CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

7.1 References

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SUMMARY

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

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

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

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

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

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

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

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