CHAPTER 5

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

5.1 Abstract

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

5.2 Introduction

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

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

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

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

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

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

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

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

5.3 Materials and methods

5.3.1 Plant material

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

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

5.3.2 Experiment 1

5.3.2.1 Rooting of stem cuttings, rooting medium and growth conditions

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

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

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

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

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

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

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

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

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

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

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

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

5.3.3 Experiment 2

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

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

5.3.3.2 Determination of total soluble phenols

a) Sample preparation

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

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

b) Reagents

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

c) Extraction and quantification of total soluble phenols

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

5.3.3.3 Analysis of starch content

a) Sample preparation

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

b) Reagents for procedure

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

c) Procedure for extraction and starch determination

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

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

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

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

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

where,

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

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

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

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

5.3.4 Statistical analysis

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

5.4 Results

5.4.1 Experiment 1

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

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

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

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

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

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

5.4.2 Experiment 2

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

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

5.4.2.2 Determination of total soluble phenols

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

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

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

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

5.4.2.3 Analysis of starch content

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

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

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

5.5 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

5.6 Conclusion

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

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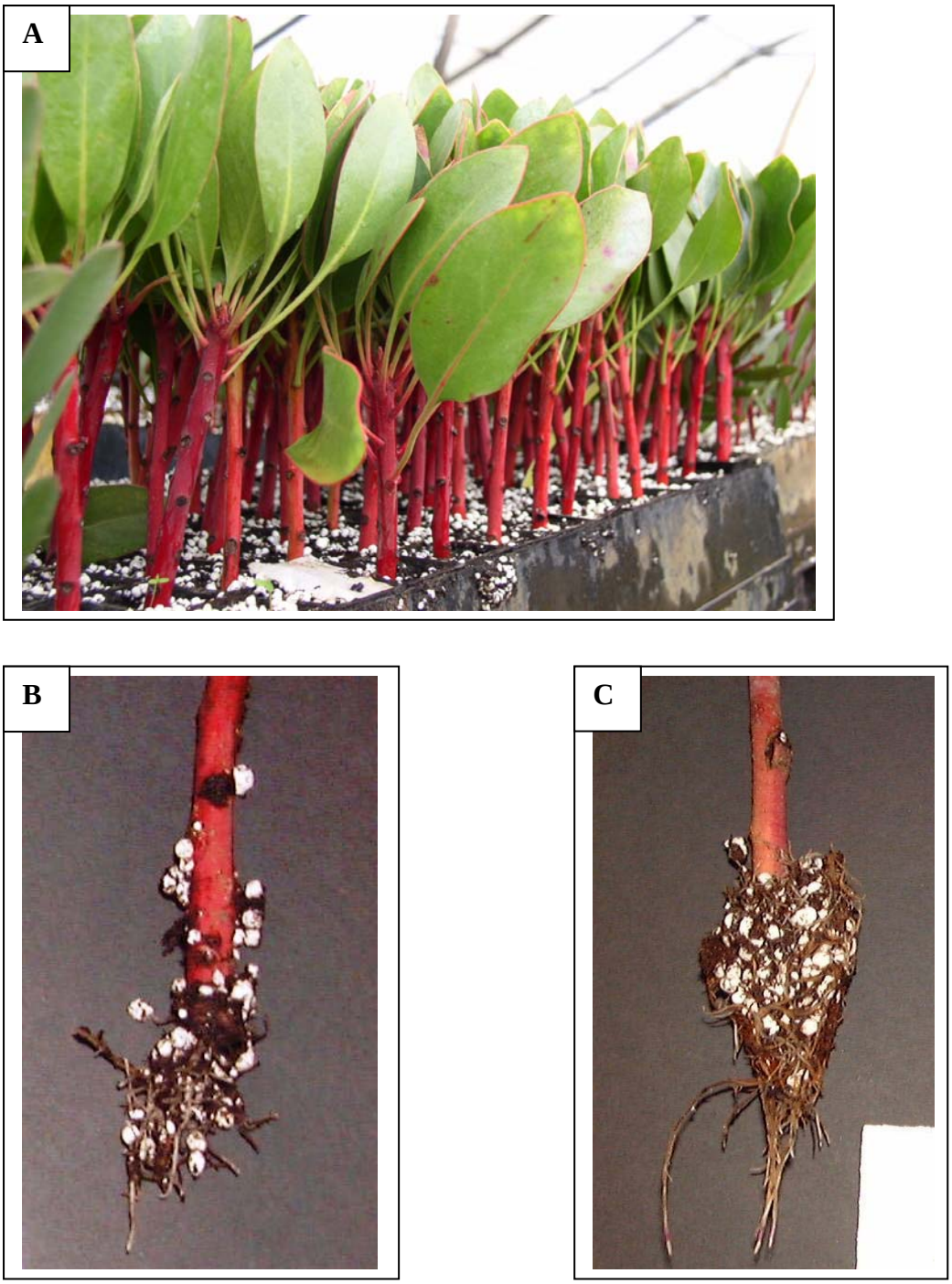

Figure 5.1. (A) Rooting of *P. cynaroides* cuttings in seedling trays placed in a mistbed; **(B)** Rooting of untreated and **(C)** blanched *P. cynaroides* cuttings after 90 days.

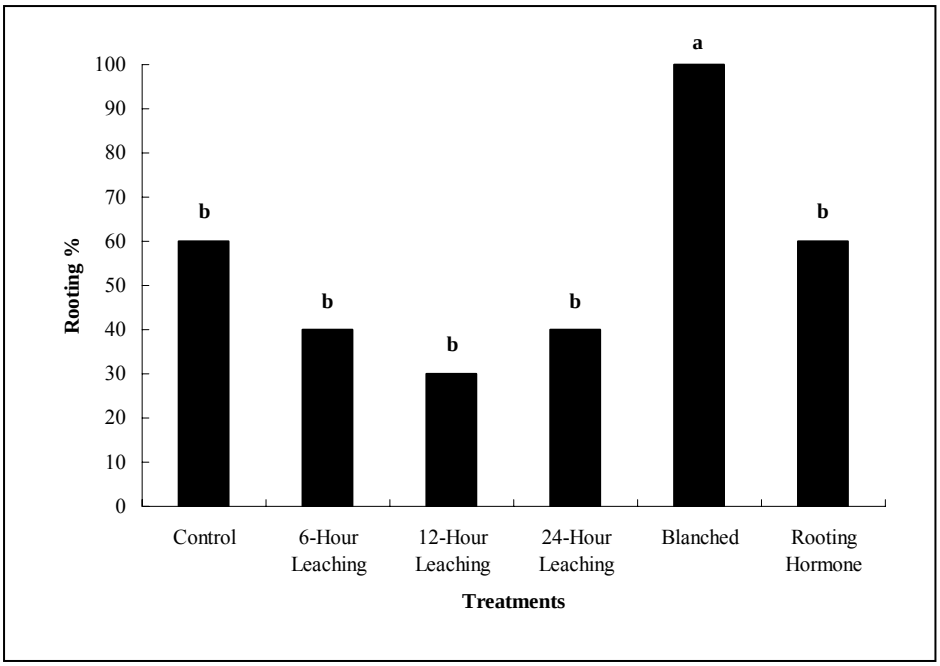

Figure 5.2. The effects of pre-treatments on the rooting % of *P. cynaroides* cuttings after 90 days. Different letters indicate significant differences at $P \leq 0.05$ according to Chi-square.

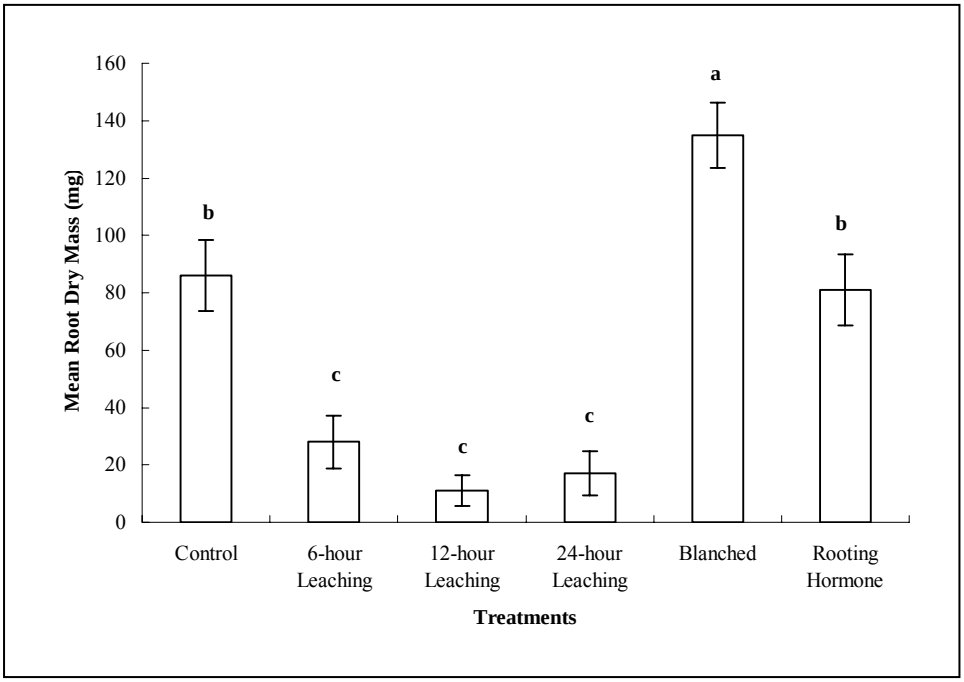

Figure 5.3. The effects of pre-treatments on the root dry mass of *P. cynaroides* cuttings after 90 days. Different letters indicate significant differences at $P \leq 0.001$ according to Tukey's studentised test. (LSD = 42.72).

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

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

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

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

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

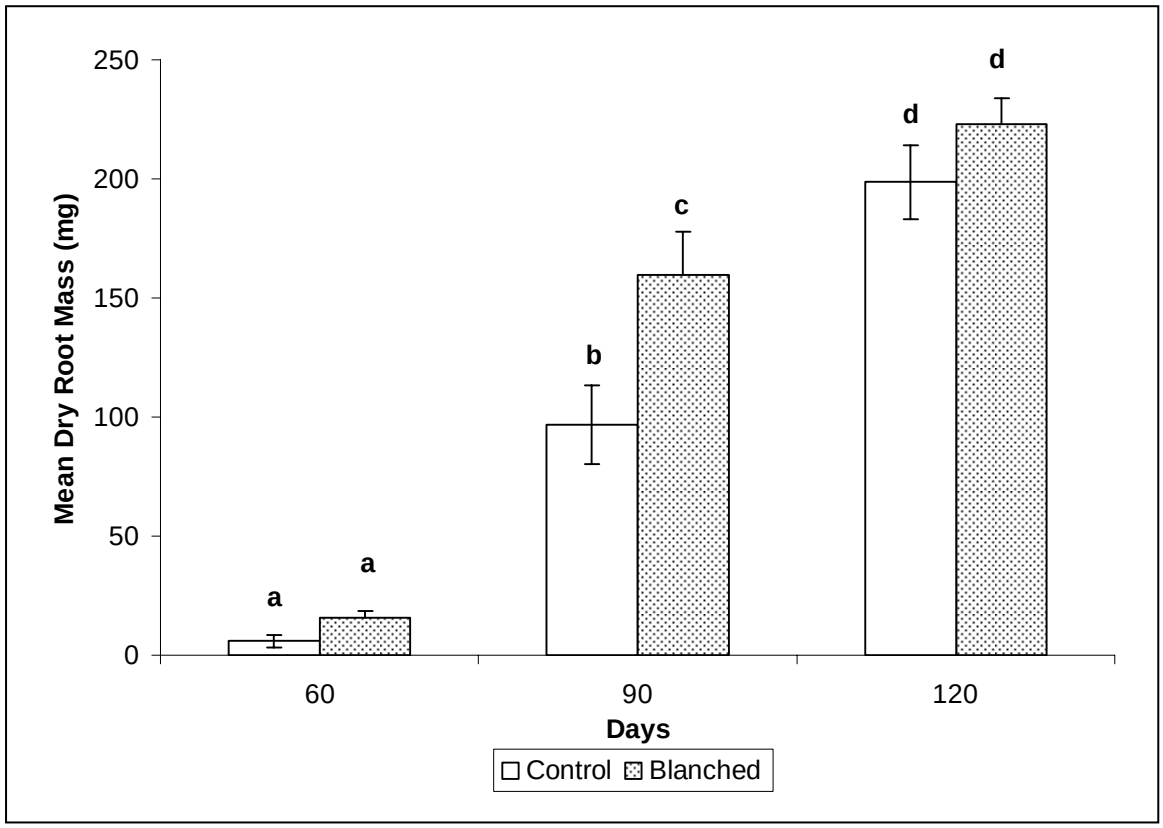

Figure 5.4. Dry root mass of cuttings during rooting period (60, 90 and 120 days after planting). Treatment means with different letters are significantly different at $P \leq 0.05$ according to Tukey's studentised test.

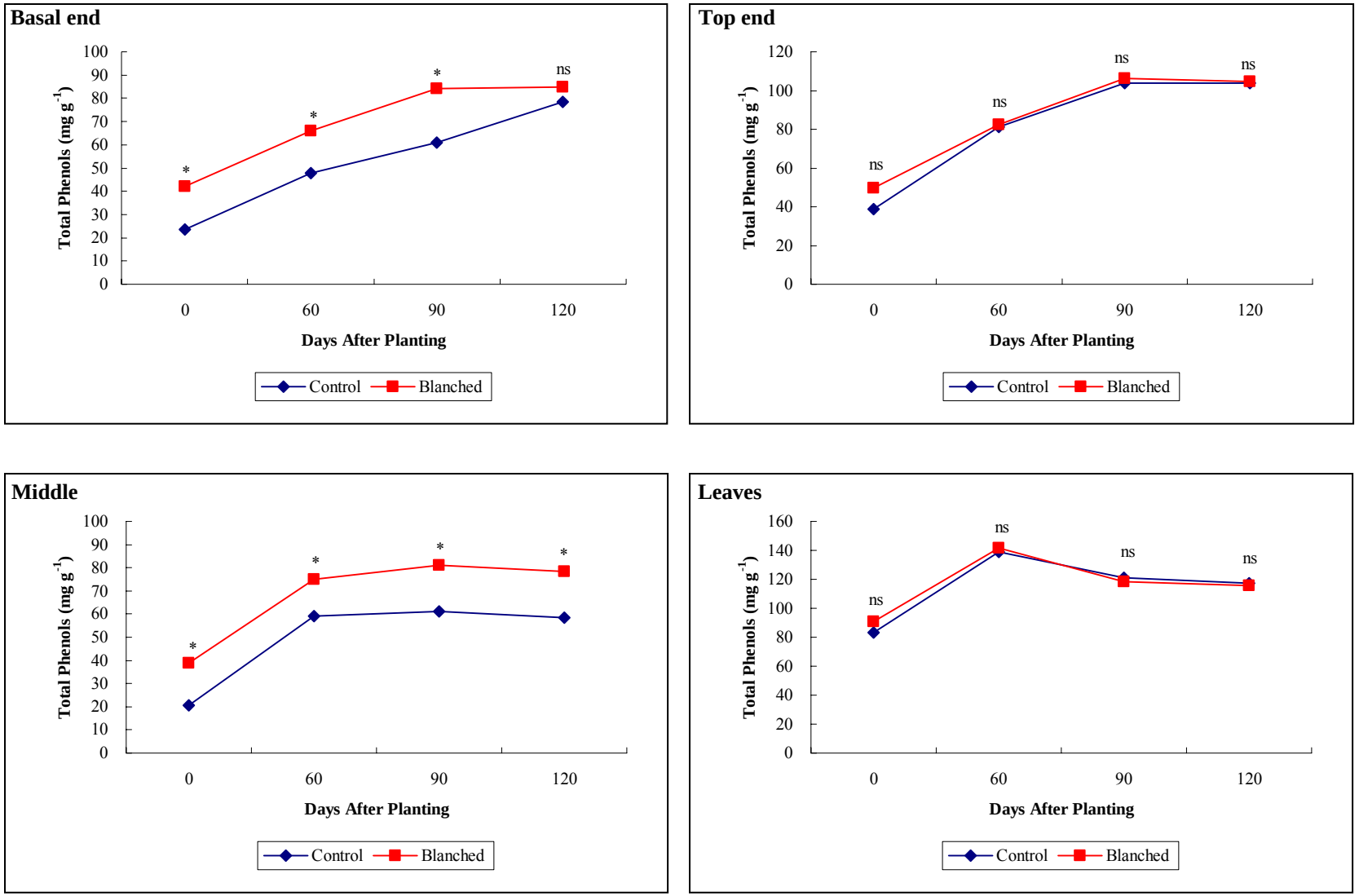

Figure 5.5. Changes in the total soluble phenolic content in different parts of *P. cynaroides* cuttings, during rooting. Means tested for significance at the same time period within each plant part according to Tukey's studentized test.
*: significant ($P \leq 0.05$); ns: not significant.

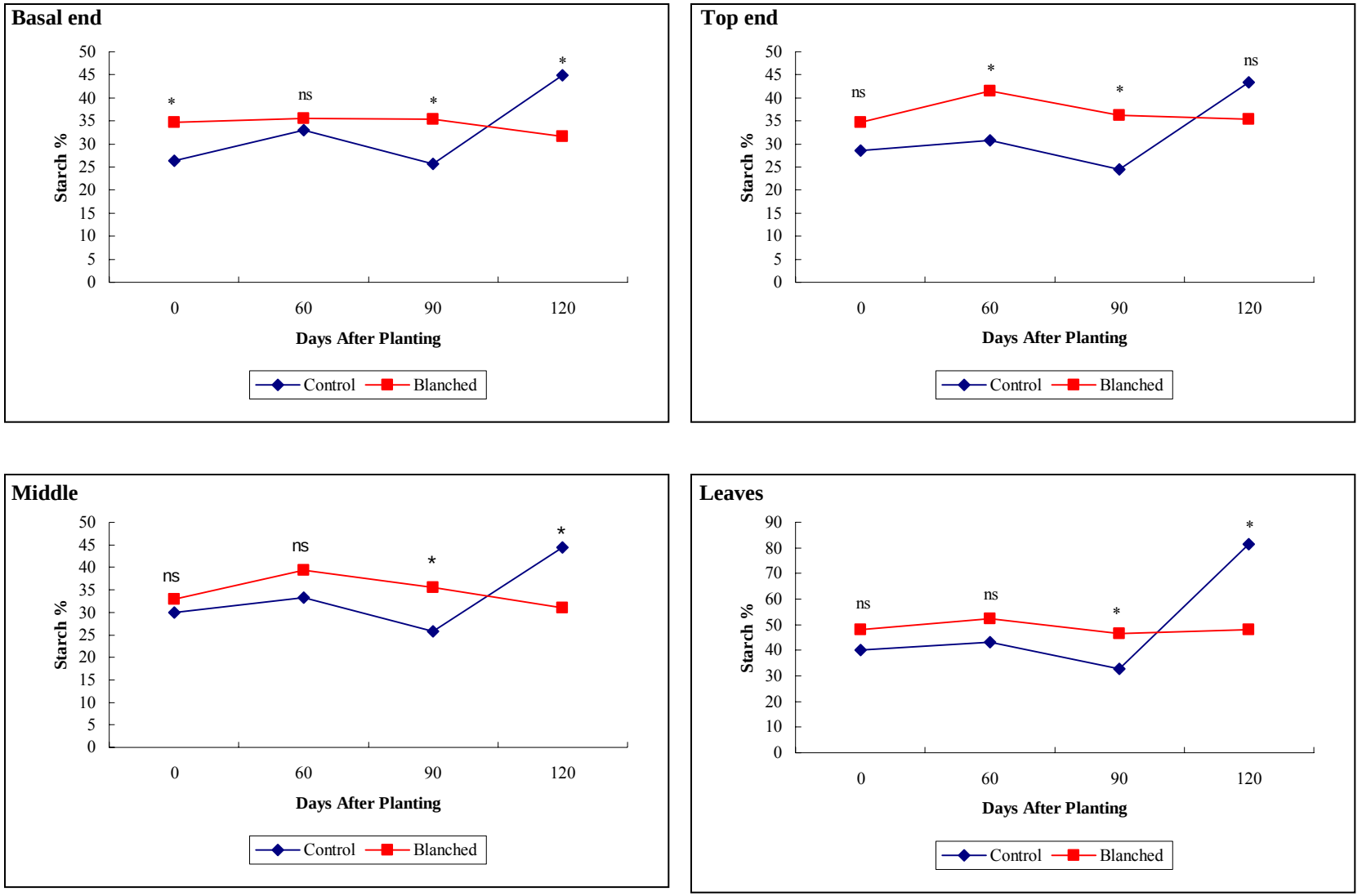

Figure 5.6. Changes in the starch content in different parts of *P. cynaroides* cuttings, during rooting. Starch percentages tested for significance at the same time period within each plant part according to Tukey's studentized test.
*: significant ($P \leq 0.05$); ns: not significant.