

## CHAPTER 3

### MICROGRAFTING OF *PROTEA CYNAROIDES*

#### 3.1 Abstract

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

#### 3.2 Introduction

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

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

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

### 3.3 Materials and methods

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

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

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

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

### 3.4 Results

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

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

### 3.5 Discussion

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

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

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

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

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

### 3.6 Conclusion

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

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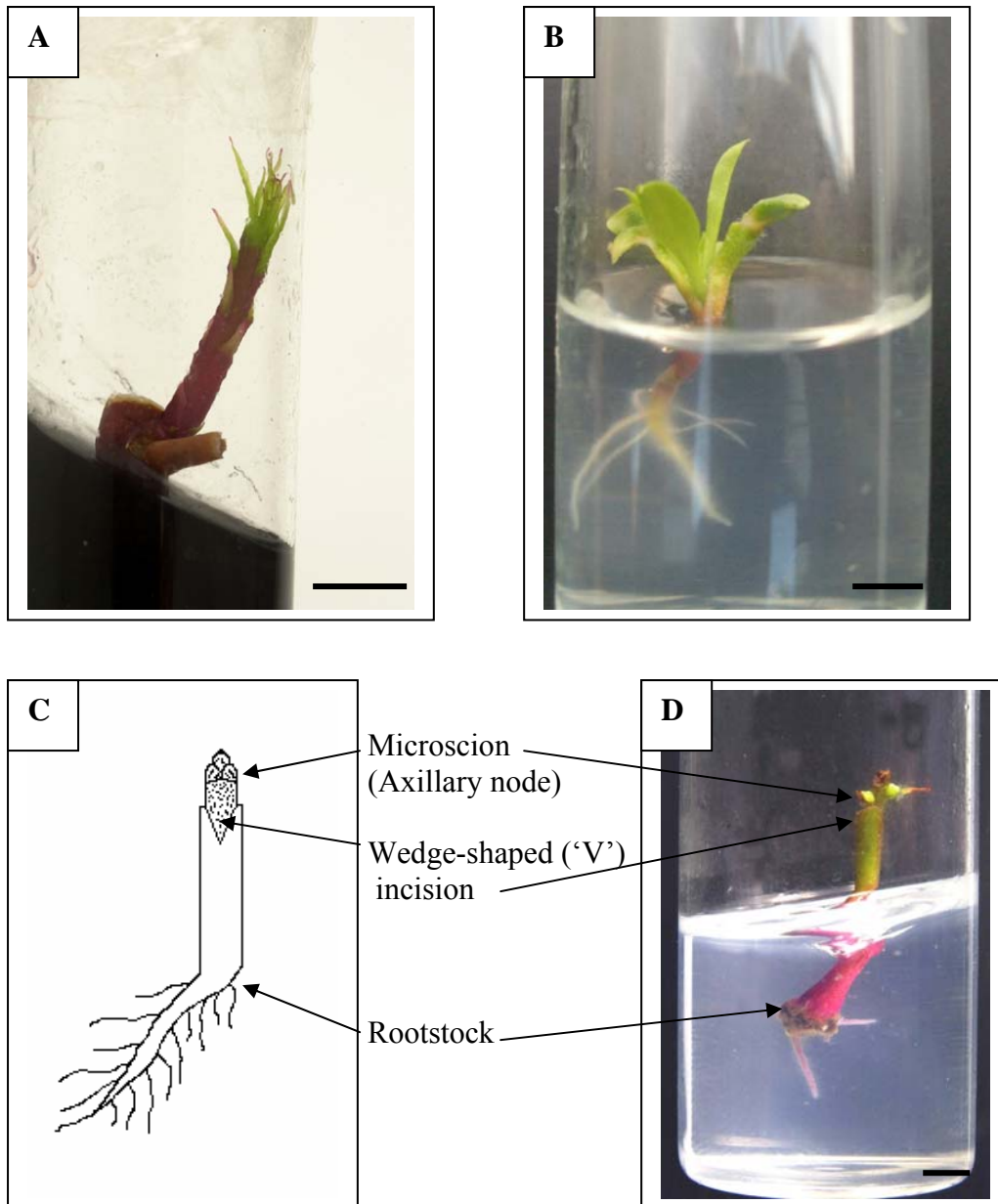
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**Figure 3.1.** (A) Thirty-day old sprouted axillary bud on established nodal explant (Wu, 2001) was used as microscion; (B) Roots of 30-day old *in vitro*-germinated *P. cynaroides* seedling were used as rootstock; (C) A diagrammatic representation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

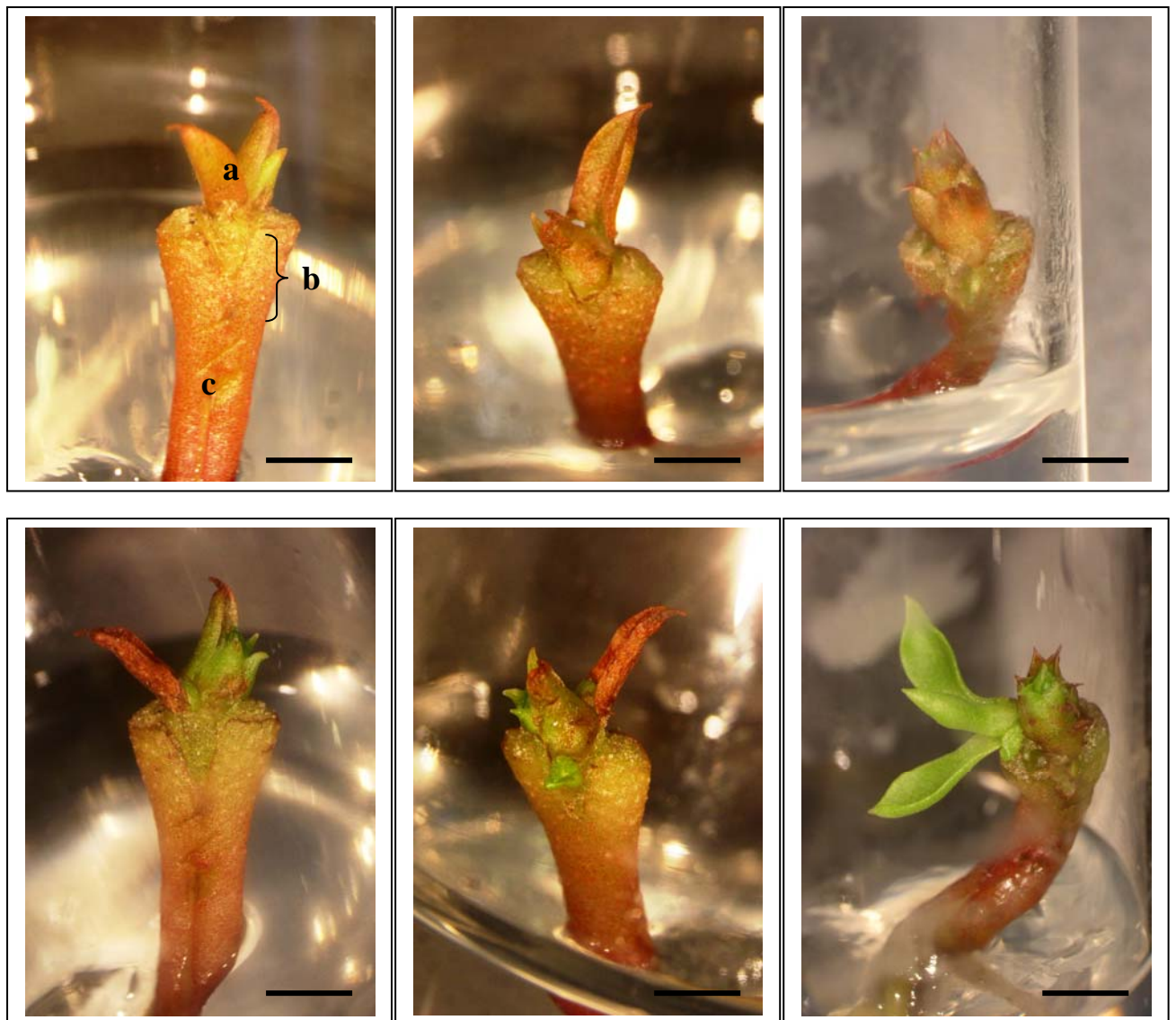
**Table 3.1.** Effects of pre-treatments on grafting success, indicated by the formation of graft union and the growth of buds.

| Treatment                                     | Grafting success (%) |
|---|----------------------|
| Control                                       | 80 a                 |
| Antioxidant Pre-treatment <sup>1</sup>        | 10 b                 |
| Medium application at graft area <sup>2</sup> | 10 b                 |

<sup>1</sup> Ascorbic acid (100 mg l<sup>-1</sup>) and citric acid (150 mg l<sup>-1</sup>)

<sup>2</sup> Half-strength MS medium

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



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