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# ***In Vitro* Organogenesis of *Protea cynaroides* L. Shoot-Buds Cultured Under Red and Blue Light-Emitting Diodes**

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## **1. Introduction**

*Protea cynaroides* L. (King Protea), which belongs to the Proteaceae family, is a slow-growing, semi-hardwood shrub. The *Protea* genus has the widest distribution area of all the southern Africa Proteaceae, ranging from the predominantly winter or all-year round rainfall area of the Cape in South Africa to the subtropical and tropical areas of southern Africa (Paterson-Jones, 2007). They occupy a variety of habitats from sea level to up to 1500 metres. *P. cynaroides* species vary widely in colour, shape and flowering time (Matthews, 1993). Its growth habits vary from dwarf variants to dense, bushy forms reaching heights of 2 m, which are commonly used in cultivation. The most characteristic feature of its blossom is its flowerhead, which typically consists of hundreds of flowers (Rebelo, 2000). Its flowerhead shape ranges from small, narrow, goblet-shapes to large, wide, flat types. Their colours range from greenish-white to deep pink and red (Matthews, 1993). Due to their wide variability, flowers can be seen throughout the year, depending on the variety. In their natural habitat, *P. cynaroides* are found in well-drained, acidic, nutrient-deficient soils. Their ability to thrive in soil with low nutrients is assisted by the growth of proteoid roots, which are specialized roots that look like very fine bottlebrushes, and are very efficient at absorbing nutrients. The King Protea is a well-known cut flower in many parts of the world, and is a highly sought after commodity in the international flower market due to its attractive flowerhead and long vase life. The demand for the King Protea has remained consistent on the international market and its market price has remained relatively high over the years. Current important production areas include: Australia, South Africa, California, Portugal, Israel, Zimbabwe, Hawaii, Chile, New Zealand, and Ecuador (Dorrington, 2008). Due to its popularity, production areas are expanding in Europe, with new plantations being established in Portugal and Spain (Leonardt, 2008).

King Proteas are plants that are difficult to grow and fertilize (Littlejohn et al., 2003). The major factors identified for successful cultivation are well-drained, sandy acidic soils with low phosphor content and pH ranging from 3.5 to 5.8 (Silber, et al., 2001). Although higher pH levels can be tolerated, these plants have low mineral requirements and are therefore not

tolerant to salt concentrations that would appear normal to other plants (Montarone & Allemand, 1995). Stem cuttings are commonly used to vegetatively propagate *P. cynaroides*, however, root formation usually needs several months to take place, and typically have low success rates. *In vitro* propagation techniques are widely used to propagate numerous economically important plants. Under *in vitro* conditions, growers are able to mass-produce plants in a relatively short period of time. In addition, *in vitro* propagation is also used to overcome problems that are found in traditional vegetative propagation, such as poor root formation of cuttings, slow growth rates, and susceptibility of cuttings to diseases. In traditional sexual propagation, problems such as seed dormancy and low germination rate are often overcome via *in vitro* propagation. The significant successes in this field have been extensively reported, which in most cases have dramatically changed the way plants are propagated.

Over the years, very few studies investigating the *in vitro* propagation of *P. cynaroides* have been reported. According to Tal et al. (1992), recurrent difficulties encountered in the propagation of proteas *in vitro* include phenolic oxidation and necrosis of clonal explants. These factors have resulted in limited success and prevented progress in this area of research. The first attempt to propagate *P. cynaroides in vitro* was carried out by Ben-Jaacov & Jacobs (1986). In their study, growth of axillary buds was successful through the establishment of nodal stem segments. More recently, advances were made in the *in vitro* establishment of *P. cynaroides* nodal explants by treating shoot segments with antioxidants (ascorbic acid and citric acid) after surface sterilization to reduce oxidative browning and increase axillary bud growth (Wu & du Toit, 2004). In another study, *P. cynaroides* apical buds were used as explants and successfully established *in vitro* by Thillerot et al. (2006). Proliferation of buds were subsequently achieved in the multiplication stage, however, it was reported that bud growth was slow, possibly due to apical dominance. Most importantly, *in vitro* rooting of *P. cynaroides* explants in the studies described above was never achieved. Moreover, growth of *P. cynaroides* shoots *in vitro* remains to be slow and inconsistent. With the aim to produce complete plantlets more efficiently, somatic embryogenesis of *P. cynaroides* was studied. Results showed that somatic embryos were able to form directly on mature zygotic embryos and cotyledons (Wu et al., 2007b), and germinate into plantlets. While studying the induction of somatic embryos in *P. cynaroides* cotyledons, it was discovered that the cotyledonary nodes possessed a high organogenic potential to produce shoot-buds. However, the growth rates of the axillary buds and shoot-buds were slow, and subsequent attempts to multiply these explants were not successful. The slow growth rate of these buds may be attributed to the absence of a root system, since the growth rate of *P. cynaroides* somatic embryos, which possessed a root system, was relatively high. It is likely that the uptake of nutrients by rootless *P. cynaroides* buds were highly inefficient. Therefore, in order to increase the growth rates of axillary buds and shoot-buds in the multiplication stage, induction of adventitious roots is required. The use of growth regulators to promote rooting of buds has been ineffective (Wu et al., 2007b).

Light is an important stimulus for plant development. It is also widely known that spectral quality is a key factor in plant morphogenesis (Okamoto et al., 1997). Conventional fluorescent lamps, which have a wide range of wavelengths from 350 to 750 nm, are the most commonly used light source in plant tissue culture (Economou & Read, 1987). Due to the difficulty in controlling the light quality of fluorescent lamps, and with technological

advances in recent years, the use of light emitting diodes (LEDs) as an alternative light source for explants cultured *in vitro* has attracted considerable interest. The advantages that LEDs have over fluorescent lamps are their wavelength specificity, light intensity adjustability, low thermal energy output, small mass, and long life (Bula et al., 1991; Brown et al., 1995; Okamoto et al., 1997). Numerous studies have been conducted to investigate the effectiveness of specific light qualities emitted by LEDs in promoting growth and morphogenesis of different plants. An overview of the available literature shows that red LEDs (620-680 nm), blue LEDs (420-480), a combination of red and blue LEDs, and LEDs emitting far-red light (735 nm), at various wavelengths and intensities are commonly used as light sources in research studies.

Light quality studies have been carried out on important agricultural crops such as banana (Nhut et al., 2002), lettuce (Okamoto et al., 1996), pepper (Brown et al., 1995), potato (Jao & Fang, 2004), spinach (Yanagi & Okamoto, 1997) and wheat (Goins et al., 1997). In addition, floral plants such as anthurium (Budiarto, 2010), calla lily (Chang et al., 2003; Jao et al., 2005), gerbera (Wang et al., 2011), *Lilium* (Lian et al., 2002; Lin et al., 2008) and *Pelargonium* (Appelgren, 1991) amongst others, have also been studied. Results from different studies have shown that red and blue lights in particular, have a significant influence on plant photomorphogenesis. However, the responses of plants to different light qualities vary widely. Studies showed that culturing *Lilium* explants under a combination of red and blue LEDs produced larger bulblets, and a higher number of roots (Lian et al., 2002). Findings by Appelgren (1991) revealed that growing *Pelargonium* plantlets *in vitro* under red light significantly stimulated stem elongation, while inhibition of stem elongation was found under blue light. In a recent study, red and blue LEDs were found to induce root formation in anthuriums (Budiarto, 2010). Similarly, a higher rooting percentage and higher root numbers of grape explants were obtained when cultured under red LEDs (Poudel et al., 2008).

From the literature described above, it is clear that LEDs have numerous advantages over conventional fluorescent lamps, and that light emitted by LEDs are highly beneficial to the growth and morphogenesis in a wide range of plant species. In order for *in vitro* propagation to become an alternative method of propagation for *P. cynaroides*, stimulating adventitious root formation and promoting vegetative growth of *P. cynaroides* explants *in vitro* must be achieved. Adventitious root formation in *P. cynaroides* explants has never been reported before. The use of LEDs as a light source is ideal to study the effects of specific wavelengths on organogenesis, particularly adventitious root formation, in difficult-to-grow plants such as *P. cynaroides*. Therefore, the aim of this study was to investigate the effects of light quality emitted by light-emitting diodes (LEDs) on the induction of adventitious roots and bud growth of *P. cynaroides* shoot-buds.

## 2. Materials and methods

### 2.1 Embryo excision and culture conditions

*P. cynaroides* seedlings were established using mature embryos excised from seeds. Surface-sterilization of the seeds and excision of the embryos was done according to Wu et al. (2007a) with modifications. Hairs on *P. cynaroides* seeds were first removed by hand and only plump-looking, healthy seeds were selected for germination. For surface sterilization,

the seeds were placed in 99% sulphuric acid ( $\text{H}_2\text{SO}_4$ ) for 30 seconds. The seeds were then immediately transferred to sterilized distilled water and rinsed for 5 mins to remove traces of sulphuric acid. This was repeated twice. Afterwards, the embryo was removed from the seed by carefully cutting open the seed coat with a scalpel. After excision, the embryos were placed into the growth medium in an upright position. Only the bottom half of the embryo was in direct contact with the growth medium. Half-strength Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with  $30 \text{ g L}^{-1}$  sucrose and  $9 \text{ g L}^{-1}$  agar was used for germinating the embryos. Ten mL of growth medium were dispensed into glass test tubes ( $25 \text{ mm} \times 150 \text{ mm}^2$ ). The pH of the medium was adjusted to 5.8 prior to adding agar. The medium was autoclaved for 20 min at  $121^\circ\text{C}$  and 104 KPa. Embryos in the test tubes (one embryo/tube) were placed in a growth chamber with a 16-h photoperiod. An alternating temperature regime of  $21^\circ\text{C}/12^\circ\text{C}$  (light/dark) was used throughout the germination period. Cool white fluorescent tubes provided  $50 \mu\text{mol m}^{-2} \text{ sec}^{-1}$  photosynthetically active radiation (PAR). The PAR was measured at plant height with a light meter (LI-1800, LI-COR Inc.). Separation of the two cotyledons was observed after approximately 10 days (Fig. 1A). Growth and greening of the cotyledons occurred after 20 days (Fig. 1B). After 40 days, germinated embryos (Fig. 1C), which consisted of two cotyledons and a radicle, were subcultured to fresh medium for the induction of adventitious bud formation.

## 2.2 Induction of adventitious bud formation

Germinated seedlings were transferred to half-strength MS medium media containing  $2 \text{ mg L}^{-1}$  benzyladenine (BA) and  $0.5 \text{ mg L}^{-1}$  naphthalene acetic acid (NAA) to induce the formation of shoot-buds. Glass culture vessels ( $100 \text{ mm} \times 150 \text{ mm}^2$ ) containing 50 mL of growth medium were used. The pH of the growth medium was adjusted to 5.8 before autoclaving. Each glass vessel contained five explants. The cultures were placed in a growth room with the temperature adjusted to  $25 \pm 2^\circ\text{C}$ . Cool white fluorescent tubes provided  $50 \mu\text{mol m}^{-2} \text{ sec}^{-1}$  PAR with a 16-h photoperiod. Direct formation of shoot-buds on the cotyledons was observed after approximately 40 days (Fig. 1D). Almost identical shoot-buds were selected, removed and transferred to fresh medium for the light quality experiment.

## 2.3 Light quality treatments and culture conditions

After each shoot-bud was removed from the cotyledons, they were weighed under sterile conditions. Shoot-buds with similar weights (10 mg) were selected for this experiment. The shoot-buds were grown in glass test tubes placed in customized LED lighting systems. The explants were exposed to the following light treatments: red LEDs (660 nm), blue LEDs (450 nm), and total darkness. Conventional cool white fluorescent lamps were used as the control. The LEDs were purchased from Ryh Dah Inc. (Taiwan). The lighting systems were constructed with aluminum boxes ( $50 \text{ cm (L)} \times 50 \text{ cm (W)} \times 25 \text{ cm (H)}$ ), and equipped with three hundred red or blue LEDs spaced 2 cm apart, on the cover of the box. A temperature sensor, timer and two fans were also installed on each lighting system. The LED lighting systems were placed in a growth room throughout the entire duration of the experiment. In all treatments, the PAR, photoperiod and temperature were adjusted to  $50 \mu\text{mol m}^{-2} \text{ sec}^{-1}$ , 16 h, and  $25 \pm 2^\circ\text{C}$ , respectively. The wavelengths of the light sources were measured with a spectroradiometer (International Light Technologies, ILT900). The spectral distributions of red, blue LEDs and fluorescent lights are shown in Fig. 2. Shoot-buds were cultured in 10

mL of growth medium in test tubes (1 shoot-bud/tube) containing half-strength MS media supplemented with 0.1 mg L<sup>-1</sup> NAA, 30 g L<sup>-1</sup> sucrose and 9 g L<sup>-1</sup> agar.

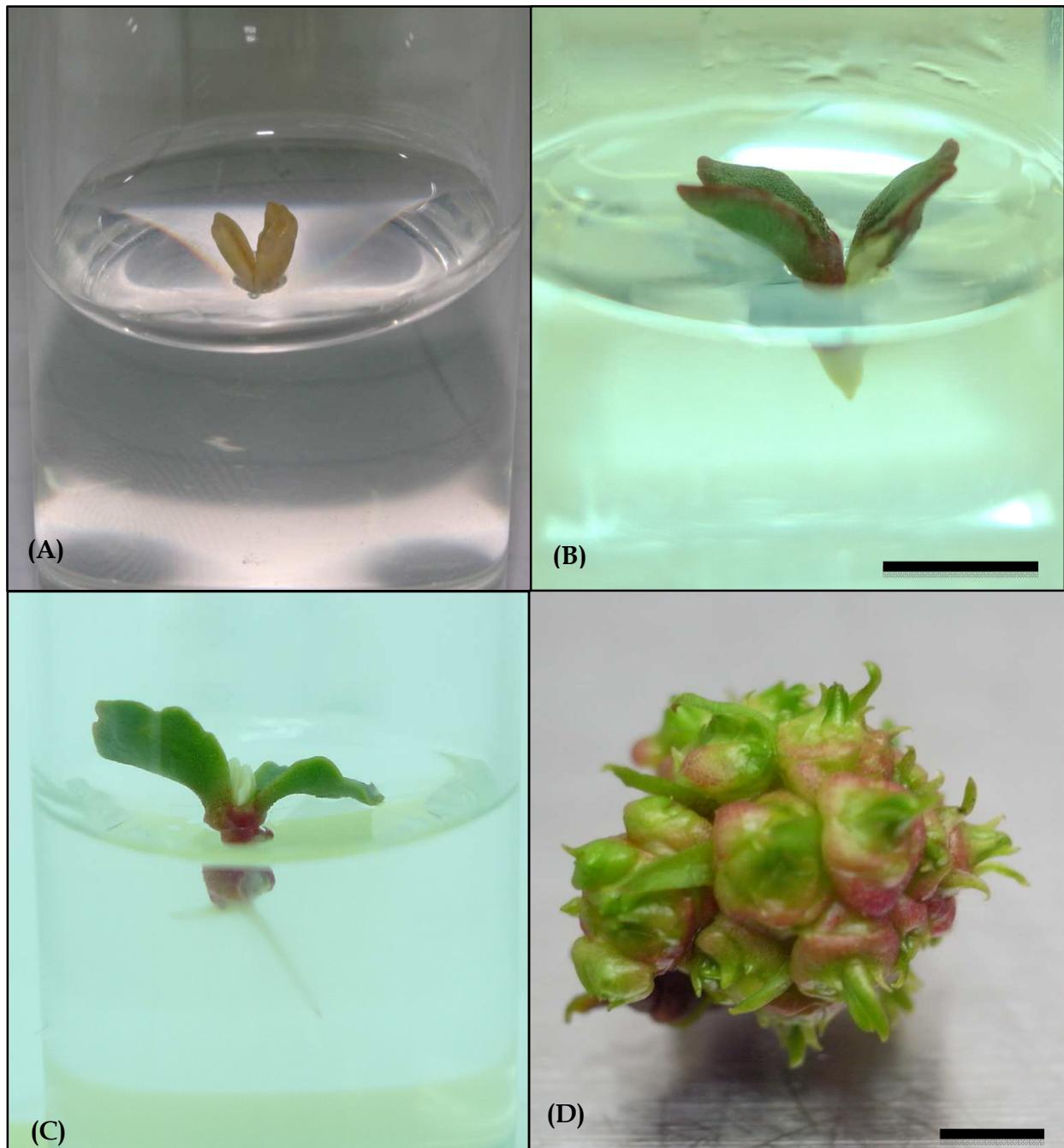
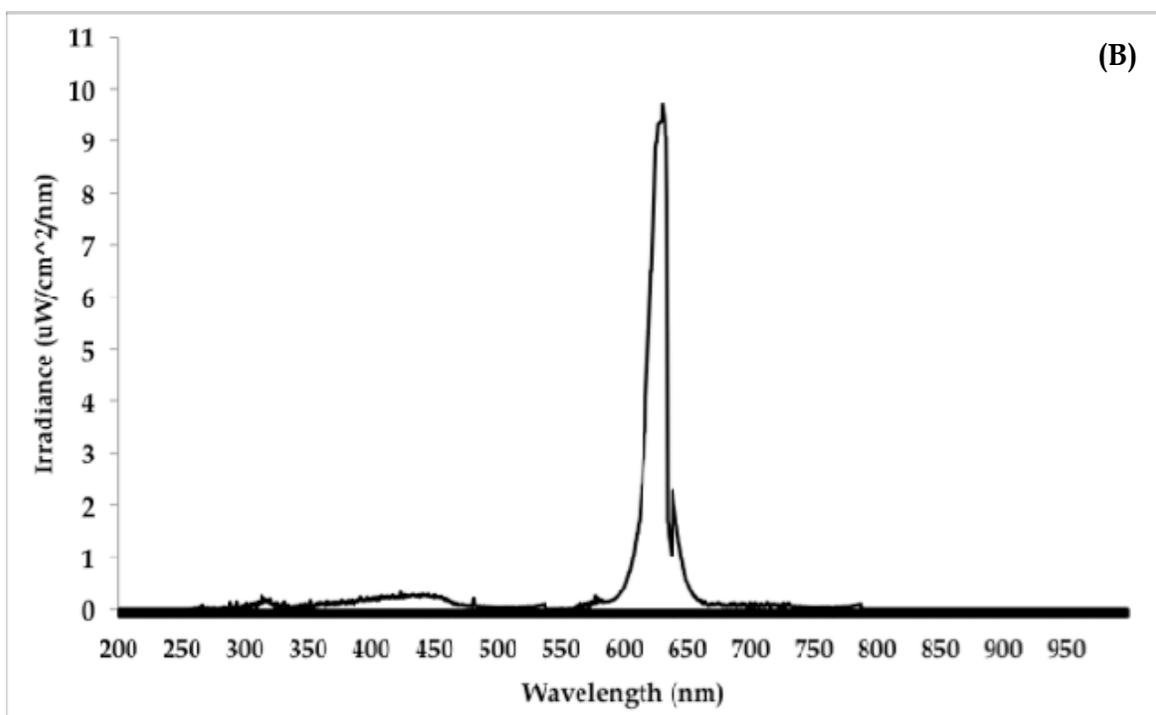
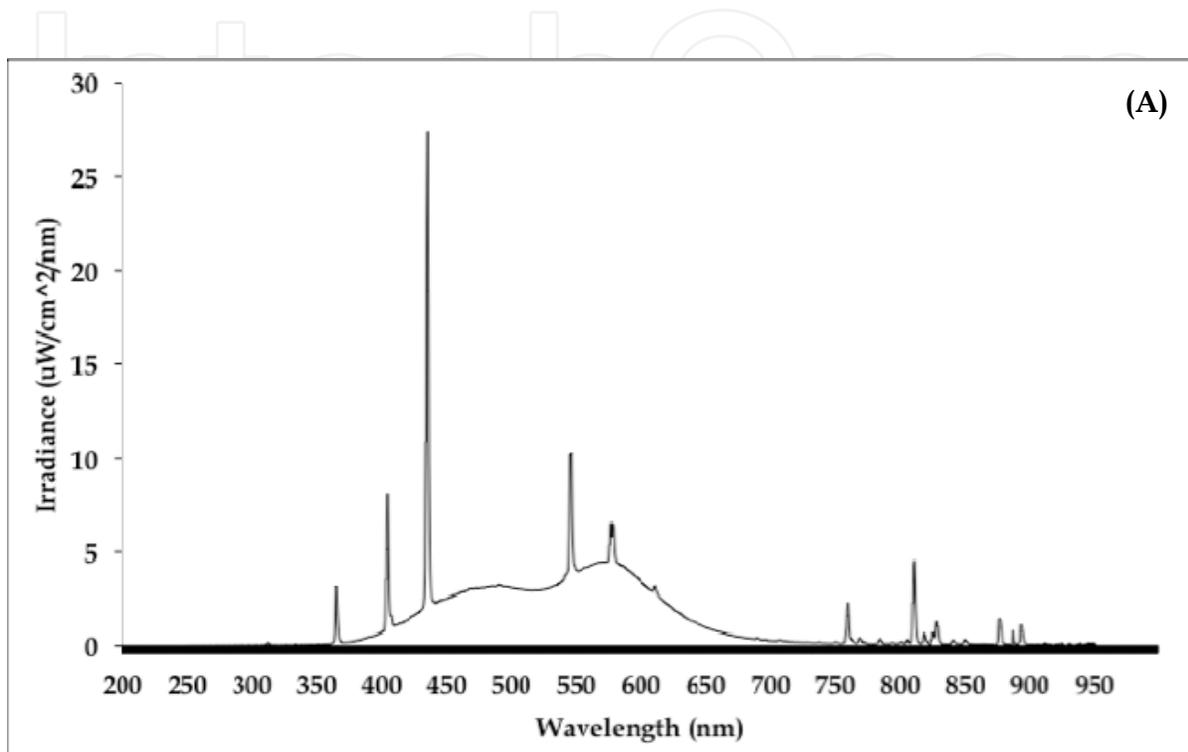


Fig. 1. Germination of *P. cynaroides* zygotic embryo after (A) 10 days, (B) 20 days, and (C) 40 days. (D) Direct shoot-bud formation on cotyledonary node 40 days after subculturing to half-strength MS medium with 2 mg L<sup>-1</sup> BA and 0.5 mg L<sup>-1</sup> NAA (Bar = 0.5 cm).

#### 2.4 Statistical analysis

A completely randomized design was used in all treatments. Eight replications per treatment were used. Data for rooting percentage, number of roots, root length, root fresh

weight, number of leaves, and bud fresh weight were recorded after 45 days in culture. The experiment was repeated twice. Data were analyzed using Duncan's Multiple Range test to compare treatment means. Differences were considered significant when  $P < 0.001$ . Statistical analyses were done using the Statistical Analysis System (SAS) program (SAS Institute Inc., 1996).



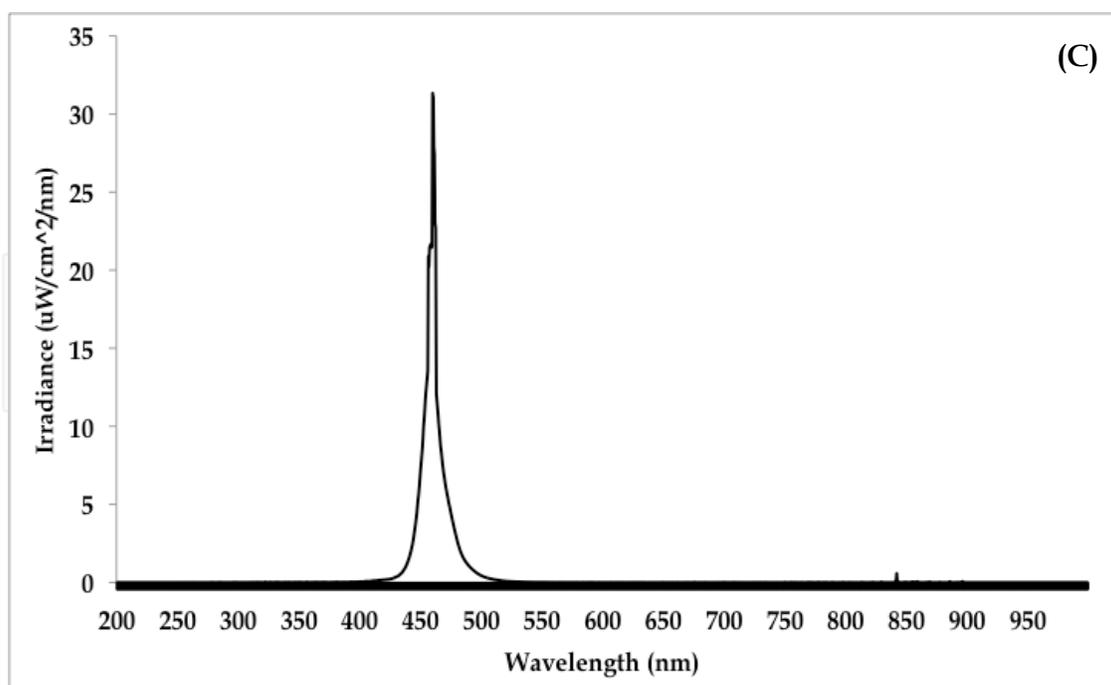


Fig. 2. Spectral distribution of (A) fluorescent lamp, (B) red LED, and (C) blue LED.

### 3. Results

Results of the study are shown in Fig. 3 and Fig. 4. Vegetative growth of explants in all treatments was very slow. As Fig. 3 shows, elongation of the shoot-buds did not take place. However, root formation, growth of new leaves and increase in bud weight occurred after 30 days in culture. From a visual observation of the buds, browning of the leaves and bud tissues of explants cultured under conventional white fluorescent light (control) were clearly evident (Fig. 3A). On the other hand, very little to no browning of the leaves or tissues was observed on explants grown under red LEDs, blue LEDs or in the dark (Fig. 3B, 3C, 3D). In addition, adventitious buds exposed to blue light seemed to possess the greenest leaves (Fig. 3C), while those grown in the dark exhibited light green leaves and tissues (Fig. 3D). In terms of root formation, adventitious roots were found on explants cultured under red LEDs after 30 days (Fig. 3B), while root formation on explants grown under the other light conditions were only evident towards the end of the study at day 45 (Fig. 4A).

Results of the analyses of the different growth parameters after 45 days in culture are shown in Fig. 4. A significantly higher rooting percentage was observed in adventitious buds cultured under red LEDs (Fig. 4A). Furthermore, the rooting percentage of explants irradiated by white fluorescent light and those grown in the dark were similar. In contrast, the rooting percentage of buds cultured under blue LEDs was significantly lower than all the other light treatments. In terms of the number of roots formed on explants, results showed a similar trend to that of rooting percentage (Fig. 4B). Adventitious buds irradiated by red LEDs produced the highest mean number of roots, while those exposed to light emitted by blue LEDs produced a significantly lower number of roots. No significant differences were observed between the number of roots formed by explants under white fluorescent lights and in the dark (Fig. 4B). In addition, results showed that although red LEDs induced the highest rooting percentage and root numbers, the lengths of these roots

were comparable to those formed in the dark (Fig. 4C). Moreover, the roots formed on buds irradiated by white fluorescent light and blue LEDs were similar in length.

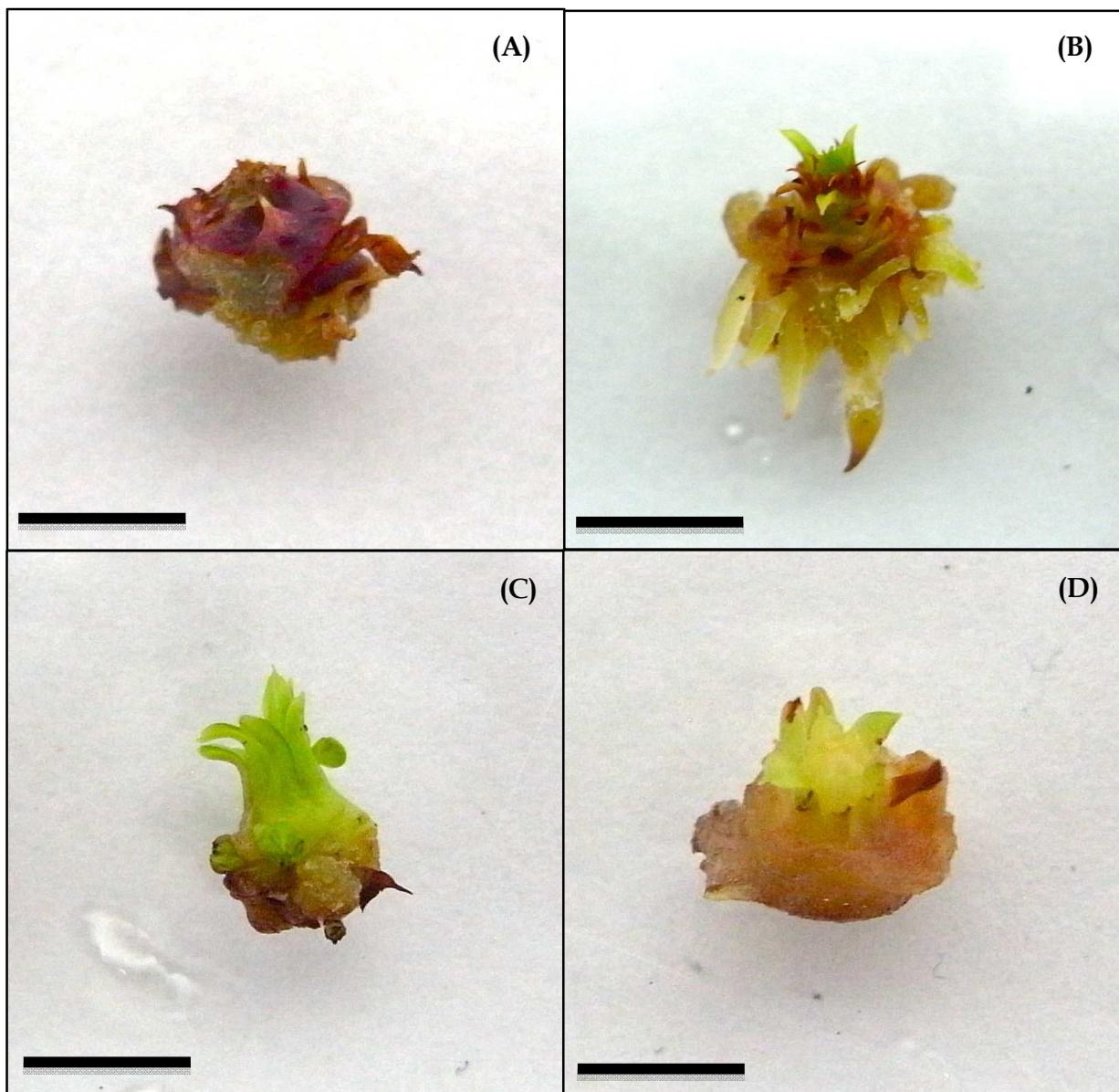


Fig. 3. Response of explants to (A) white fluorescent light, (B) red LED light, (C) blue LED light, (D) total darkness, after 30 days in culture (Bar = 0.5 cm).

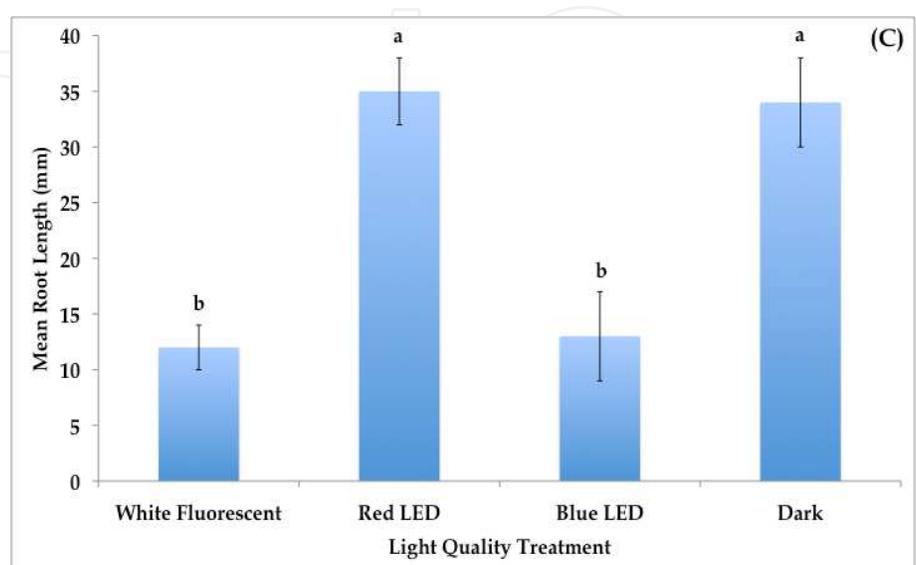
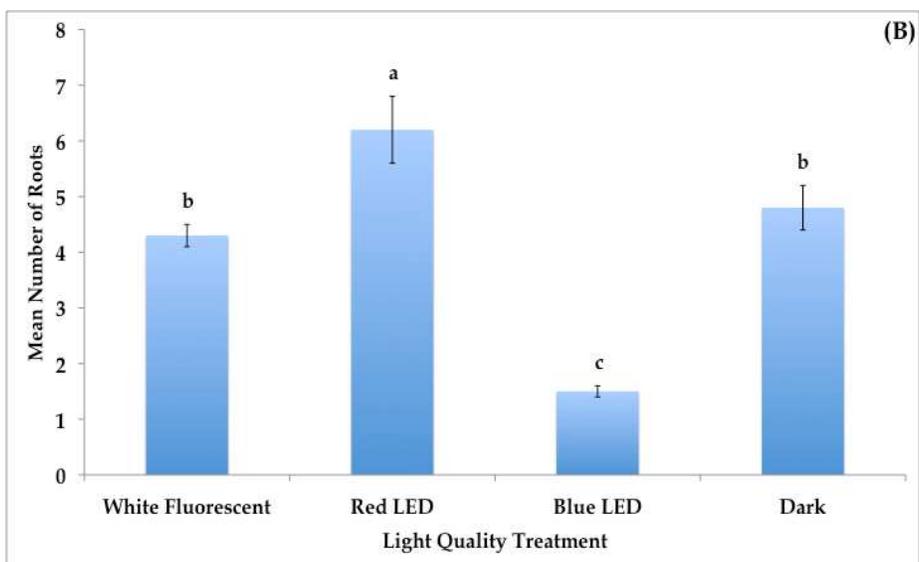
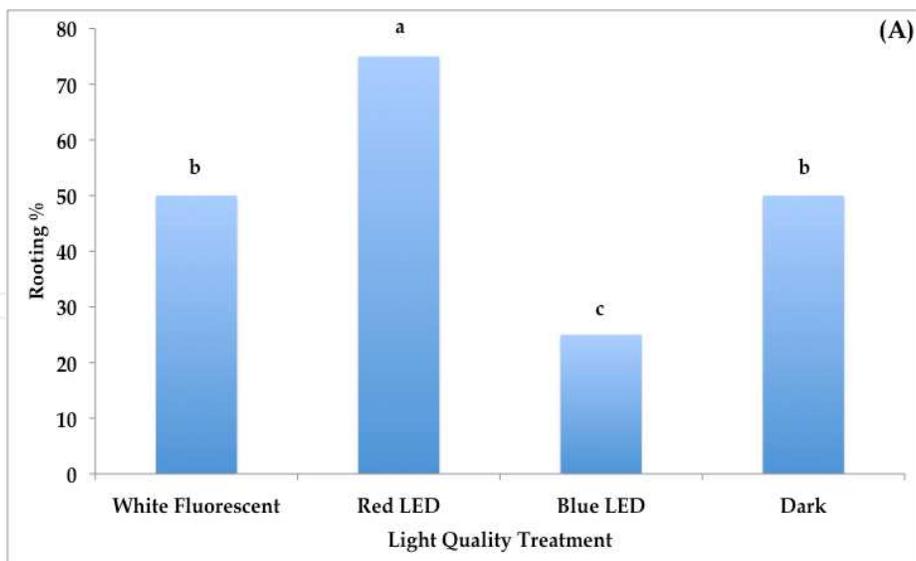
As a result of the similar root lengths found between explants grown under red LEDs and those grown in the dark, the root fresh weight (per root) of these two treatments were not significantly different (Fig. 4D). Furthermore, the root fresh weight of buds exposed to light emitted by white fluorescent lamps and blue LEDs were similar, and were significantly lower than those cultured under red LEDs and in the dark. Results of this study showed that

red LEDs also induced the formation of the highest number of new leaves on the buds (Fig. 4E). However, compared to those cultured under blue LEDs and in the dark, the leaf numbers were not significantly different. Surprisingly, buds cultured under conventional white fluorescent light produced the least number of new leaves, which were significantly lower than those exposed to red LEDs, blue LEDs or those grown in the dark. The fresh weight of buds was found to be the highest when irradiated by red LEDs or grown in the dark (Fig. 4F). The lowest bud fresh weight was found in explants cultured under either white fluorescent light or blue LEDs.

#### 4. Discussion

Overall results of this study demonstrated the difficulties in propagating *P. cynaroides* explants *in vitro*. Besides the direct formation of a high number of adventitious buds without an intervening callus phase on cotyledons (Fig. 1D), the subsequent vegetative growth of these buds were limited. Although the rate and severity of phenolic oxidation of the adventitious shoot-buds were not analysed, phenolic oxidation of explants were visually evident in buds cultured under conventional fluorescent lamps, while those irradiated by light emitted by LEDs or grown in the dark were less pronounced (Fig. 3). Phenolic oxidation has been previously reported to be one of the recurrent difficulties faced by researchers attempting to propagate *Protea* species *in vitro* (Tal et al., 1992; Thillerot et al., 2006). An important finding of this study is the poor overall performance of conventional white fluorescent lamps compared to monochromatic light or growing explants in the dark. Although it is commonly known that growing plants in the absence of light reduces phenolic oxidation (Sivaci et al., 2007), it is morphogenetically and physiologically disadvantageous for explants to be exposed to total darkness for a prolonged period of time. Based on visual observation of the buds in this study, it seems that individual light quality plays an important role in oxidation process of *P. cynaroides* explants. Results of these observations indicate that monochromatic light may be the answer to reducing phenolic oxidation, which has so often been described by other authors as a barrier to successful propagation of the *Protea* species. A detailed study on the influences of individual light quality on phenolic oxidation is needed.

With regard to the organogenic growth and development of *P. cynaroides* adventitious shoot-buds, overall findings from this study showed that the buds responded positively to light emitted by red LEDs. On the other hand, buds cultured under white fluorescent lamps, which are commonly used as a light source for explants, showed poor growth in all parameters measured (Fig. 4). In the initial stages of the experiment, new vegetative growths were evident in these explants, however, as time progressed and browning of the buds took place, further growth of leaves and buds were severely limited (data not shown). A comparison of the overall root growth of *P. cynaroides* buds between red and blue LEDs showed a clear beneficial effect of red LEDs over blue LEDs in all root growth parameters. In literature, wide-ranging responses to different light qualities by various plant species have been reported. For example, no differences were found in rooting percentage, root number and root length between red and blue LEDs in two of the three grape cultivars tested (Poudel et al., 2008). Similarly, findings by Wang et al. (2011) showed that no significant differences in root number and root length were observed between *Gerbera* plantlets cultured under red LEDs and blue LEDs.



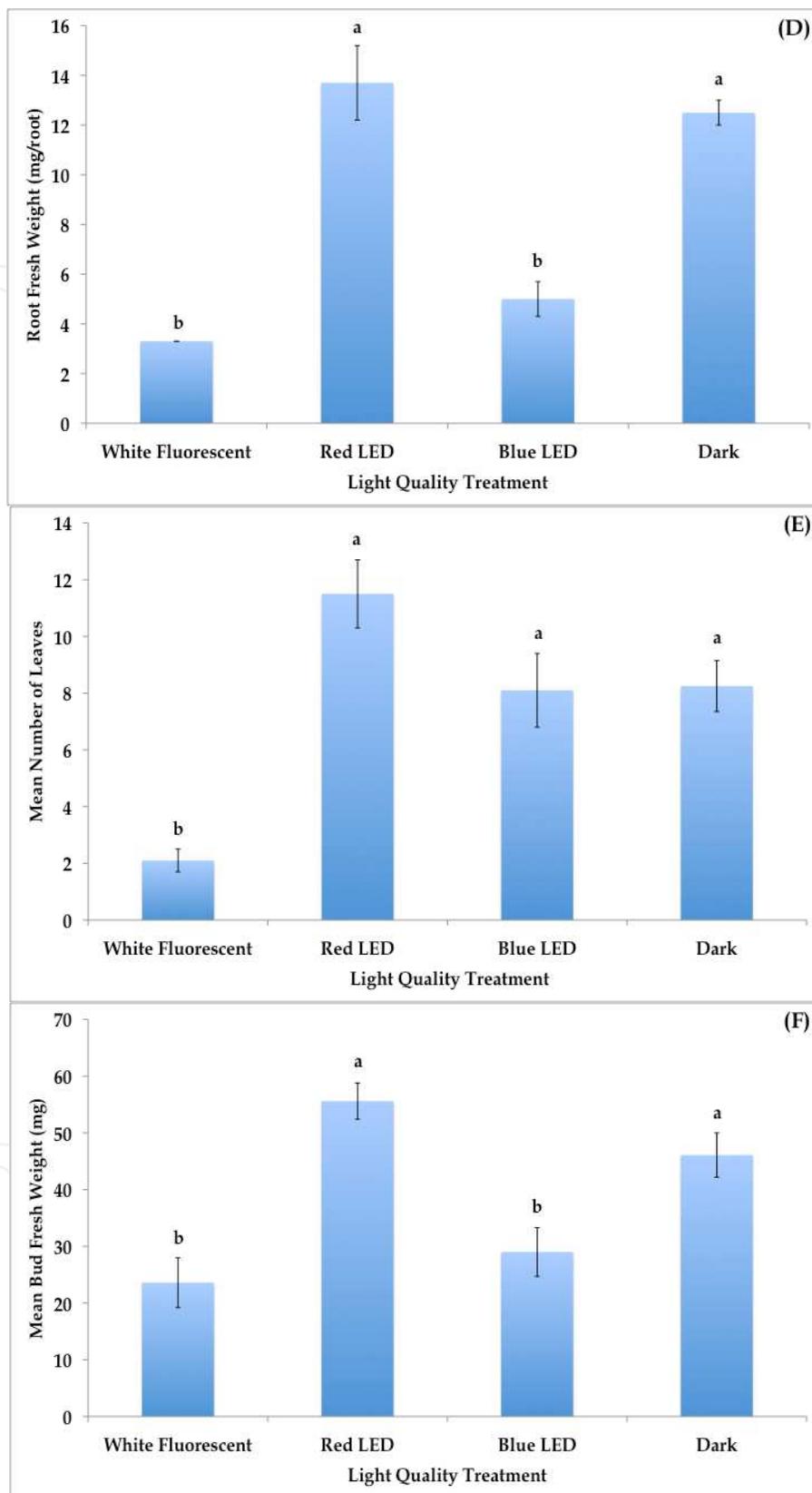


Fig. 4. Response of adventitious buds to light quality treatments after 45 days in culture. (A) Rooting percentage; (B) Root number; (C) Root length; (D) Root fresh weight; (per root) (E) Leaf number; (F) Bud fresh weight.

On the other hand, red light was found to be inhibitory toward the formation of roots in *Cattleya* microcuttings (Cybularz-Urban et al., 2007). In their study, the number of roots and root length produced by the microcuttings under red light were significantly lower than those exposed to blue light. Similar results were also found in cherry plantlets where, compared to red light, irradiation by blue lights significantly increased the root numbers (Iacona & Muleo, 2010). Nevertheless, an overview of literature seems to indicate that red light in general are stimulatory to root formation. The positive effects of red LEDs in the present study are in agreement with this trend. For example, the number of roots formed in *Dieffenbachia* explants significantly increased under red light, while root growth of explants cultured under blue light were similar to those irradiated by conventional white fluorescent tubes, which were found to produce significantly lower number of roots (Gabarkiewicz et al., 1997). Red light was also found to stimulate root formation in anthurium (Budiarto, 2010), cotton (Li et al., 2010) and strawberry (Nhut et al., 2003) explants.

In terms of leaf growth, results of the present study seem to be in agreement with those reported in other plant species. Poudel et al. (2008) reported that in their *in vitro* propagation of three grape cultivars, no significant differences were found in the number of leaves formed by shoots cultured under red and blue LEDs. Similar findings were also reported in *Gerbera jamesonii* where an almost identical number of leaves was found in plantlets cultured under red LEDs and blue LEDs (Wang et al. (2011). However, according to Nhut & Nam (2010), red LEDs promote leaf growth, but the amount of chlorophyll decreases, thereby reducing the quality of the leaves. This statement is supported by results of a study by Chang et al. (2003) where the chlorophyll content of calla lily leaves were found to be significantly higher when grown under blue lights compared to those cultured under red lights. This however, is in contrast to results of a study by Kim et al. (2004), who found the chlorophyll content (SPAD value) to be similar between chrysanthemum plantlets cultured under red LEDs and blue LEDs. It is almost certain that, in terms of chlorophyll content, the response of different plant species to red and blue LEDs varies widely. Further studies are needed to analyse the leaves of *P. cynaroides* explants to clarify the relationship between light quality and chlorophyll content.

The poor leaf growth of *P. cynaroides* buds under conventional fluorescent lamps was, to a certain extent, expected. As mentioned above, phenolic browning is a problem that has not been totally resolved. The results of this study showed the severity of this problem in the browning of leaves and tissues (Fig. 3A). When compared to explants in the other light treatments, the negative effects of browning is clearly evident in explants grown under white fluorescent lights, and analyses of the growth parameters further illustrates its negative influence on the overall growth of the shoot-buds. The lack of elongated growth of *P. cynaroides* shoot-buds in the LED treatments is an issue that needs to be resolved. A possible explanation for the lack of shoot-bud elongation could be due monochromatic lights causing an imbalance of light energy distribution available for photosystems I and II, which inhibits shoot growth (Kim et al., 2004). However, this does not explain the lack of elongation of shoot-buds cultured in the dark, which were less affected by phenolic browning, and is known to induce cell elongation. It is therefore probable that different growth regulator concentrations in the medium are needed to promote cell elongation in *P. cynaroides*. Growth regulators alone, or in combination with light quality could improve the growth of *P. cynaroides* explants.

Results of this study indicate that the significantly higher bud weight of shoot-buds cultured under different light conditions (Fig. 4F) is related to their overall root growth (Fig. 4A-C). As suggested earlier, the formation of roots is vital for the efficient absorption of nutrients, and thus is directly related to explant growth. Results showed that dark-grown buds and those irradiated by red LEDs produced the most roots, which in turn resulted in the highest bud weight. In contrast, poor root growth of buds cultured under blue LEDs resulted in lower bud weight. Under white fluorescent lamps, although rooting percentage and the number of roots were similar to those grown in the dark, however, due to phenolic browning, growth and development of the shoot-buds were severely inhibited.

## 5. Conclusion

The induction of adventitious root formation on *P. cynaroides* buds was achieved for the first time. In sharp contrast to blue LEDs, red LEDs were found to be the most suitable light source for root induction. Phenolic browning of shoot-buds cultured under conventional white fluorescent lamps resulted in poor overall vegetative growth, as are commonly reported. Of particular interest was that phenolic browning of *P. cynaroides* shoot-buds does not seem to occur under monochromatic red or blue lights. This finding could be an important breakthrough in reducing browning of *P. cynaroides* explants *in vitro*. In addition, the results of this study suggest that the light quality emitted by red and blue LEDs were both beneficial to the vegetative growth of *P. cynaroides* shoot-buds *in vitro*. Successful induction of adventitious roots on this difficult-to-grow plant has provided a step closer to the realization of micropropagation as an alternative means for propagating *P. cynaroides*. However, further studies are needed to investigate the effects of red and blue LED combinations at different ratios on the growth and development of *P. cynaroides* explants. In addition, the induction of shoot elongation and development through the use of growth regulators is required. Further analysis of the relationship between light quality and phenolic browning, is needed.

## 6. Acknowledgement

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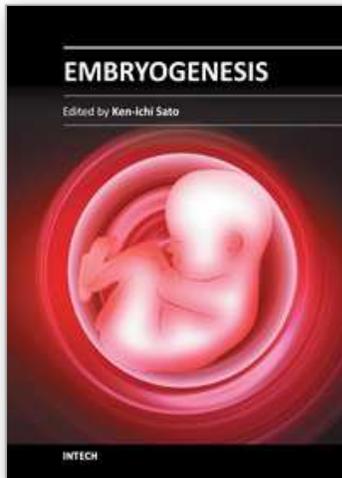
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