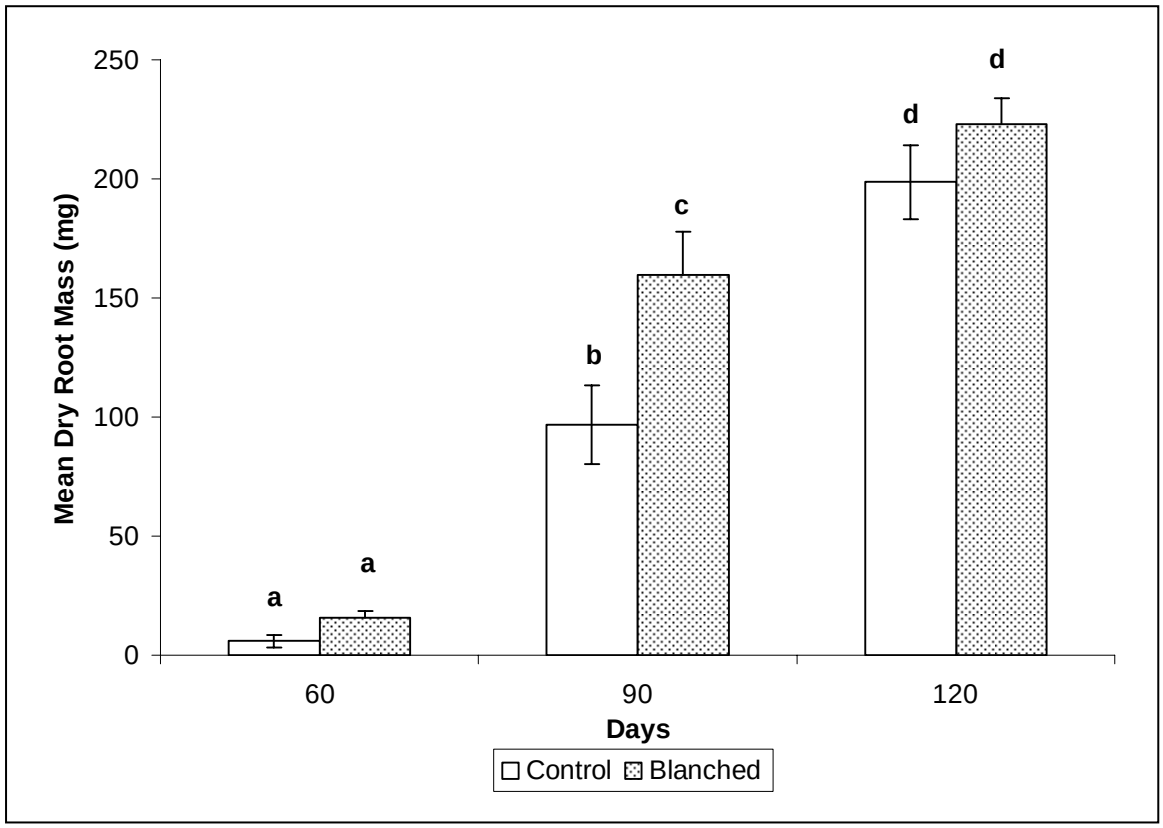

Figure 5.4. Dry root mass of cuttings during rooting period (60, 90 and 120 days after planting). Treatment means with different letters are significantly different at $P \leq 0.05$ according to Tukey's studentised test.

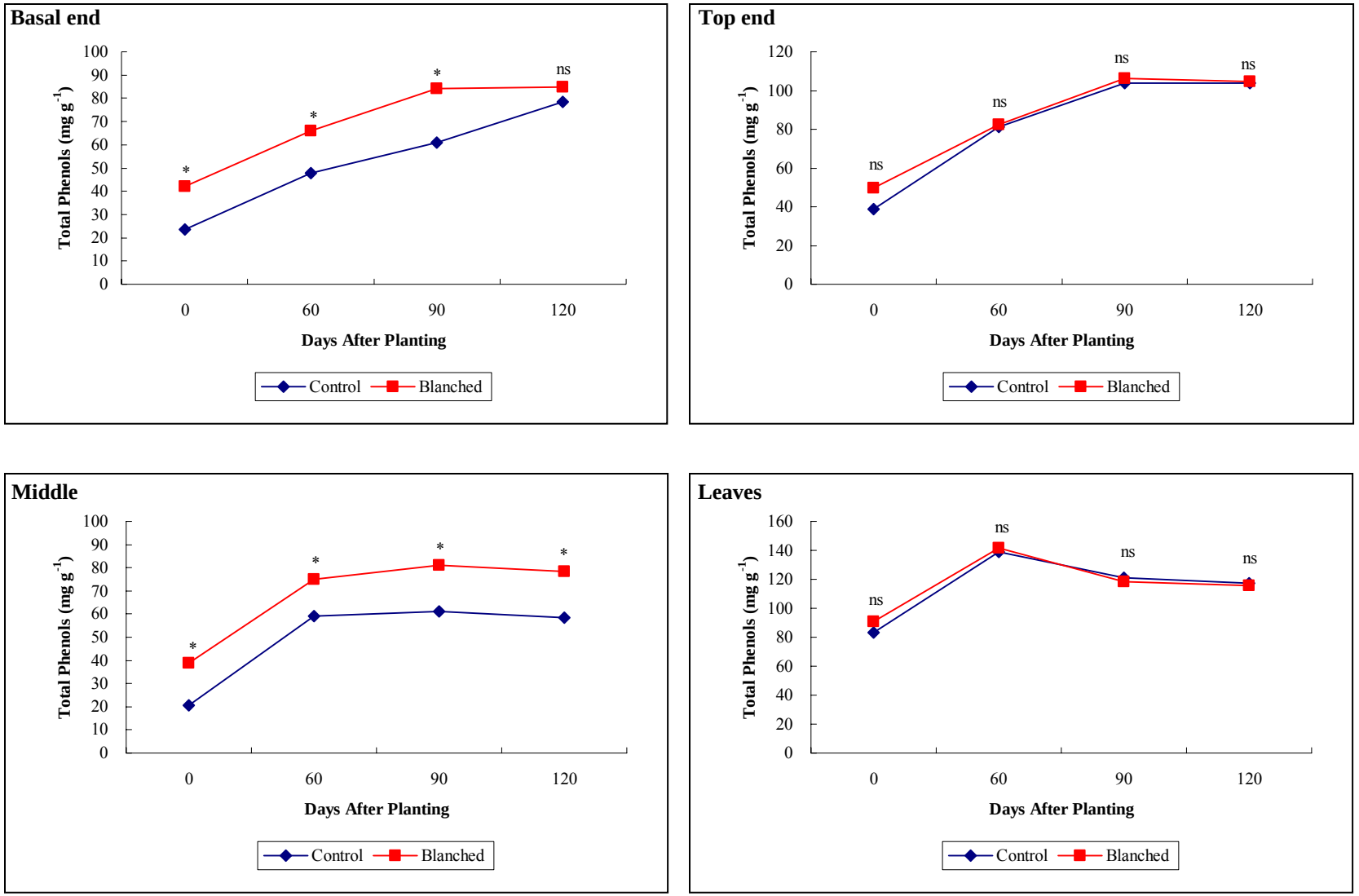

Figure 5.5. Changes in the total soluble phenolic content in different parts of *P. cynaroides* cuttings, during rooting. Means tested for significance at the same time period within each plant part according to Tukey's studentized test.
*: significant ($P \leq 0.05$); ns: not significant.

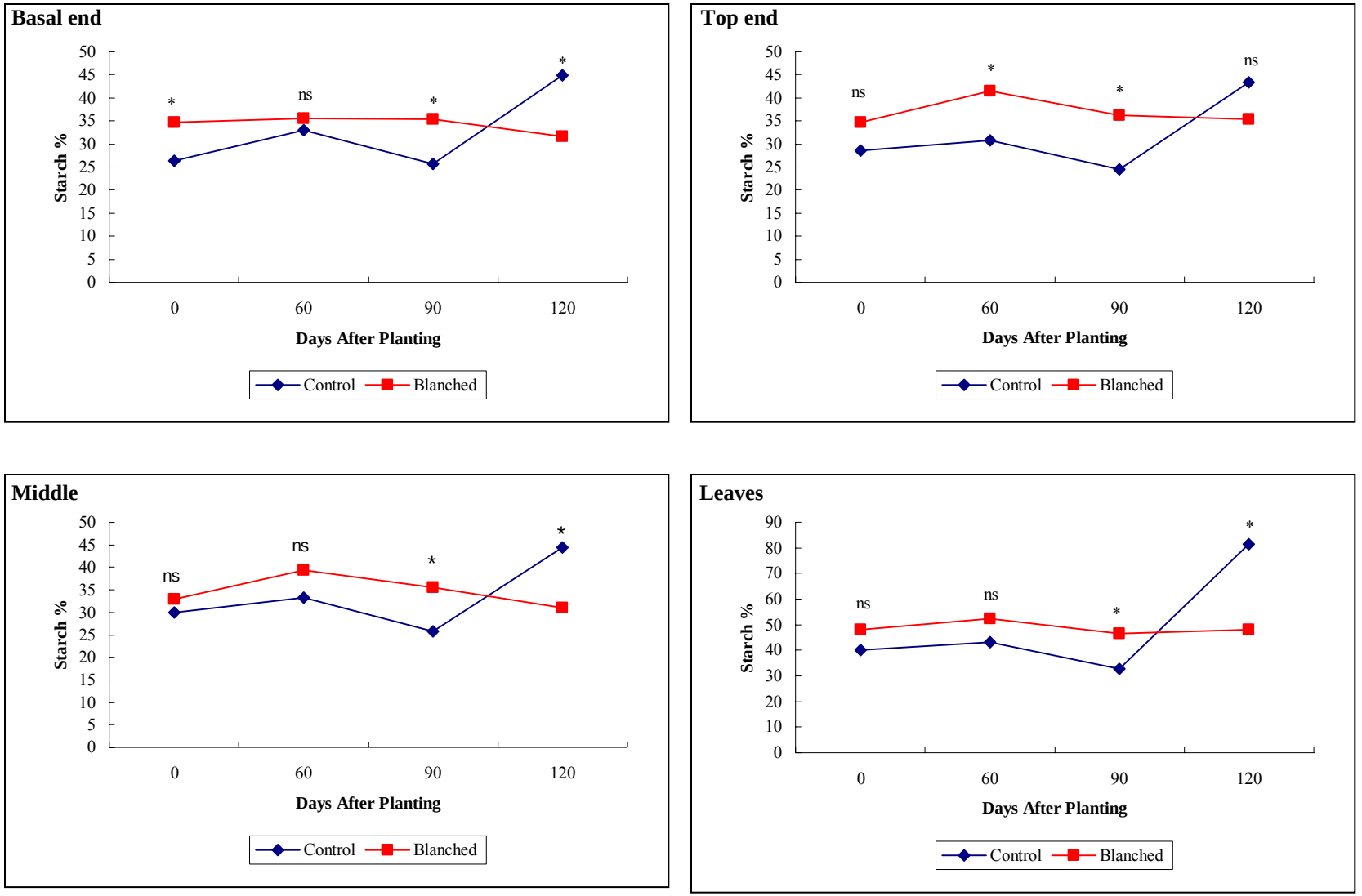

Figure 5.6. Changes in the starch content in different parts of *P. cynaroides* cuttings, during rooting. Starch percentages tested for significance at the same time period within each plant part according to Tukey's studentized test.
*: significant ($P \leq 0.05$); ns: not significant.

CHAPTER 6

ALLELOPATHIC ACTIVITY OF PHENOLICS FROM *PROTEA CYNAROIDES* STEMS, AND THEIR ROLE AS ENDOGENOUS ROOTING REGULATORS *IN VIVO*

6.1 Abstract

Allelopathy bioassay indicated the presence of allelochemicals in *Protea cynaroides* stem cuttings. Further analysis of stem extracts identified large quantities of 3,4-dihydroxybenzoic acid and other similar phenolics in the stem. Phytotoxicity bioassay showed that 3,4-dihydroxybenzoic acid both stimulated and inhibited root growth of lettuce seedlings, depending on the concentration applied. The exogenous application of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants *in vitro* stimulated root growth at 100 mg l^{-1}, but not at lower concentrations, while root inhibition was observed at toxic levels (500 mg l^{-1}). HPLC analysis of cuttings during vegetative propagation showed a considerable increase in 3,4-dihydroxybenzoic acid levels from initial planting to when root formation took place, indicating that 3,4-dihydroxybenzoic acid may be an important phenolic compound in regulating root formation in *P. cynaroides* cuttings. HPLC analysis also identified caffeic, ferulic, gallic and salicylic acids in the cuttings.

6.2 Introduction

The quantification of total phenols in cuttings was reported in Chapter 5, which showed that the total phenol levels in *P. cynaroides* cuttings increased during root formation. In addition, the results showed that rooting took place when the total phenol content increased to more than three times the original level in untreated cuttings. Although very few studies have analyzed the phenolic contents of commercially important proteas, several phenolic compounds have been extracted and identified from *Protea rubropilosa* (Perold, Beylis and Howard, 1973), *Protea obtusifolia, Protea eximia* and *Protea neriifolia* (Verotta, Orsini, Pelizzoni, Torri and

Rogers, 1999). However, no research has been done on the effects of phenolic compounds on the rooting of proteas.

Phenolic compounds such as caffeic acid, ferulic acid, protocatechuic acid, *p*-hydrobenzoic acid and vanillic acid have all been identified as potential allelopathic agents (Einhellig, 2004). Dose-response data often show, depending on the concentration, both inhibitory and promotory effects on root growth of test species. Concentrations which exhibit root stimulation are usually a narrow band of doses at low levels, while root inhibition is found at higher concentrations.

In addition to the roles phenolic compounds play in allelopathy, they have also been identified as endogenous promoters and inhibitors of adventitious rooting. Their effects on the rooting of cuttings have been studied in numerous plant species, particularly in difficult-to-root plants. Various studies on this subject suggest that difficult-to-root stem cuttings tend to contain higher amounts of endogenous rooting inhibitors, which inhibits or delays root formation, compared to easy-to-root stems which have high promotory activity due to the presence of rooting promoters (Fadl and Hartmann, 1967; Richards, 1964; Taylor and Odon, 1970; Biran and Halevy, 1973; Reuveni and Adato, 1974). Furthermore, a number of known phenolic compounds such as catechol (Hackett, 1970), chlorogenic acid (Hammerschlag, 1982), phloroglucinol (James and Thurbon, 1981; Zimmerman, 1984) and phloretic acid (Jones and Hatfield, 1976) have been used to stimulate root formation, while rutin and tannic acid (Still, Dirr and Gartner, 1976) inhibit root formation in cuttings.

The poor rooting of protea cuttings has often been linked to them being hardwood plants, and therefore, inherently difficult to root. However, as mentioned above, because phenolics have been found to be important in the rooting of numerous other plant species, it is likely that phenolic compounds may indeed play a significant role as endogenous rooting regulators in *P. cynaroides*. The main objective of this study was to determine whether phenolic compounds found in *P. cynaroides* stems are the causal factors of rooting inhibition and/or stimulation. The phytotoxicity of aqueous stem extracts from *P. cynaroides* was assessed on lettuce seeds in a dose-response bioassay. Chemical analysis of compounds contained in stem extracts was done to establish the identity of phenolic compounds, and to determine if and to what extent

they may contribute to rooting inhibition and/or stimulation during *in vitro* and *in vivo* propagation.

6.3 Materials and methods

6.3.1 Collection of plant material

Plant material was obtained during autumn from *P. cynaroides* motherplants grown in an open field near Cullinan (25º40'32S; 28º31'20E; Altitude 1482 metres), situated in the Highveld region (summer rainfall) of South Africa. Semi-hardwood stems from the current season's growth, which were suitable to be used as cuttings, were removed from the motherplants and used for the bioassay studies, phenolic compound analyses and *in vivo* propagation.

6.3.2 Lettuce seed bioassay of stem extract

A dilution series of crude aqueous stem extract was prepared by soaking *P. cynaroides* stems in one-litre of distilled water for 24 hours in the dark at room temperature. Ten lettuce (*Lactuca sativa*) cv. 'Great Lakes' seeds were evenly spread on filter paper (Whatman No. 1) lining 9-cm Petri dishes. Five ml of the stem extracts, ranging from 200 to 5000 mg l^{-1}, were added to each Petri dish. Distilled water was used as the control. Each extract solution was replicated five times. After sealing the Petri dishes with Parafilm® to prevent moisture loss, they were placed in a growth chamber. The temperature of the growth chamber was kept at 25±2°C. Cool, white fluorescent tubes provided 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR) with a 12-hour photoperiod. After 6 days, the root lengths of germinated seedlings were measured and the mean root length from each treatment was calculated. A dose-response curve was drawn using the Curve-Fitting to Allelochemical Response Data (CARD) program (Liu, An, Johnson and Lovett, 2003).

6.3.3 Osmotic interference

The determination of osmotic potential of the extracts was done to exclude the possibility of osmotic inhibition in the bioassay, and to ensure that it is indeed the allelochemicals in the extract causing a reduction in the root growth of test species. The osmotic potentials of the stem extracts were measured with a Roebling digital micro-osmometer (Bothma, 2002).

6.3.4 Extraction, separation, isolation and characterization procedures

Fresh stems (100 g) of *P. cynaroides* were homogenized in 70% aqueous acetone for 6 hours. The extraction solution was then placed into a flask and dried under vacuum by using a Rotavapour (BUCHI® Rotavapor R-114) and a waterbath (BUCHI® Waterbath B-480). The fraction (2995 mg) was dissolved in water and submitted to a Diaion HP 20 chromatographic column (2.5 cm i.d. x 29 cm length). Elution was performed in a step-wise gradient of water:MeOH (100:0, 90:10, 80:20, 70:30, 60:40, 50:50. 40:60, 30:70, 20:80, 90:10), collecting 24 fractions of 100 ml and monitoring them by UV spectrophotometry (Pharmacia LKB Ultrospec III spectrophotometer) at 280 nm. A chromatogram was built from this data (Figure 6.1) and those fractions giving a chromatographic peak were pooled and concentrated in vacuum.

The combined fraction of the main peak (Fractions 16 – 20) was sent to the Natural Products Utilization Research Unit, USDA, USA, for separation and purification. Peaks were separated by high performance flash chromatography (HPFC) on a Horizon High Performance Flash Chromatography system (Biotage, Inc., Charlottesville, VA), using a reversed phase (12+ M C18, Biotage) column with the solvent gradient program set from 5% methanol to 45% methanol over 48 tubes (3 ml per tube), and finally washing the column with 100% methanol. The fractions were spotted on a reversed phase thin layer chromatography (TLC) plate (RP-18 F_{254s}, 10 x 20 cm, EM Science, Gibbstown, NJ) with developing solvent 20% methanol : 80% water. Spots were detected by observing under UV at 254 nm. Fractions with similar spots were combined into four semi-crude fractions: Fractions 1, 2, 3 and 4. Fraction 2 was further subjected to purification on RP-18 plate using developing solvent 15%

methanol:85% water. Four bands were collected from the plate. From these four bands, the one that was in the largest quantity, named Compound 1 (9.4 mg), was subjected to high performance liquid chromatography (HPLC) to confirm its purification, after which it was analyzed in the nuclear magnetic resonance (NMR) and mass spectrophotometer (MS) for characterization.

NMR data

The compound was dissolved in 0.5 ml of CD3OD. The proton spectra were acquired on a 200MHz Varian Mercury PLUS spectrometer at 199.97 MHz and Carbon 13 on 50.28 MHz.

MS data

The molecular mass of the compound was determined on a Shimadzu 2010EV spectrometer. For ionization, an atmospheric pressure chemical ionisation probe was used. The sample was dissolved in methanol and directly injected into the probe.

6.3.5 Lettuce seed bioassay of 3,4-dihydroxybenzoic acid

3,4-Dihydroxybenzoic acid was purchased from Merck®. A dilution series was prepared, ranging from 1 to 500 mg l^{-1}. Lettuce (*L. sativa*) cv. 'Great Lakes' was used as test species. The experimental design and growth conditions were the same as described in section 6.3.2. A dose-response curve was generated from the data using the CARD program (Liu *et al.*, 2003).

6.3.6 *In vitro* bioassay of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants

A concentration range similar to the one used in the allelopathic bioassay above (6.3.5) was incorporated into Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) to determine the effect of 3,4-dihydroxybenzoic acid on *in vitro* root growth of *P. cynaroides* seedlings. The MS medium consisted of sucrose (3%), 3 g l^{-1} Gelrite® and the respective concentrations of 3,4-dihydroxybenzoic acid. The pH of the medium was adjusted to 5.7 before autoclaving. In order to determine whether

3,4-dihydroxybenzoic acid was degraded during the medium sterilization procedure (autoclaving), 3,4-dihydroxybenzoic acid was autoclaved and analyzed in the HPLC.

Thirty-day old *in vitro*-germinated seedlings (sterilization and excision method described in Chapter 2) had their radicles removed at the hypocotyl and placed in the growth medium containing the different concentrations of 3,4-dihydroxybenzoic acid. Twenty explants per treatment medium were used, which were cultured in a growth chamber under a 12-hour photoperiod illuminated with cool, white fluorescent tubes providing 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR). The temperature of the growth chamber was adjusted to 25±2ºC.

6.3.7 Analysis of phenolic compounds by HPLC

Phenolic compound analyses were done on semi-hardwood stems taken from the motherplant, which were suitable to be used as cuttings, and on those that had been planted in the mistbed and rooted after 90 and 120 days. Phenolic compounds were analyzed using a HPLC (Hewlett Packard Agilent 1100 series) with DAD detection (diode array detector, 280, 325, 340 nm). A Luna 3u C-18 (Phenomenex®) reverse phase column (250 mm by 4.6 mm) was used. A gradient elution was performed with water (pH 2.6 adjusted with H_3PO_4) and acetonitrile (ACN) as follows: 0 min, 7% ACN; 0 – 20 min, 20% ACN; 20 – 28 min, 23% ACN; 28 – 40 min, 27%, ACN; 40 – 45 min, 29%, ACN; 45 – 47 min, 33%, ACN. The flow rate was 1 ml min^{-1}, and the injection volume was 20 µl. Identification of the phenolic compounds was done by comparing their retention times and UV apex spectrum to those of standards (purchased from Sigma Chemical Company, USA), which included syringic, gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, ferulic, caffeic, and chlorogenic acids. The amount of each identified compound was expressed in µg mg^{-1} of dry sample.

6.3.8 Statistical analysis

In the *in vitro* study on the effects of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants' root length and root fresh mass, twenty uniform explants were used in each medium treatment. Tukey's Studentized test was used to compare treatment means.

Statistical analyses were done in the SAS program (SAS Institute Inc, 1996). ANOVA are shown in Tables C44 and Table C45, Appendix C.

6.4 Results

6.4.1 Osmotic potential of stem extracts

Results from the osmometer readings showed that the osmolalities of the aqueous stem extracts ranged from 0 to 11 mOsmol.kg^{-1}. Data from experiments with polyethylene glycol (PEG-6000) solutions of increasing osmolality showed that no osmotic interference of lettuce seed germination or root growth occurred up to 53 mOsmol.kg^{-1} (Bothma, 2002). Therefore, the results for the stem extract concentrations which were used in the lettuce seed bioassay are considered to be indicative of allelopathic effects, and not distorted by osmotic effects.

6.4.2 Bioassay of stem extract

Results shown in Figure 6.2 illustrate a typical dose-response curve where aqueous solutions prepared from the stem crude extracts were able to both promote and inhibit rooting, depending on the extract concentration. At lower concentrations (200 - 800 mg l^{-1}), lettuce root growth was stimulated, while at higher concentrations of between 1000 mg l^{-1} and 5000 mg l^{-1}, reduction in root growth was observed. At the 400 mg l^{-1} stem extract solution, stimulation of root growth was the highest, which was 28% higher than the control. Although total inhibition was not observed, strong root inhibition was evident at the highest concentration (5000 mg l^{-1}).

6.4.3 Extraction, separation, isolation and characterization

Compound 1 was identified as 3,4-Dihydroxybenzoic acid (Protocatechuic acid): APCI-MS *m/z*: 153 $[M - H]^-$; ^{1}H NMR (CD_3OD, 200MHz): 6.75 (H5, 1H, d, J = 8.0 Hz, H-5), 7.39 (H6, 1H, dd, J = 2.0, 8.0 Hz, H-6), 7.43 (H2 1H, d, J = 2.0 Hz, H-2); ^{13}C NMR (CD_3OD, 200MHz): 115.7 (C-5), 117.7 (C-2), 123.9 (C-6), 146.03 (C-4), 151.52 (C-3), 170.28 (C-1).

The NMR spectral analysis of 3,4-dihydroxybenzoic acid, and its chemical structure are shown in Figure 6.3 and Figure 6.4, respectively.

6.4.4 Bioassay of 3,4-dihydroxybenzoic acid

Figure 6.5 illustrates the response of lettuce root growth to a concentration series of 3,4-dihydroxybenzoic acid. The effects of 3,4-dihydroxybenzoic acid on the lettuce root growth were typical of an allelochemical, with stimulation at low concentrations and inhibition at high concentrations. The highest stimulation was recorded at 100 mg l^{-1}, where the mean root length was 23% longer than the control. The ED_{50} value of 3,4-dihydroxybenzoic acid for lettuce, which was calculated from the CARD program (dose-response curve), was 339.1 mg l^{-1}. The ED_{50} value represents the effective dosage of 3,4-dihydroxybenzoic acid to cause 50% inhibition in lettuce root growth. The sharp drop in the dose-response curve from the concentration of 200 mg l^{-1} onwards indicates the contrasting activity of root stimulation and inhibition within a relatively narrow concentration range of between 100 mg l^{-1} and 200 mg l^{-1} (Figure 6.5).

6.4.5 Effects of 3,4-dihydroxybenzoic acid on *P. cynaroides in vitro*

Results from the HPLC analysis showed that the concentration of 3,4-dihydroxybenzoic acid remained unchanged after autoclaving, which indicated that the compound was not affected by high temperatures during the sterilization procedure. Table 6.1 shows the effects of 3,4-dihydroxybenzoic acid on the rooting of rootless seedlings after 3 weeks in culture. High biological variation was noted in the *P. cynaroides* explants, where the rooting percentage was relatively low and inconsistent throughout the concentration range. The inherent variation of *P. cynaroides* may have partly contributed to the differences in response to particular treatments. The other factor which contributed to the low rooting percentage recorded was the exclusion of roots which were less than 3 mm in length. These roots were extremely thin and barely visible to the naked eye, and explants with these roots were not considered rooted.

Despite the low rooting percentages, the amount of rooting by the *P. cynaroides* explants was affected by the concentration of 3,4-dihydroxybenzoic acid (Table 6.1). The response of the explants to 3,4-dihydroxybenzoic acid at the lower concentration range between 1 and 50 mg l^{-1} is noteworthy in that non-observable effects were obtained, i.e., root growth was similar to the control in terms of mean root length and root mass. However, an increase in mean root length and root mass was noticeable from 75 mg l^{-1}, even though the mean root length and root mass was not significantly higher (Table 6.1). Of importance was that the roots which formed on explants cultured on medium supplemented with 75 and 100 mg l^{-1} of 3,4-dihydroxybenzoic acid, were visibly thicker and fleshier (Figure 6.6). This was particularly evident at the 100 mg l^{-1} concentration, which coupled with longer roots, resulted in significantly higher mean root mass. Coincidentally, at the concentration level in which root growth was stimulated (100 mg l^{-1}), root stimulation was also observed in the lettuce bioassay.

Although a few explants produced roots in the medium containing the highest concentration (500 mg l^{-1}) of 3,4-dihydroxybenzoic acid, it was clear that the concentration was toxic to the explants. Rapid browning of cut surfaces of the explants was observed immediately after planting, which soon spread to other parts of the explant (Figure 6.6). This browning effect is also often seen when explants containing high phenol contents are cultured *in vitro*, where large amounts of phenols are leached from the explants and inhibit the formation of roots and ultimately causes the death of the explant (Debergh and Read, 1991; George, 1993).

6.4.6 Analysis of phenolic compounds by HPLC

From the HPLC analysis, 3,4-dihydroxybenzoic acid, caffeic, ferulic, gallic and salicylic acids were identified (Figure 6.7). In the stems taken from the motherplant, 3,4-dihydroxybenzoic acid (12.2 µg g^{-1}), caffeic (11 µg g^{-1}) and salicylic acids (32 µg g^{-1}) were found in relatively low amounts, while traces of ferulic and gallic acids were also detected. However, in the analyses of the basal end of cuttings taken from the mistbed when rooting began (after 90 days), high quantities of 3,4-dihydroxybenzoic acid was found (180.2 µg g^{-1}), which remained at a similar level after 120 days (188

µg g^{-1}) (Figure 6.7). At the same time, the levels of caffeic acid (28.6 µg g^{-1}) and salicylic acid (16.8 µg g^{-1}) remained low during the same time period.

Furthermore, from the results of the *in vitro* propagation study (6.4.5) and this study, a link between the concentration levels of 3,4-dihydroxybenzoic acid and root formation can be made. The concentration level (100 mg l^{-1} = 100 ppm) of 3,4-dihydroxybenzoic acid at which root stimulation of the *P. cynaroides* explants was observed, corresponded with the amount of 3,4-dihydroxybenzoic acid (180.2 µg g^{-1} = 180.2 ppm) found in the stem cuttings during rooting in the mistbed. It can therefore be deduced that once the concentration level of 3,4-dihydroxybenzoic acid in cuttings reached 100 mg l^{-1} or more, root formation was stimulated. In the *in vitro* propagation study, although the exact concentration between 100 mg l^{-1} and 500 mg l^{-1} where root stimulation would have ceased and inhibition commenced is not known, nevertheless, from the results for cuttings in the mistbed, it can be assumed that up to at least 180 mg l^{-1}, root formation would still be stimulated.

6.5 Discussion

Numerous papers have reported that phenolic compounds are both promoters and inhibitors of root formation, based on results of dose-response bioassays (Haissig, 1974; Still, Dirr and Gartner, 1976; Kling and Meyer, 1983). Based on results from the initial lettuce seed bioassay, where *P. cynaroides* crude stem extracts were used, root stimulation and inhibition occurred (Figure 6.1). Through further analyses of *P. cynaroides* stems, the phenolic allelochemical 3,4-dihydroxybenzoic acid was identified. Its identification was confirmed by comparing NMR and MS data with those reported in literature (Sang, Lapsley, Jeong, Lachance, Ho and Rosen, 2002), as well as with a reference standard. In total, twenty-eight fractions were isolated, of which the majority was found in very small amounts. Furthermore, NMR data showed that several of these fractions contained compounds which were similar in structure, and therefore, were derivatives of 3,4-dihydroxybenzoic acid, indicating that phenolics found in the stems of *P. cynaroides* are made up mostly of 3,4-dihydroxybenzoic acid and related compounds.

3,4-Dihydroxybenzoic acid is a common and widespread allelopathic agent that is able to influence growth at various stages of plant development (Rice, 1984). 3,4-Dihydroxybenzoic acid and other phenolic compounds have also been isolated from plant species such as *Arctostaphylos glandulosa* (Chou and Muller, 1972), *Chrysanthemum morifolium* (Kil and Lee, 1987), *Pennisetum clandestinum* and *Cunninghamia lanceolata* (Chou, Hwang and Peng, 1987), *Rumex japonicus* (Elzaawely, Xuan and Tawata, 2005) and *Vulpia myuros* (An, Haig and Pratley, 2000). Allelopathic bioassays of phenolics in these reports showed phytotoxicity to root growth. In addition, other chemically-related compounds such as 2,5-dihydroxybenzoic acid have also been reported to affect the rooting of *Tilia americana* (Morsink and Smith, 1975). Furthermore, from the report by Bär, Pfeifer and Dettner (1997), 3,4-dihydroxybenzoic acid extracted from three *Kalanchoe* spp. had non-observable effects at lower concentrations on the root growth of *Kalanchoe* cuttings, while root growth was inhibited at higher levels.

With regard to ED_{50} value, it is known that differences in the sensitivity of receiving plant species are keys to the inherent phytotoxicity of allelochemicals (Einhellig, 2004). Although no research paper could be found that reported the ED_{50} value of 3,4-dihydroxybenzoic acid on lettuce seeds, dose-response bioassays of other phytotoxins revealed that lettuce was less sensitive than other test species. For instance, with parthenin, the ED_{50} value for lettuce roots was four times higher than the dose level necessary to give the same response on the most sensitive species *Ageratum conyzoides* (Belz, Reinhardt, Foxcroft and Hurle, 2006). Thus, the effective dosage of 3,4-dihydroxybenzoic acid can be even more clearly shown when test species of different sensitivities are used. Further studies in this regard are necessary to allow comparisons between the effective dosages of 3,4-dihydroxybenzoic acid and other phytotoxins.

Results of the *in vitro* study further illustrated the effects of 3,4-dihydroxybenzoic acid on the root growth of plant species. No observable effects on the rooting of *P. cynaroides* were found at the lower concentrations, while stimulation of root growth was apparent when the concentration reached 100 mg l^{-1}. Although the exact concentration range of root stimulation is not known, it is clear that at 500 mg l^{-1}, 3,4-dihydroxybenzoic acid was toxic to the *P. cynaroides* explants, which was

demonstrated by root inhibition and browning of explants. Few studies have used *in vitro* conditions to determine the effects of plant-extracted phenolics on root growth. Nevertheless, Mucciarelli, Scannerini, Gallino and Maffei (2000) reported that root growth of *Nicotiana tabacum* explants, which were cultured on MS medium containing 3,4-dihydroxybenzoic acid, was also stimulated at low concentrations, while root inhibition was observed at the higher concentration level. In addition, several other phenolic compounds such as phloretic acid, phloroglucinol and chlorogenic acid are often incorporated into growth media at specific concentrations to induce *in vitro* rooting of explants as a substitute to conventional rooting hormones. This further demonstrates the importance of phenolics compounds, in general, in the propagation of plants.

Considerable differences in the level of 3,4-dihydroxybenzoic acid were found between stems taken from the motherplant and the basal ends of rooted cuttings in the mistbed after 90 days and 120 days. This indicated that the endogenous levels of 3,4-dihydroxybenzoic acid of the cuttings increased after they were planted, and that this high level was maintained during the rooting period. Although no reports have shown changes in endogenous levels of 3,4-dihydroxybenzoic acid during rooting of cuttings, Pellissier (1994) reported that root formation was stimulated by higher concentrations of 3,4-dihydroxybenzoic acid in *Picea abies*. Furthermore, the presence of other phenolic compounds such as caffeic, ferulic, gallic and salicylic acids, which were detected by HPLC analysis, is noteworthy. It is likely that the presence of these phenolics in the cuttings played a role in promoting rooting, since numerous allelopathy research papers have also identified caffeic, ferulic and gallic acids together with 3,4-dihydrixybenzoic acid in plants such as *C. lanceolata* (Zhiqun, Haig, Silong and Sijie, 2002), *Phytolacca* spp. (Kim, Johnson and Lee, 2005) and *R. japonicus* (Elzaawely *et al*, 2005). Furthermore, in the *C. lanceolata* extracts, which contained 3,4-dihydrixybenzoic acid, ferulic acid and gallic acid, stimulation of root growth of test species at high concentrations was observed (Zhiqun *et al*, 2002). Moreover, salicylic acid was also found to be stimulatory to root initiation of *Phaseolus aureus* cuttings (Kling and Meyer, 1983). It can therefore be assumed that this group of phenolic compounds has an important role to play in regulating root formation, particularly in terms of stimulation.

It is well known that several compounds usually act together in controlling many functions of a plant, including root formation. However, based on the relatively large amounts of 3,4-dihydroxybenzoic acid found in the stem extract, and on the stimulatory effects on root growth in *P. cynaroides* explants *in vitro*, as well as on the high endogenous levels of the compound in cuttings during rooting in the mistbed, it is proposed that 3,4-dihydroxybenzoic acid could play a primary role in root formation, depending largely on its endogenous concentration.

6.6 Conclusion

The current findings showed that a crude aqueous extract containing phenolic compounds from *P. cynaroides* stem cuttings exhibited allelopathic activity against root growth of lettuce. Chemical analyses revealed the presence of 3,4-dihydroxybenzoic acid in these stem cuttings. In addition, results from the dose-response bioassay and the *in vitro* experiment showed that 3,4-dihydroxybenzoic acid could either promote or inhibit rooting depending on its concentration. Large increases of 3,4-dihydroxybenzoic acid levels in cuttings during vegetative propagation suggests that root formation might be dependent on its endogenous levels. It was established that, in terms of the amount of 3,4-dihydroxybenzoic acid required for root formation to be stimulated, root formation took place when relatively high concentration levels (100 – 180 ppm) were reached. The results of this study provide a better understanding of how phenolic compounds might affect the rooting of *P. cynaroides* cuttings. Further investigations are needed to determine the full extent of the influence of 3,4-dihydroxybenzoic acid and other phenolics on root formation.

6.7 References

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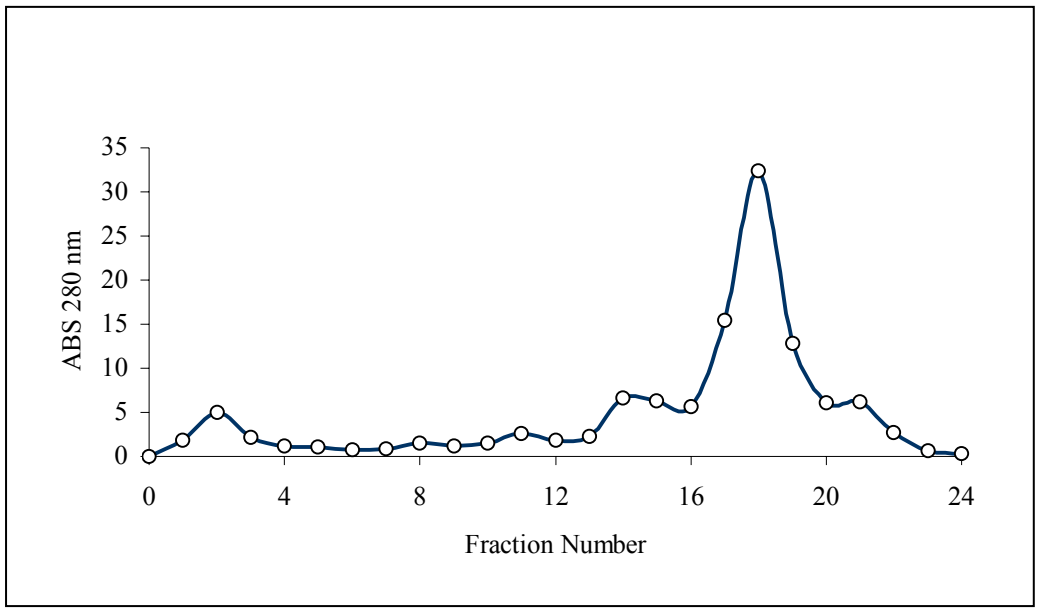

Figure 6.1. Column chromatography of 70% aqueous acetone extract from *P. cynaroides.*

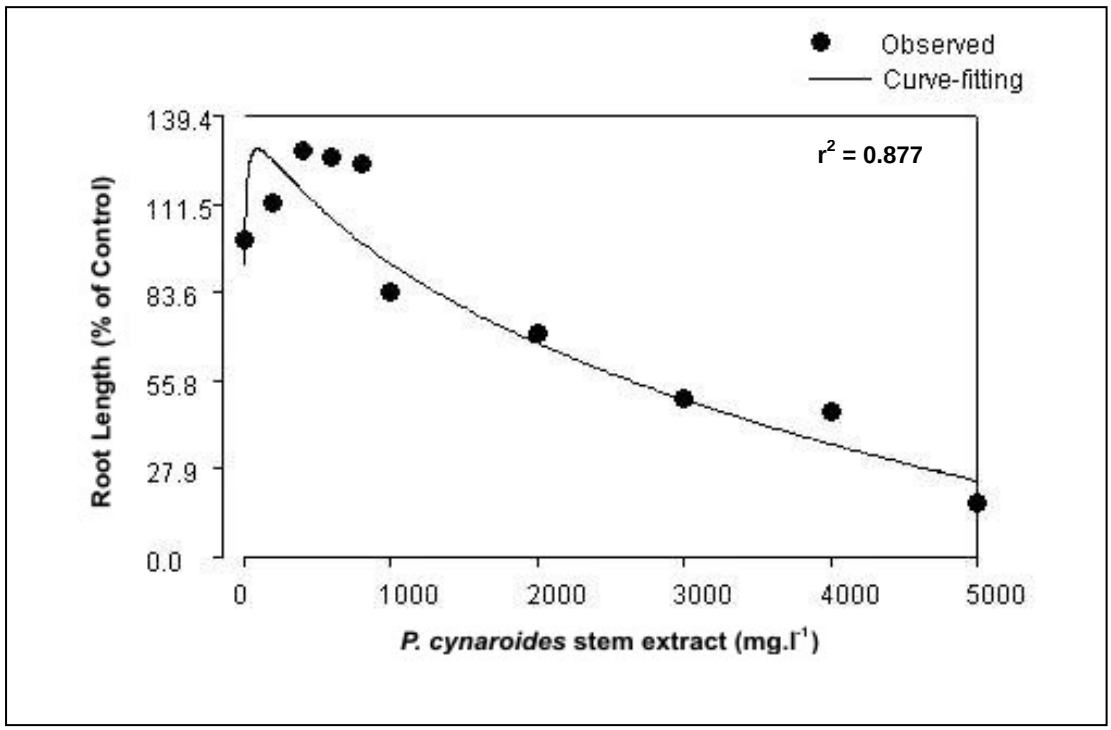

Figure 6.2. Dose-response of stem extract on lettuce root growth.

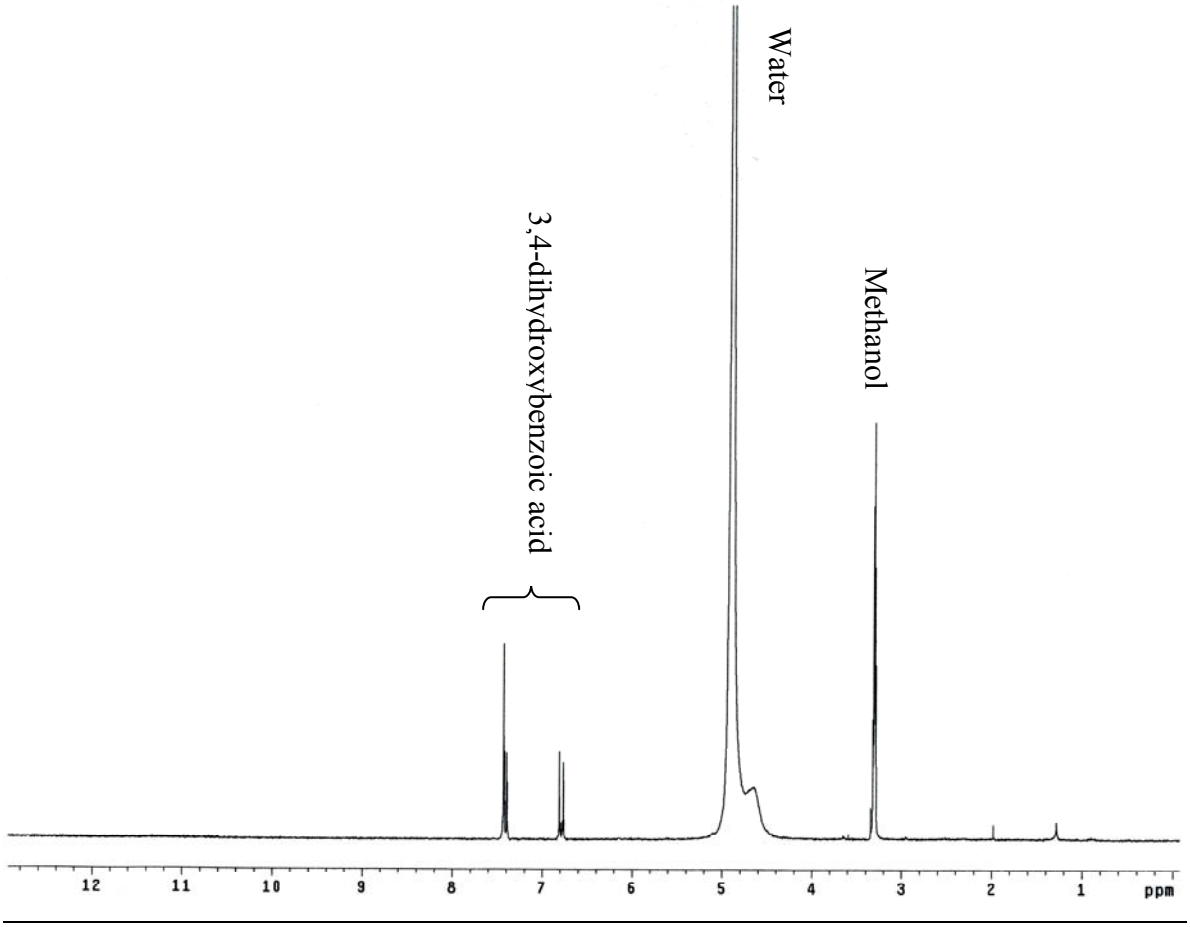

Figure 6.3. NMR spectral analysis of Compound 1 showing pure 3,4-dihydroxybenzoic acid.

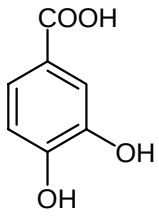

Figure 6.4. Chemical structure of 3,4-dihydroxybenzoic acid.

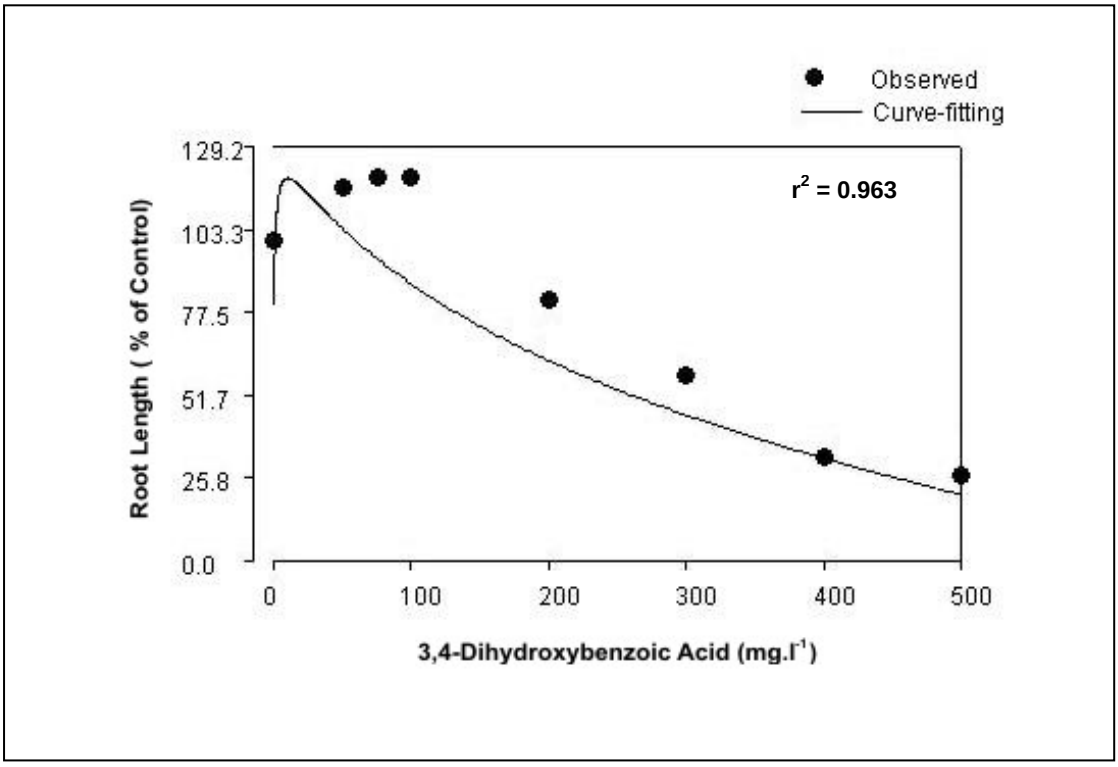

Figure 6.5. Dose-response of 3,4-dihydroxybenzoic acid on the root growth of lettuce seedlings.

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

MS medium + 3,4-dihydroxybenzoic acid (mg l^{-1})	Rooting %	Mean root length (mm)	Mean root fresh mass (mg)
0	40	5.5 ± 0.93 ab	0.55 ± 0.17 b
1	40	5.25 ± 0.46 ab	0.58 ± 0.15 b
5	30	5.0 ± 0 ab	0.50 ± 0.06 b
25	10	5.0 ± 0 ab	0.55 ± 0.07 b
50	30	5.3 ± 0.52 ab	0.57 ± 0.08 b
75	20	5.75 ± 0.5 ab	0.88 ± 0.15 b
100	50	7.76 ± 2.79 a	2.66 ± 1.40 a
500	20	3 ± 0 b	0.3 ± 0.00 b

Means in each column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's studentised test.

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).

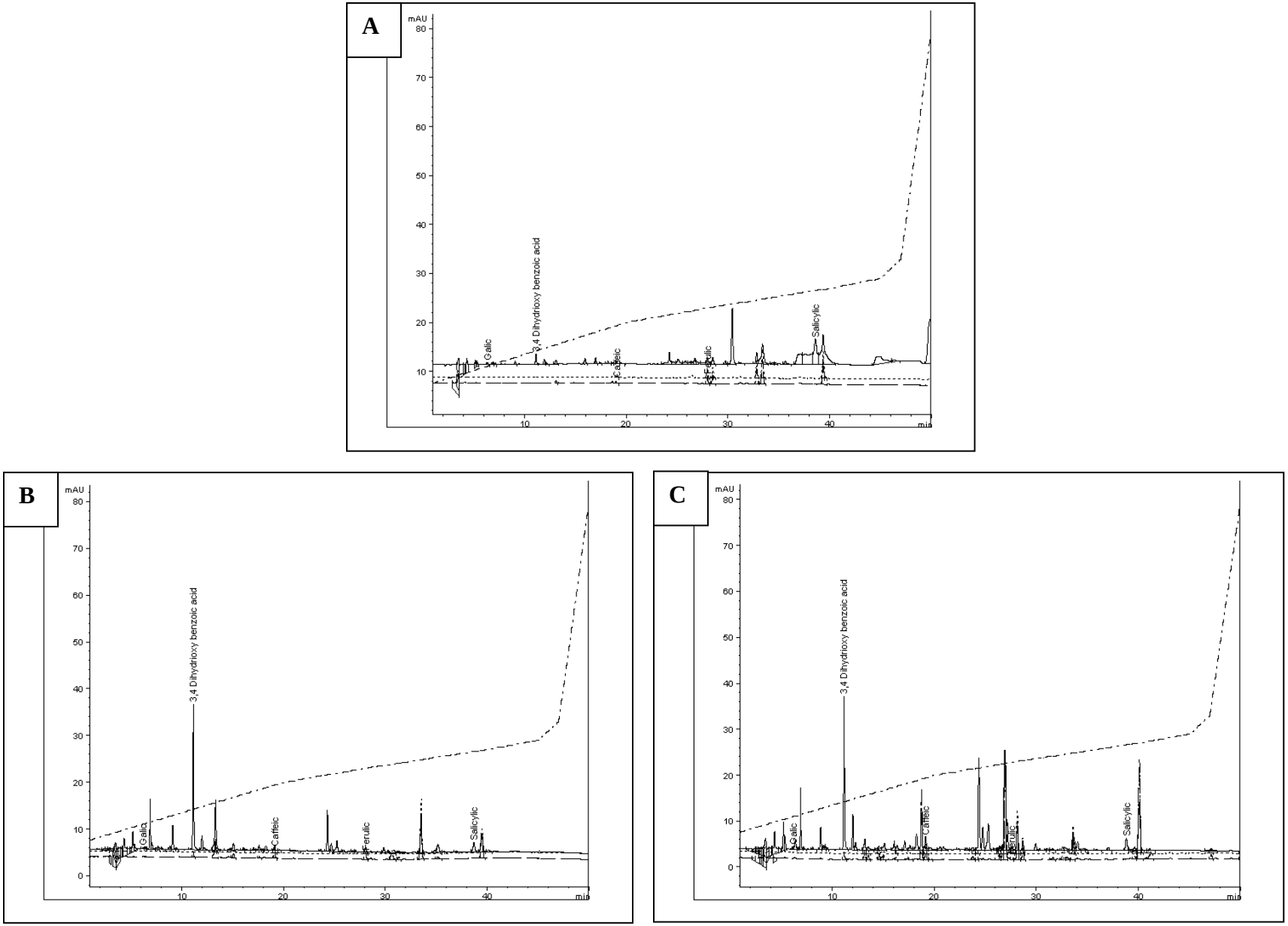

Figure 6.7. HPLC analysis of the basal end of stems. **(A)** Stems taken from motherplant; **(B)** After 90 days in mistbed when rooting was observed; **(C)** After 120 days when root growth was high.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

Protea cynaroides is one of many species in the Proteaceae family with high commercial value. It is also the most recognizable and undoubtedly has one of the most magnificent inflorescences. Intensive research of *P. cynaroides* and other closely-related species is required to achieve advancements in its propagation and to discover new varieties that may be of commercial importance. *In vitro* propagation can be used to not only rapidly produce disease-free plantlets, but also used in breeding programs to regenerate varieties that are rare and endangered.

Although *in vitro* propagation is used to produce a wide range of plant species, it has not been extensively used to propagate proteas in general, particularly on a commercial scale. In addition, as a result of very little research done on the *in vitro* propagation of *P. cynaroides*, information regarding its physiological development and growth *in vitro* is lacking. Consequently, little is known regarding the potential of micropropagation technologies to mass produce *P. cynaroides*. The main objective in the *in vitro* propagation section (*in vitro* germination of zygotic embryos, micrografting and somatic embryogenesis) of this study (Chapters 2, 3 and 4) was to provide a basis for a more comprehensive understanding of *P. cynaroides* in an *in vitro* environment. The results of the *in vitro* studies have shown the potential of *in vitro* propagation as a viable alternative to conventional propagation methods.

For *in vitro* germination of excised *P. cynaroides* zygotic embryos (Chapter 2), the use of alternating temperature (21±2ºC/12±2ºC) was the most important factor for increasing the germination percentage of zygotic embryos. Alternating temperature treatments are not often applied during micropropagation, and are sometimes not considered to be a vital factor in controlling the growth and development of explants. This is probably partly due to the emphasis being placed on the response of explants to the effects of different types of growth regulators and their applied concentrations. Furthermore, the addition of GA_3 into the germination media did not improve the germination percentage of zygotic embryos. However, malformation of the seedlings was observed, with cotyledons abnormally elongated and twining. This agrees with

reports that GA_3 causes the development of elongated and narrow leaves (De Fossard and de Fossard, 1988). Seedlings that were cultured in the dark were pale and elongated, nevertheless, the germination percentage of the embryos was similar in both light and dark conditions. Overall, the results of the germination study illustrated an important role in which the alternating temperatures of 21±2ºC/12±2ºC played. At this temperature regime germination of the zygotic embryos was highest, irrespective of the GA_3 or light treatments. Further studies are required to obtain the optimum temperature for the germination of *P. cynaroides* zygotic embryos.

Micrografting is a technique that requires precise manipulation of small tissues and plant organs. It was first developed to eliminate viruses in citrus (Murashige, Bitters and Rangan, 1972). More recently, micrografting has been found to be useful in obtaining rooted microshoots in plant species that are difficult to induce rooting under *in vitro* conditions (Thimmappaiah, Puthra and Anil, 2002). *P. cynaroides* explants are known to be a difficult-to-root species. Although *P. cynaroides* explants were established *in vitro* by Ben-Jaacov and Jacobs (1986), and later successfully multiplied *in vitro* (Wu, 2001), the production of rooted plantlets were not achieved. Micrografting has proved to be a suitable technique to produce rooted microshoots, as discussed in Chapter 3. Phenolic oxidation was found to be the main cause of death of microscions. This was apparent when the microscions were treated with antioxidant solutions or when solidified nutrient agar was applied to the graft area to prevent moisture loss. However, both these treatments worsened the oxidative browning, which was aggravated by the excessive wetness of the microscions. A dry microscion and the establishment of good contact between tissues of the microscion and rootstock ensured minimal oxidative browning, which resulted in callus growth at the graft area. Once the graft union was formed, vascular connections were made, which allowed the translocation of water and nutrients from the rootstock to the microscion, as indicated by the sprouting of buds and growth of new leaves.

Cultivar improvement by micrografting has also been studied by horticulturists and geneticists. Benefits include improved growth rates, improved nutritional and water use efficiencies, as well as improved flowering characteristics (Burger, 1985). Considering that it is a slow-growing plant by nature, the use of micrografting to improve the growth rates of *P. cynaroides* plants could be highly beneficial to

growers. In addition, the improvement of flowering characteristics could potentially be rewarding in the highly competitive and well-priced cutflower market. In fact, the successful micrografting achieved in this study can be a valuable tool for breeders of proteas to improve cultivars with poor characteristics.

Somatic embryogenesis is an ideal propagation method for producing large amounts of plantlets from very few source plants. Findings of the somatic embryogenesis study (Chapter 4) showed that *P. cynaroides* explants have an inherently-high regeneration capacity, which allowed somatic embryos to be produced in a relatively short period of time through direct somatic embryogenesis. Full-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without growth regulators, were suitable for the induction of somatic embryos, while a low concentration of gibberellic acid (0.1 mg l^{-1}) was found to be essential for the somatic embryos to germinate normally without malformation. Of particular interest was the inhibition of embryogenesis by auxins such as NAA and 2,4-D, which are commonly used in the induction of somatic embryos. It is seldom reported that auxins are not required for somatic embryogenesis. In certain cases, although growth regulators are not essential for the induction of somatic embryos, auxins are usually reported to increase the capacities of explant tissues to form somatic embryos (George, 1996).

With regard to conventional root formation of stem cuttings in the mistbed, the rooting problems associated with *P. cynaroides* cuttings and other proteas are well documented. Loss of nutrients from deliberate leaching of the cuttings resulted in very poor rooting (Chapter 5). Analyses of the leached cuttings and leachate solution showed that nutrients, particularly nitrogen, in *P. cynaroides* cuttings, were easily leached. This suggests that natural leaching of nutrients in the mistbed through the leaves and stems may be a contributing factor to slow rooting, since the cuttings require additional time to recover from the nutrient-deficient state (Good and Tukey, 1964). Therefore, further research is required to investigate the extent of nutrient-loss during rooting of *P. cynaroides* cuttings in the mistbed, and whether liquid fertilization should be applied through the irrigation systems to replenish cuttings with nutrients to compensate for their loss. Another factor that may have influenced the poor rootability of the leached cuttings is the loss of water-soluble phenolics from the cuttings, causing a reduction in the endogenous concentration levels of phenolic

compounds required to stimulate root formation. Further investigation into the loss of phenolic compounds during rooting of cuttings is required.

The findings of Chapter 5 also showed that blanching of stems on the motherplant for 30 days before removing and planting them in the mistbed, improved rooting of the cuttings. Analyses of starch content and total soluble phenols of the cuttings showed higher accumulation of these compounds in the etiolated areas of the blanched cuttings than in untreated areas. In addition, this study has established that the endogenous concentrations of starch and phenolics are directly linked to the time of rooting. It seems that once the total phenol content in the basal-end of a cutting has reached a certain level, root formation is stimulated. Starch grains are known to serve as the energy source for root formation (Veierskov, 1988). Thus, since etiolation accelerated the accumulation of starch, more starch was available in the cutting, which resulted in earlier rooting. The current findings have contributed towards a better understanding of the compounds responsible for root formation in *P. cynaroides*. Furthermore, methods applied in this study can also be used to improve the rooting of other commercially important proteas such as *Protea magnifica* and *Leucospermum cordifolium* cv. 'Vlam', which are also known to be difficult to root. Future studies should investigate the roles of insoluble and cell-wall bound phenolics and other forms of carbohydrates during rooting of *P. cynaroides* cuttings. In addition, it is conceivable that phenolics and carbohydrates have a closer relationship during rooting than currently reported in literature, thus, investigations into a possible relationship between phenolic compounds and carbohydrates during root induction and initiation should be done to further increase our understanding of adventitious root formation. Studies of endogenous auxins and rooting co-factors are also needed.

The roles which phenolic compounds play in various functions during plant growth and development, as well as their response to wounding and infection (Poapst and Durkee, 1967; Kosuge, 1969; Bassuk, Hunter and Howard, 1981) have been extensively researched. Most notably, the effects of phenolic compounds on endogenous root initiation of cuttings and their allelopathic activity on plant species, have been widely reported. Moreover, in addition to regulating root formation, their effects on photosynthesis (Nyberg, 1986) and mineral uptake have also been reported (Einhellig, 1986). The present studies on *P. cynaroides* stem cuttings (Chapters 5 and

6) have shown that the stimulatory effect of phenolic compounds on rooting is influenced by their endogenous concentration levels. Of particular importance is 3,4-dihydroxybenzoic acid, which was identified as one of the phenolic compounds that played a prominent role during root formation of *P. cynaroides* cuttings. The effects of 3,4-dihydroxybenzoic acid on root growth was shown in the *in vitro* rooting of *P. cynaroides* explants, where stimulation of root growth was observed on MS medium containing 100 mg l^{-1}. Results of the HPLC analysis of stems confirmed the presence of 3,4-dihydroxybenzoic acid in the cuttings during rooting, which were detected in much lower concentrations in unrooted stems. Other phenolics such as caffeic, ferulic and salicylic acids were also detected at very low concentrations in the cuttings. However, it is likely that interactions of several phenolics are responsible for root initiation, with 3,4-dihydroxybenzoic acid perhaps playing a primary role. The results of this study are particularly important in terms of identifying the specific compound that plays a prominent role in the rooting of *P. cynaroides* cuttings. In this regard, the identification of 3,4-dihydroxybenzoic acid as an important phenolic in the rooting of *P. cynaroides* will enable future research to investigate possible methods to counteract the inhibitory nature of 3,4-dihydroxybenzoic acid at low concentrations. A possible method to improve the rooting *P. cynaroides* could include the use of exogenous application of 3,4-dihydroxybenzoic acid onto the cuttings in an attempt to increase the endogenous 3,4-dihydroxybenzoic acid concentration at an early stage during vegetative propagation. Furthermore, interactions between 3,4-dihydroxybenzoic acid and other phenolics such as caffeic, ferulic, gallic and salicylic acids, which were found in smaller amounts in *P. cynaroides* cuttings, need to be established.

For the first time, complete plantlet regeneration from *P. cynaroides* explants through *in vitro* germination, micrografting and direct somatic embryogenesis is reported. In my view, the high germination percentage of excised zygotic embryos of *P. cynaroides* and the high regeneration rate of *P. cynaroides* somatic embryos achieved in this study is a critical development in pursuing the ultimate goal of mass production of *P. cynaroides* plantlets in a commercial environment. In addition, the aim of this study, which was also to contribute new knowledge towards understanding the roles of starch and phenolic compounds in rooting of *P. cynaroides* cuttings, was achieved. Knowledge gained from this study can serve as a basis for future research of other protea species.

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SUMMARY

P. cynaroides L. (King Protea) is an important cutflower in the South African floriculture industry. Conventional propagation by seeds and stem cuttings is inconsistent and slow. This thesis reports on the potential of *in vitro* propagation as an alternative method to produce *P. cynaroides* plantlets, and the roles of phenolic compounds and starch in the rooting of stem cuttings. *In vitro* studies consisted of *in vitro* germination of mature zygotic embryos, micrografting and direct somatic embryogenesis, where mature zygotic embryos and excised cotyledons were used to induce somatic embryos. In the study of rooting of cuttings, total soluble phenols and starch content in cuttings were analyzed. In addition, phenolic compounds were identified, and their effect on rooting was investigated.

The effects of GA_3, temperature and light on the *in vitro* germination of *Protea cynaroides* zygotic embryos were studied. Temperature was the most important factor in obtaining a high germination percentage. Alternating temperatures of 21±2ºC/12±2ºC (light/dark) was optimal for germination and over 90% of embryos germinated, while the germination percentage of embryos at 25±2ºC was poor. The incorporation of GA_3 into the growth medium had no effect on germination percentage, and the cotyledons of seedlings germinated in this medium were long and abnormal, while the roots were stunted. The presence of light was not necessary since the embryos germinated similarly in a 12-hour photoperiod and in total darkness. The roots of the seedlings formed *in vitro* were incapable of functioning in *ex vitro* conditions. However, the plantlets were able to produce new roots in *ex vitro* conditions. A higher percentage of plantlets survived when transferred to the medium containing a peat/coir/sand mixture than those planted in silica sand.

A successful shoot-tip micrografting technique was developed using *in vitro*-germinated *P. cynaroides* seedlings as rootstocks and axenic microshoots established from pot plants as microscions. Thirty-day old seedlings, germinated on growth-regulator-free, half-strength Murashige and Skoog medium, were decapitated and a vertical incision made from the top end. The bottom ends of microshoots established on modified Murashige and Skoog medium were cut into a wedge (‘V’) shape, and

placed into the incision. The micrografted explants were cultured in a growth chamber with the temperature adjusted to 25±2°C, with a 12-hour photoperiod. Best results were obtained by placing the microscions directly onto the rootstock without any pre-treatments. Dipping the explants in anti-oxidant solution or placing a layer of medium around the graft area led to the blackening of the microscion.

A protocol to induce direct somatic embryogenesis was developed. Somatic embryos formed directly on both *P. cynaroides* mature zygotic embryos and excised cotyledons cultured on MS medium without growth regulators. The addition of growth regulators such as NAA (1; 2.5 and 5 mg l^{-1}) and 2,4-D (1; 2.5 and 5 mg l^{-1}), in combination with TDZ (0.2 mg l^{-1}), BAP (0.2 mg l^{-1}) or kinetin (0.2 mg l^{-1}) suppressed the formation of somatic embryos. After eight weeks in culture, formation of somatic embryos was observed. Zygotic explants formed the most embryos when cultured in a 12-hour photoperiod in comparison to explants cultured in the dark. Up to 83% of these embryos germinated after transferal to the germination medium containing 0.1 mg l^{-1} GA_3. Significantly fewer embryos germinated in MS medium with no growth regulators, or supplemented with higher concentrations of GA_3, while low germination percentages were also observed in MS media containing casein hydrolysate and coconut water. The germination of normal embryos was observed only in medium containing either no growth regulators, 0.1 mg l^{-1} GA_3 or 0.5 mg l^{-1} GA_3. All embryos that germinated in high concentrations of GA_3 were malformed.

To improve the rooting percentage and rooting rate of *P. cynaroides* stem cuttings, cuttings were treated either by blanching, leaching or rooting hormone before planting into the mistbed. The rooting percentage and the mean root dry mass of *P. cynaroides* cuttings were significantly improved by the blanching treatment. Starch and total phenol analyses results revealed a positive correlation between high root formation and increased starch and phenolic content by the blanching treatment. Significantly higher amounts of starch and total phenols were found in the basal-end of blanched cuttings than the control, from planting time until the cuttings were well-rooted after 90 days. The blanched cuttings were ready to be transplanted after 90 days, compared to the control, which was only ready at day 120. Leaching of cuttings in water resulted in poor rooting percentage and low root dry mass, which may have been

caused by the loss of macro- and micro-nutrients from the cuttings. Analyses of the leachate showed that significant amounts of N were leached from the cuttings, while a lesser amount of P, K Ca and Mg were also leached. It is possible that phenolic compounds, which may be responsible for stimulating root formation, were also leached from the cuttings.

Allelopathy bioassay indicated the presence of allelochemicals in *P. cynaroides* stem cuttings. Further analysis of stem extracts identified 3,4-dihydroxybenzoic acid and other similar phenolics in the stem. Phytotoxicity bioassay showed that 3,4-dihydroxybenzoic acid both stimulated and inhibited root growth of lettuce seedlings, depending on the concentration applied. The highest stimulation was recorded at 100 mg l^{-1}, where the mean root length of lettuce seedlings was 23% longer than the control. The exogenous application of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants *in vitro* stimulated root growth at 100 mg l^{-1}, but not at concentrations below this, while root inhibition was observed at toxic levels (500 mg l^{-1}). HPLC analysis of cuttings during vegetative propagation showed a considerable increase in 3,4-dihydroxybenzoic acid levels from initial planting (12.2 µg g^{-1}) to when root formation took place (180.2 µg g^{-1}) A link can therefore be made between the concentration levels of 3,4-dihydroxybenzoic acid and root formation, where the concentration level (100 mg l^{-1} = 100 ppm) of 3,4-dihydroxybenzoic acid at which root stimulation of the *P. cynaroides* explants was observed, corresponded with the amount of 3,4-dihydroxybenzoic acid (180.2 µg g^{-1} = 180.2 ppm) found in the stem cuttings during rooting in the mistbed. It can be deduced that once the concentration level of 3,4-dihydroxybenzoic acid in cuttings reached 100 mg l^{-1}, root formation was stimulated. HPLC analysis also identified caffeic, ferulic, gallic and salicylic acids in the cuttings.

APPENDIX A

(Murashige and Skoog medium)

Medium composition	mg l^{-1}
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_47H_2O$	370
KH_2PO_4	170
KI	830
H_3BO_3	6200
$MnSO_4.4H_2O$	22300
$ZnSO_4.7H_2O$	8600
$Na_2MoO_4.2H_2O$	250
$CuSO_4.5H_2O$	25
$CoCl_2.6H_2O$	25
$FeSO_4.7H_2O$	27850
$Na_2EDTA.2H_2O$	37250
Myo-inositol	100
Nicotinic acid	500
Pyridoxine-HCl	500
Thiamine-HCl	100
Glycine	2

APPENDIX B

(Starch analysis reagents)

1) Acetate buffer

Dissolve the following in 5.72 ml of acetic acid (99.8%):

a) $CaCl_2.2H_2O$ (0.2198 g)
b) NaOH (first dissolve approximately 10 pellets in 20 ml water)

Adjust pH to 5 with NaOH and then make up to one litre with distilled water.

2) Glucose oxidase colour solution

Dissolve the following in 500 ml distilled water in a two litre volumetric flask.

a) 24.8 g disodium hydrogen orthophosphate (AR grade)
b) 12.4 g sodium dihydrogen orthophosphate (AR grade)
c) 4.0 g benzoic acid (GPR grade), dispersed in a small volume of ethanol
d) 0.2 g 4-amino-antipyrine (Sigma Chemical Co., A-4382)
e) 3.0 g p-hydroxybenzoic acid (Sigma Chemical Co., H-5376)
f) 0.04 g glucose oxidase (Sigma Chemical Co., G-2133)
g) 0.01 g peroxidase (Sigma Chemical Co., P-8375)

Afterwards, make up the solution to two litres and stored at 4°C in the dark. Only remove the solution from refrigerator before use.

3) Glucose standard solution

Add 1 ml of glucose solution (reagent 5) to 9 ml distilled water and vortex. This solution is used in the standard for test tubes 1 to 8. For test tubes 9 and 10, the undiluted glucose solution (reagent 5) is used.

The glucose standard curve is drawn from the glucose standard solution according to the volumes listed below.

Test Tube	Glucose Standard Solution (µl)	Glucose Oxidase Colour Solution (ml)	Glucose Concentration (mg 5 ml^{-1})
1	100	4.90	0.01
2	200	4.80	0.02
3	300	4.70	0.03
4	400	4.60	0.04
5	500	4.50	0.05
6	600	4.40	0.06
7	800	4.20	0.08
8	1000	4.00	0.10
9	150	4.85	0.15
10	200	4.80	0.20

APPENDIX C

Abbreviated analysis of variance (ANOVA) tables

Table C1. Analysis of variance of cotyledon fresh mass of *P. cynaroides* zygotic embryos germinated on MS medium (Figure 2.2).

Source	DF	SS	MS	F-value	Pr > F
Treatment	11	318583.16	28962.11	29.01	< 0.0001
Error	132	131778.45	998.32		
Total	143	450361.61			
Media	2	196948.43	98474.21	98.64	< 0.0001
Light	1	1730.39	1730.39	1.73	0.1903
Temperature	1	217.02	217.02	0.22	0.6418
Media*Light	2	11122.59	5561.30	5.57	0.0048
Media*Temperature	2	6203.23	3101.62	3.11	0.0480
Light*Temperature	1	2020.34	2020.34	2.02	0.1572
Media*Light*Temperature	2	1157.90	578.95	0.58	0.5614
CV = 25.73	R^2 = 0.71				

Table C2. Analysis of variance of root fresh mass of *P. cynaroides* zygotic embryos germinated on MS medium (Figure 2.3).

Source	DF	SS	MS	F-value	Pr > F
Treatment	11	775.06	70.46	48.92	< 0.0001
Error	132	190.12	1.44		
Total	143	965.17			
Media	2	546.88	273.44	189.85	< 0.0001
Light	1	2.07	2.07	1.43	0.2332
Temperature	1	31.96	31.96	22.19	< 0.0001
Media*Light	2	23.46	11.73	8.14	0.0005
Media*Temperature	2	18.36	9.18	6.37	0.0023
Light*Temperature	1	5.81	5.81	4.03	0.0466
Media*Light*Temperature	2	2.68	1.34	0.93	0.3964
CV = 27.18	R^2 = 0.80				

Table C3. Analysis of variance for the mean number of somatic embryos formed on *P. cynaroides* explants on MS medium after 8 weeks in culture (Table 4.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	3	242.28	80.76	40.22	0.0001
Error	58	116.46	2.01		
Total	61	358.74			
CV	21.59				
R^2	0.67				

Table C4. Analysis of variance for the effect of pre-treatments on the mean root dry mass of *P. cynaroides* cuttings after 90 days (Figure 5.3).

Source	DF	SS	MS	F-value	Pr > F
Treatment	5	143489.14	28697.83	8.72	< 0.0001
Error	60	197414.15	3290.24		
Total	65	340903.29			
CV	73.75				
R^2	0.42				

Table C5. Analysis of variance for the effect of pre-treatments on the number of roots formed between 1 and 10 mm in length (Group 1) (Table 5.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	5	266.27	53.25	5.98	0.001
Error	24	213.60	8.9		
Total	29	479.87			
CV	52.03				
R^2	0.55				

Table C6. Analysis of variance for the effect of pre-treatments on the number of roots formed between 11 and 20 mm in length (Group 2) (Table 5.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	5	313.60	62.72	24.60	< 0.0001
Error	24	61.20	2.55		
Total	29	374.80			
CV	38.02				
R^2	0.84				

Table C7. Analysis of variance for the effect of pre-treatments on the number of roots formed between 21 and 30 mm in length (Group 3) (Table 5.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	5	342.80	68.56	24.93	< 0.0001
Error	24	66.00	2.75		
Total	29	408.80			
CV	34.55				
R^2	0.84				

Table C8. Analysis of variance for the effect of pre-treatments on the number of roots formed between 31 and 40 mm in length (Group 4) (Table 5.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	5	45.77	9.15	10.36	< 0.0001
Error	24	21.20	0.88		
Total	29	66.97			
CV	35.69				
R^2	0.68				

Table C9. Analysis of variance for the effect of pre-treatments on the number of roots formed between 41 and 50 mm in length (Group 5) (Table 5.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	5	294.80	58.96	16.38	< 0.0001
Error	24	86.40	3.60		
Total	29	381.20			
CV	52.70				
R^2	0.77				

Table C10. Analysis of variance for the effect of pre-treatments on the number of roots formed that were >51 mm in length (Group 6) (Table 5.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	5	122.7	24.54	17.32	< 0.0001
Error	24	34.00	1.41		
Total	29	156.70			
CV	62.64				
R^2	0.78				

Table C11. Analysis of variance of dry root mass of cuttings during rooting period (60, 90 and 120 days after planting) (Figure 5.4).

Source	DF	SS	MS	F-value	Pr > F
Treatment	5	226888.63	45377.73	7.73	< 0.0001
Error	35	205569.28	5873.41		
Total	40	432457.92			
CV	51.73				
R^2	0.52				

Table C12. Analysis of variance of total phenolic content in the basal part of *P. cynaroides* cuttings taken from motherplants (0 days) (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	677.68	677.68	86.13	< 0.0001
Error	6	47.21	7.87		
Total	7	724.89			
CV	8.53				
R^2	0.93				

Table C13. Analysis of variance of total phenolic content in the basal part of *P. cynaroides* cuttings during rooting, after 60 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	669.32	669.32	84.91	< 0.0001
Error	6	47.29	7.88		
Total	7	716.62			
CV	4.93				
R^2	0.93				

Table C14. Analysis of variance of total phenolic content in the basal part of *P. cynaroides* cuttings during rooting, after 90 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	1086.41	1086.41	38.79	0.0008
Error	6	168.05	28.01		
Total	7	1254.46			
CV	7.3				
R^2	0.87				

Table C15. Analysis of variance of total phenolic content in the basal part of *P. cynaroides* cuttings during rooting, after 120 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	81.78	81.78	2.49	0.1654
Error	6	196.80	32.80		
Total	7	278.59			
CV	7.02				
R^2	0.29				

Table C16. Analysis of variance of total phenolic content in the middle part of *P. cynaroides* cuttings taken from motherplants (0 days) (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	644.19	644.19	52.97	< 0.0003
Error	6	72.96	12.16		
Total	7	717.16			
CV	11.72				
R^2	0.90				

Table C17. Analysis of variance of total phenolic content in the middle part of *P. cynaroides* cuttings during rooting, after 60 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	502.90	502.90	82.96	< 0.0001
Error	6	36.37	6.06		
Total	7	539.27			
CV	3.67				
R^2	0.93				

Table C18. Analysis of variance of total phenolic content in the middle part of *P. cynaroides* cuttings during rooting, after 90 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	790.92	790.92	100.06	< 0.0001
Error	6	47.43	7.90		
Total	7	838.34			
CV	3.96				
R^2	0.94				

Table C19. Analysis of variance of total phenolic content in the middle part of *P. cynaroides* cuttings during rooting, after 120 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	795.28	795.28	47.22	< 0.0005
Error	6	101.04	16.84		
Total	7	896.32			
CV	6.00				
R^2	0.89				

Table C20. Analysis of variance of total phenolic content in the top part of *P. cynaroides* cuttings taken from motherplants (0 days) (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	22.18	22.18	2.47	< 0.1674
Error	6	53.96	8.99		
Total	7	76.14			
CV	7.41				
R^2	0.29				

Table C21. Analysis of variance of total phenolic content in the top part of *P. cynaroides* cuttings during rooting, after 60 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	3.16	3.16	0.13	< 0.7318
Error	6	146.97	24.49		
Total	7	150.13			
CV	6.06				
R^2	0.02				

Table C22. Analysis of variance of total phenolic content in the top part of *P. cynaroides* cuttings during rooting, after 90 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	9.19	9.19	0.29	< 0.6081
Error	6	188.51	31.42		
Total	7	197.71			
CV	5.33				
R^2	0.05				

Table C23. Analysis of variance of total phenolic content in the top part of *P. cynaroides* cuttings during rooting, after 120 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	0.82	0.82	0.03	< 0.8795
Error	6	196.34	32.72		
Total	7	197.16			
CV	5.49				
R^2	0.004				

Table C24. Analysis of variance of total phenolic content in the leaves of *P. cynaroides* cuttings taken from motherplants (0 days) (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	105.70	105.70	2.11	< 0.1968
Error	6	300.89	50.15		
Total	7	406.58			
CV	8.14				
R^2	0.26				

Table C25. Analysis of variance of total phenolic content in the leaves of *P. cynaroides* cuttings during rooting, after 60 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	17.05	17.05	0.27	< 0.6188
Error	6	372.17	62.03		
Total	7	389.22			
CV	5.61				
R^2	0.04				

Table C26. Analysis of variance of total phenolic content in the leaves of *P. cynaroides* cuttings during rooting, after 90 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	13.39	13.39	0.21	< 0.6592
Error	6	373.70	62.28		
Total	7	387.09			
CV	6.58				
R^2	0.03				

Table C27. Analysis of variance of total phenolic content in the leaves of *P. cynaroides* cuttings during rooting, after 120 days (Figure 5.5).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	6.26	6.26	0.38	< 0.5619
Error	6	99.75	16.63		
Total	7	106.02			
CV	3.50				
R^2	0.06				

Table C28. Analysis of variance of starch content in the basal part of *P. cynaroides* cuttings taken from motherplants (0 days) (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	101.11	101.11	12.49	< 0.0241
Error	4	32.37	8.09		
Total	5	133.48			
CV	9.31				
R^2	0.76				

Table C29. Analysis of variance of starch content in the basal part of *P. cynaroides* cuttings during rooting, after 60 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	9.20	9.20	0.64	< 0.4681
Error	4	57.39	14.35		
Total	5	66.59			
CV	11.04				
R^2	0.14				

Table C30. Analysis of variance of starch content in the basal part of *P. cynaroides* cuttings during rooting, after 90 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	142.79	142.79	149.40	< 0.0003
Error	4	3.82	0.96		
Total	5	146.61			
CV	3.20				
R^2	0.97				

Table C31. Analysis of variance of starch content in the basal part of *P. cynaroides* cuttings during rooting, after 120 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	260.70	260.70	44.92	< 0.0026
Error	4	23.21	5.80		
Total	5	283.91			
CV	6.30				
R^2	0.92				

Table C32. Analysis of variance of starch content in the middle part of *P. cynaroides* cuttings taken from motherplants (0 days) (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	13.35	13.35	0.95	< 0.3856
Error	4	56.38	14.10		
Total	5	69.73			
CV	11.97				
R^2	0.19				

Table C33. Analysis of variance of starch content in the middle part of *P. cynaroides* cuttings during rooting, after 60 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	57.54	57.54	2.78	< 0.1705
Error	4	82.63	20.66		
Total	5	140.19			
CV	12.50				
R^2	0.41				

Table C34. Analysis of variance of starch content in the middle part of *P. cynaroides* cuttings during rooting, after 90 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	140.17	140.17	136.93	< 0.0003
Error	4	4.09	1.02		
Total	5	144.26			
CV	3.30				
R^2	0.97				

Table C35. Analysis of variance of starch content in the middle part of *P. cynaroides* cuttings during rooting, after 120 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	273.11	273.11	79.47	< 0.0009
Error	4	13.75	3.44		
Total	5	286.85			
CV	4.90				
R^2	0.95				

Table C36. Analysis of variance of starch content in the top part of *P. cynaroides* cuttings taken from motherplants (0 days) (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	56.61	56.61	4.19	< 0.1102
Error	4	54.10	13.53		
Total	5	110.71			
CV	11.62				
R^2	0.51				

Table C37. Analysis of variance of starch content in the top part of *P. cynaroides* cuttings during rooting, after 60 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	174.21	174.21	47.72	< 0.0023
Error	4	14.60	3.65		
Total	5	188.81			
CV	5.28				
R^2	0.93				

Table C38. Analysis of variance of starch content in the top part of *P. cynaroides* cuttings during rooting, after 90 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	207.92	207.92	12.47	< 0.0242
Error	4	66.70	16.67		
Total	5	274.61			
CV	13.45				
R^2	0.76				

Table C39. Analysis of variance of starch content in the top part of *P. cynaroides* cuttings during rooting, after 120 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	96.16	96.16	7.41	< 0.0529
Error	4	51.89	12.97		
Total	5	148.05			
CV	9.14				
R^2	0.65				

Table C40. Analysis of variance of starch content in the leaves of *P. cynaroides* cuttings taken from motherplants (0 days) (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	93.22	93.22	4.62	< 0.0982
Error	4	80.80	20.20		
Total	5	174.02			
CV	10.17				
R^2	0.54				

Table C41. Analysis of variance of starch content in the leaves of *P. cynaroides* cuttings during rooting, after 60 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	127.70	127.70	9.44	< 0.0572
Error	4	54.13	13.53		
Total	5	181.83			
CV	7.71				
R^2	0.70				

Table C42. Analysis of variance of starch content in the leaves of *P. cynaroides* cuttings during rooting, after 90 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	290.51	290.51	15.35	< 0.0173
Error	4	75.71	18.93		
Total	5	366.23			
CV	10.97				
R^2	0.79				

Table C43. Analysis of variance of starch content in the leaves of *P. cynaroides* cuttings during rooting, after 120 days (Figure 5.6).

Source	DF	SS	MS	F-value	Pr > F
Treatment	1	1695.12	1695.12	7.44	< 0.050
Error	4	911.52	227.88		
Total	5	2606.64			
CV	23.31				
R^2	0.65				

Table C44. Analysis of variance of root length of *P. cynaroides* explants cultured on MS medium containing 3,4-dihydroxybenzoic acid concentrations (Table 6.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	7	78.09	11.15	5.63	< 0.0001
Error	40	79.25	1.98		
Total	47	157.33			
CV	24.97				
R^2	0.50				

Table C45. Analysis of variance of root fresh mass of *P. cynaroides* explants cultured on MS medium containing 3,4-dihydroxybenzoic acid concentrations (Table 6.1).

Source	DF	SS	MS	F-value	Pr > F
Treatment	7	34.66	4.95	10.39	< 0.0001
Error	38	18.12	0.48		
Total	45	52.78			
CV	67.30				
R^2	0.66				