# CHAPTER 2

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

## 2.1 Abstract

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

## 2.2 Introduction

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

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

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

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

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

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

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

## 2.3 Preliminary trial

### 2.3.1 Materials and methods

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

### 2.3.2 Results

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

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

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

## 2.4 Main trial

### 2.4.1 Materials and methods

#### 2.4.1.1 Sterilization procedure

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

#### 2.4.1.2 Medium treatments

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

### 2.4.1.3 Growth condition treatments

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

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

### 2.4.1.4 Transfer to *ex vitro* environments

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

### 2.4.1.5 Statistical analysis

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

### 2.4.2 Results

#### 2.4.2.1 Effects of treatments on germination percentage

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

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

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

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

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

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

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

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

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

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

#### 2.4.2.3 Establishment in mistbed

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

### 2.4.3 Discussion

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

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

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

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

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

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

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

### 2.4.4 Conclusion

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

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

### 2.4.5 References

Brits, G. J., 1986. The effect of hydrogen peroxide treatment on germination in Proteaceae species with serotinous and nut-like achenes. *South African Journal of Botany* 52: 291 – 293.

Brown, N. A. C. and Dix, L., 1985. Germination of the fruits of *Leucadendron tinctum*. *South African Journal of Botany* 51(6): 452.

Brown, N. A. C. and van Staden, J., 1971. Germination inhibitors in aqueous seed extracts of four South African Proteaceae. *Journal of South African Botany* 37(4): 305 – 315.

Brown, N. A. C. and van Staden, J., 1973. The effect of scarification, leaching, light, stratification, oxygen and applied hormones on germination of *Protea compacta* R. Br. and *Leucadendron daphnoides* Meisn. *Journal of South African Botany* 39(2): 185 – 195.

Brown, N. A. C. and van Staden, J., 1975a. Inhibitors are not responsible for poor germination of *Protea compacta* seed. *Journal of South African Botany* 41(4): 225 – 230.

Brown, N. A. C. and van Staden, J., 1975b. The effect of temperature and various gases on the germination and endogenous hormones levels of seed. *Zeitschrift für Pflanzenphysiologie* 75: 31 – 37.

Bunn, E. and Dixon, K. W., 1992. *In vitro* propagation of the rare and endangered *Grevillea scapigera* (Proteaceae). *HortScience* 27(3): 261 – 262.

Deall, G. B. and Brown, N. A. C., 1981. Seed germination in *Protea magnifica* Link. *South African Journal of Science* 77: 175 – 176.

De Fossard, R. A. and de Fossard, H., 1988. Micropropagation of some members of the Myrtaceae. *Acta Horticulturae* 227: 346 – 351.

George, E. F., 1993. Plant propagation by tissue culture. Part 1: The Technology, 2nd Ed. Exegetics Ltd. Edington, Wilts. BA13 4QG, England.

George, E. F., 1996. Plant propagation by tissue culture. Part 2: In Practice, 2nd Ed. Exegetics Ltd. Edington, Wilts. BA13 4QG, England.

Kunisaki, J. T., 1989. *In vitro* propagation of *Leucospermum* hybrid 'Hawaii Gold'. *HortScience* 24(4): 686 – 687.

Kursanov, A. L., Kulaeva, O. N. and Mikulovich, T. P., 1969. Combined effect of 6-benzylaminopurine, gibberellic acid, and 3-indoleacetic acid on the expansion of isolated pumpkin cotyledons. *American Journal of Botany* 56(7): 767 – 772.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Plantarum* 15, 473-497.

Rodríguez-Pérez, J. A., 1995. Effects of treatment with gibberellic acid on germination of *Protea cynaroides, P. eximia, P. neriifolia* and *P. repens* (Proteaceae). *Acta Horticulturae* 387: 85 – 89.

SAS Institute Inc., 1996. The SAS system for Windows. SAS Institute Inc. SAS Campus drive, Cary, North Carolina, USA.

Schöner, S. and Reinhard, E., 1982. Clonal multiplication of *Digitalis lanata* by meristem culture. *Planta Medica* 45: 155.

Van Staden, J. and Brown, N. A. C., 1972. Characterization of germination inhibitors in seed extracts of four South African species of Proteaceae. *Journal of South African Botany* 38(3): 135 – 150.

Van Staden, J. and Brown, N. A. C., 1973. The role of covering structures in the germination of seed of *Leucospermum cordifolium* (Proteaceae). *Australian Journal of Botany* 21: 189 – 192.

Van Staden, J., Brown, N. A. C. and Button, J., 1972. The effects of applied hormones on germination of excised embryos of *Protea compacta* R. Br. *in vitro*. *Journal of South African Botany* 38(4): 211 – 214.

Vogts, M., 1982. South Africa's Proteaceae. Know them and grow them. C. Struik, Cape Town.

Wareing, P. F., 1969. Germination and dormancy. In: Wilkins, M. J. (Ed). The physiology of plant growth and development. London, McGraw-Hill.

Wu, H. C., 2001. Micropropagation of *Protea cynaroides* L. Master's thesis. Department of Plant Production and Soil Science, University of Pretoria, Pretoria, South Africa.

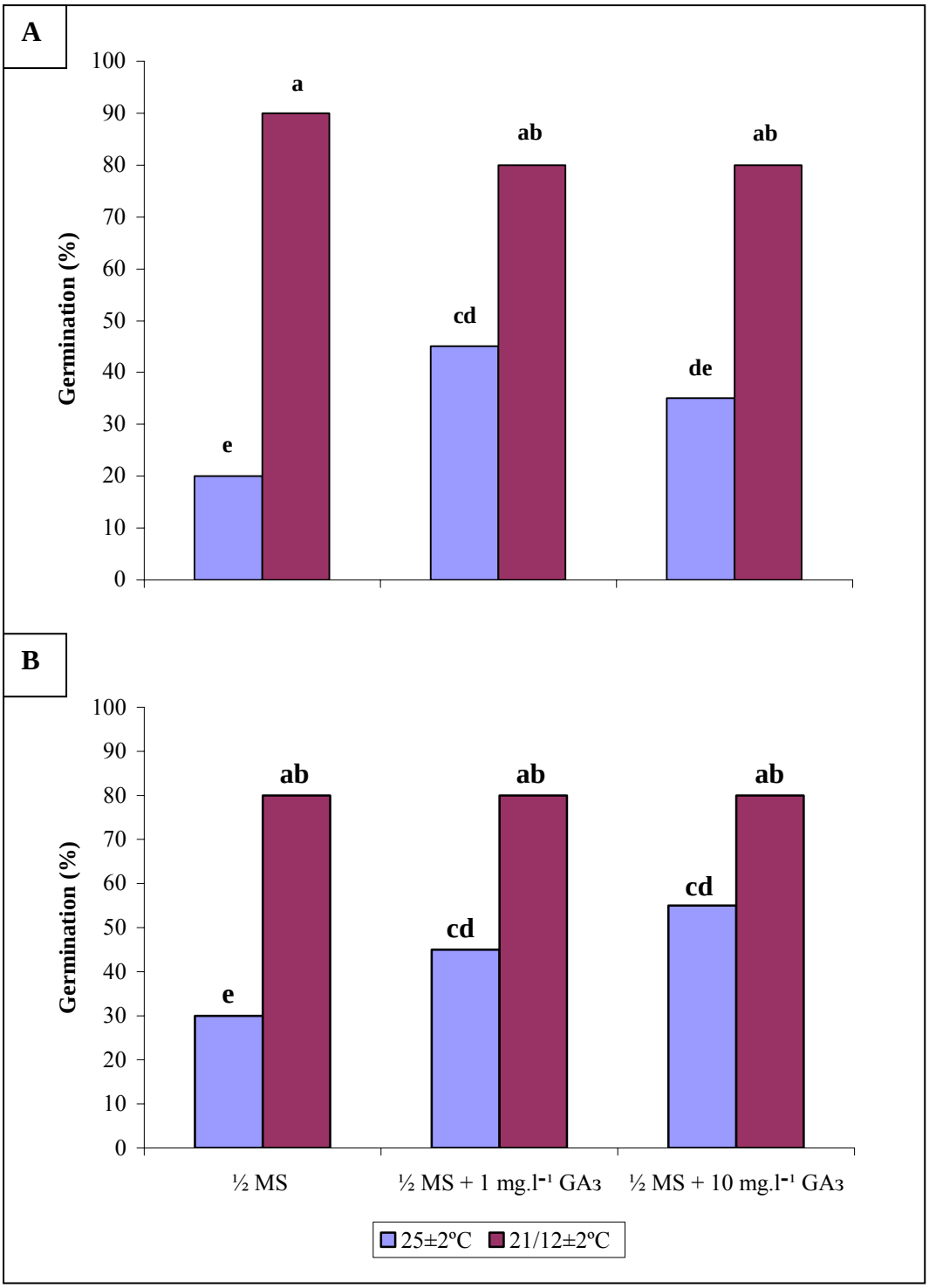


**Figure 2.1.** The effects of two temperature regimes and different media on the germination percentage of *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. Means with different letters differ significantly at according to Chi-square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

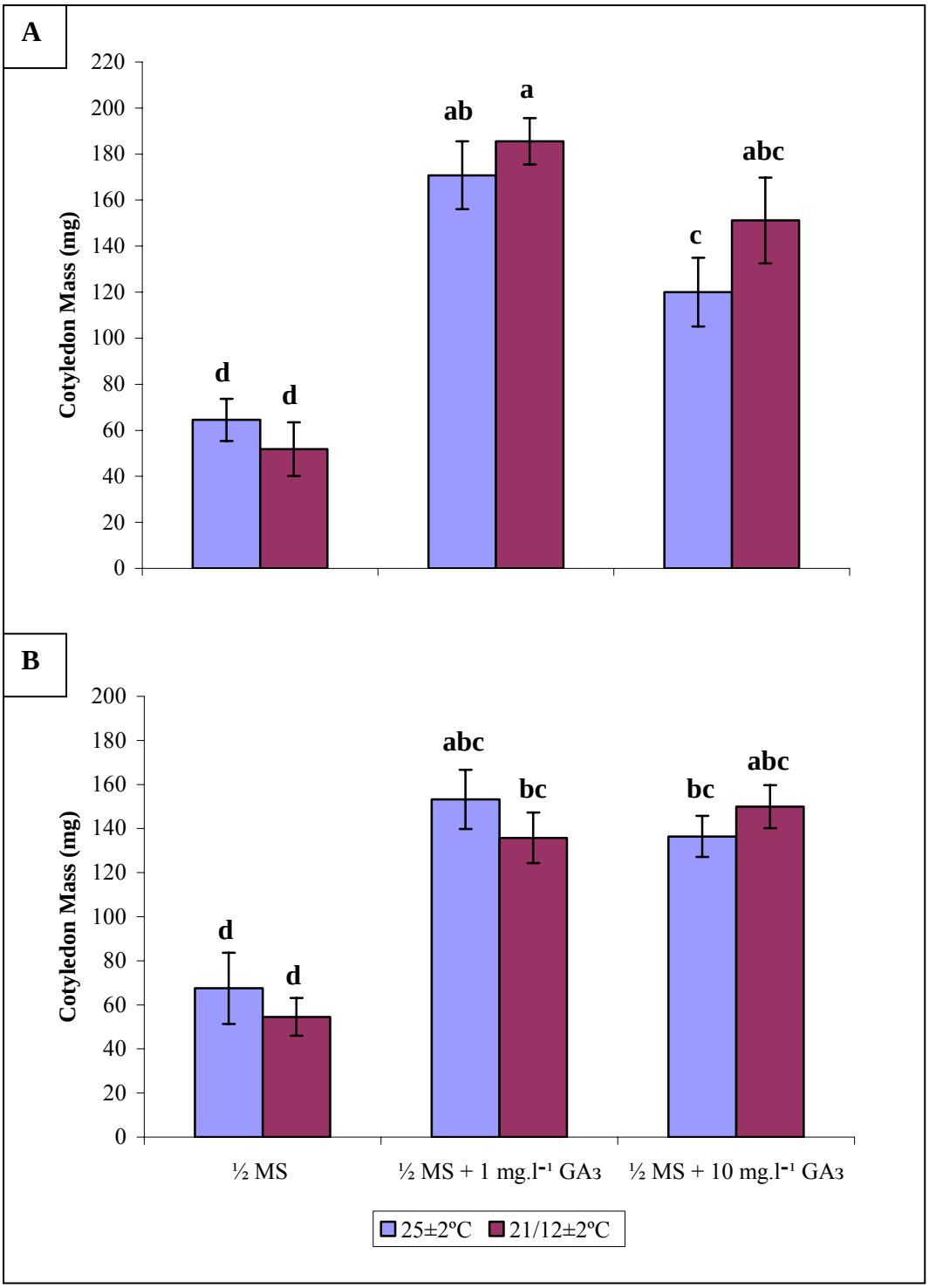


**Figure 2.2.** The effects of two temperature regimes and different media on the growth of cotyledons in *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. Means with different letters differ significantly according to Tukey's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

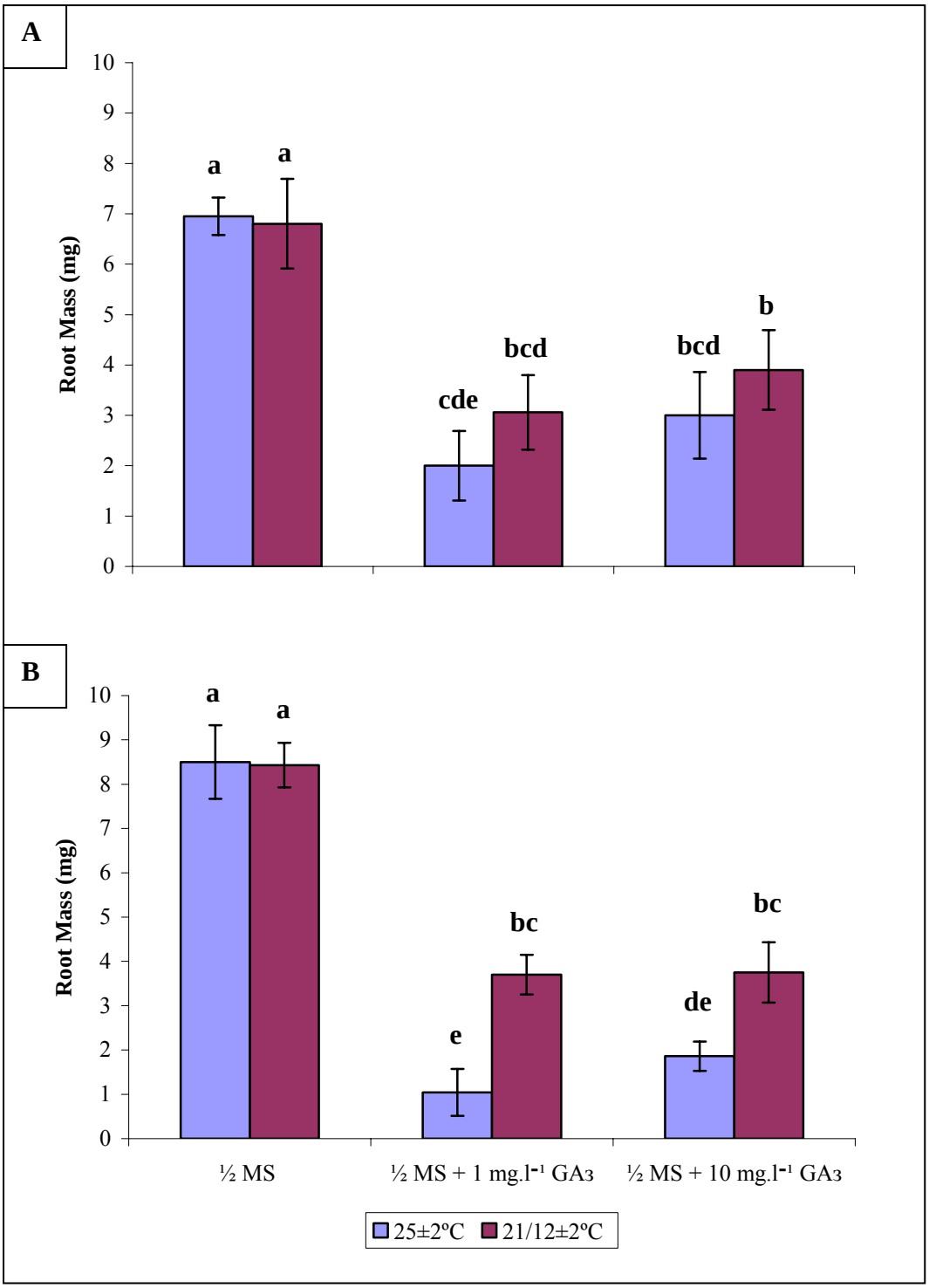


**Figure 2.3.** The effects of two temperature regimes and different media on root growth of *P. cynaroides* excised embryos after 30 days in culture, grown under: **(A)** a 12-hour photoperiod; **(B)** total darkness. Means with different letters differ significantly according to Tukey's studentised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

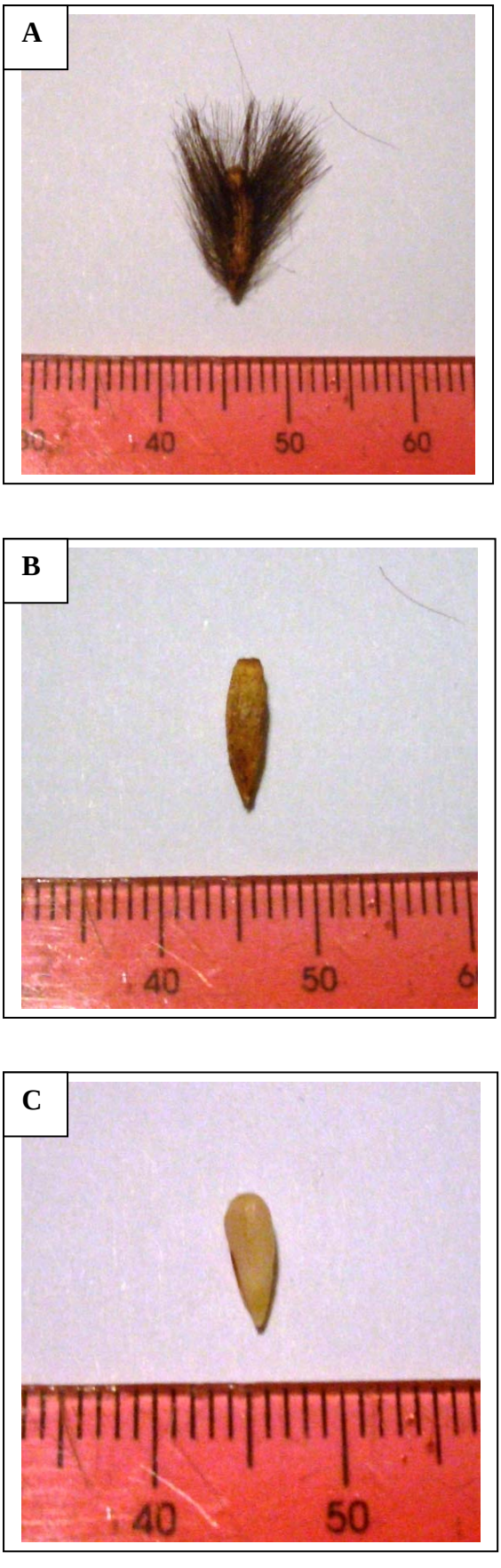


**Figure 2.4. (A)** *P. cynaroides* seed (=achene); **(B)** *P. cynaroides* seed with hairs removed; **(C)** Excised *P. cynaroides* embryo.

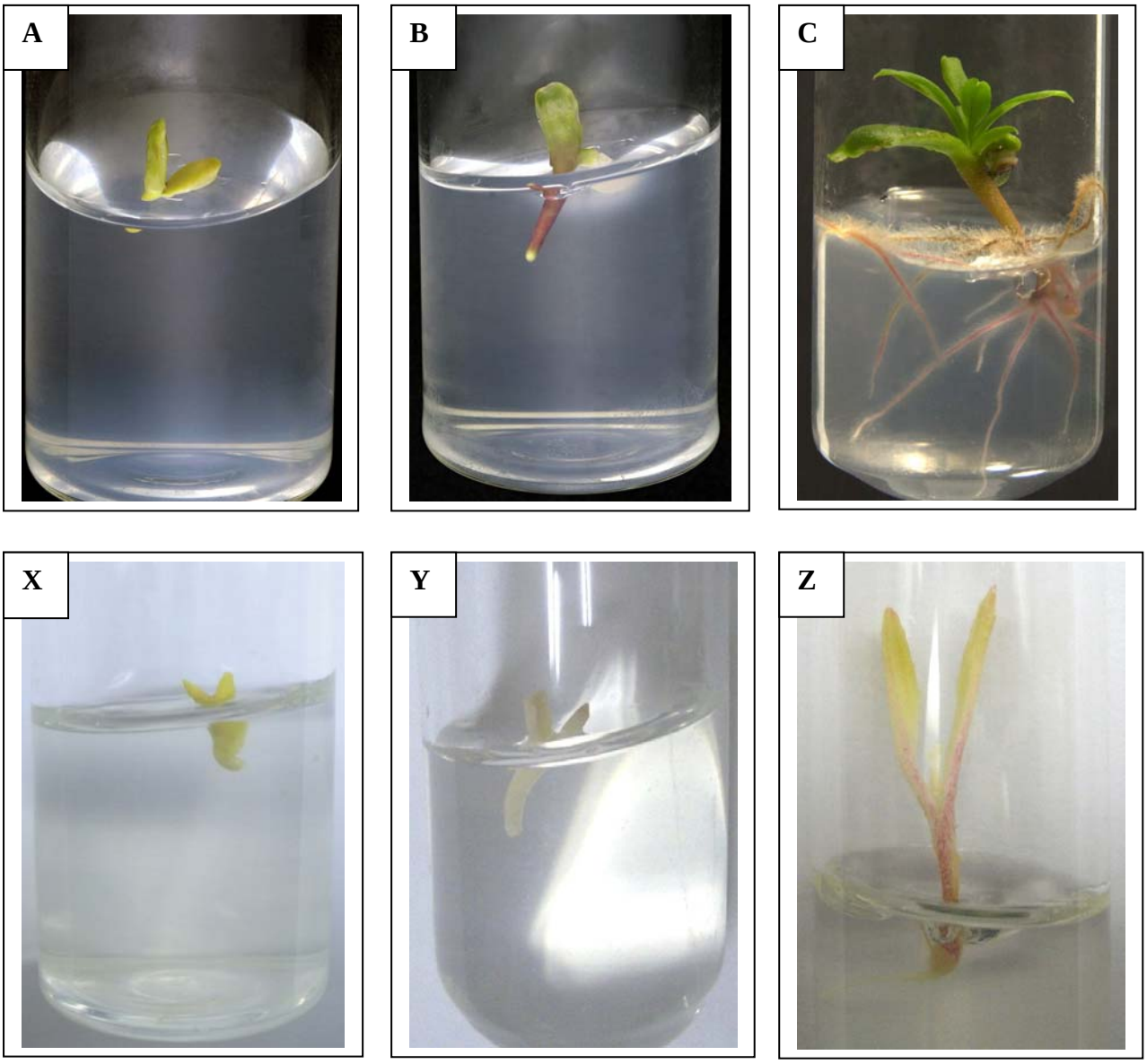


**Figure 2.5.** *In vitro* germination of excised *P. cynaroides* embryos cultured on MS medium grown under light (12-hour photoperiod), after **(A )** 9 days; **(B)** 14 days; **(C)** 30 days, and in total darkness, after **(X)** 9 days; **(Y)** 14 days; **(Z)** 30 days.

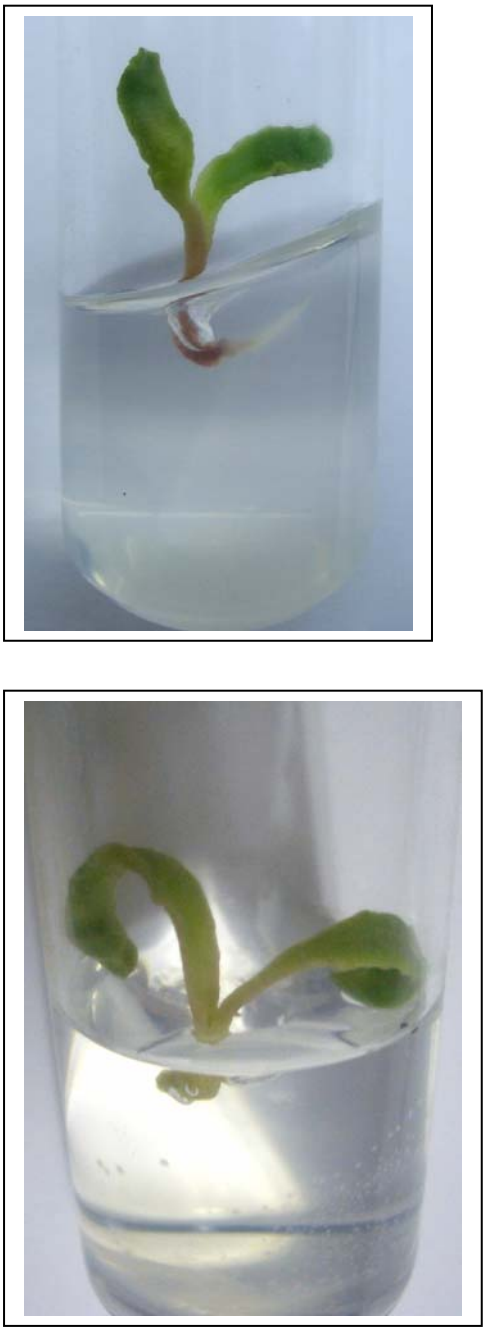

**Figure 2.6.** Growth of long and twining cotyledons on *P. cynaroides* seedlings germinated in media containing $GA_3$.

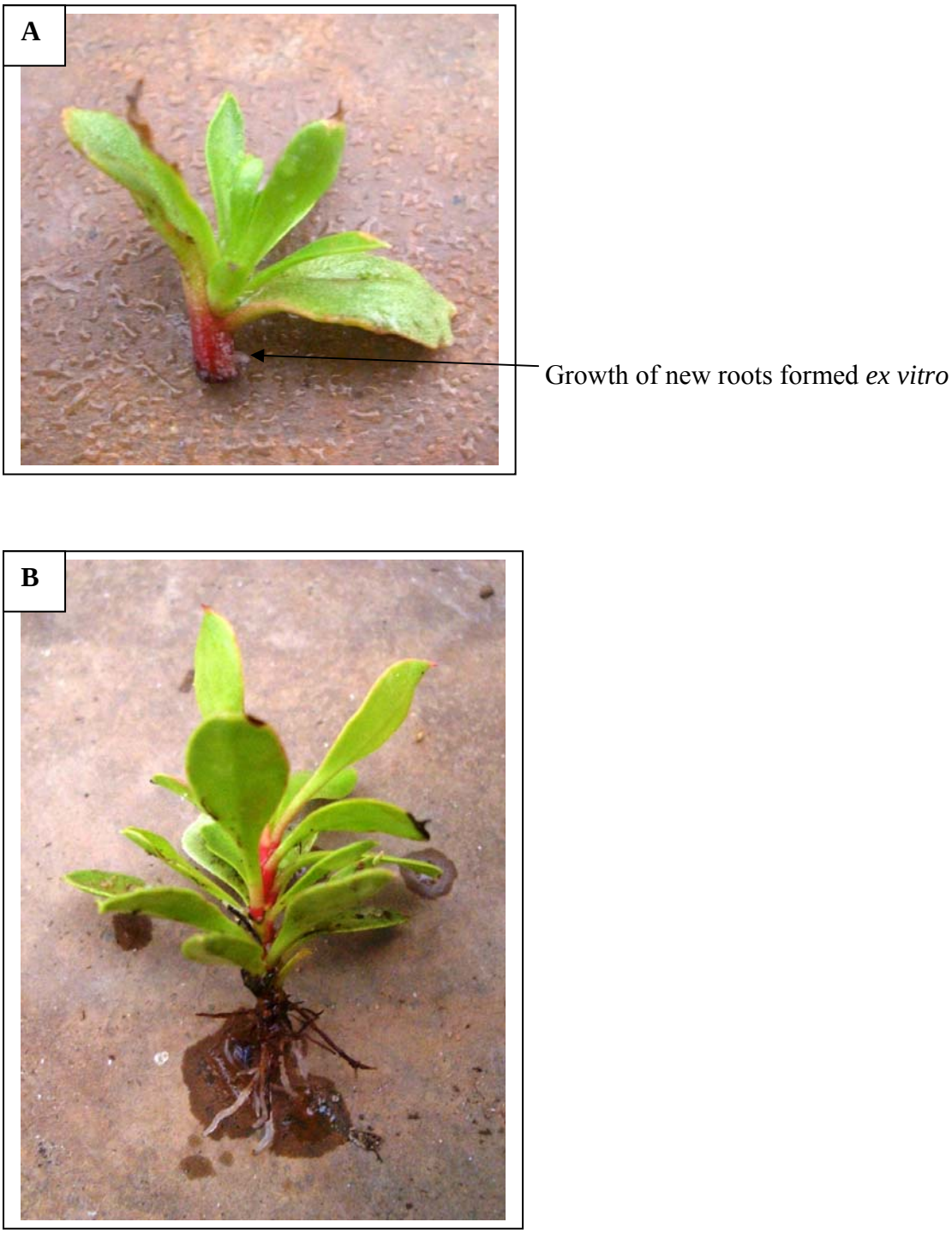


**Figure 2.7. (A)** Two weeks after transferring to the mistbed, *in* vitro-formed roots have died, and the emergence of new roots formed *ex vitro* is visible; **(B)** A seedling with healthy roots after 60 days in the mistbed.

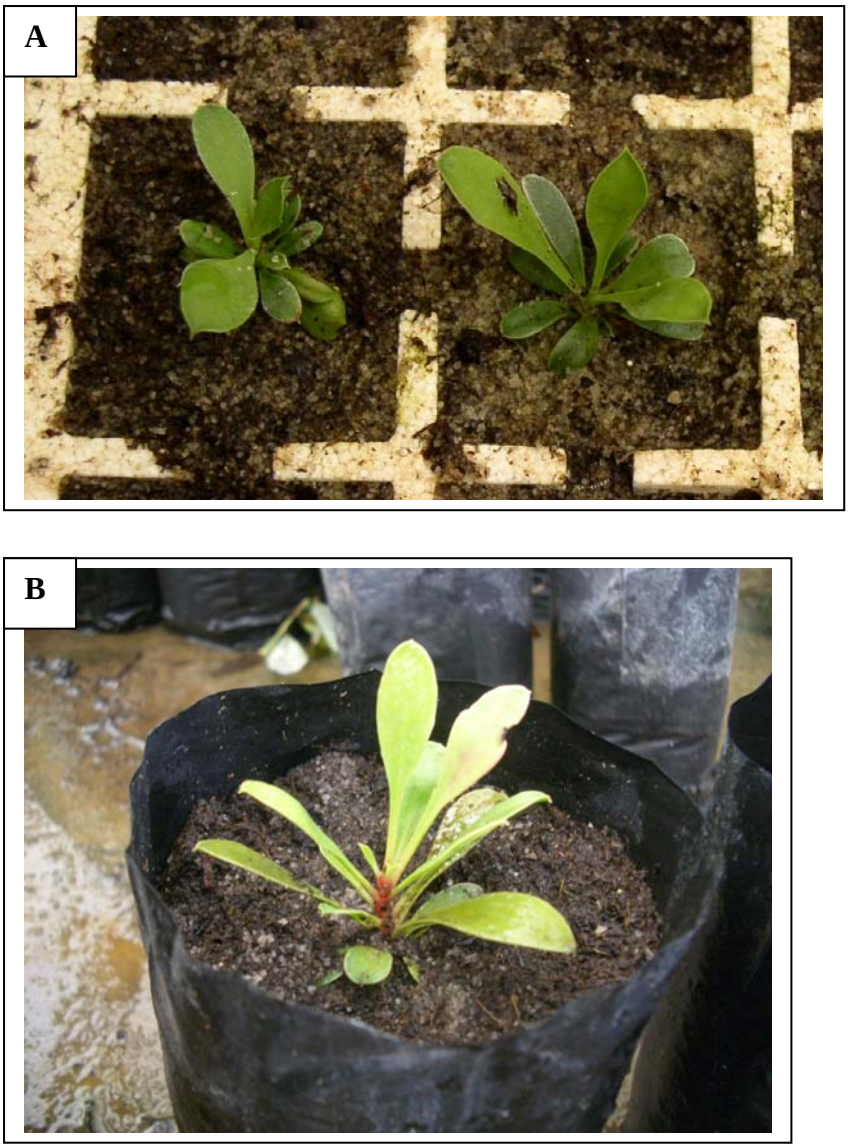


**Figure 2.8.** Establishment of *in vitro*-germinated *P. cynaroides* seedlings in the mistbed. **(A)** After 30 days; **(B)** Transferred to black bag after 60 days.