African Journal of Biotechnology Vol. 9(47), pp. 8032-8037, 22 November, 2010

Full Length Research Paper

Effects of temperature, light conditions and gibberellic acid on the *in vitro* germination of *Protea cynaroides* L. embryos

H. C. Wu[1]* and E. S. du Toit[2]

[1]Department of Natural Biotechnology, College of Science and Technology, Nanhua University, No. 55, Section 1, Nanhua Rd., Dalin Township, Chiayi 62248, Taiwan (R.O.C.).
[2]Department of Plant Production and Soil Science, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa.

Accepted 1 November, 2010

Poor and inconsistent germination of *Protea cynaroides* seeds are often observed in soil. A protocol based on embryo culture was developed for efficient *in vitro* propagation of *P. cynaroides*. The effects of temperature, light conditions and gibberellic acid (GA_3) on the *in vitro* germination of *P. cynaroides* embryos were studied. The results showed that the use of alternating temperatures of 21/12°C (light/dark) significantly improved the germination percentage of the embryos, where 90% of embryos germinated, compared to a maximum of 55% when grown under a constant temperature of 25°C. Mean cotyledon fresh mass of embryos that germinated on media containing gibberellic acid (2.89, 28.89 µM GA_3) were significantly higher than those cultured on media without growth regulators. Conversely, root growth was severely inhibited in embryos germinated on media containing gibberellic acid. The *in vitro*-germinated seedlings were successfully transplanted to a peat/coir/sand mixture in the mist bed.

Key words: Alternating temperature, embryo culture, gibberellic acid, King Protea, Proteaceae.

INTRODUCTION

Protea cynaroides (King Protea) is endemic to South Africa. It is an important cut flower in the floriculture industry. Seeds are used by Protea growers to obtain new plants. However, seed propagation of members of the Proteaceae are known to be difficult. This is because Proteaceae seeds germinate poorly and erratically (Deall and Brown, 1981). In addition, germination usually takes a long period of time. Several studies have shown that endogenous inhibitors contribute to poor germination of Proteaceae 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, lack of promoters rather than the presence of inhibitors was responsible for poor germination of *P. compacta* (Brown and van Staden, 1975).

Several methods have been used to improve germination percentage. These include using chemicals or growth regulators, either as a pre-treatment 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 transferring to Petri dishes. Also, higher seed germination percentages

*Corresponding author. E-mail: hcwu@mail.nhu.edu.tw. Tel: +886 5 272 1001 Ext. 5441. Fax: +886 5 242 7195.

Abbreviations: GA_3, Gibberellic acid; **PAR,** photosynthetically active radiation.

of *Protea eximia* and *Protea neriifolia* were reported for GA_3-soaked seeds (Rodriguez-Perez, 1995). However, conflicting results in the same experiment were observed for *P. cynaroides* when GA_3 did not significantly improve its germination percentage.

In vitro embryo culture is commonly used 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 (Raghavan, 2003). The main objective of this study is to develop a method for rapid germination of *P. cynaroides* embryos *in vitro*, from which a large number of healthy plantlets could be obtained. An investigation into the effects of different temperature regimes, light conditions and GA_3 on germination percentage, cotyledon mass and root mass was conducted.

MATERIALS AND METHODS

In order to better expose the seed coat to sterilants during surface sterilization, hairs on the seeds were removed by hand. The seeds were surface-sterilized in 99% sulphuric acid (H_2SO_4) for 30 s. Sulphuric acid also softens the seed coat to make embryo excision easier. Thereafter, the seeds were rinsed in sterilized distilled water for 10 min. Each seed coat was then cut open using a scalpel and a pair of forceps to remove the embryo then carefully placed into the medium in an upright position.

Three different types of media were used in this experiment, which all contained half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) as the basal medium. Each medium treatment contained 0 (control), 2.89 or 28.89 µM GA_3. All the media were also supplemented with sucrose (3%) and Gelrite® (3 g/L). The pH of the media was adjusted to 5.7 before autoclaving and 10 ml of each medium were dispensed into glass test tubes and capped. The media were autoclaved at 104 KPa at 121°C for 20 min.

Excised embryos were germinated in two light conditions. For one treatment, a 12 h photoperiod was used. Cool, white fluorescent tubes provided 60 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) to the explants. In the other treatment, explants were grown in continuous darkness for the entire germination period (30 days). In addition, two different temperature regimes were used. For the first temperature treatment, the embryos were kept in a growth chamber at 25°C for the entire germination period. In the other treatment, alternating temperatures of 21/12°C (light/dark) were used. The embryos were considered germinated when the growth of the radicle took place.

In vitro-germinated embryos were planted out into a mixture of peat, coconut coir and silica sand (1:1:1, v:v:v). After removing the plantlets from the test tubes, they were washed with tap water and transplanted to a mist bed facility where the relative humidity was kept above 95%. The PAR and temperature of the mist bed was 400 µmol m^{-2} s^{-1} and 28±2°C, respectively.

Statistical analysis

A 3 × 2 × 2 factorial experiment with a completely randomized design was conducted. The factorial combinations were three growth media (0, 2.89 and 28.89 µM GA_3), two temperature regimes (25 and 21/12°C) and two light conditions (12 h photoperiod and continuous darkness). Forty explants were used in each treatment. Data were collected after 30 days in culture. Significant differences in the germination percentage between treatments were tested using Chi-square analysis. Treatment means for root and cotyledon fresh mass were separated using Tukey's studentised test at 5% level of significance. All statistical analyses were performed using the Statistical Analysis System (SAS) program (SAS Institute Inc., 1996).

RESULTS

Effects of treatments on germination percentage

The germination trend was similar in all treatments. The first visible change noted was the separation of the cotyledons from each other (Figure 1a), followed by the growth of the radicle (Figure 1b). The cultures then developed into complete plantlets (Figure 1c). The results showed that temperature had the most influence on the germination percentage of embryos. Irrespective of the light conditions and media treatments, significant differences were found between embryos grown in the alternating temperature regime of 21/12°C and the constant temperature of 25°C (Figure 2). The largest difference in germination percentage was observed under the 12 h photoperiod on basal ½ MS medium without GA_3, where 90% of embryos germinated under the 21/12°C temperature regime compared to only 20% under 25°C. With regard to the media treatments, in the 21/12°C temperature regime, no significant differences were found in the germination percentage between the basal ½ MS medium and those supplemented with GA_3, irrespective of whether it was under the 12 h photoperiod or continuous darkness (Figure 2). However, in the 25°C temperature treatment, the germination percentage of explants grown on basal ½ MS medium without GA_3 was significantly lower than those grown in medium containing 2.89 µM GA_3 in the 12 h photoperiod treatment, as well as both media containing either 2.89 or 28.89 µM GA_3 in continuous darkness (Figure 2). Furthermore, an increasing trend in germination percentage of seedlings grown in the different media under 25°C in continuous darkness seemed to indicate that the addition of GA_3 improved germination, however, germination percentage between seedlings grown on media containing 2.89 and 28.89 µM GA_3 were not significantly different. Within the same temperature and growth media, germination percentage was not influenced by the light treatments. No significant differences were observed between the explants cultured in the 12 h photoperiod and those in continuous darkness (Figure 2).

Effects of treatments on cotyledon and root growth of embryos

For cotyledon fresh mass, significant interaction effects were found between media and light treatments, and media and temperature regimes (Figure 3). Cotyledons of seedlings grown on medium without GA_3 were regular in size (Figure 1c), compared to those grown on 2.89 and

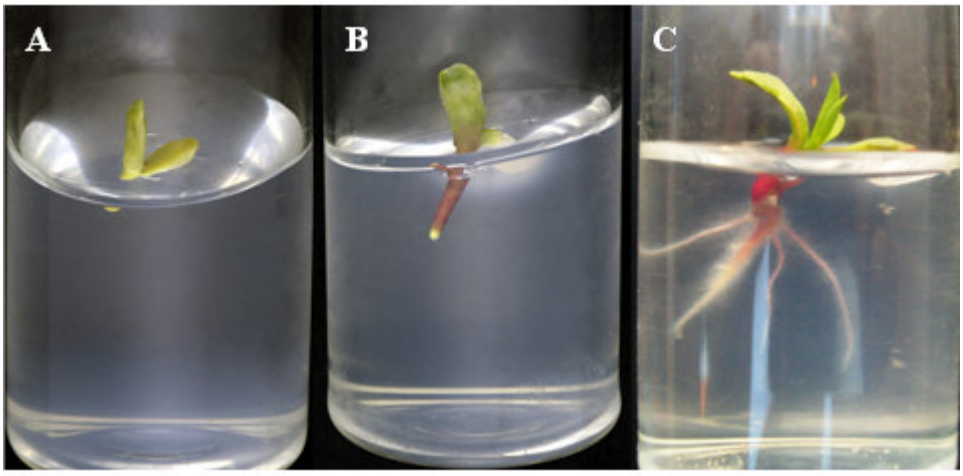

Figure 1. *In vitro* germination of excised *P. cynaroides* embryos cultured on half-strength MS basal medium grown under a 12-h photoperiod, after (A) 9 day, (B) 14 days, (C) 30 days.

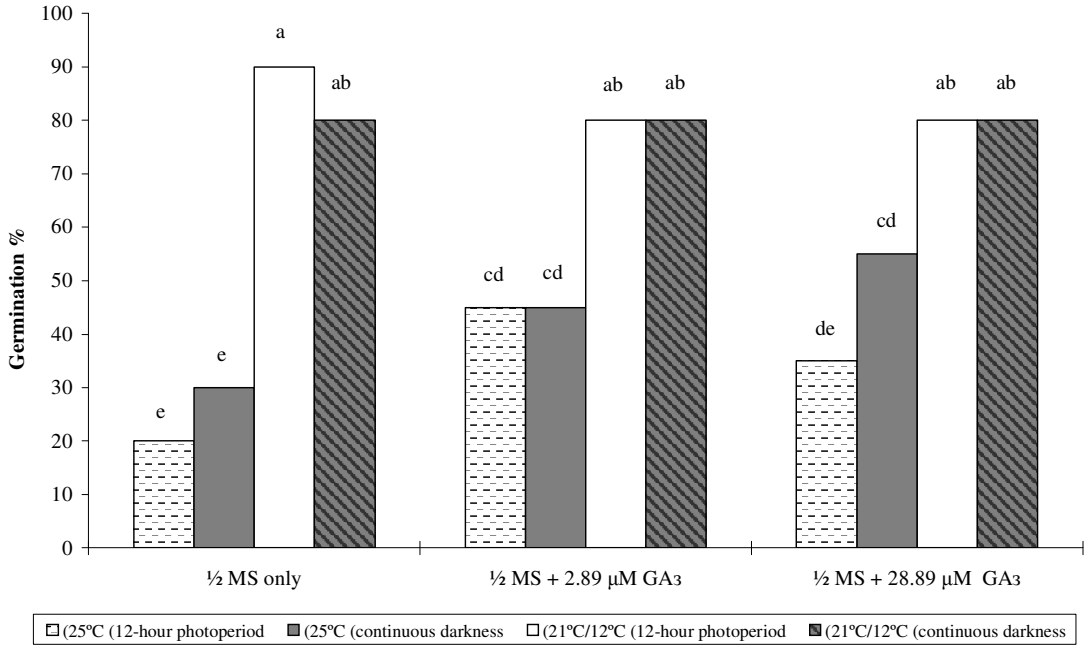

Figure 2. The effects of temperature regimes, light conditions and media compositions on the germination percentage of *P. cynaroides* excised embryos. Data were recorded after 30 days in culture. Means with different letters are significantly different based on Chi-square analysis ($p \leq 0.05$).

28.89 µM GA_3, which were elongated (Figure 4a). Although, no significant differences were found between cotyledon fresh mass of seedlings grown on 2.89 and 28.89 µM GA_3, the cotyledon fresh mass of seedlings germinated on the basal MS medium without GA_3 was significantly lower than those exposed to GA_3 (Figure 3). In certain cases, the cotyledon fresh mass of seedlings cultured in media containing GA_3 were up to three times higher than those cultured on MS basal medium only. Although, temperature influenced the germination percentage of embryos, cotyledon development was not significantly affected. Despite the poor germination percentage of embryos at 25°C, within the same medium treatment, the cotyledon fresh mass of those that germinated was similar to those cultured at 21/12°C (Figure 3). Cotyledons of seedlings that germinated in continuous darkness were pale in colour (Figure 4b). Even though these cotyledons were longer, their mean

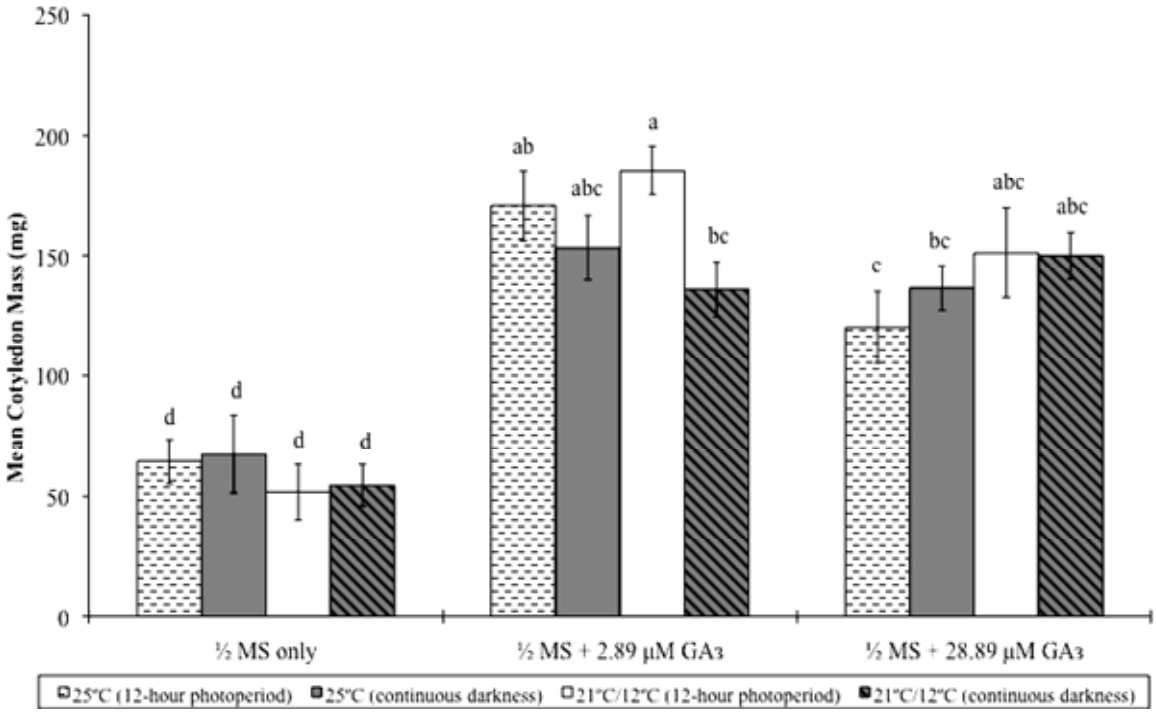

Figure 3. The effects of temperature regimes, light conditions and media compositions on the growth of cotyledons in *P. cynaroides* excised embryos. Data were recorded after 30 days in culture. Means with different letters are significantly different according to Tukey's studentised test ($p \leq 0.05$).

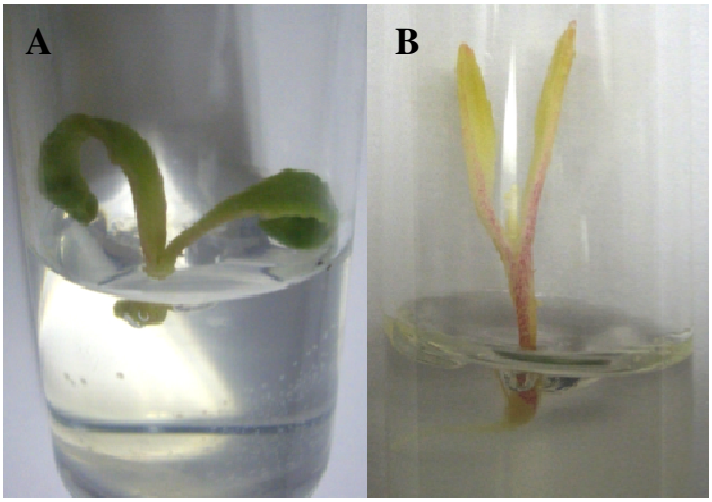

Figure 4. (A) Development of elongated cotyledons on *P. cynaroides* seedlings germinated in media containing 28.89 µM GA3; (B) growth of slender, light-coloured cotyledons on seedlings germinated in continuous darkness.

fresh mass was not significantly different to those grown in the 12 h photoperiod treatment (Figure 3). Cotyledons of dark-germinated seedlings were narrow and slender.

Root growth of the embryos was significantly inhibited on growth media containing GA_3. Interaction effects between media and light, media and temperature, and light and temperature, were significant (Figure 5). Root fresh mass of germinated seedlings grown on the ½ MS basal medium without GA_3 was significantly higher than that of seedlings on media supplemented with either 2.89

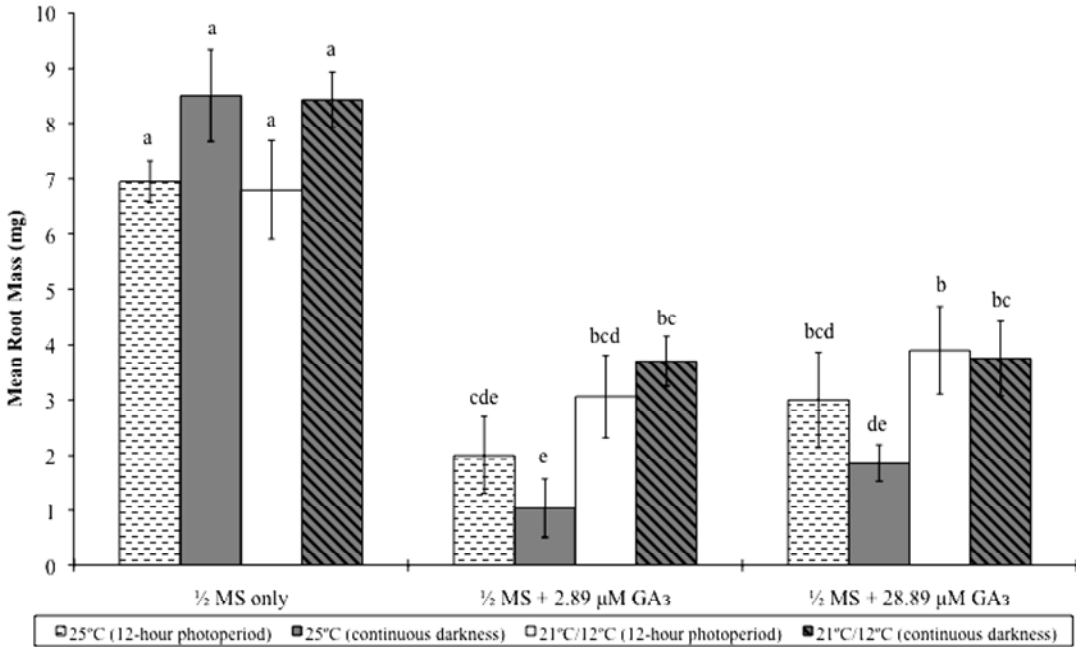

Figure 5. The effects of temperature regimes, light conditions and media compositions on root growth of *P. cynaroides* excised embryos. Data were recorded after 30 days in culture. Means with different letters are significantly different according to Tukey's studentised test ($p \leq 0.05$).

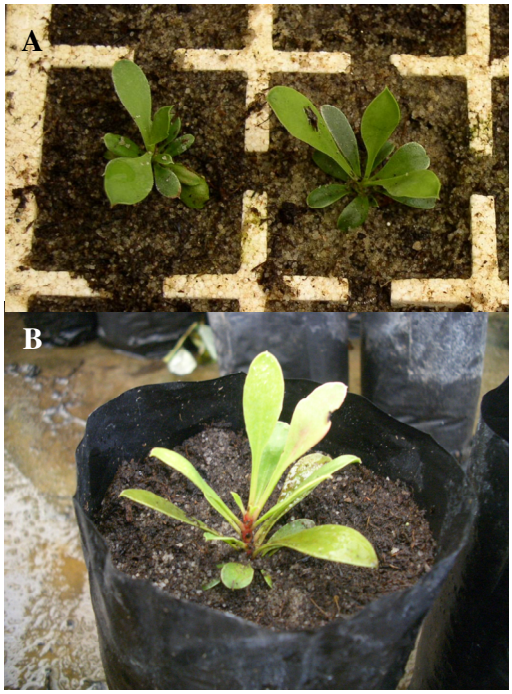

Figure 6. Establishment of *in vitro*-germinated *P. cynaroides* plantlet in soil after (A) 30 days, (B) 60 days.

or 28.89 µM GA_3, irrespective of light conditions or temperature regimes (Figure 5). Within the same medium treatment in continuous darkness, significant differences were only observed when they were cultured on ½ media supplemented with 2.89 (25˚C) or 28.89 µM GA_3 (21/12°C).

Healthy seedlings grown on basal ½ MS medium without GA_3 under alternating temperatures of 21/12°C, which had developed true leaves were selected and transplanted to a peat/coir/sand mixture in the mist bed. New leaves and roots developed on these seedlings after 30 days (Figure 6a). After 60 days, acclimatized plantlets were transplanted to larger bags (Figure 6b).

DISCUSSION

Results from this study showed that the use of alternating temperatures is the most important factor in controlling germination of *P. cynaroides* embryos. This finding contrasts, to a certain extent, with suggestions that endogenous inhibitors (Brown and van Staden, 1971) or the lack of promoters (Brown and van Staden, 1975) may be partly responsible for poor germination of Proteaceae seeds. It is probable that plants belonging to the Proteaceae family may have similar temperature requirements since the low germination percentage of these excised embryos at 25°C cultured in 2.89 µM GA_3 (45% germination) was similar to that reported by Van Staden et al. (1972). In their study, where *P. compacta* excised embryos were also germinated *in vitro* at 25°C with a 12 h photoperiod, the highest germination obtained

was 43% on MS medium containing 2.89 µM GA_3.

Constant temperatures are normally used in growth rooms. However, the importance of using alternating temperature regimes in *in vitro* propagation should not be underestimated. According to George (1993), adjusting the temperature of the growth room to that of its natural habitat can be advantageous in stimulating growth of explants of a particular species. Therefore, in the case of *P. cynaroides*, the importance of using alternating temperatures lies in the fact that its seeds apparently germinate best during autumn where fluctuating diurnal temperatures are prevalent (Vogts, 1982). Similar results were reported by Carpenter and Gilman (1988), where an alternation between 35 and 25°C was found to be the optimum germination temperatures for *Thrinax morrisii* seeds. The use of alternating temperatures (32/19°C) in the germination of *Musa* seeds was also reported to yield the highest germination percentage (99%) (Stotzky and Cox, 1962). In addition, *Digitalis lanata* explants were reported to root better when cultured in a 19/14°C (day/night) temperature regime (Schoner and Reinhard, 1982).

GA_3 is known to play an important role in germination by initiating the mobilization of nutrient reserves stored in the endosperm (Hopkins and Huner, 2009). The addition of GA_3 in growth media has often successfully induced or improved germination (Pech et al., 2007; de Nazaré et al., 2009). However, results of this study show that GA_3 plays a secondary role to temperature in the germination process of *P. cynaroides* embryos, since at the less favourable temperature (25°C), GA_3 was able to only improve the germination percentage from 20 (control) to 45% (2.89 µM GA_3) in the 12 h photoperiod, and from 30 (control) to 55% (28.89 µM GA_3) in continuous darkness, while at the temperature regime of 21/12°C, up to 90% germination was achieved in the absence of GA_3 (Figure 2). Furthermore, it is reported that the addition of GA_3 to the medium results in the production of elongated and narrow leaves (De Fossard and de Fossard, 1988). In this study, the effect of GA_3 in promoting the formation of abnormally long cotyledons was very distinctive in the seedlings (Figure 4a). Similar effects were found on pumpkin cotyledons where fresh mass cultured in 2.89 µM GA_3 were more than 40% higher than the control (Kursanov et al., 1969). The inhibitory effect of GA_3 on root growth was clear. The stunted growth of radicles in media containing either 2.89 or 28.89 µM GA_3 support reports that GA_3 diminishes or prevents the formation of roots *in vitro* (George, 1993). Although, GA_3 did not inhibit the vegetative growth of the seedlings, their stunted roots led to poor anchorage of the seedlings in the growth media, and due to their over-developed cotyledons, the seedlings were top-heavy and abnormal.

A protocol for the *in vitro* germination of *P. cynaroides* embryos has been developed. A high germination percentage (90%) was obtained in a relatively short period of time (30 days) when grown in basal ½ MS medium without GA_3 under an alternative temperature regime of 21/12°C (light/dark). These plantlets were successfully acclimatized and established in *ex vitro* conditions. The *in vitro* embryo culture described above could be used as an alternative propagation method to mass produce *P. cynaroides* plantlets in a commercial setting.

REFERENCES

Brits GJ (1986). The effect of hydrogen peroxide treatment on germination in Proteaceae species with serotinous and nut-like achenes. S. Afr. J. Bot. 52: 291-293.

Brown NAC, van Staden J (1971). Germination inhibitors in aqueous seed extracts of four South African Proteaceae. J. S. Afr. Bot. 37(4): 305-315.

Brown NAC, van Staden J (1973). The effect of scarification, leaching, light, stratification, oxygen and applied hormones on germination of *Protea compacta* R. Br. cccand *Leucadendron daphnoides* Meisn. J. S. Afr. Bot. 39(2): 185-195.

Brown NAC, van Staden J (1975). Inhibitors are not responsible for poor germination of *Protea compacta* seed. J. S. Afr. Bot. 41(4): 225-230.

Carpenter WJ, Gilman EF (1988). Effect of temperature and desiccation on the germination of *Thrinax morrisii*. Proc. Fla. State Hort. Soc. 101: 288-290.

De Fossard RA, de Fossard H (1988). Micropropagation of some members of the Myrtaceae. Acta Hort. 227: 346-351

De Nazaré Oliveira Ribeiro N, Pasqual M, Villa F, Aparecida Salles Pio L, William Maria Hilhorst H (2009). *In vitro* seed germination and seedling development of *Annona crassiflora* Mart. Sci. Agric. 66(3): 410-413.

Deall GB, Brown NAC (1981). Seed germination in *Protea magnifica* Link. S. Afr. J. Sci. 77: 175-176.

George EF (1993). Plant propagation by tissue culture. Part 1: The Technology, 2nd Ed. Edington, Wilts. Exegetics Ltd.

Hopkins WG, Huner NPA (2009). Introduction to plant physiology, 4th Edition. John Wiley and Sons, Inc., New Jersey, USA.

Kursanov AL, Kulaeva ON, Mikulovich TP (1969). Combined effect of 6-benzylaminopurine, gibberellic acid, and 3-indoleacetic acid on the expansion of isolated pumpkin cotyledons. Am. J. Bot. 56(7): 767-772.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Pech y Aké A, Maust B, Orozco-Segovia A, Oropeza C (2007). The effect of gibberellic acid on the *in vitro* germination of coconut zygotic embryos and their conversion into plantlets. *In Vitro* Cell. Dev. Biol.: Plant 43(3): 247-253.

Raghavan V (2003). One hundred years of zygotic embryo culture investigations. In Vitro Cell. Dev. Biol.: Plant 39(5): 437-442.

Rodriguez-Pérez JA (1995). Effects of treatment with gibberellic acid on germination of *Protea cynaroides, P. eximia, P. neriifolia* and *P. repens* (Proteaceae). Acta Hort. 387: 85-89.

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

Schoner S, Reinhard E (1982). Clonal multiplication of *Digitalis lanata* by meristem culture. Planta Med. 45: 155.

Stotzky G, Cox EA (1962). Seed germination studies in Musa. II. Alternating temperature requirement for the germination of *Musa balbisiana*. Am. J. Bot. 49(7): 763-770.

Van Staden J, Brown NAC (1972). Characterization of germination inhibitors in seed extracts of four South African species of Proteaceae. J. S. Afr. Bot. 38(3): 135-150.

Van Staden J, Brown NAC, Button J (1972). The effects of applied hormones on germination of excised embryos of *Protea compacta* R. Br. *in vitro*. J. S. Afr. Bot. 38(4): 211-214.

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