Micropropagation of *P. cynaroides* L.

by

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Protea cynaroides (Source: Patterson-Jones, 2000)

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Abstract

The exotic blooms of the indigenous South African flowering plants and the biodiversity of the floral material available, have led to a high demand for these flowers on the international market. A wide variety of high-quality flowers, of which *Protea cynaroides* L. is an important component, are exported from South Africa. However, because of the high demand for the export market the producers are unable to supply enough plant material. Therefore, due to the limited supply, poorer quality flowers are exported which results in lower prices obtained. One of the reasons for the lack of flowers available for export is that being a woody perennial, *P. cynaroides* require a longer period of time to grow when conventional propagation methods are used. Micropropagation of *P. cynaroides* may become the most suitable alternative propagation method for rapid production and to ensure high standards of quality. In this study, Stage 1 (establishment) and Stage 2 (multiplication) of micropropagation were investigated.

Establishment (Stage 1) of the explants was achieved by using Murashige and Skoog medium with the addition of 30 $mg.l^{-1}$ GA_3. However, it was found that browning of the explant tissues caused by phenolic oxidation was a major problem during the establishment stage, as the browning of many explants led to the inability of the axillary buds to sprout which resulted in death. Browning of tissues occurs when phenolic compounds are oxidized, this usually happens when the plant tissues are stressed or injured during explant preparations.

Therefore, methods to control oxidation were tested in an experiment which included the use of sterilants (mercuric chloride and sodium hypochlorite) and antioxidants (ascorbic and citric acid). The selection of a few treatments which showed potential in controlling oxidation, followed by further tests, led to positive results. Phenolic oxidation was reduced by stirring the explants in 100 $mg.l^{-1}$ ascorbic acid and 1500 $mg.l^{-1}$ citric acid for 1 hour. This was followed by growing the explants in a 16-hour photoperiod which was suitable for the axillary buds to sprout.

Subsequently, Stage 2 of micropropagation (multiplication) was successfully done by subculturing the explants from the establishment stage onto the multiplication media. The effects of phosphorous on the growth of the explants were tested by using two media, where no ammonium phosphate was added into one medium, while 1400 $mg.l^{-1}$ ammonia phosphate was added to the other. Surprising results were obtained when explants in both media grew well, illustrating that *P. cynaroides* may be tolerant to high levels of phosphorous. However, a possible reason for this is that because no roots were formed by the explants in the multiplication medium, the phosphorous in the medium were not taken up by the explants. These results also illustrated that two-budded explants achieved a higher survival rate and longer mean length than one-budded explants. Investigation into the rooting requirements of the explants, Stage 3 (rooting) of micropropagation must still be achieved.

Keywords: Proteaceae, tissue culture, oxidation, antioxidants, sterilants

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INTRODUCTION

Protea cynaroides L., known as the King Protea, is endemic to South Africa. The flowers are an export commodity, and form an important part of the floriculture industry in South Africa. Therefore, its taxonomy, distribution and habitat, and morphology will be described to allow readers to familiarize themselves with the flower. Furthermore, the definitions, terminology and importance of *in vitro* propagation of *P. cynaroides* will also be discussed to ensure that the concepts described in this study are fully understood. Finally, phenolic compounds and oxidation will be discussed in detail, including the causes of phenolic oxidation and methods for their control.

1.1 Taxonomy

Protea cynaroides, named by the renowned botanist Linnaeus, belongs to the Proteaceae family (Rourke, 1980). The family is subdivided into 14 genera comprising an estimated 1700 species. Of the 14 genera, seven are commercially important. These are *Protea*, *Leucospermum*, *Leucadendron*, *Serruria*, *Aulax*, *Mimetes* and *Paranomus*. The flower of *P. cynaroides* resembles an artichoke and was therefore named *cynaroides* by Linnaeus meaning 'like cynara' – a globe artichoke (Vogts, 1982). The flower of the *P. cynaroides* plant, is in fact, an inflorescence. But in the rest of this thesis, it will be referred to as a flower.

1.2 Distribution and Habitat

Most plants belonging to the Proteaceae family are found in the southern hemisphere, with the richest concentration of species in eastern and western Australia and the western

Cape region of South Africa (Paterson-Jones, 2000). The King Protea grows best in well drained, nutrient deficient, sandy acid soils (Vogts, 1982). These plants have low mineral requirements and are therefore not tolerant to salt concentrations that would appear normal to other plants (Montarone and Allemand, 1995). Although all species belonging to this genus survive in dry summer conditions, *P. cynaroides* is more water-loving than others, therefore in areas more prone to long periods of drought, they are usually found near river banks (Vogts, 1982). In South Africa, *P. cynaroides*, occupies a variety of habitats ranging from the Cederberg in the northern parts of western Cape to the Cape Peninsula as well as eastwards to Grahamstown (Paterson-Jones, 2000). The majority of these areas are characterized by mountainous landscapes with hot, dry summers. However, in the southeastern coast near Port Elizabeth, the rainfall is spread more evenly throughout the year.

1.3 Morphology

P. cynaroides is a shrub which can grow up to a height of two metres. The leaf stalks are reddish and can reach lengths of 50 to 100 mm. The plant's 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 120mm to 300mm in diameter and the colour of the bracts, which are either hairy or hairless, range from pink to creamy-white (Patterson-Jones, 2000).

1.4 Economic Importance

The total international flower industry is estimated at US$ 45 billion for the year 2000 (Coetzee, 2000). The majority of flowers (88%) are marketed in Europe. Although Europe imports about $800 million worth of flowers, South Africa only constitutes 3.3% of this import market. The positive aspect about the South African export market is that there is a high demand for high quality indigenous South African flowers on the

international market, and in contrast to other flower prices, their prices have remained constant over the years (Coetzee, 2000).

In South Africa, Proteas are sold mainly as a fresh cut flower (Coetzee, 2000). Although cultivation of fresh King Protea cut flowers are mostly from seeds, which takes place on a large scale, some are still harvested from their natural habitats. However, a transition phase of harvesting from the open fields to large-scale cultivation is currently under way. The aim of the transition is to firstly allow South Africa to compete in the fresh flower industry with other countries by cultivating high quality flowers and secondly to conserve the flowers in its natural habitat.

1.5 Tissue culture propagation

1.5.1 Terminology

Tissue culture is used as a collective term for the culture of seeds, embryos, organs, tissue, cells and protoplasts *in vitro*. *In vitro* means literally, 'in a glass' because, initially at least, glass vessels were used as containers for the cultures (Pierik, 1987). *In vitro* propagation consists of culturing plant materials on nutrient media under sterile conditions, with fully controlled environmental conditions.

Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* propagation techniques (Debergh and Read, 1991). Cultures are started with very small pieces of plants (explants), and thereafter small shoots or embryos are propagated, hence the term 'micropropagation' to describe the *in vitro* methods. Since Murashige (1974a, b) proposed a three stage protocol for micropropagation of plants, many discoveries have been made. As more information evolved, it is now agreed that five stages are involved in the micropropagation process (Debergh and Read, 1991), ie. stage 0 (mother plant selection and preparation), stage 1 (establishment of aseptic culture), stage 2

(multiplication), stage 3 (elongation and root induction) and stage 4 (transfer to greenhouse conditions).

1.5.2 Advantages

When conventional methods of vegetative propagation such as stem cuttings, grafting, budding and layering (Hartmann *et al.*, 1997) are ineffective in propagating certain plant material, *in vitro* propagation can be an alternative method to overcome this problem. Factors influencing vegetative growth such as nutrients and growth regulator levels as well as light and temperature can be adjusted to suit the type of explant used. This results in a much higher propagation rate in a given time (George, 1993). Rapid multiplication of new cultivars is possible by plant breeders, allowing earlier entry of the new cultivar into the market than when using conventional propagation methods. The technique is very suitable when high volume production is important. *In vitro* propagation is carried out in aseptic conditions, free from contaminants. Also, methods are available to free plants from virus diseases. Once the plant materials are virus-free, certified virus-free plants can be produced in large numbers. The production of disease-free plants overcomes the quarantine requirements for international exchange of plant materials (Pierik, 1988).

Physiologically mature material often cannot be propagated *in vivo*, but can be rejuvenated *in vitro* and then subsequently micropropagated. The creation of gene banks (preservation of valuable plant material) and the storage of plants under disease-free conditions in a relatively small space are possible (Pierik, 1988). Techniques for genetic manipulation (engineering) of plants also depend upon *in vitro* propagation for regeneration and multiplication of the new characteristics. In contrast to *in vivo* propagation, plants can be propagated *in vitro* throughout the year and become independent of the seasons.

1.5.3 Disadvantages

Contamination of the explants, whether it is by internal or external contaminants, are very problematic. The explants are said to be liable to three kinds of contamination (George, 1993). The first kind occurs during the establishment of a culture probably caused by ineffective surface sterilization. The second kind occurs after establishment and is either caused by endogenous micro-organisms that were concealed within the explant or exogenous micro-organisms that were introduced during subculturing. The third kind of contamination is of long-lived nature which occurs in a batch of cultures after a long period of sterility.

In addition, when the plant is of a woody species, such as those belonging to the Proteaceae family, there are more problems to overcome than when herbaceous plants are used. One of the main differences between herbaceous and woody plants is that the latter are more difficult to propagate in *in vitro* (Pierik, 1987). For example, when using nodal cultures, it is difficult to induce the axillary buds from woody plants to sprout. The multiplication rate of woody plants is also much lower than herbaceous species. This may be due to woody plants having relatively weak regenerative capacity compared to herbaceous plants (Pierik, 1987).

Woody species are more easily affected by excretion of toxic substances into the media such as phenolic compounds (Hartmann *et al.*, 1997). The oxidation of phenolic compounds on wounded surfaces is a very common problem. The products produced from the oxidation process may be toxic to the explants and causes browning of the tissues and medium which generally inhibits growth or result in death of the explants (George, 1993; Debergh & Read, 1991). Phenolic compounds will be discussed in greater detail below.

1.5.4 Phenolic compounds

The subject of phenolic compounds includes a whole range of various chemical compounds which all fall under the same category. In this review the basic definition of phenolic compounds will be discussed. The distribution and the causes of phenolic compound production will be described to a lesser extent. Therefore, the majority of this review will be based on phenolic oxidation but more importantly, the methods to limit their production.

1.5.4.1 Definition and Distributions

The term phenolic is defined chemically as a substance which possesses an aromatic ring bearing a hydroxyl substituent, including functional derivatives (Harborne, 1989). Table 1.1 illustrates the major classes of phenolics in plants, while Figure 1.1 shows the structures of six basic phenols. Phenolic compounds are very labile products which are easily oxidized (Debergh and Read, 1991). After oxidation, these products can be phytotoxic or even enhance the oxidation process, because after they are oxidized, they become very strong oxidants themselves. In a microscopical study, Mueller and Beckman (1974) indicated that phenolic compounds are probably synthesized primarily in the chloroplasts and transported via the endoplasmic reticulum to the vacuole for storage. However, according to Harborne (1989), a wide range of phenolics of all types are found in the cell wall lignins of all flowering plants, including Proteaceae plants.

1.5.4.2 Causes of phenolic oxidation

Plant tissues that are exposed to factors causing stress or injury such as mechanical damage, chemical treatments with heavy metals or ethylene or infection by fungi, bacteria or viruses, can stimulate the metabolism of phenolic compounds (Kosuge, 1969). The stress or injury caused by these factors may lead to hypersensitive reactions in the

tissues. There are three hypersensitive reactions. Firstly, the loss of contents from broken cells following mechanical damage. Secondly, metabolic responses in cells close to the site of injury which do not themselves shows signs of major damage. Thirdly, the premature death of some cells in the vicinity of the wound or infection (Rhodes and Wooltorton, 1978).

In general, phenolic metabolism has three possible types of reactions in response to stress and injury: The first involves that oxidation of preformed phenolic components. The remaining two involves either the synthesis of monomeric or the synthesis polymeric phenolic derivatives (Rhodes and Wooltorton, 1978).

1.5.4.3 Enzymes

The tendency of *P. cynaroides* plant tissues to turn brown easily seems to be mainly due to relatively high amounts of phenolic compounds that are found within the plant. The loss of its natural colour due to browning is, as described above, a physiological phenomenon (Vogts, 1982). Because of the fact that relatively high amounts of phenolic compounds are stored or produced by *P. cynaroides*, the responses involving the oxidation of these pre-existing phenolic compounds will be discussed.

There are basically three enzymes that are known to be able to oxidize phenolic compounds, namely polyphenol oxidase (PPO) (also known as phenolase), peroxidases and laccases. The copper-containing enzyme polyphenol oxidase (Mason, 1955), is responsible for the oxidation of o-diphenols to o-quinones when oxygen and a suitable substrate are present (Marks and Simpson, 1990). The o-quinone products are very powerful oxidizing agents which, after undergoing polarmerization reactions, are responsible for producing the brown or black products which are characteristic of phenolic oxidation. Peroxidase is another enzyme whose primary function is to oxidize hydrogen donors at the expense of peroxides to produce products which have lignin-like properties (Rhodes and Wooltorton, 1978). Hydrogen peroxide is the most common

donor of hydrogen, and therefore their activity depends on the internal level of hydrogen peroxide. Laccases catalyzes the oxidation of p-diphenols to p-quinones. Laccases are less widely distributed and besides oxidation reactions, they may also play a role in lignin formation (Freudenberg, 1968).

1.5.4.4 Control methods

Many experiments have been carried out and papers published in various species of Proteaceae showing different methods to overcome this problem. For example, *P. obtusifolia* (Watad *et al.*, 1992), *P. repens* (Rugge, 1995) and *Leucadendron* (Perez-Frances *et al.*, 1995).

The methods of minimizing and slowing down the phenolic oxidation process ranges from applying various treatments to the mother plant to frequent transfers of the cultured explants. George (1993) has given a comprehensive review of ways to prevent phenolic oxidation. These include:

a) Minimizing the damage caused to the explant
b) Removing the phenolic compounds produced (leaching or dispersal, adsorption with activated charcoal)
c) Modifying the environment (etiolated stock plants, culture in darkness or in low light irradiance and in low temperatures)
d) Modifying redox potential (use of antioxidants and reduced oxygen availability)
e) Reduction in phenolase activity and substrate availability (elements involved in oxidative reactions, chelating agents and lowering of pH)
f) Altering the composition of the medium.

These methods are discussed in greater detail below.

a) Minimizing the damage caused to the explant

The extent of phenolic oxidation which causes browning of the explant can be controlled by reducing the amount of wounding during excision and sterilization (George, 1993). The rate of browning of wounded tissues is also dependent on the redox potential of the cut surfaces of excised tissue. Therefore, oxidative damage can be reduced by rapid excision of the explant (Guerra and Handro, 1988). Regarding sterilization, in some cases, the type of sterilant (sterilizing agent) used can help to reduce phenolic browning. The common sterilants that are used are NaOCl, $Ca(ClO)_2$ and to a lesser extent, HgCl. NaOCl is most widely used because it is found in household bleaches where it is easily obtainable. The concentrations at which all of these are used would have an effect on the amount of shock and damage the explant would receive. Tissues often become brown due to the presence of activated oxygen in which sterilants such as NaOCl and $Ca(OCl)_2$ are a source (George, 1993). This may not seem to be a point for concern, but in cases where plant tissues are easily oxidized such as *P. cynaroides* explants, every effort should be made to reduce this problem. Such was the case in *Strelitzia reginae* (Ziv and Halevy, 1983) where browning was less severe when 0.3% mercuric chloride was used instead of 9% calcium hypochlorite.

b) Removing the phenolic compounds produced (leaching or dispersal, adsorption with activated charcoal)

Damage caused by phenolic oxidation is most severe during the initial stages of a culture (George, 1996). When secretion of phenolic compounds shortly after explant isolation occurs, growth is generally prevented. Therefore one of the methods to control this, would be to leach out as much phenolic compounds as possible from the explants, to an extent where the presence of these compounds in the explants are reduced substantially so that minimal damage is done to them. Various procedures have been followed by different researchers regarding the leaching of phenolic compounds. However, what seems to be common to many explant preparation procedures is the rinsing of the excised

explants in either running tap water or sterilized distilled water. The length of time in which the explants are rinsed ranges from a few minutes to several hours. The specific stage at which this is carried out is also varied, ie. either before or after the explants have been agitated in certain concentrations of sterilant solutions (mainly NaOCl or $Ca(OCl)_2$) but in all cases, the objective is to leach out phenolic compounds. Frequent subculturing of explants is probably the most widely used methods for preventing browning. However, the severity of the problem often determines the interval between transfers. (George, 1993).

Another common practice for the control of phenolic browning is the addition of activated charcoal to the medium. Activated charcoal has many functions, the important one being its ability to absorb phenolic compounds. But because activated charcoal can also absorb growth regulators and other components of the medium, it should be used with caution (George, 1993). Hence, when activated charcoal is added in the medium, growth regulators (especially auxins) needs to be incorporated at high levels to obtain beneficial effects on the explants (Zaid, 1987). However, this was not the case in an experiment carried out by Ben-Jaacov and Jacobs (1986) where *P. cynaroides* explants were grown in liquid AND (Anderson, 1975) medium with 2.5% activated charcoal. The factorial experiment included 1, 10 and 100 $mg.l^{-1}$ of BA and 0.3, 3 and 30 $mg.l^{-1}$ of NAA. They reported that all the explants placed on the highest levels of BA and NAA died, while those explants grown on the lowest levels of BA and NAA survived the best. Therefore, this indicates that there are some contradictions regarding the addition of activated charcoal and their effect on growth regulators.

c) Modifying the environment (etiolated stock plants, culture in darkness or in low light irradiance and in low temperatures)

Treating the mother plants or modifying the surrounding environment of the mother plants and the cultured explants can help in reducing phenolic browning. The etiolation treatment of actively growing shoots on the mother plants was found to be effective in

reducing phenolic oxidation on *P. obtusifolia* (Watad *et al.*, 1992) explants. This is because young juvenile shoots or shoots that have been etiolated are less prone to browning than others due to less phenolic compounds being found in these tissues than older ones (George, 1996). Etiolation is defined as placing plants in darkness or light of low irradiance which leads to the formation of pale elongated shoots and small unexpanded leaves (George, 1993). According to Rugge (1995), treating *P. repens* mother plants was also beneficial in reducing browning, but in this case, the application of 200 $mg.l^{-1}$ benzyladenine (BA) on actively growing axillary shoots reduced browning.

The activity of enzymes concerned with both the biosynthesis and oxidation of phenolic compounds can be reduced by maintaining the explants in the dark (etiolation) and also by lowering the temperature (George and Sherrington, 1984). The length of time in which the explants are placed in the dark is dependent on the type of species and the severity of the oxidation problem. By keeping the explants in the dark also reduced the diffusion of oxidized compounds into the growing medium (Ziv and Halevy, 1983; Durand-Cresswell and Nitsch, 1977).

d) Modifying redox potential (use of antioxidants and reduced oxygen availability)

An antioxidant is defined as an electron donor that inhibits the oxidation of labile substrates with a high stoichiometric efficiency (Siegel and Porto, 1961). Antioxidants can act as reducing agents and remove oxygen from other molecules (George, 1996). Antioxidants are often assumed to prevent the oxidation of phenolic compounds by lowering the redox potentials of solution mixtures. The explants taken from mother plants which turn brown easily are often rinsed in antioxidant solutions, followed by culturing them on media containing antioxidants. (Hartmann *et al.*, 1997; Murashige, 1974a). Ascorbic acid and citric acid are the most common antioxidants used for these purposes. However, because ascorbic acid is heat labile, therefore they should rather be filter sterilized and not autoclaved. By rinsing freshly isolated explants in a solution containing ascorbic acid and citric acid, it allows the phenolic compounds in the explant tissues to

diffuse into the solution and thus, prevent the browning process (Ziv and Halevy, 1983). These types of mixtures have been used in Proteaceae experiments by Perez-Frances *et al.* (1995) where *Leucadendron* 'Safari Sunset' was treated. Another use of antioxidants is by excising the explants on filter paper which was soaked in antioxidant solution (Jarret, *et al.*, 1985a; Visseur, 1987; Ziv and Halevy, 1983).

e) Reduction in phenolase activity and substrate availability (elements involved in oxidative reactions, chelating agents and lowering of pH)

When the activity of oxidative enzymes and the substrate available for oxidation is reduced, the rate of phenolic oxidation will clearly be less (George, 1993). Some microelements such as manganese and copper can stimulate the oxidation of phenolic compounds, therefore it is advised that the use of these elements be kept to a minimum (Debergh and Read, 1991). Certain brands of agar may also introduce large amounts of copper into the medium (George, 1996). For example, 6 $g.l^{-1}$ Difco Bacto agar increased the copper concentration in the MS medium 60 – 100 times. Chelating agents such as Ethylenediaminetetraacetate (EDTA) can be used to interfere with the action of peroxidase enzymes. It was discovered that EDTA could inhibit the polyphenol oxidase activity of sunflower leaf tissues *in vitro* by removing metals essential for the oxidase enzyme activity (Weinstein *et al.*, 1951). In addition, EDTA was used by Smith (1968) to prevent blackening of shoot tips in propagation of *Carex*. This was due to the chelation of copper required by functional phenolase enzymes.

It has also been suggested that oxidation is the greatest at the pH of 6.5 and decreases at a lower pH (Ichihashi and Kako, 1977). During *in vitro* propagation of *Strelitzia reginae*, Ziv and Halevy (1983) reported that soaking the explants in a low pH solution, further reduced oxidation of polyphenols released by the injured tissue. This was probably due to the inhibition of phenolases activities. (Kahn, 1977).

f) Altering the composition of the medium.

The majority of researchers have used Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium to micropropagate Proteaceae. The majority of the experiments used either half strength or less of MS medium or half strength of the macroelements only. A possible reason for this could be because less browning is experienced when the KNO_3 and NH_4NO_3 are reduced to lesser strengths (Anderson, 1975; George, 1993).

It should be kept in mind that no single method is effective on all species and with some plants, the problem may still persist. It is recommended that a combination of treatments is adopted (George, 1993). Common practices include the etiolation of actively growing shoots on the mother plant, the use of antioxidants during the explant preparation stage and the incorporation of activated charcoal and antioxidants into the media.

The use of tissue culture methods in propagating *P. cynaroides* would assist researchers in better understanding their nutritional requirements. In addition, information regarding their physiology and growth patterns under such conditions could also benefit those that are attempting to micropropagate them. However, experimental results that contain information which maybe helpful to those in this field of work are hard to come by, simply because very few experiments that deals specifically with this topic, have been carried out. There may be two reasons for this Firstly, as discussed before, *P. cynaroides* are woody plants and they are much more difficult to propagate. Secondly, *Protea* species compared to other species of the Proteaceae family are probably the most difficult and slowest plants to propagate, partly due to its tissues being easily-oxidized (Malan, 1992).

1.6 Aim of this study

P. cynaroides (King Protea), as a cut flower is in high demand overseas and this has led to the need to explore the applicability of tissue culture for mass production of *P. cynaroides* plantlets for establishment in soil media. The hypothesis is that through the

application of specific techniques, the problem of phenolic browning can be prevented and through the manipulation of various elements in the media, the optimum media for rapid vegetative growth can be achieved. Therefore, the aim of this study was:

- to develop a medium suitable for establishing *P. cynaroides* explants;
- to develop pretreatment procedures that are able to control phenolic oxidation;
- to subculture the established explants to multiplication media for explant multiplication.

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Table 1.1: The major classes of phenolics in plants (Adapted from Harborne, 1989)

Number of carbon atoms	Basic Skeleton	Class
6	C_6	Simple phenols, Benzoquinones
7	$C_6 - C_1$	Phenolic acids
8	$C_6 - C_2$	Acetophenones, Phenylacetic acids
9	$C_6 - C_3$	Hydroxycinnamic acids, Phenypropenes, Coumarins
10	$C_6 - C_4$	Naphthoquinones
13	$C_6 - C_1 - C_6$	Xanthones
14	$C_6 - C_2 - C_6$	Stilbenes, Anthraquinones
15	$C_6 - C_3 - C_6$	Flavonoids, Isoflavonoids
18	$(C_6 - C_3)_2$	Lignans, Neolignans
30	$(C_6 - C_3 - C_6)_2$	Biflavonoids
n	$(C_6 - C_3)_n$ $(C_6)_6$ $(C_6 - C_3 - C_6)_n$	Lignins, Catechol melanins Flavolans (condensed tannins)

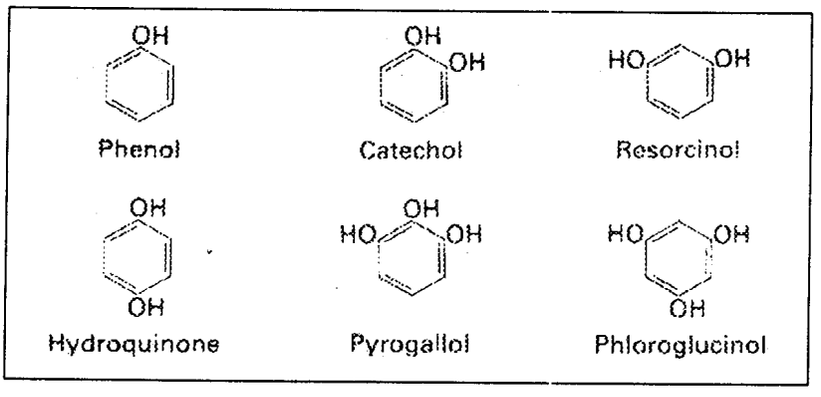

Figure 1.1: Structures of six simple phenols. (Source: Harborne, 1989)

CHAPTER 2

ALTERNATIVE ESTABLISHMENT MEDIUM FOR *PROTEA CYNAROIDES*

2.1 Summary

P. cynaroides explants were successfully established on a modified Murashige and Skoog medium. Changes made to the medium included adding gibberellic acid (GA_3), 6-benzyl-aminopurine (BAP) and ascorbic acid. Additional myo-inositol and thiamine as well as activated charcoal were added to stimulate the growth of the explants and control phenolic oxidation. Gibberellic acid (GA_3) was identified as an important growth regulator in the initial stages of micropropagation of *P. cynaroides*. GA_3 at 30 $mg.l^{-1}$ was found to be optimal for the establishment of *P. cynaroides*, whilst 10 $mg.l^{-1}$ GA_3 was insufficient.

2.2 Introduction

Being woody plants, *Protea cynaroides* are inherently difficult to micropropagate. Reasons related to this have been discussed in Chapter 1. Nevertheless, attempts have been made to establish these explants in *in vitro*. One such attempt was successfully carried out by Ben-Jaacov and Jacobs (1986). However, according to 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 into Anderson (AND) medium was the most suitable combination for establishment of *P. cynaroides*.

GA_3 incorporated into tissue culture media induces the growth of undifferentiated callus cells (Schroeder and Specter, 1957). Furthermore, in combination with auxins and cytokinin, it promotes the growth of these cells (Engelke *et al.*, 1973). However, it has

generally been found to inhibit somatic embryogenesis (Fujimura and Komamine, 1975; Tisserat and Murashige, 1977b,c) and prevent the formation of adventitious roots and shoots (George, 1993).

Nevertheless, in most papers where the propagation of Proteaceae plants via tissue culture were reported, shoot or node cultures were usually used for initiation. Thus, when these types of plant parts are being used as starting material for initiation, GA_3 plays a different role. For example, it may assist the growth and development of preformed organs such as axillary and terminal buds (George, 1993). However, a condition that is caused by GA_3 is the attenuation of leaves, where leaves that are formed are abnormally thin and slender (Jacobs *et al.*, 1969, 1970; Elliott, 1970). Despite this phenomenon, in shoot and node cultures, the addition of GA_3, in combination with cytokinin to the Stage 1 (initiation) media can improve the establishment of these cultures (George, 1993). In addition to the research done by Ben-Jaacov and Jacobs (1986) on *P. cynaroides*, similar results were also reported in *Protea obtusifolia* (Watad *et al.*, 1992), *Protea repens* (Rugge, 1995), *Grevillea scapigera* (Bunn and Dixon, 1992) and *Telopea speciosissima* (Seelye *et al.*, 1986).

The role GA_3 played in the establishment of *P. cynaroides* (Ben-Jaacov and Jacobs, 1986) was very significant and this has led to further investigation on this compound. Therefore, the aim of this study was to test the effect of GA_3 on the establishment of *P. cynaroides* by inducing the explants' buds to sprout and grow on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962).

2.3 Materials and Methods

Plant material was taken from one year-old *P. cynaroides* pot plants. The temperature of the greenhouse in which the plants grew was maintained at ±23°C throughout the duration of the experiment.

Shoots of between 10 and 20 cm in length were removed from the plants and cut into shorter lengths containing several nodes. The shoot's leaves were then removed, leaving only a short petiole. These shoots were then placed under running water for approximately 10 minutes to wash away excessive contaminants. The shoots were further sterilized by stirring them in a beaker filled with distilled water and 0.5g/l Benlate (a commercial fungicide) for 1 hour. Afterwards, they were cut into even shorter segments containing one or two nodes and dipped into 2% sodium hypochlorite (a surface sterilant) for 20 minutes under sterile conditions. This was followed by 3 successive rinses in sterile distilled water for 15 minutes each. Each segment was then inserted into test tubes (2.5 cm x 9.9 cm) containing the formulated Murashige and Skoog (MS) media.

Two different media treatments comprising of Murashige and Skoog (MS) medium were used. For both media, the NH_4NO_3 and KNO_3 were reduced to half strength, while the remaining salts were kept at full strength (Table 2.1). Two different concentrations of GA_3 were incorporated into the media, namely 10 $mg.l^{-1}$ and 30 $mg.l^{-1}$ to test which concentration is most suitable for establishment of *P. cynaroides* explants.

Additional growth regulators and vitamins which were added into the media were 6-benzyl-aminopurine (BAP) (2 $mg.l^{-1}$), myo-inositol (100 $mg.l^{-1}$) and thiamine (0.1 $mg.l^{-1}$). Ascorbic acid was also added to function as an antioxidant to control phenolic oxidation. In addition, because ascorbic acid is a vitamin (Vitamin C), together with myo-inositol and thiamine, they can assist in the growth of the explants by stimulating cell division and promote tissue growth (George, 1993). Activated charcoal (3 $g.l^{-1}$) was included to control phenolic oxidation. The pH of the medium was adjusted to five and gelrite (2.5 $g.l^{-1}$) (a synthetic agar) was added to solidify the media.

After planting the explants into the test tubes, they were placed in the growing chamber with a 16-hour photoperiod provided by white fluorescent tubes placed at 30 cm above plant level. The temperature was adjusted to 25°C ± 3°C for the duration of the experiment which lasted 40 days.

2.4 Results and Discussion

The explants cultured on 30 $mg.l^{-1}$ GA_3 responded much better than those of the control (10 $mg.l^{-1}$ GA_3). The shoots that were planted in the control medium either remained dormant or turned brown after 14 days whilst those cultured on the medium containing 30 $mg.l^{-1}$ GA_3 sprouted and continued to grow and reached lengths of between 1.5 cm and 2.5 cm within 30 days (Figure 2.1). These results differ from Ben-Jaacov and Jacobs (1986), where 10 $mg.l^{-1}$ GA_3 added to the Anderson medium was found to be optimum. Higher concentrations of GA_3 were not tested in this experiment because previous research showed that high amounts of GA_3 did not necessarily result in stronger and more robust growth (Ben-Jaacov and Jacobs, 1986).

Regarding the attenuation of leaves caused by the addition of GA_3, this was not the case in the establishment of these explants. As Figure 2.1 below illustrates, normal growth of the leaves from the shoot was evident. This is similar to the results of Ben-Jaacov and Jacobs (1986) where after sprouting and growth of the axillary bud, it was reported that expansions of leaves were observed.

The plant material used in this trial was shoots with one or two nodes containing axillary buds. This choice was made since the results from the experiments by Ben-Jaacov and Jacobs (1986) showed that seeds should not be used for initiation because of its low germination rate. Terminal buds were also not used, because they remain dormant or turned brown which resulted in death (Ben-Jaacov and Jacobs, 1986). Therefore, axillary buds are recommended for future establishment of *P. cynaroides*.

Factors that are also of importance are the formulation of the medium used. Murashige and Skoog medium was chosen because its mineral salt compositions are basically the same as that of Anderson's medium. In addition, Murashige and Skoog medium has been the most successful and most widely used medium for establishment of Proteaceae in *in vitro* propagation. Furthermore, it is the most common and easily obtainable medium available.

For establishment of *P. cynaroides* explants, solid media containing activated charcoal are more suitable according to Ben-Jaacov and Jacobs (1986). In the micropropagation of other *Protea* species such as *P. obtusifolia* (Watad *et al.*, 1992) and *P. repens* (Rugge, 1995), solid media were also used rather than liquid media. In this trial, the use of solid media proved to be suitable for *P. cynaroides*, since growth of the explants was achieved and therefore confirms abovementioned statements.

Another finding that was made was the detrimental effects of phenolic oxidation on the *P. cynaroides* explants. Activated charcoal was used throughout this trial to absorb the phenolic compounds. The inclusion of ascorbic acid in the medium was also to reduce phenolic oxidation. These methods however, have failed to control phenolic oxidation sufficiently since, over 50% of the explants died due to browning.

2.5 Conclusion

A Murashige and Skoog medium, comprising of half strength NH_4NO_3 and KNO_3, and full strength of the remaining salts, in combination with 30 $mg.l^{-1}$ GA_3, 100 $mg.l^{-1}$ myo-inositol, 1 $mg.l^{-1}$ ascorbic acid and 0.1 $mg.l^{-1}$ thiamine is an alternative medium for the establishment of *P. cynaroides*. However, a problem which affected the experiment as a whole was phenolic oxidation. In addition to the methods used in this trial to address the problem, other methods such as etiolation, and use of antioxidants and chelating agents which are known to be able to control phenolic oxidation needs to be studied. Future experiments should concentrate more in the application of combined methods that are effective in reducing phenolic oxidation. This will ensure that less explants are lost to phenolic oxidation which will result in an increased survival rate.

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Table 2.1: The composition of Murashige and Skoog (MS) and Anderson (AND) media and the additional growth regulators and vitamins included in this trial. (Adapted from George, 1993)

Medium Composition	MS ($mg.l^{-1}$)	AND ($mg.l^{-1}$)
MS medium		
NH_4NO_3	1650	2000
KNO_3	1900	950
$CaCl_2.2H_2O$	440	440
$MgSO_47H_2O$	370	370
KH_2PO_4	170	170
$NaH_2PO_4.H_2O$	-	170
KI	830	830
H_3BO_3	6200	6200
$MnSO_4.4H_2O$	22300	22300
$ZnSO_4.7H_2O$	8600	8600
$Na_2MoO_4.2H_2O$	250	250
$CuSO_4.5H_2O$	25	25
$CoCl_2.6H_2O$	25	25
$FeSO_4.7H_2O$	27850	27850
$Na_2EDTA.2H_2O$	37250	37250
Myo-inositol	100	100
Nicotinic acid	500	-
Pyridoxine-HCl	500	-
Thiamine-HCl	100	400
Glycine	2	-
Additional growth regulators and vitamins		
Gibbelleric acid (GA_3)	10[a] 30[b]	
6-benzylaminopurine (BAP)	2	
Ascorbic Acid	1	
Myo-inositol	100	
Thiamine	0.1	
Others	($g.l^{-1}$)	
Activated Charcoal	3	
Sucrose	30	
Gelrite	2.5	

[a] Concentration used for medium 1 (Control)
[b] Concentration used for medium 2

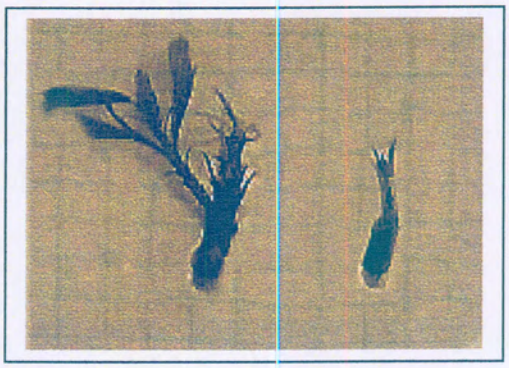

Figure 2.1: Photographical illustration of axillary bud on an explant grown on medium containing 30 mg.l^{-1} GA_3 (Left) and an explant unresponsive to 10 mg.l^{-1} GA_3 (Right).

CHAPTER 3

EFFECT OF PRETREATMENTS ON PHENOLIC OXIDATION IN *PROTEA CYNAROIDES* DURING MICROPROPAGATION

3.1 Summary

Phenolic oxidation is one of the reasons why *Protea cynaroides* are not being extensively propagated via tissue culture techniques. The leaching of the oxidized phenolic compounds into the medium causes the explant and the medium to turn brown. This is detrimental to the growth of the explants. Therefore, methods to reduce the oxidation of phenolic compounds are needed to ensure that explants remain green to allow growth to commence. Axillary buds that were sterilized with 0.35% sodium hypochlorite and stirred in an antioxidant solution of 100 $mg.l^{-1}$ ascorbic acid for 24 hours had the highest survival rates. The explants that were sterilized with 0.3% mercuric chloride and stirred in the same antioxidant solution were all killed.

3.2 Introduction

As mentioned in Chapter 1, plants belonging to the *Protea* genus are more difficult to micropropagate when compared to other genera of the Proteaceae family (Malan, 1992). In addition to this, phenolic oxidation is another stumbling block that *P. cynaroides* is faced with in *in vitro* propagation (Malan, 1992). The main problem encountered in the studies done in Chapter 2 was phenolic oxidation, which confirms the difficulties in which *in vitro* propagation of *P. cynaroides* may have. The abovementioned problems are therefore seen as one of the main reasons for the lack of progress in formulating a complete procedure for the micropropagation of these *Protea* species.

In this trial, essential pretreatments for *in vitro* propagation, namely sodium hypochlorite and mercuric chloride and/or antioxidants (ascorbic acid and citric acid) were evaluated for phenolic oxidation reduction in *in vitro* propagation of *P. cynaroides*.

3.3 Materials and Methods

In this trial, 32 treatments with five replicates each were used. These treatments were made up of 16 factorial combinations of ascorbic acid and citric acid solutions, and two sterilants (Table 3.1). Shoot segments (± 10 cm) were taken from one-year old *P. cynaroides* plants grown in black plastic bags, containing sandy loam medium, in a greenhouse. The average greenhouse temperature was maintained at ±23°C throughout the duration of the trial.

In the tissue culture laboratory the leaves were removed and thereafter the explants were cut into smaller segments of ±1 cm in length, containing one or two nodes each, and then rinsed in running water for 2 hours. The explants were then dipped into 70% ethanol for 10 seconds. Further surface sterilization took place when the explants were either sterilized with 0.3% mercuric chloride or 0.35% sodium hypochlorite for 7 minutes and 10 minutes respectively. The explants of both treatments were then stirred in solutions containing different concentrations of ascorbic acid and citric acid for 24 hours (Table 3.1).

The explants were then planted into test tubes containing half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Sucrose (20 $g.l^{-1}$), activated charcoal (3 $g.l^{-1}$) and gelrite (3 $g.l^{-1}$) were also added. Ethylenediaminetetraacetate (EDTA) (50 $mg.l^{-1}$) was specifically added to the medium of all the treatments as they are chelating agents and are known to reduce oxidation in some plants (George, 1996). Additional growth regulators such as gibberellic acid (GA_3), 6-benzylaminopurine (BAP) and myo-inositol were incorporated into the medium in concentrations of 30 $mg.l^{-1}$, 2 $mg.l^{-1}$ and 100 $mg.l^{-1}$ respectively. These growth regulators were added into the medium because

they assist the explants to establishment in the initial stages of micropropagation (See Chapter 2). The pH was adjusted to five before autoclaving.

After planting, the explants in the test tubes were etiolated in the dark for 7 days according to George (1996) to reduce phenolic oxidation in the growth chamber, after which they were grown under lights with a 16-hour photoperiod. Fluorescent tubes which were 30 cm above plant level were used, providing 60µmol/m^2/sec photosynthetically active radiation (PAR), and the temperature was adjusted to 28°C±2 in the growth chamber.

Fortunately, due to the success of the sterilization process, no contamination of the explants were observed. This led to all treatments being equally replicated with five replications each.

3.4 Results and Discussion

Figure 3.1 clearly illustrates that sodium hypochlorite-sterilized explants did not turn brown as opposed to those sterilized by mercuric chlorite, which turned brown after 48 hours. The use of sodium hypochlorite proved to be more suitable as the concentration was optimal enough to decontaminate the explant and at the same time limit any injuries which may have enhanced oxidative browning. *P. cynaroides* explants failed to grow when they were sterilized with 0.3% mercuric chloride. These results are in contradiction with Ziv and Halevy (1983) where oxidation was reduced when *S. reginae* explants were sterilized with the same concentration of mercuric chloride. Therefore, even though mercuric chloride has been successfully used as a sterilant in other plant species, this sterilant is not recommended for *P. cynaroides*.

Following the sterilization process was the stirring of the explants in solution mixtures of ascorbic acid and citric acid for 24 hours. Most of the treated explants turned brown and failed to grow after 48 hours (Figure 3.2). However, Figure 3.2 illustrates that explants

which were stirred in Treatment 3 had the highest percentage that remained green (60%), this was followed by Treatment 1 and 5 with 40% survival, whilst Treatment 25 had only 20% of its explants remaining green.

The length of time in which the explants were in the antioxidant solution (24 hours) seemed to be too long. For *P. cynaroides*, this probably caused it to enhance the oxidation process that might have already started after the sterilizing stage because as mentioned before, ascorbic acid can be oxidized to become dehydro-ascorbic acid, depending on the copper and iron concentrations in the medium (Elmore *et al.*, 1990). However, submerging the explants for 24 hours in a solution of antioxidants had a positive effect on phenolic oxidation of *S. reginae*, where oxidative browning was reduced considerably (Ziv and Halevy, 1983). Similar results were found by Zaid and Tisserat (1983) in *Phoenix dactylifera*.

3.5 Conclusion

P. cynaroides explants are more suited to being sterilized by 0.35% sodium hypochlorite than 0.3% mercuric chloride, probably because their tissues are more easily stressed or injured by these chemicals than other plant species such as *S. reginae*. Similarly, stirring the explants in antioxidant solutions for 24 hours was also too long and this injured the explant tissues even more. Nevertheless, concerning the antioxidants, the fact that several explants have survived is an indication that ascorbic acid and citric acid could have a role to play in controlling phenolic oxidation. Further adjustments in terms of their concentrations as well as the length of stirring time will probably result in more positive results. Furthermore, comparisons need to be made between explants that are etiolated after excision and those that are not. This could then allow researchers to better understand the light requirements of *P. cynaroides* explants. Future research is needed to further study the importance of these factors in reducing phenolic oxidation during micropropagation of *P. cynaroides*.

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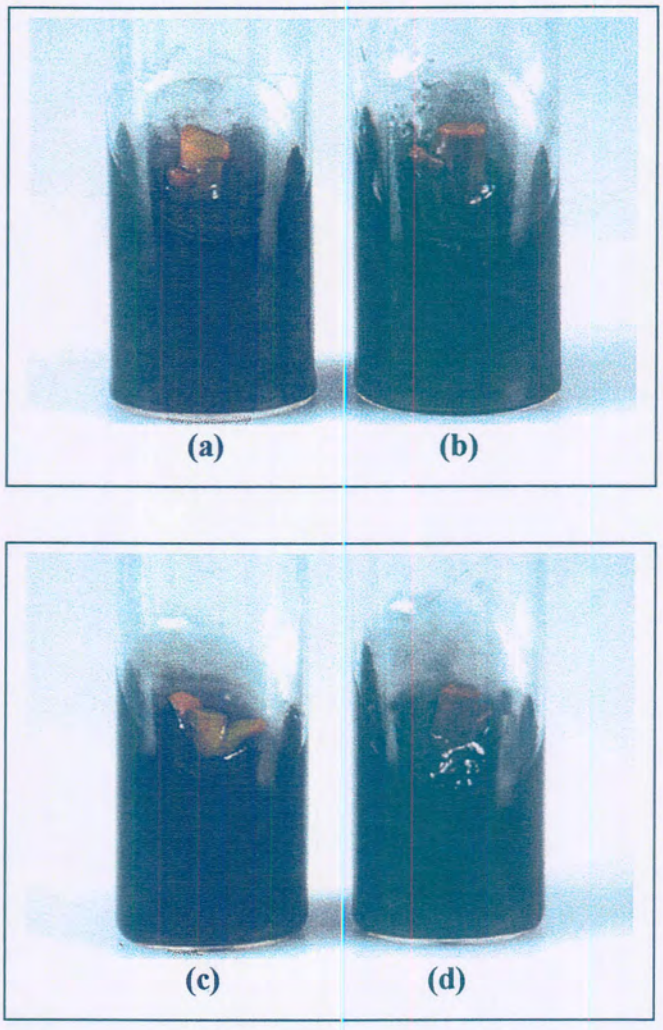
Table 3.1: Thirty-two treatments comprising of 16 factorial combinations of ascorbic acid and citric acid as well as two sterilants.

Treatments	Ascorbic Acid ($mg.l^{-1}$)	Citric Acid ($mg.l^{-1}$)	Sterilants
1	0	0	Sodium Hypochlorite
2	0	0	Mercuric Chloride
3	100	0	Sodium Hypochlorite
4	100	0	Mercuric Chloride
5	500	0	Sodium Hypochlorite
6	500	0	Mercuric Chloride
7	1000	0	Sodium Hypochlorite
8	1000	0	Mercuric Chloride
9	0	500	Sodium Hypochlorite
10	0	500	Mercuric Chloride
11	100	500	Sodium Hypochlorite
12	100	500	Mercuric Chloride
13	500	500	Sodium Hypochlorite
14	500	500	Mercuric Chloride
15	1000	500	Sodium Hypochlorite
16	1000	500	Mercuric Chloride
17	0	1000	Sodium Hypochlorite
18	0	1000	Mercuric Chloride
19	100	1000	Sodium Hypochlorite
20	100	1000	Mercuric Chloride
21	500	1000	Sodium Hypochlorite
22	500	1000	Mercuric Chloride
23	1000	1000	Sodium Hypochlorite
24	1000	1000	Mercuric Chloride
25	0	1500	Sodium Hypochlorite
26	0	1500	Mercuric Chloride
27	100	1500	Sodium Hypochlorite
28	100	1500	Mercuric Chloride
29	500	1500	Sodium Hypochlorite
30	500	1500	Mercuric Chloride
31	1000	1500	Sodium Hypochlorite
32	1000	1500	Mercuric Chloride



* Treatment 1: 0 mg.l^{-1} ascorbic acid & 0 mg.l^{-1} citric acid (sodium hypochlorite as sterilant)
* Treatment 2: 0 mg.l^{-1} ascorbic acid & 0 mg.l^{-1} citric acid (mercuric chlorite as sterilant)
* Treatment 3: 100 mg.l^{-1} ascorbic acid & 0 mg.l^{-1} citric acid (sodium hypochlorite as sterilant)
* Treatment 4: 100 mg.l^{-1} ascorbic acid & 0 mg.l^{-1} citric acid (mercuric chlorite as sterilant)

Figure 3.1: Illustrations of explants after treating in Treatment 1* (a), Treatment 2* (b), Treatment 3* (c) and Treatment 4* (d).

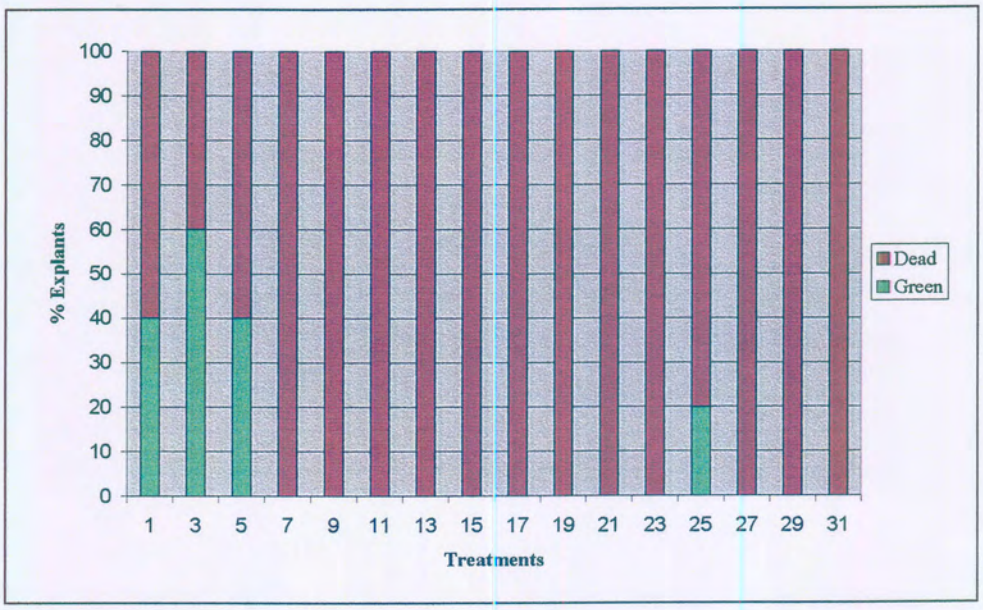


Figure 3.2: The percentages of explants of various treatments which died or remained green after 48 hours, using sodium hypochlorite as sterilant and stirring in antioxidant solutions for 24 hours.

CHAPTER 4

IMPROVEMENT IN *PROTEA CYNAROIDES* ESTABLISHMENT BY USE OF ANTIOXIDANTS TO REDUCE PHENOLIC OXIDATION

4.1 Summary

The reduction of phenolic oxidation was best achieved by stirring the explants for 1 hour in an antioxidant solution mixture containing 100 $mg.l^{-1}$ ascorbic acid and 1500 $mg.l^{-1}$ citric acid. In combination with this antioxidant solution, establishment was highly successful where 100% bud growth was achieved by growing the explants under 16-hours photoperiod. Seven-days etiolation of the explants resulted in browning of the explant tissues leading to failure of buds to grow.

4.2 Introduction

From the results of the first trial in Chapter 3, progress has been made in two areas. Firstly, which combination of ascorbic acid and citric acid solution has beneficial effects with the explants in controlling phenolic oxidation, and secondly, which sterilant is more suitable for sterilizing *P. cynaroides* explants.

Therefore, the main objective of this trial was to use similar treatments to those from trial 1 (Chapter 3), and with a few adjustments, to further improve the conditions in which these explants were exposed to, which will eventually reduce phenolic browning and result in bud sprouting.

4.3 Materials and Methods

Shoot segments (± 10 cm) were taken from one-year old mother plants grown in black plastic bags containing sandy loam medium in the greenhouse. The growing conditions of the mother plants in the greenhouse were similar to the first trial in Chapter 3. The sterilization procedure, pre-treatments used in this trial were adapted from the first trial which was described in Chapter 3. After the leaves were removed from the explants, 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. Afterwards, the explants were surface sterilized with 0.35% sodium hypochlorite for 6 minutes.

The 16 treatments used in this trial are given in Table 4.1. These treatments comprised of four different ascorbic acid and citric acid solution mixtures, selected from the first trial (Chapter 3). Two time lengths in which the explants were stirred in (1 hour and 12 hours), and two light conditions in which the explants were exposed to (seven-days etiolation period and 16-hours photoperiod) were added to this trial. The seven-days etiolation culturing condition indicates that the explants were grown under total darkness for the first seven days after planting in test tubes. Afterwards, they were grown under 16-hours photoperiod. The other culturing condition of 16-hours photoperiod indicates that the explants were grown in 16-hour photoperiod since planting into test tubes.

The medium used in this trial was identical to the one in the first trial. See Chapter 3 for the medium composition. Observations were taken from the 14th day after the trial started and then in three-day intervals thereafter because this was approximately the time when the reactions of the explants to the treatments could be clearly seen.

Each treatment consisted of five replications and the data were analyzed using the PROC. GLM (General Linear Models) procedure in the S. A. S (Statistical Analysis System) program.

4.4 Results and Discussion

Figure 4.1 illustrates the results of control explants stirred in solution 1 (0 $mg.l^{-1}$ ascorbic acid & 0 $mg.l^{-1}$ citric acid) for 1 hour and 12 hours stirring periods and under seven-days etiolation and 16 hours-photoperiod. The explants in Treatments 1, 2 and 4 (Figures 4.1a, b and d respectively) showed bud growth. The highest percentage of bud growth was achieved (40% bud growth after 17 days) when the explants were stirred in the solution for 1 hour and etiolated for 7 days (Figure 4.1a). This was followed by Treatments 2 and 4, which had 20% bud growth (Figures 4.1b and d). However, as Figures 4.1a and 4.1c clearly illustrates, at least 40% of the etiolated explants were dead in the first few weeks compared to the explants that were grown in 16-hours photoperiod (Figures 4.1b and d). In addition, the latter started to die off towards the end of the experiment between days 50 and 62, irrespective of whether it has been stirred in the solution for 1 or 12 hours. This could be an indication that newly cultured explants require light conditions to grow. This result is in contradiction with findings in *in vitro* propagation of *Rhododendron* (Meyer, 1981) that browning of tissues was often reduced or prevented by dark treatments. However, this result is similar to reports mentioned by Marks and Simpson (1990) that phenolic oxidation of several woody plants are not prevented by culturing explants in darkness.

Nevertheless, treating explants with solution 1 (control: 0 $mg.l^{-1}$ ascorbic acid & 0 $mg.l^{-1}$ citric acid) certainly had beneficial effects due to the fact that sprouting and growth of buds occurred. A possible reason for this could be because this solution had only sterilized distilled water, which may have diluted the phenolic compounds that leached from the excised tissues when the explants were submersed, to an extent where browning of the tissues was prevented. This process is similar to leaching and dispersal of phenolic compounds described by George (1996), to control phenolic oxidation. In this case, George (1996) emphasized that dispersion of the products released from damaged cells is effective in controlling phenolic oxidation. In addition, findings made from *in vitro* propagation of *Eucalyptus grandis* (Cresswell and Nitsch, 1975) and regeneration of

apple plants (Lane, 1978) recommended that explants should be rinsed or left in sterile water for several hours before transferring to the test tubes.

Figures 4.2a, b, c and d shows the results for the effect of antioxidant solution 2 which only had 100 $mg.l^{-1}$ ascorbic acid added in it. This seems to have caused more explants to turn brown and die than in those treated with solution 1 (Figures 4.1a, b, c and d). In general, all the treatments (Figures 4.2a, b, c and d) using antioxidant solution 2 had 100% explant death by the end of the trial. Even the explants in Treatment 8 (Figure 4.2d) which sprouted and grew from day 17 to 20 ceased to continue growing due to phenolic oxidation. This confirms that ascorbic acid can be oxidized to dehydro-ascorbic acid depending on the concentration of copper and iron in the medium and thus enhancing oxidative browning rather than reducing it (Elmore *et al.*, 1990). In this trial, after the explants were planted into the test tubes, the presence of ascorbic acid in the medium was probably from residues left on the pretreated explants, which were oxidized after coming in contact with copper and iron compounds in the medium. In Figure 4.2b, it is also important to notice that Treatment 6 gave the best results on restricting phenolic oxidation (100% survival rate for 29 days). Probably the low stirring time, without the etiolation treatment was the main reason for controlling phenolic oxidation. As illustrated in Figure 4.1b, these two factors improved explant establishment.

The results of the explants that were stirred in antioxidant solution 3 (1500 $mg.l^{-1}$ citric acid) are shown in Figures 4.3a, b, c and d. The antioxidant solution which was made up of high amounts of citric acid alone without any ascorbic acid did not seem to be the correct concentration in reducing phenolic browning, especially in the 12 hours stirring time periods. However, as illustrated in Figure 4.3b, the explants from Treatment 10 yielded positive results, as only 20% of the explants died while 80% of the explants' buds sprouted. Opposite results were found in Treatment 11 (Figure 4.3c) where all the explants turned brown resulting in death since observations of the explants began.

Figure 4.4 illustrates the effects of antioxidant solution 4 (100 $mg.l^{-1}$ ascorbic acid & 1500 $mg.l^{-1}$ citric acid), the different stirring time periods (1 hour & 12 hours) as well as

the culturing conditions (seven-days etiolation & 16-hours photoperiod), on the survival of *P. cynaroides* explants. Treatment 14 (Figure 4.4b) was undoubtedly the most suitable for reducing phenolic oxidation and promoting bud sprouting. These explants had a 100% survival rate. Coincidentally, this ascorbic acid and citric acid solution mixture is the same as the one used in the multiplication of *Leucadendron* 'Safari Sunset' (Perez *et al.*, 1995). In their experiment, the explants were kept in shaking antioxidant solution for 1 hour to prevent oxidation. The same concentrations of ascorbic acid and citric acid were also added into their establishment medium.

From previous research where antioxidant solutions were used, although varied in their concentrations, a similarity which exists is that the solutions usually contain a combination of ascorbic acid and citric acid. For example, *Musa textilis* (1000 $mg.l^{-1}$ ascorbic acid & 1500 $mg.l^{-1}$ citric acid) (Mante and Tepper, 1983), *Aechmea fasciata* (150 $mg.l^{-1}$ ascorbic acid & 100 $mg.l^{-1}$ citric acid) (Jones and Murashige, 1974), *Rhododendron* (150 $mg.l^{-1}$ ascorbic acid & 150 $mg.l^{-1}$ citric acid) (Anderson, 1975) and *S. reginae* (100 $mg.l^{-1}$ ascorbic acid & 150 $mg.l^{-1}$ citric acid) (Ziv and Halevy, 1983). A reason why this combination seems to be common is because according to George (1996), citric acid could act as a chelating agent which sequesters metal ions and therefore delay the oxidation of ascorbic acid to dehydro-ascorbic acid, reducing browning of the explants.

Table 4.2 summarizes the survival percentages of each treatment from the figures as well as the mean length of those buds which sprouted. Regarding the mean length of the shoots, an important point that should be mentioned is the etiolation of the explants. As mentioned before, etiolation did not assist in reducing phenolic oxidation. However, there seems to be an overall indication that when there are buds sprouting from etiolated explants, their mean length are much longer than those which were grown under 16-hours photoperiod (Table 4.2). Although the percentage of the etiolated explants that survived is small, it seems as if the etiolation process has an effect on the elongation of the shoots after the buds have sprouted. This result is in agreement with the well known fact that

etiolation causes anatomical and physiological changes in the stem tissues causing the elongation of shoots (Hartmann *et al.*, 1997).

Figure 4.5 shows a photographical illustration of treated explants that was exposed to a 16-hour photoperiod. The explants that were etiolated had no sign of any growing axillary buds, while the axillary bud of explant, that was grown under 16-hour photoperiod, started to sprout after 14 days. Figures 4.6 and 4.7 show the elongation of the shoots after bud sprouting from the 30th day to the 62nd day in Treatment 14 (100 mg.l^{-1} ascorbic acid and 1500 mg.l^{-1} citric acid for 1 hour and grown under a 16-hour photoperiod).

4.5 Conclusion

It is clear that it is not a single factor that affects phenolic oxidation of the explants, but rather a number of factors. Firstly, the concentration of the antioxidants should be correct, because as mentioned before, ascorbic acid can induce oxidation once it is oxidized. Closely associated with the antioxidant concentration is the length of time in which the explants are stirred. The *P. cynaroides* explants are too soft to be submerged in the solution for long periods of time. Therefore, the shorter time period (1 hour) was more effective. However, the age of the explant may also play a role, since older plant materials are woodier. Secondly, etiolation of the explants has showed that it does not reduce oxidation, while a 16-hour photoperiod seems to be a better light condition for the explants. In conclusion, the procedures discussed in this paper are adequate in controlling phenolic oxidation and the medium used are suitable for establishing *P. cynaroides*.

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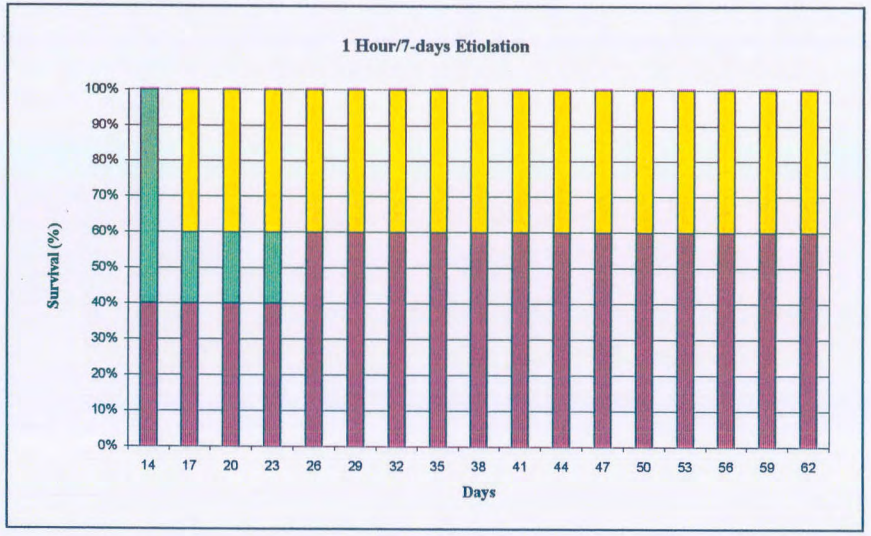
a) Treatment 1



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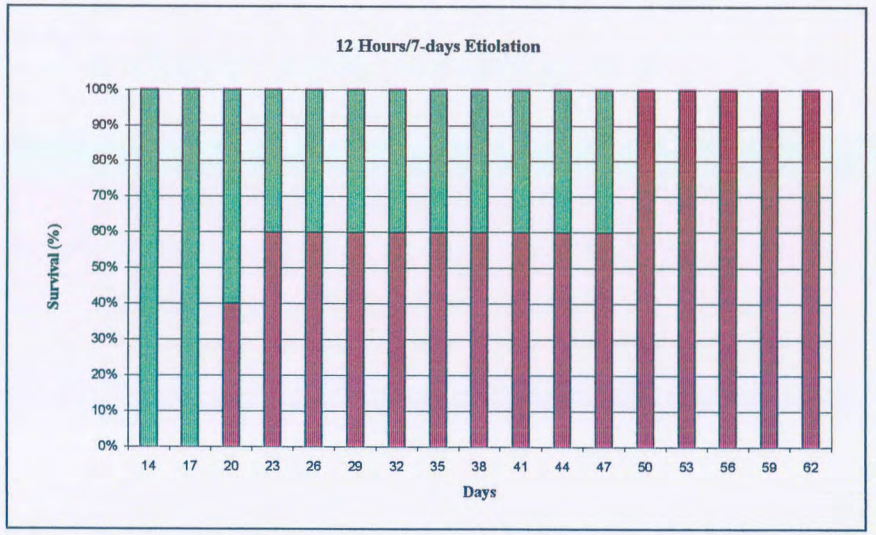

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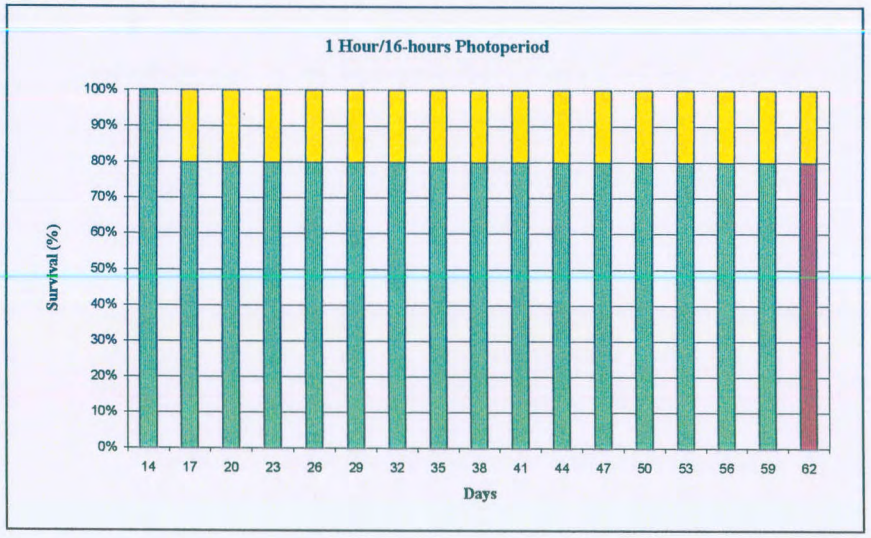

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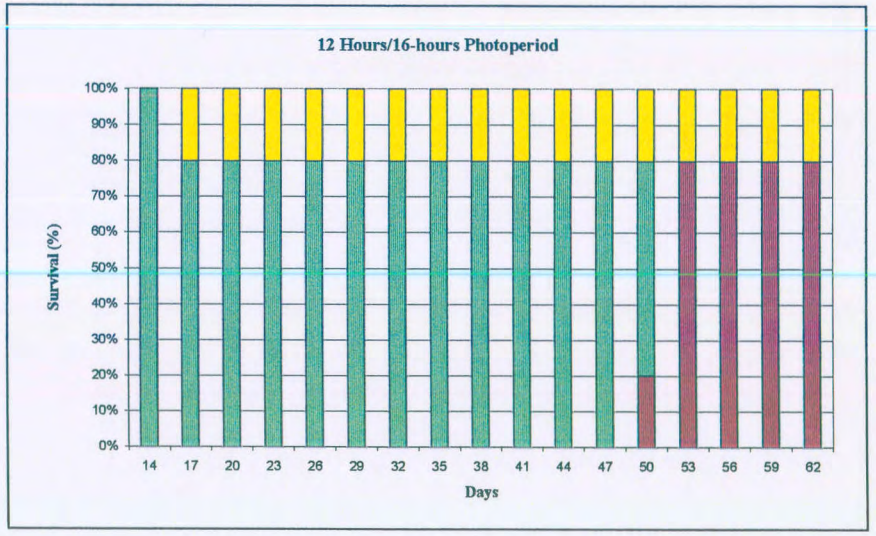


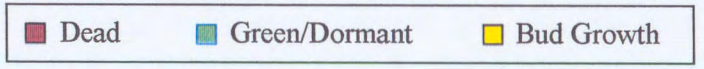
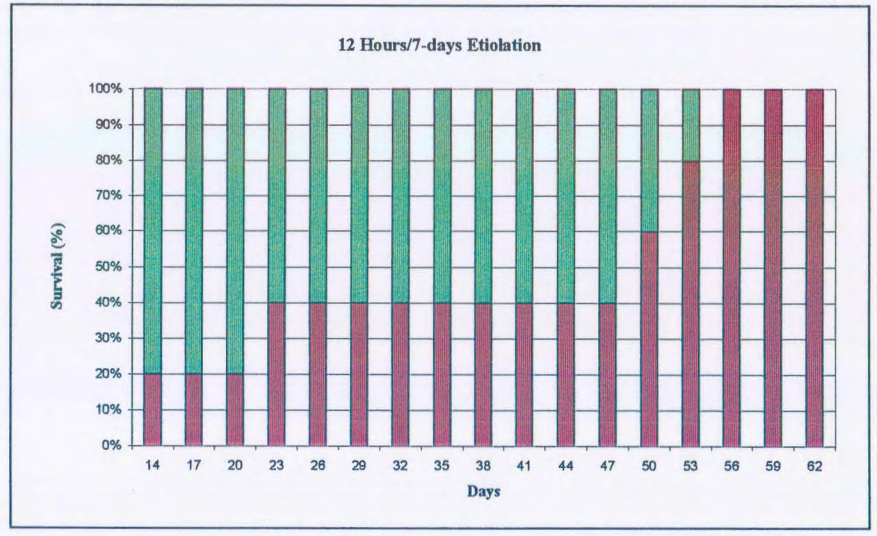


Figure 4.1: The effects of antioxidant solution 1 (control: 0 mg.l^{-1} ascorbic acid & 0 mg.l^{-1} citric acid), the different stirring time periods (1 hour & 12 hours) and culturing conditions (7-days etiolation & 16-hours photoperiod), on the survival of *P. cynaroides* during establishment.

a) Treatment 5

c) Treatment 7



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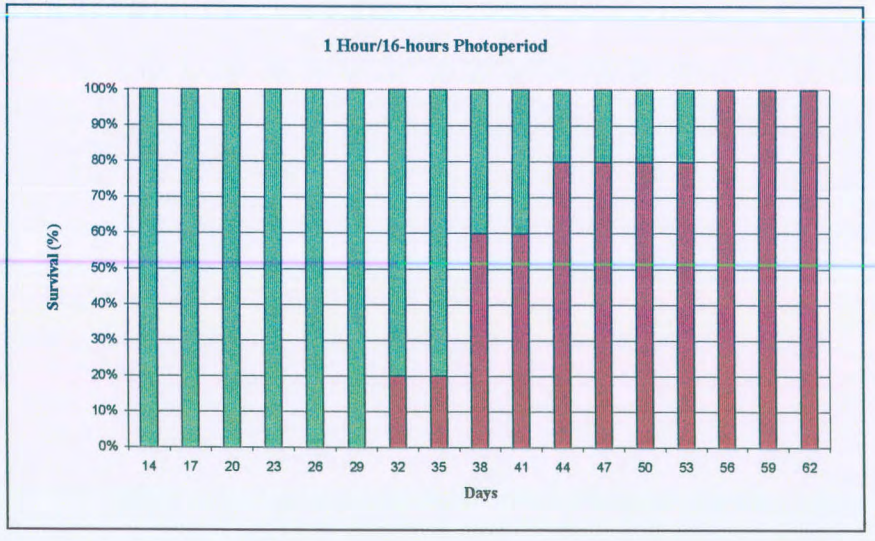
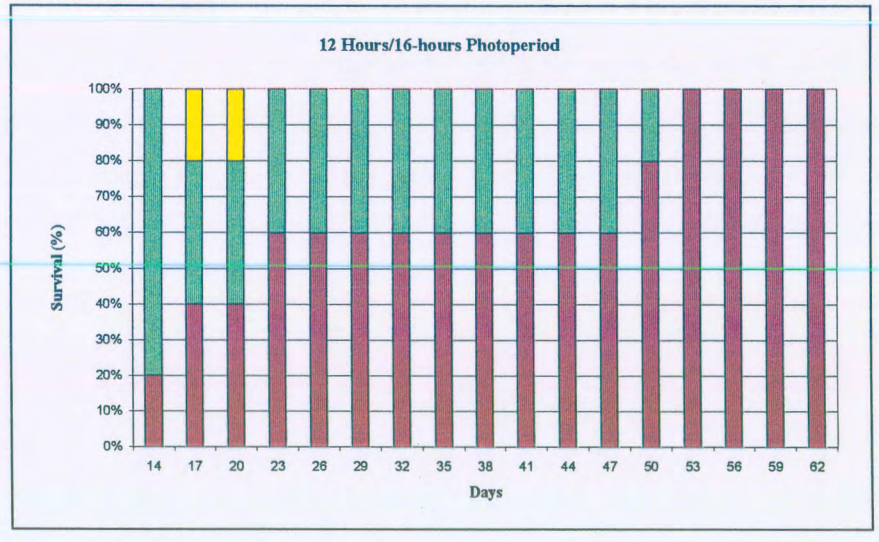


d) Treatment 8



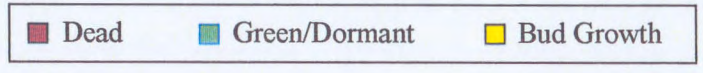
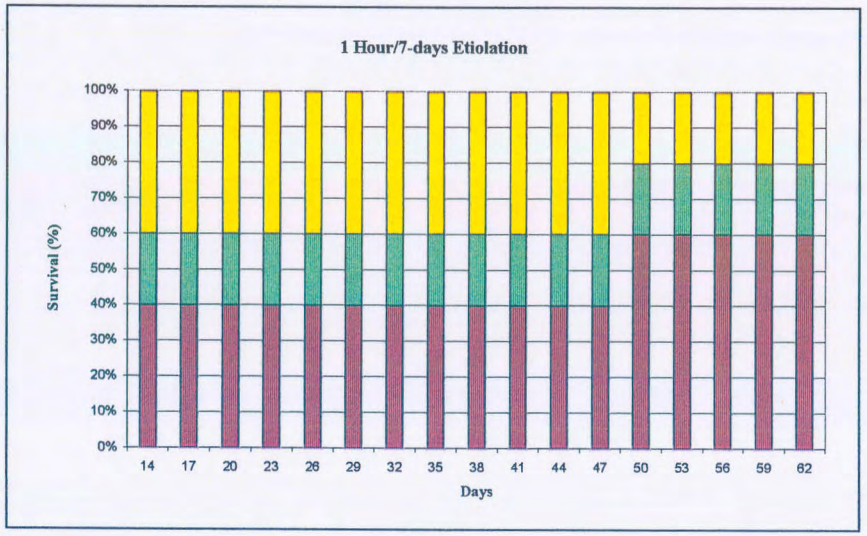
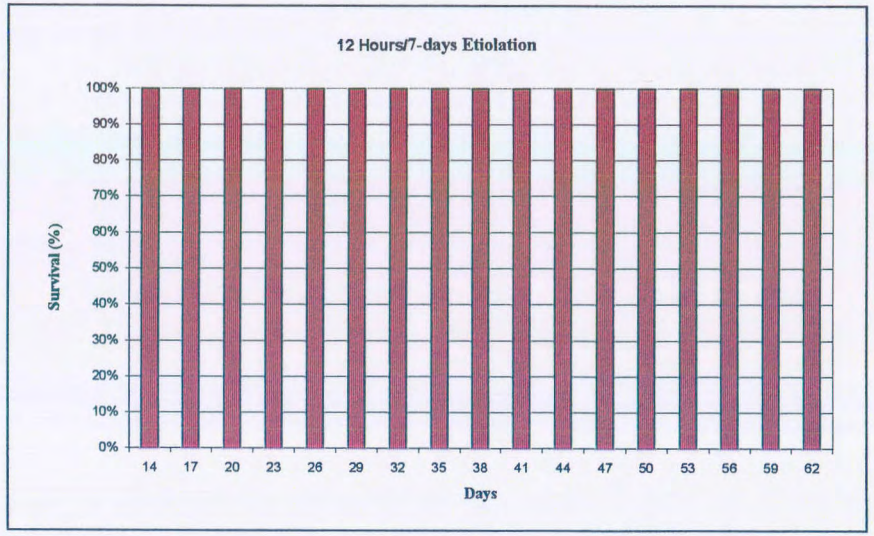


Figure 4.2: The effects of antioxidant solution 2 (100 mg.l^{-1} ascorbic acid & 0 mg.l^{-1} citric acid), different stirring time periods (1 hour & 12 hours) and culturing conditions (7-days etiolation & 16-hours photoperiod), on the survival of *P. cynaroides* during establishment.

a) Treatment 9

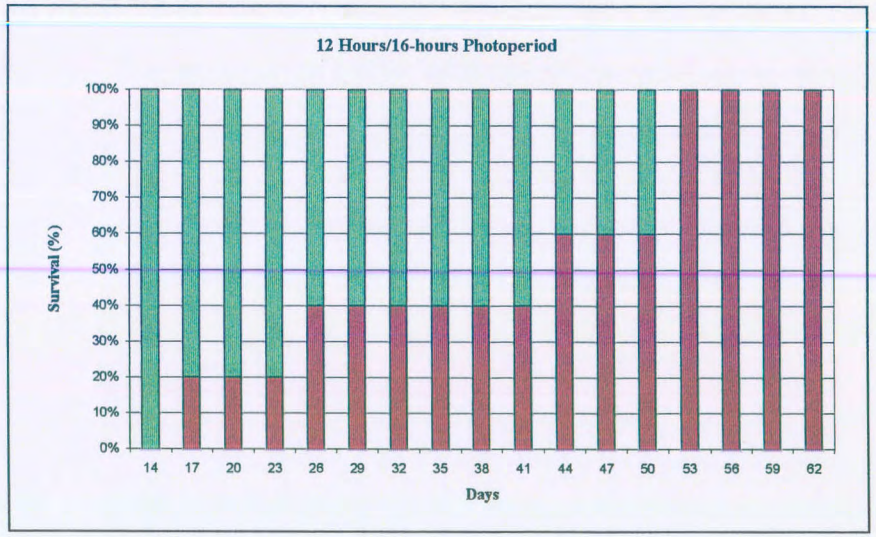


c) Treatment 11



b) Treatment 10

d) Treatment 12



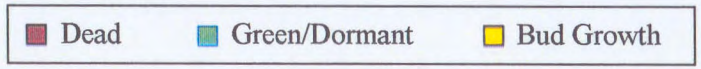
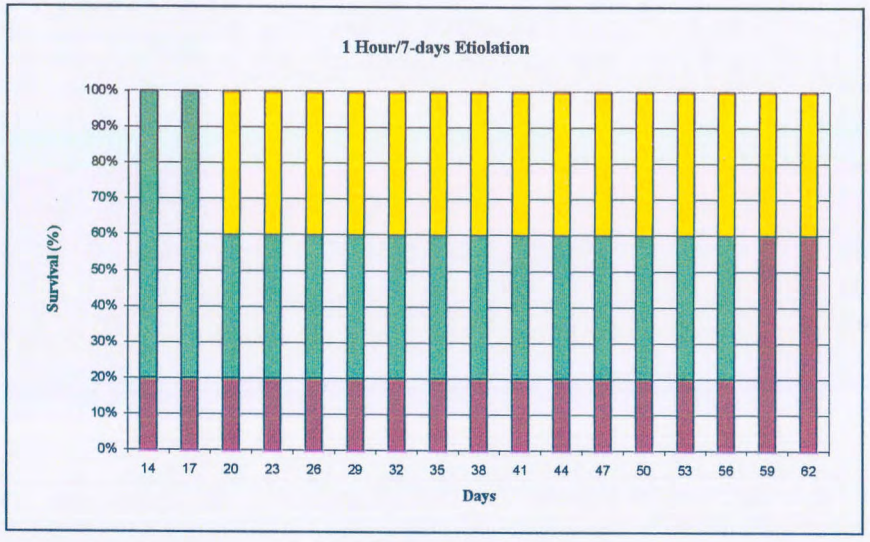
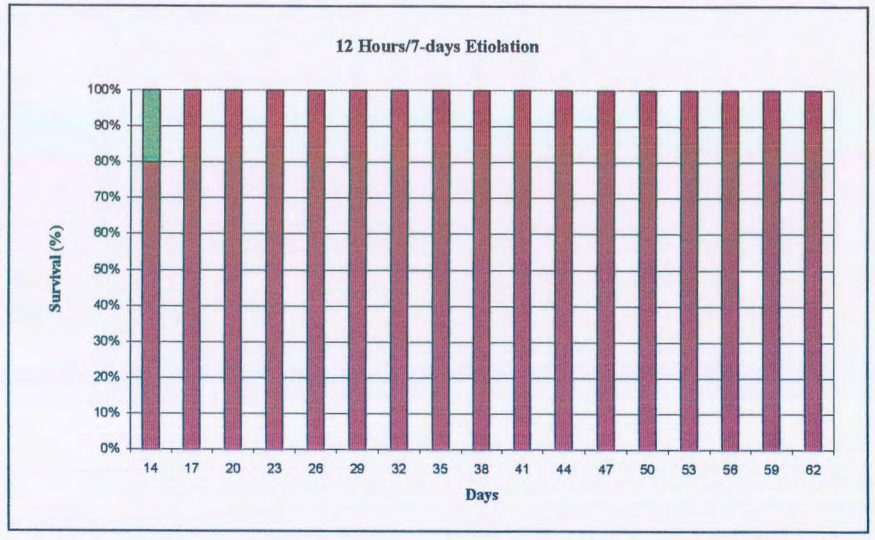


Figure 4.3: The effects of antioxidant solution 3 (0 mg.l^{-1} ascorbic acid & 1500 mg.l^{-1} citric acid), different stirring time periods (1 hour & 12 hours) and culturing conditions (7-days etiolation & 16-hours photoperiod), on the survival of *P. cynaroides* during establishment.

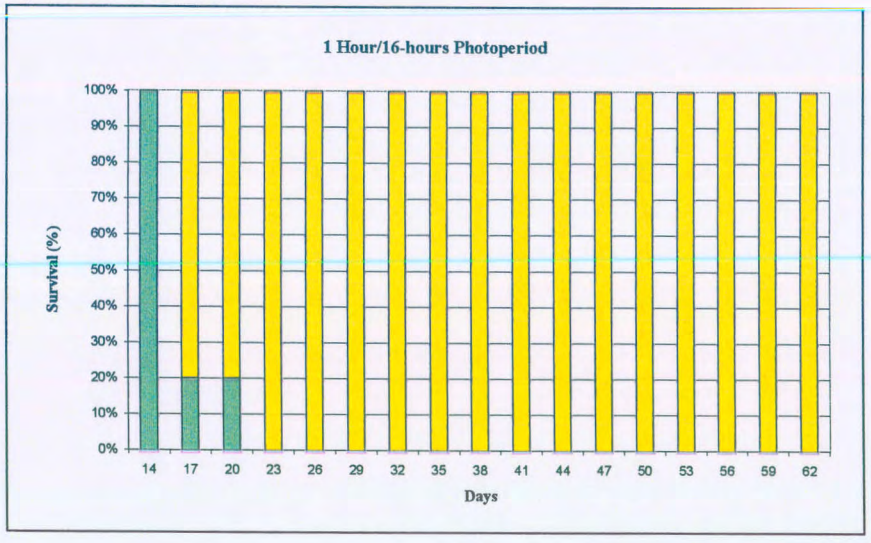
a) Treatment 13



c) Treatment 15



b) Treatment 14



d) Treatment 16

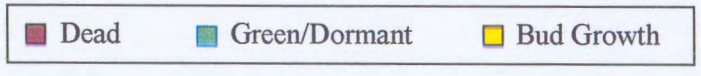


Figure 4.4: The effects of antioxidant solution 4 (100 mg.l^{-1} ascorbic acid & 1500 mg.l^{-1} citric acid), different stirring time periods (1 hour & 12 hours) and culturing conditions (7-days etiolation & 16-hours photoperiod), on the survival of *P. cynaroides* during establishment.

Table 4.1: Sixteen treatments, made up of four ascorbic acid and citric acid solution mixtures, stirred for one hour and 12 hours, and exposed to seven-days etiolation and 16-hours photoperiods.

Treatments	Antioxidant Solution	Ascorbic Acid ($mg.l^{-1}$)	Citric Acid ($mg.l^{-1}$)	Length of Stirring Time	Culturing Conditions
1	1	0	0	1	7-days Etiolation
2	1	0	0	1	16-hours Photoperiod
3	1	0	0	12	7-days Etiolation
4	1	0	0	12	16-hours Photoperiod
5	2	100	0	1	7-days Etiolation
6	2	100	0	1	16-hours Photoperiod
7	2	100	0	12	7-days Etiolation
8	2	100	0	12	16-hours Photoperiod
9	3	0	1500	1	7-days Etiolation
10	3	0	1500	1	16-hours Photoperiod
11	3	0	1500	12	7-days Etiolation
12	3	0	1500	12	16-hours Photoperiod
13	4	100	1500	1	7-days Etiolation
14	4	100	1500	1	16-hours Photoperiod
15	4	100	1500	12	7-days Etiolation
16	4	100	1500	12	16-hours Photoperiod

Table 4.2: Survival percentages and the mean length of *P. cynaroides* explants which survived in each treatment at the 62nd day.

DAY 62			
Treatment No.	**Composition**	**Survival (%)**	**Mean Length (mm)**
1	Solution 1; 1 Hour; 7-days Etiolation	40	8
2	Solution 1; 1 Hour; 16-hours Photoperiod	20	3
3	Solution 1; 12 Hours; 7-days Etiolation	0	-
4	Solution 1; 12 Hours; 16-hours Photoperiod	20	54
5	Solution 2; 1 Hour; 7-days Etiolation	0	-
6	Solution 2; 1 Hour; 16-hours Photoperiod	0	-
7	Solution 2; 12 Hours; 7-days Etiolation	0	-
8	Solution 2; 12 Hours; 16-hours Photoperiod	0	-
9	Solution 3; 1 Hour; 7-days Etiolation	20	26
10	Solution 3; 1 Hour; 16-hours Photoperiod	80	4.5
11	Solution 3; 12 Hours; 7-days Etiolation	0	-
12	Solution 3; 12 Hours; 16-hours Photoperiod	0	-
13	Solution 4; 1 Hour; 7-days Etiolation	40	38
14	Solution 4; 1 Hour; 16-hours Photoperiod	100	14.6
15	Solution 4; 12 Hours; 7-days Etiolation	0	-
16	Solution 4; 12 Hours; 16-hours Photoperiod	0	-

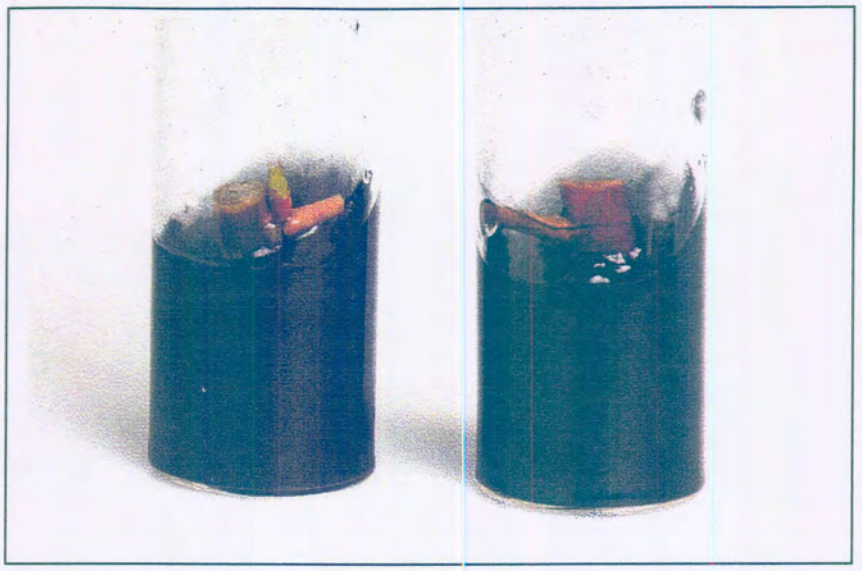


Figure 4.5: *P. cynaroides* axillary bud sprouting after 14 days under light conditions (left) and no sprouting under 7 days of darkness (etiolation) when treated at the same stage (right) in solution 4 (100 $mg.l^{-1}$ ascorbic acid & 1500 $mg.l^{-1}$ citric acid) before establishment.

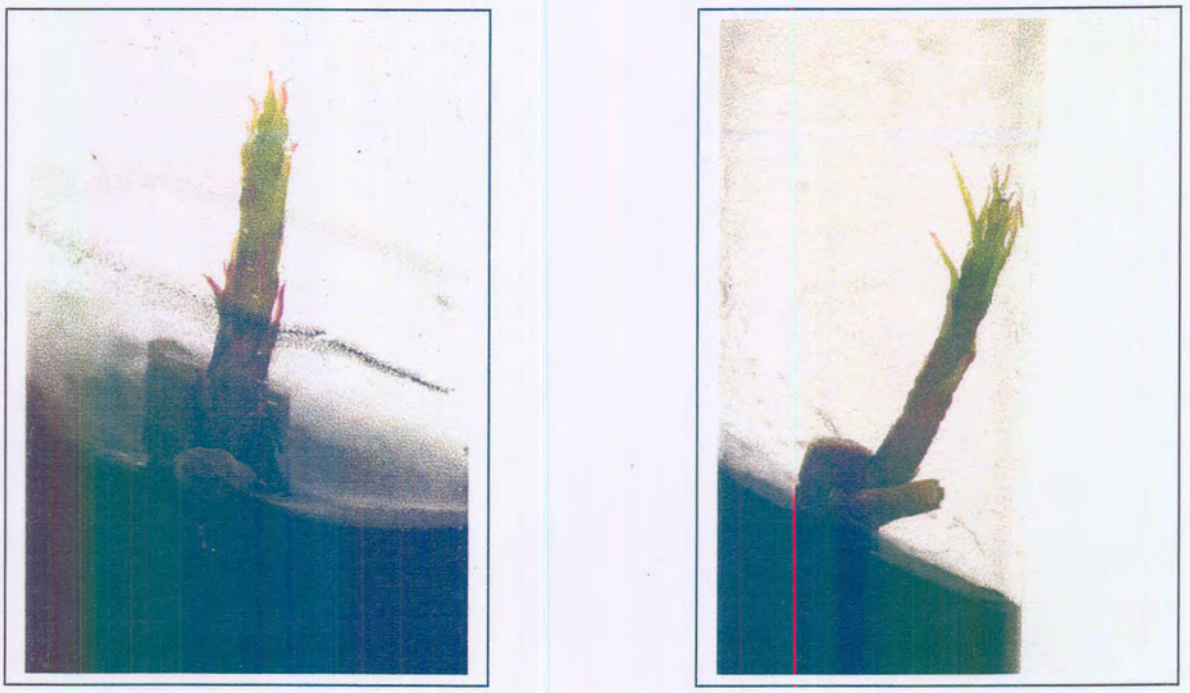


Figure 4.6: Elongation of *P. cynaroides* axillary buds after 30 days when treated in antioxidant solution 4 (100 $mg.l^{-1}$ ascorbic acid & 1500 $mg.l^{-1}$ citric acid) before establishment.

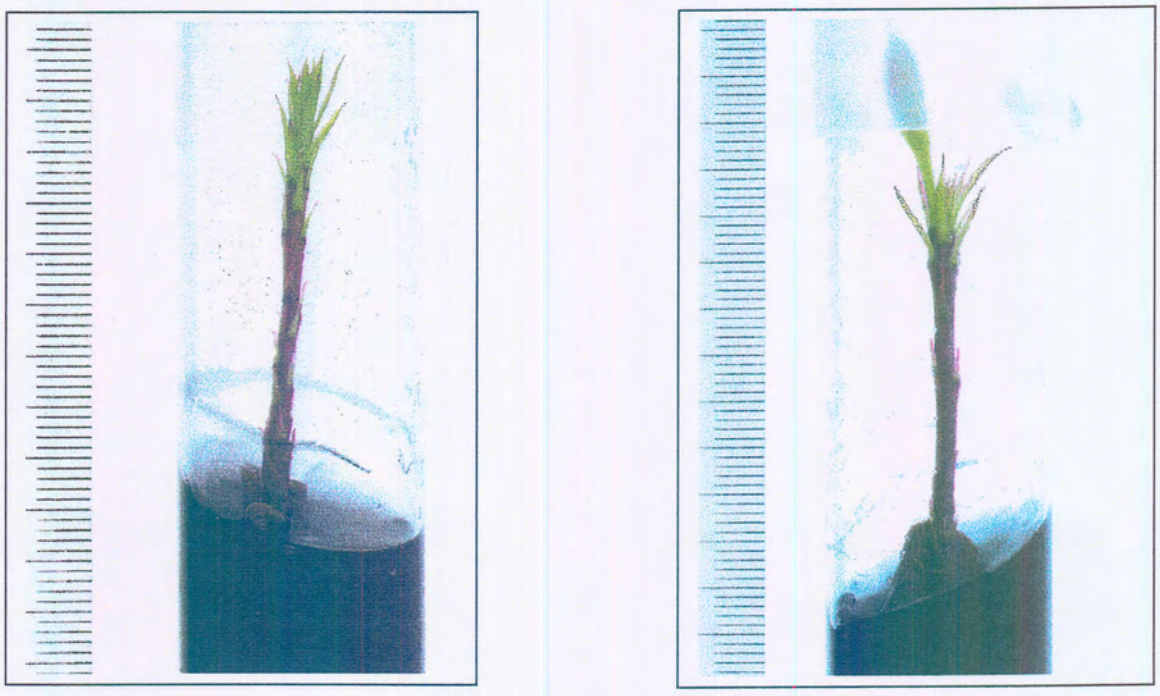


Figure 4.7: Elongation of *P. cynaroides* axillary buds after 62 days.

CHAPTER 5

MULTIPLICATION OF *PROTEA CYNAROIDES*

5.1 Summary

In general, plants of the Proteaceae family require low amounts of mineral nutrients. Their nutritional requirements, compared to other plants, are to some extent quite different and unique. A high phosphorous concentration in the growth media can be harmful to these plants. Therefore, due to the lack of information on the requirements of phosphorous in the micropropagation of *P. cynaroides*, it has led to the need for an investigation into this aspect. Two modified Murashige and Skoog media were used in which 0 and 1400 $mg.l^{-1}$ ammonia phosphate were added instead of potassium phosphate. Results showed that shoot multiplication were possible on the explants from both media. In addition, the explants with two or more buds were able to grow and multiply, whilst those with a single node were unable to survive and several died. A possible reason could be because explants with two or more nodes contain more actively growing tissues and therefore were able to survive. The number of buds on the explants also affected the overall mean length, where longer mean lengths were achieved with explants consisting of two or more buds than those with one bud.

5.2 Introduction

After the establishment of explants in Stage 1 (Chapter 4), the multiplication stage (Stage 2) commences which consists of subculturing the explants to another medium. This medium generally contains nutrients suitable for bud formation and shoot elongation. The amount of nutrients in this medium is therefore of utmost importance, as it will determine whether or not multiplication of the explant will occur.

As mentioned in Chapter 1, *P. cynaroides* which is from the Proteaceae family, generally require low concentrations of mineral nutrients and, compared to other plants, are quite different and unique (Montarone and Allemand, 1995). It seems that different phosphorous concentrations affect the growth of various plant parts differently. For example, Claassens and Folscher (1980) reported that phosphorous can reduce proteoid root production of *Leucospermum*. In addition, Barrow (1977) mentioned that a low phosphorous concentration is necessary in leaves of some species for optimal photosynthesis. According to Montarone and Allemand (1995) and Nichols *et al.* (1979) it is also generally known that high phosphorous concentrations are harmful to Proteaceae plants which in some cases, may cause leaf damage.

The aim of this study was to determine the effects of ammonia phosphate on *P. cynaroides* explants during the multiplication stage and to see whether ammonia phosphate is indeed important to the explants during this stage. In addition, the effects of the type of explants (one-budded or two or more budded) used on the survival rate and mean length were evaluated.

5.3 Materials and Methods

Two treatments of ten replicates each were used in this trial. Nodal cuttings, which were taken from microshoots (15 mm to 50 mm in length) of previously established explants (see Chapter 4), were used as explants and subcultured onto the multiplication media to induce more bud formation and shoot elongation. The lengths of the nodal cuttings were approximately 10 mm, consisting of between one to three nodes.

These explants were planted into 25 x 100 mm test tubes which contained two media with different nutrient compositions. A Murashige and Skoog medium (Murashige and Skoog, 1962) was used in which the concentrations of the macroelements, microelement and vitamins were reduced to half strength, except for potassium phosphate which was replaced with ammonia phosphate. The concentrations of ammonia nitrate and potassium

nitrate were further adjusted accordingly to balance the medium (Table 5.1). The elements were reduced to half strength because results from past experiments indicate that lower strength concentrations are more suitable (See Chapter 1). Due to the lack of plant material available, only two concentrations of ammonia phosphate were tested in this trial, namely 0 $mg.l^{-1}$ and 1400 $mg.l^{-1}$. The use of such a high level of ammonia phosphate was to test the *P. cynaroides* explants' tolerance to phosphate, as they have been known to be more phosphorous tolerant than other Proteas (Littlejohn, 2000). At the same time, the growth-promoting effect of ammonium will also be evaluated. The additional growth regulators that were added to both media were as follows: GA_3 (30 $mg.l^{-1}$), myo-inositol (100 $mg.l^{-1}$), BAP (2 $mg.l^{-1}$), EDTA (50 $mg.l^{-1}$) and IBA (0.5 $mg.l^{-1}$). Sucrose and gelrite at 30 $g.l^{-1}$ and 3 $g.l^{-1}$ respectively, were added. The pH was adjusted to five before autoclaving.

The subcultured explants were placed into a growth chamber with a photoperiod adjusted to 16 hours. Fluorescent tubes were used as the light source providing 60$\mu mol.m^{-2}.sec^{-1}$ photosynthetically active radiation (PAR) at 30 cm above plant height and the temperature was adjusted to 28°C±2.

Data were analyzed using the PROC. GLM (General Linear Models) procedure in the S. A. S. (Statistical Analysis System) program. The ANOVA (Analysis of Variance) was done to determine whether there are significant differences in the mean length, survival rates and explant types between the treatments.

5.4 Results and Discussion

After 30 days from subculturing, the buds on the explants in both treatments started growing and no differences in growth between the explants, which were cultured on the media containing no ammonia phosphate (control) and those containing 1400 $mg.l^{-1}$ ammonia phosphate, were observed. Figures 5.1a and 5.1b show photographs of subcultured explants at day 40 grown into young developing shoots and after 60 days,

further growth was achieved with no significant differences observed between the two treatments (Figures 5.1c and d). At day 60, the medium containing 1400 $mg.l^{-1}$ ammonia phosphate had a longer overall mean length than the control medium, as Figure 5.2 illustrates. This may be due to the fact that a higher ammonium concentration is present in the medium of the treatment, which may have increased the growth of the explant.

These results demonstrate that phosphorous is not required in the multiplication medium since the explants which were on the medium with no phosphorous grew just as well as those with 1400 $mg.l^{-1}$. This is in contrast to the general belief that high phosphorous concentrations are detrimental to the growth of plant in the Proteaceae family. However, according to Chin and Miller (1982), potassium deficiency in the media causes a decrease in the rate of phosphate absorption. Due to the fact that potassium phosphate was replaced by ammonia phosphate in this trial, there was probably a potassium deficiency in the medium causing fewer phosphates being taken up. This could explain the success in multiplication of explants in the medium containing 1400 $mg.l^{-1}$ ammonia phosphate. The results of this trial seemed to agree with the results of the experiment done by Oosthuizen *et al.* (2000) where *P. cynaroides* plants were successfully grown in diluted Hoagland nutrient solutions which contained relatively low levels of potassium. In addition, Nichols and Beardsell (1981) reported that in *Grevillea* cv. 'Poorinda Firebird', high levels of nitrogen alleviated phosphorous toxicity. Similar findings were also found by Grundon (1972) where increasing the nitrogen levels in nutrient solution cultures helped reduce phosphorous toxicity in *Banksia* and *Hakea* species of the Proteaceae family. However, in *Banksia serrata*, Groves and Keraitis (1976) found that high nitrogen levels induced phosphorus toxicity in seedlings grown in sand culture. Another explanation for this phosphorous tolerance is that because no roots were formed by the explants, probably higher concentrations of phosphorous in the medium were not taken up by the explants. This explanation is supported by Purnell (1960) where proteoid roots were reported to be important on the uptake of phosphorous.

The different results discussed by various researchers seem to suggest that different genera and species of Proteaceae react differently to phosphorous where in one species, a

certain mineral nutrient may alleviate the phosphorous toxicity while in another species, it may aggravate it. Furthermore, studies by Prasad and Dennis (1986) found that *Leucadendron* 'Safari Sunset' was tolerant to high levels of phosphorous, indicating that specific cultivars may be tolerant to phosphorous, irrespective of the levels of other nutrients.

In addition, the survival rate of the explants of both treatments was affected by the number of buds which were initially on the explants. As Figure 5.3 shows, the explants with two or more buds had 100% survival rate while the explants with only one bud had a lower survival rate. A possible reason could be because explants with two or more nodes contain more actively growing tissues which gave the explants a better chance of surviving. Furthermore, the mean lengths of the explants in both treatments were also affected by the number of buds on the explant. As Figure 5.4 illustrates, the mean length of the explants with two or more buds is longer than those with only one bud. This may be due the two-budded explants being physiologically stronger resulting in more growth. The results of this analysis show that significant differences were found between the mean lengths these explants at a 5% level (Figure 5.4).

On the economical side, for multiplication of *P. cynaroides* explants in a commercial laboratory, the omission of phosphorous may be advantageous since it means that there would be a reduction in the chemicals needed to make up the media which will result in a reduction of the total costs.

5.5 Conclusion

P. cynaroides explants multiplied in *in vitro* were not affected by high phosphorous concentrations. The low concentration of potassium in the medium may have contributed to the abovementioned result. In addition, it is recommended that explants with two or more nodes should be used for multiplication since these seemed to survive better than those with a single node. The results of this pilot trial will serve as a starting point for

future *in vitro* multiplication of *P. cynaroides*. Further studies involving the relationship between phosphorous, potassium and ammonium should be carried out with regards to the growth of *P. cynaroides* explants.

5.6 References

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Table 5.1: The composition of the two media used consisting of 0 $mg.l^{-1}$ and 1400 $mg.l^{-1}$ ammonia phosphate

Salt/Vitamins	Control ($mg.l^{-1}$)	Treatment ($mg.l^{-1}$)
NH_4NO_3	0	1400
KNO_3	23	
$MgSO_4.7H_2O$	51	
$NH_4H_2PO_4$	370	
$MnSO_4.H_2O$	22300	
$ZnSO_4.7H_2O$	8600	
$CuSO_4.5H_2O$	25	
$CaCl_2.2H_2O$	440	
KI	830	
$CoCl_2.6H_2O$	25	
H_3BO_3	6200	
$Na_2MoO_4.2H_2O$	250	
$FeSO_4.7H_2O$	27850	
$Na_2EDTA.H_20$	37250	
Myo-inositol	100	
Glycine	2	
Nicotinic acid	500	
Pyridoxine-HCl	500	
Thiamine-HCl	100	
Additional growth regulators		
Gibbelleric Acid	30	
Myo-inositol	100	
BAP	2	
IBA	0.5	
Others	**($g.l^{-1}$)**	
Sucrose	30	
Gelrite	3	

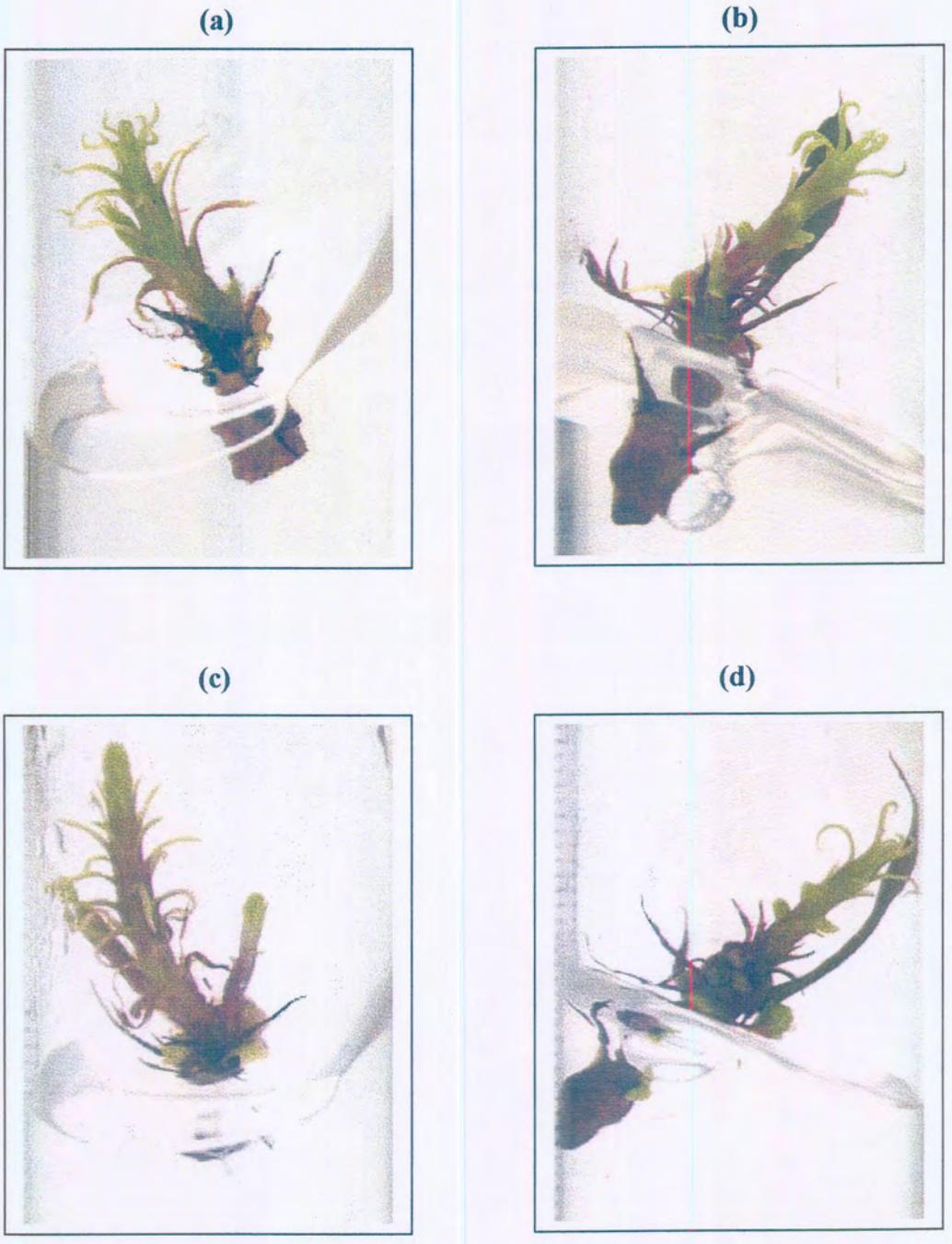
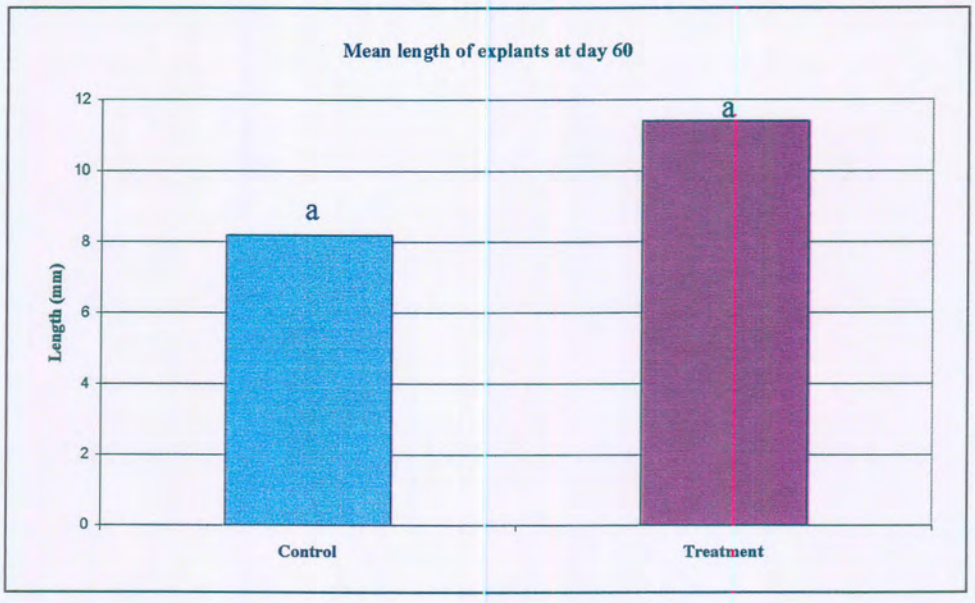
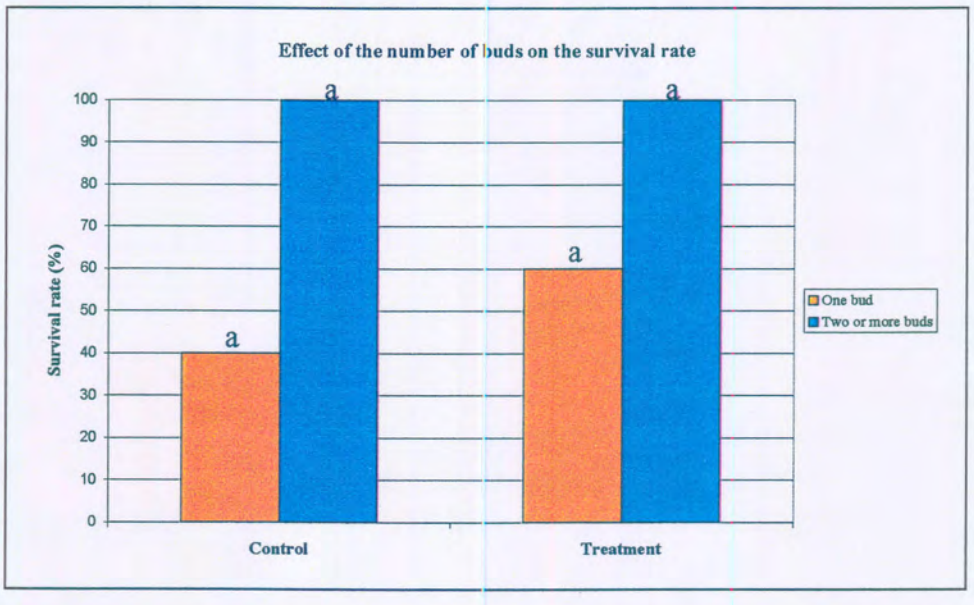


Figure 5.1: The response of the explants cultured on two different media after 40 days (a, b) and 60 days (c, d). Explants growing on the medium (a) and (c) contains no ammonia phosphate, while the other contains 1400 mg l^{-1} ammonia phosphate (b, d).



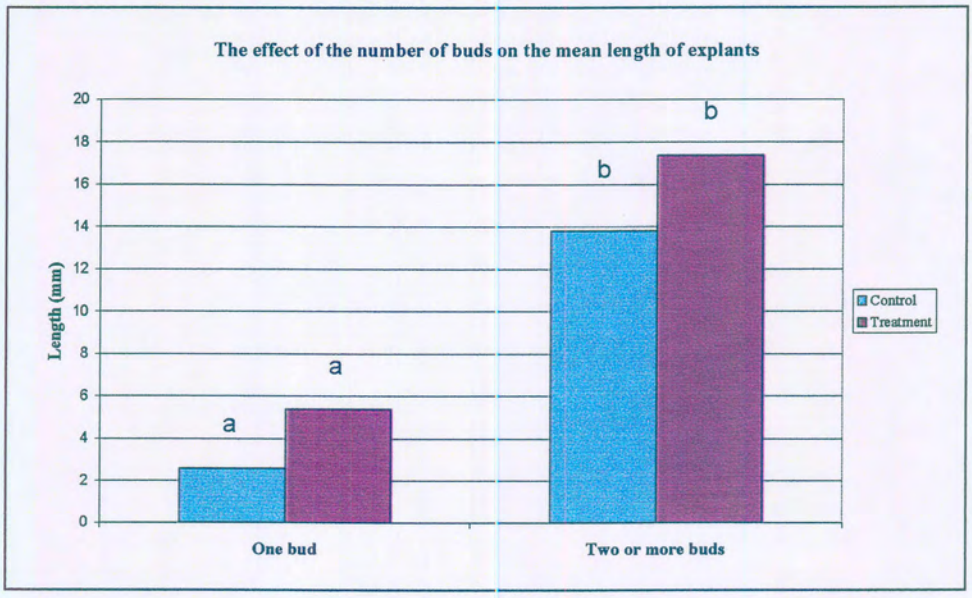
Treatment means with different letters are significantly different at P < 0.05

Figure 5.2: Graphical illustration of the mean length of explants grown on the control medium (0 mg.l^{-1} ammonia phosphate) and the treated medium (1400 mg.l^{-1} ammonia phosphate) (ten replications each) at day 60.



Treatment means with different letters are significantly different at P < 0.05

Figure 5.3: The survival rates of both treatments (0 mg.l^{-1} and 1400 mg.l^{-1} ammonia phosphate) affected by the number of buds present on the explants.



Treatment means with different letters are significantly different at $P < 0.05$

Figure 5.4: The number of buds affecting the overall mean length of both treatments (0 $mg.l^{-1}$ and 1400 $mg.l^{-1}$ ammonia phosphate).

CHAPTER 6

GENERAL DISCUSSION

In the world today, the demand for high quality cut flowers is high, including indigenous South African flowers such as *P. cynaroides* (Coetzee, 2000). However, competition between growers is also increasing, and therefore the availability of the most recent technologies and methods to allow large-scale production of high quality Proteas may be advantageous to the growers and the floriculture industry. The advantages of *in vitro* propagation in general have been discussed and the successes of numerous examples of its application on other flowering plants have illustrated its potential to be a viable alternative to the conventional methods. In addition, the reason for the use of *in vitro* methods to propagate *P. cynaroides* not only lies with the possibility of mass production in a shortened time period, but equally important, also to allow the rapid multiplication of new cultivars that are coming onto the market.

In vitro propagation of woody plants has been more difficult than herbaceous plants because of the many more problems that the former is faced with. This has however, not prevented the numerous attempts that have been made to propagate these type of plants by tissue culture methods. *P. cynaroides*, a member of the Proteaceae family, is probably one of the most difficult species in this plant family to propagate by tissue culture. The limited information available and small number of articles published on it, is a clear indication to this. Nevertheless, the initial work by Ben-Jaacov and Jacobs (1986) has illustrated the potential for a success in this particular species. The results obtained in this study may have further helped to achieve the ultimate aim of large-scale commercial production of *P. cynaroides* via rapid micropropagation consisting of establishment, multiplication, rooting and planting out into field conditions.

From the results of this study, several findings were made which have solved some of the problems that have been known to be associated with *P. cynaroides*. The importance of

gibberellic acid in the establishment of *P. cynaroides* explants was shown in the early work by Ben-Jaacov and Jacobs (1986). In their study, the addition of GA_3 in the Anderson medium (Anderson, 1975) proved to be vital to the success rate. Their results were confirmed in this study where GA_3 was included into a medium that is very similar to the Anderson medium, the more well known Murashige and Skoog medium (Murashige and Skoog, 1962). However, as one problem may have been solved in terms of establishment, another arises. The problem of phenolic oxidation which causes the browning of the explant tissues and the medium, leading to the death of the explants was, according to Malan (1992), solved by Ben-Jaacov (1986). However, methods of overcoming the browning problem could not be found in the article concerned. In addition, it is surprising that no further in-depth study was ever done on methods to reduce phenolic oxidation in *P. cynaroides* since it has been known for a long time that oxidation is a major problem during establishment of this species. The need to develop methods for controlling phenolic oxidation can never be underestimated because although establishment of *P. cynaroides* was successful as described in Chapter 1 of this dissertation, the survival rate was below 50% due to death of explants caused by tissue browning. This has therefore prompted the urgent need to solve the phenolic oxidation problem.

Hence, for the first time, by collecting the information available concerning browning, including among others, *Leucadendron* (Perez *et al.*, 1995), *S. reginae* (Ziv and Halevy, 1983) and *Phoenix dactylifera* (Zaid and Tisserat, 1983), a method was developed to control phenolic oxidation in *P. cynaroides* during establishment. The pilot trial which involved a factorial experiment consisting of two different sterilants (mercuric chloride and sodium hypochlorite), and 16 ascorbic acid and citric acid combinations, gave a clear illustration of which treatments had a potential to succeed (Chapter 3). Therefore, from these results, a selection of a few treatments was done and extra additions were included for the next experiment. In the following experiment (Chapter 4), the best pretreatment combination to control browning was obtained by treating the explants in sodium hypochlorite (0.35%) and ascorbic acid and citric acid (100 $mg.l^{-1}$ and 1500 $mg.l^{-1}$ respectively) for 1 hour. Furthermore, in addition to the satisfactory control of explant

browning by using these pretreatments, the use of a 16-hour photoperiod as the light condition, allowed the growth of the axillary bud to commence.

From the abovementioned results, it was concluded that mercuric chloride, as an alternative sterilant, was too toxic to *P. cynaroides* because it resulted in all the explants being killed. However, although sodium hypochlorite was the more suitable sterilant, its oxidation capacity (George, 1993) is seen as a probable source to the cause of phenolic oxidation. This is one of the reasons for the relatively low concentration and short exposure time used in the experiments compared to its use in experiments of other plants belonging to the Proteaceae family. For example, in *in vitro* propagation of *P. obtusifolia* (Watad *et al.*, 1992) and *P. repens* (Rugge, 1995), the explants were sterilized for 15 minutes in 1.75% and 2% sodium hypochlorite respectively.

After this investigation, a final conclusion could be drawn from the results in terms of the importance of ascorbic acid and citric acid in reducing phenolic oxidation. The use of these antioxidants for controlling oxidation, although different in concentrations to other plants, has added *P. cynaroides* into the lengthy list of plants with browning problems, which are controlled by ascorbic acid and citric acid. However, from recent developments, the use of glucose instead of sucrose as the energy source has resulted in a decrease in leaf blackening during post-harvest treatments (Tahir and Ericsson, 2001). Therefore, further investigation into this finding could be carried out in the tissue culture medium to test whether similar effects are present.

After the browning of the explant was controlled, followed by the successful establishment of the explants, the next step was to induce the explants to multiply by encouraging bud formation and subsequent shoot elongation. The primary factor which directly affected these processes was the composition of the medium. Therefore, by manipulating the medium composition, the explants were, for the first time, successfully subcultured onto a multiplication medium (Chapter 5).

The general belief regarding the nutritional requirements, phosphorous in particular, of plants belonging to the Proteaceae family, is the need for low concentration applications (Claassens, 1986; Nichols *et al.*, 1979). However, as an increasing number of studies have been done in this regard, more information is available which are not in agreement with this statement. For example, according to Prasad and Dennis (1986) and Silber *et al.* (1997), *Leucadendron* 'Safari Sunset' was found to be tolerant to high concentrations of phosphorous. In addition, the plant growth and yield was reported to have improved with increasing phosphorous applications (Silber *et al.*, 1997). Furthermore, *P. cynaroides* was found to be growing in soils containing phosphorous levels of up to 230 $mg.kg^{-1}$ (Littlejohn, 2000).

However, in this study, the success of multiplication in a medium containing 1400 $mg.l^{-1}$ ammonia phosphate (Chapter 5) has illustrated the tolerance of *P. cynaroides* plants to even higher levels of phosphorous. Furthermore, surprising results obtained from the multiplication stage was that no differences were observed between the control medium containing no ammonia phosphate and the treated medium with 1400 $mg.l^{-1}$ ammonia phosphate, which suggests that the range of phosphorous to which *P. cynaroides* is tolerant, is in fact, very wide. A possible explanation for this phosphorous tolerance is that because no roots were formed by the explants in the multiplication medium, the phosphorous in the medium were not taken up by the explants. This explanation is supported by Purnell (1960) where proteoid roots were reported to be important in the uptake of phosphorous.

In addition to the abovementioned findings, other results obtained from the experiments done in Chapter 5 include the importance of two-budded explants as plant material for multiplication in terms of overall mean length and survival rate. There were significant differences in the mean length obtained by the two-budded and single-budded explants in ($P < 0.05$), where the two-budded explants obtained much longer growth.

For future research, studies into the effect of different macro-element concentrations in the multiplication stage should be done. Furthermore, the effect of explant age on the

survival rate and growth rate can be studied. The next stage following shoot multiplication is rooting. *In vitro* rooting of *P. cynaroides* explants has never been achieved. Therefore, a detailed study into this aspect is needed. However, as with the problems faced in the previous micropropagation stages, inducing *P. cynaroides* explants to root in *in vitro* conditions also has problems. The main reason for this is that *P. cynaroides*, being woody plants, are notoriously difficult to root. However, several methods that are successful in rooting other woody plants, can be tested on this species. The most common method of rooting is the use of rooting hormones such as auxins which can be added into the rooting medium. However, one of the problems associated with this is that certain hormones such as cytokinin which was used in the multiplication medium, and were subsequently absorbed by the explants, can inhibit rooting (George, 1993). Hence, explants that are difficult to root may need to be subcultured several times to obtain a hormonal balance within the explants itself so that the explants are physiologically ready for rooting. In addition, one of the most recent advances in methods of inducing rooting is the use of *Bacillus subtilis*, a micro-organism. Successful applications were reported on peas and tomato cultivar Minibell (Kilian *et al.*, 2000). Furthermore, other more conventional methods for *in vitro* rooting include the manipulation of mineral elements, etiolation of the explants and the use of activated charcoal.

From the research thus far, the gradual accumulation of information regarding *in vitro* propagation of *P. cynaroides* have certainly answered numerous questions and solved several problems which may have poised as stumbling blocks in the past. The recent findings are a step closer in completing the micropropagation procedure, which will no doubt, be achieved in future research.

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