

# Red Light-emitting Diode Light Irradiation Improves Root and Leaf Formation in Difficult-to-propagate *Protea cynaroides* L. Plantlets In Vitro

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**Abstract.** The effects of light quality emitted by light-emitting diodes (LEDs) on the growth and morphogenesis, and concentrations of endogenous phenolic compounds of *Protea cynaroides* L. plantlets in vitro, were investigated. Plantlets were cultured under four light treatments: conventional fluorescent lamps (control), red LEDs (630 nm), blue LEDs (460 nm), and red + blue LEDs (1:1 photosynthetic photon flux). Four phenolic compounds extracted from the plantlets were analyzed: 3,4-dihydroxybenzoic acid, gallic acid, caffeic acid, and ferulic acid. The highest rooting percentage was observed in plantlets cultured under red LEDs (67%) compared with 7% under conventional white fluorescent light, 13% under blue LEDs, and 13% under red + blue LEDs. The highest number of roots per plantlet was also found under red LEDs, whereas a significantly lower number of roots per plantlet was obtained under the other light treatments. Furthermore, red light promoted the formation of new leaves in *P. cynaroides* plantlets. However, the highest leaf dry weight (53.8 mg per plantlet) was found in plantlets irradiated by the combination of red and blue LEDs. Phenolic analyses showed that the lowest concentrations of 3,4-dihydroxybenzoic acid (4.3 mg·g<sup>-1</sup>), gallic acid (7.0 mg·g<sup>-1</sup>), and ferulic acid (7.4 mg·g<sup>-1</sup>) were detected in plantlets exposed to red light, whereas those irradiated by white fluorescent light contained the highest concentration. A significant inverse correlation ( $r = -0.419$ ) was established between 3,4-dihydroxybenzoic acid and rooting percentage. Strong inverse correlations were also established between 3,4-dihydroxybenzoic acid and number of roots per plantlet ( $r = -0.768$ ) as well as between ferulic acid and number of roots per plantlet ( $r = -0.732$ ). These results indicate that the stimulation of root formation in *P. cynaroides* plantlets under red LEDs is the result of the low endogenous concentrations of 3,4-dihydroxybenzoic acid and ferulic acid.

*Protea cynaroides* L. (King Protea) is a slow-growing, semihardwood shrub (Patterson-Jones, 2000). It is one of the most important cut flowers belonging to the Proteaceae family, and it is notoriously known as a difficult-to-propagate ornamental plant (Littlejohn et al., 2003; Thillerot et al., 2006). In particular, it has a poor physiological capacity for adventitious root formation. Conventional propagation methods used to propagate *P. cynaroides* are slow and inconsistent and typically have low success rates (Malan, 1992). Advances in the in vitro establishment of *P. cynaroides* nodal explants (Wu and du Toit, 2004) and apical buds (Thillerot et al., 2006) have been made. However, recurrent problems such as phenolic oxidation coupled with

slow vegetative growth have resulted in limited success.

Light is an important stimulus for plant development and a key factor in morphogenesis (Okamoto et al., 1997). Responses of plants to light depend on the quantity (photon flux), quality (spectral quality), and duration (photoperiod) of the light source (Taiz and Zeiger, 1991). Cool white fluorescent lamps (FLs), which have a wide range of wavelengths from 350 to 750 nm, are the most commonly used light source in plant tissue culture (Economou and Read, 1987). However, one of the disadvantages of fluorescent lamps is the difficulty of controlling light quality, which has been shown to have significant influences on plant morphogenesis. The use of LEDs as an alternative light source for in vitro propagation has drawn considerable interest. The advantages that LEDs have over FLs are their wavelength specificity, light intensity adjustability, low thermal energy output, and long lifespan (Bula et al., 1991; Hoenecke et al., 1992; Okamoto et al., 1997). Results of numerous studies showed significant improvements in plant growth and morphogenesis when exposed to light emitted by

LEDs. The effects of LEDs on morphogenesis of crops such as banana (*Musa* spp.) (Nhut et al., 2002), lettuce (*Lactuca sativa*) (Okamoto et al., 1996), pepper (*Capsicum annuum*) (Brown et al., 1995), potato (*Solanum tuberosum*) (Jao and Fang, 2004), spinach (*Spinacia oleracea*) (Yanagi and Okamoto, 1997), wheat (*Triticum aestivum*) (Goins et al., 1997), and calla lily (*Zantedeschia jucunda*) (Jao et al., 2005) have been well documented. Although research has shown that red and blue lights in particular have a significant influence on plant morphology, responses vary according to plant species. Appelgren (1991) observed that the exposure of *Pelargonium* (*Pelargonium* × *hortorum* cv. Penny Irene) plantlets to red light (660 nm) in vitro significantly stimulated stem elongation, whereas blue light (450 nm) strongly inhibited stem elongation. In addition, findings by Poudel et al. (2008) showed that red LEDs increased rooting percentage and root numbers of grape (*Vitis vinifera*) explants. Furthermore, culturing *Lilium* hybrid explants under a combination of red and blue LEDs produced larger bulblets and a higher number of roots (Lian et al., 2002).

Phenolic compounds are known to play a role as endogenous promoters and inhibitors of adventitious root formation (Wu et al., 2007b). In difficult-to-root species such as *P. cynaroides*, the presence and concentrations of phenolic compounds are particularly important. Besides the influence of light on plant morphogenesis, light irradiation has also been shown to affect the production of phenolic compounds and other secondary metabolites in plants. Early work by Tso et al. (1970) showed that the total phenolic content of tobacco (*Nicotiana tabacum*) plants, particularly chlorogenic acid, was increased when grown under far-red light. Furthermore, the phenolic content of buckwheat (*Fagopyrum esculentum*) leaves was significantly increased when the plants received a combination of red, green, and blue light (Hossen, 2007). In *Saussurea medusa*, a positive correlation was found between blue LEDs and the biosynthesis of flavonoids, whereas red LEDs inhibited flavonoid biosynthesis (Guo et al., 2007).

Research has shown that phenolic compounds are an important factor in the rooting of stem cuttings (Wu et al., 2007b). However, little is known about how light quality influences endogenous phenolic compound concentrations in *P. cynaroides* plantlets and their relationship with explant growth. This study was conducted to investigate the effects of light quality on the growth of *P. cynaroides* plantlets in vitro as well as to establish their relationship with endogenous phenolic compound concentrations and root formation.

## Materials and Methods

**Explant establishment through embryo culture.** *P. cynaroides* seedlings were established using mature embryos excised from seeds. Surface sterilization of the seeds and

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embryo excision were done according to Wu et al. (2007a). After excision, the embryos were placed in an upright position into the growth medium in culture vessels. The growth medium contained half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962), sucrose (3%) and agar (9 g·L<sup>-1</sup>). The pH of the medium was adjusted to 5.7 before autoclaving at 104 kPa at 121 °C for 20 min. The cultures were placed in a growth cabinet with the temperature adjusted to 21 ± 2/12 ± 2 °C (16 h/8 h). Illumination for embryo germination was provided by white FLs with a photoperiod of 16 h light/8 h dark. After 30 d, germinated seedlings with two true leaves were collected.

**Explant and light treatments.** Plantlets (seedlings with cotyledons and radicle removed) were laid horizontally on growth media in petri dishes. The petri dishes containing the plantlets were positioned vertically in an upright position on transparent plastic stands. The plantlets were cultured in medium containing full-strength MS medium without growth regulators but supplemented with sucrose (3%) and agar (9 g·L<sup>-1</sup>). Five light treatments were used: cool white FLs (control), red (630 nm) LEDs, blue (460 nm) LEDs, and red + blue (R + B) LEDs [1:1 photosynthetic photon flux (PPF)]. The LEDs were purchased from Ryh Dah Inc. (Taiwan). The wavelengths of the LEDs were confirmed using a spectroradiometer (International Light Technologies; ILT900) (Fig. 1). Customized LED lighting systems were constructed with aluminum boxes (length, 50 cm; width, 50 cm; height, 25 cm) and equipped with red, blue, or a combination of red and blue LEDs; a temperature sensor; a timer; and two fans. Three hundred LEDs were installed (2 cm apart) on the cover of each lighting system. Explants in petri dishes were incubated in the lighting systems, which were placed in a growth room. In all treatments, the photoperiod and temperature were adjusted to 16 h/8 h and 25 ± 2 °C, respectively. The PPF for all the light treatments was adjusted to 50 μmol·m<sup>-2</sup>·s<sup>-1</sup>. The PPF was measured (LI-1800; LI-COR Inc.) at explant height.

**Analysis of phenolic compounds.** Shoots of plantlets that were cultured under different light sources were freeze-dried and ground into powder. An aliquot of the powder was weighed and the phenolic compounds were extracted in methanol (1:5 w/v) overnight, after which the samples were evaporated to dryness. The dried samples were prepared in deionized water and analysis was performed with a high-performance liquid chromatography (HPLC) system (Hitachi L-2130 pump and a LiChroCART column packed with Purospher STAR RP-18e resin). Elution was performed in a stepwise gradient with a water (pH 2.6 adjusted with H<sub>3</sub>PO<sub>4</sub>)/acetonitrile (ACN) volumetric ratio of 0 min, 7% ACN; 0 to 20 min, 20% ACN; 20 to 28 min, 23% ACN; 28 to 40 min, 27% ACN; 40 to 45 min, 29% ACN; and 45 to 47 min, 33% ACN. The flow rate was 1 mL·min<sup>-1</sup>, and the injection volume was 20 μL. The separated compounds (including 3,4-dihydroxybenzoic, gallic, caffeic,

and ferulic acids) were directly monitored at 270 nm by an ultraviolet spectrophotometer (Hitachi L2400) coupled in the HPLC analyzing system and then determined by a calibration curve prepared from standard solutions. Each determination was repeated three times. Results presented in this article are the average values, expressed in mg·g<sup>-1</sup>.

**Statistical analysis.** Three plantlets per petri dish were used with 15 replications per treatment. Data for rooting percentage, number of roots, number of leaves, leaf dry weight, and shoot dry weight were collected. The rooting percentage for the plantlets was obtained by first determining the number of rooted plantlets per petri dish. The total number of petri dishes with rooted plantlets per treatment was then calculated and expressed as

a percentage. For plantlets that rooted, the mean number of roots per plantlet in each treatment was calculated. All data were collected after 45 d in culture. A completely randomized design was used in all experiments. All experiments were repeated three times. Where appropriate, data were analyzed using chi square and Duncan's multiple range test to compare treatment means using SAS (SAS Institute Inc., 1996).

## Results and Discussion

**Explant growth and morphogenesis.** Results showed that adventitious root formation was highest in plantlets cultured under red LEDs (66.7%), whereas poor rooting was observed in plantlets grown under white

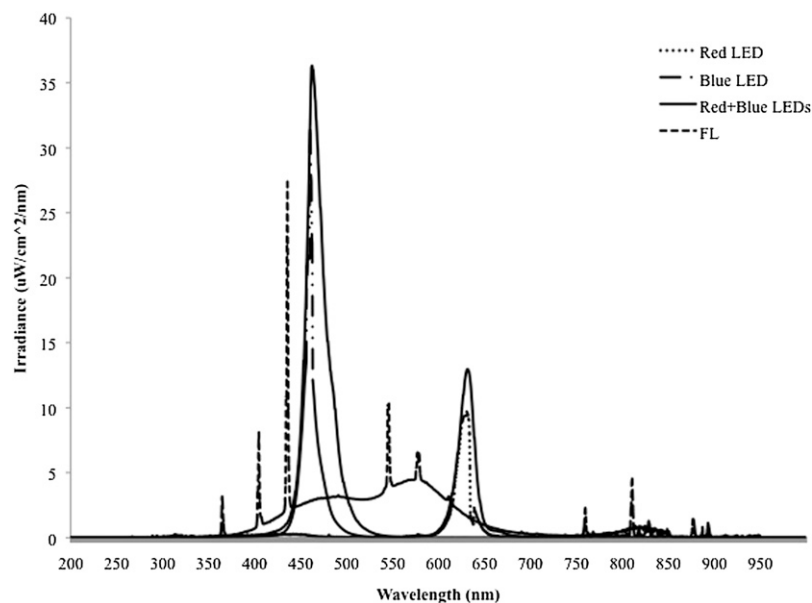


Fig. 1. Spectral distributions of light-emitting diodes (LEDs) and fluorescent lamp (FL).

Table 1. Effects of different light sources on root formation and vegetative growth of *P. cynaroides* plantlets after 45 d in culture.

Treatment	Rooting (%)	Roots/plantlet (no.)	Leaves/plantlet (no.)	Leaf dry wt (mg/plantlet)	Shoot dry wt (mg/plantlet)
FL <sup>z</sup>	6.7 b <sup>y</sup>	1.0 b <sup>x</sup>	5.6 d	29.0 b	64.8 a
Red LED	66.7 a	2.6 a	13.8 a	40.5 b	58.1a
Blue LED	13.3 b	1.0 b	11.7 b	37.0 b	55.6 a
Red + blue LED	13.3 b	1.0 b	9.5 c	53.8 a	63.8 a

<sup>z</sup>White fluorescent lamp.

<sup>y</sup>Percentages in the same column with different letters are significantly different (chi square at  $P < 0.05$ ).

<sup>x</sup>Means in the same column with different letters are significantly different (Duncan's multiple range test at  $P < 0.05$ ).

LED = light-emitting diode.

Table 2. Endogenous concentrations of phenolic compounds (mg·g<sup>-1</sup>) in *P. cynaroides* plantlets grown under different light sources after 45 d in culture.

Treatment	3,4-Dihydroxybenzoic acid (mg·g <sup>-1</sup> )	Gallic acid (mg·g <sup>-1</sup> )	Caffeic acid (mg·g <sup>-1</sup> )	Ferulic acid (mg·g <sup>-1</sup> )
FL <sup>z</sup>	8.4 a <sup>y</sup>	14.6 a	15.9 a	9.7 a
Red LED	4.3 c	7.0 c	8.4 bc	7.4 c
Blue LED	6.0 b	8.0 b	8.0 c	8.2 b
Red + blue LED	6.1 b	7.9 b	9.0 b	8.7 b

<sup>z</sup>White fluorescent lamp.

<sup>y</sup>Means in the same column with different letters are significantly different (Duncan's multiple range test at  $P < 0.05$ ).

LED = light-emitting diode.

fluorescent light (6.7%), blue LEDs (13.3%) and red + blue LEDs (13.3%) (Table 1). Furthermore, plantlets irradiated by red LEDs produced a significantly higher number of roots compared with the other light treatments (Tables 1 and 4). Similar results were reported in anthurium (*Anthurium andraeanum*) (Budiarto, 2010), cotton (*Gossypium hirtutum*) (Li et al., 2010), and chrysanthemum (*Chrysanthemum morifolium*) (Kurilčik et al., 2008), where red LEDs were also found to stimulate root formation. In addition, results showed that compared with conventional white fluorescent light, irradiation by LEDs significantly improved the formation of new leaves on *P. cynaroides* plantlets irrespective of the light quality. Moreover, these results revealed that plantlets grown under red LEDs in particular produced a significantly higher number of new leaves than any of the other LED treatments. However, the leaf dry weight of plantlets irradiated by the combination of red and blue LEDs was significantly higher than those grown under the other light treatments (Table 1). The stimulatory effect of red + blue LEDs on leaf growth is similar to those reported in chrysanthemum (Kim et al., 2004), *Doritaenopsis* (Shin et al., 2008), and strawberry (*Fragaria × ananassa* cv. Akihime) (Nhut et al., 2003). Although leaf area was not measured in this study, it is probable that the higher leaf dry weight was the result of larger leaves produced by plantlets in this treatment. According to Goins et al. (1997), the enhancement of growth and development of leaves by a combination of red and blue LEDs is attributed to their spectral energy distribution being consistent with that of chlorophyll absorption, and as a result, the net photosynthetic rate is increased. In addition, the spectral energy ratio between red and blue LEDs has been acknowledged to be of great importance in vegetative growth of plants (Okamoto et al., 1997). The presence of at least 10% blue light has been found to be beneficial to plantlet growth, whereas no differences were observed at lower percentages. It was therefore suggested that there is a minimum threshold level for blue light for optimal development under a red-based light source (Nhut and Nam, 2010). Although only a single ratio (1:1) was tested in this study, the results indicated that it is suitable for promoting the leaf growth of *P. cynaroides* plantlets.

**Endogenous concentration of phenolic compounds.** The lowest concentrations of 3,4-dihydroxybenzoic acid, gallic acid, and ferulic acid were found in plantlets irradiated by red LEDs, which were significantly lower than other light treatments (Tables 2 and 5). Although no previous information is available on the effects of light quality on these phenolic compounds, similar results have been reported for other phenolics under red light. Guo et al. (2007) showed that flavonoid biosynthesis in *Saussurea medusa* callus cultures was inhibited by red light irradiation. Similarly, leaves of *Ocimum basilicum* (Shoji et al., 2011) irradiated by red light were found to contain lower concentrations of chicoric

acid. Total phenol content has also been shown to be lower in wheatgrass (Urbonavičiute et al., 2009a) and green barley leaves (Urbonavičiute et al., 2009b) irradiated by red LEDs. These results indicate that red light inhibits the accumulation of phenolic compounds and, in our case, 3,4-dihydroxybenzoic acid, gallic acid, and ferulic acid in particular. Furthermore, analysis of the results between phenolic compounds and root growth showed a significant inverse correlation between 3,4-dihydroxybenzoic acid and rooting percentage [Pearson's coefficient ( $r$ ) = -0.419] (Table 3). In addition, strong inverse correlations were also found between 3,4-dihydroxybenzoic acid and the number of roots formed ( $r$  = -0.768) as well as between ferulic acid and the number of roots formed ( $r$  = -0.732) (Table 3). The inverse correlation between 3,4-dihydroxybenzoic acid and root number is in agreement with the results of a dose-response bioassay (Wu et al., 2007b), which showed that 3,4-dihydroxybenzoic acid at low concentration stimulates root growth of lettuce seedlings. Subsequent investigations of the same study showed that rooted *P. cynaroides* cuttings contained low endogenous concentrations of 3,4-dihydroxybenzoic acid. Similar findings were reported by Mucciarelli et al. (2000), in which low concentrations of 3,4-dihydroxybenzoic acid were found to possess auxin-like activities

by stimulating tissue dedifferentiation and significantly increased root formation in tobacco callus. It has been suggested that 3,4-dihydroxybenzoic acid acts as an indole acetic acid (IAA) synergist by counteracting IAA decarboxylation during IAA-induced growth (Tomaszewski and Thimann, 1966). However, Mucciarelli et al. (2000) suggested the possibility that 3,4-dihydroxybenzoic acid acts directly and independently on cell differentiation. In addition, low concentrations of ferulic acid have been found in cherry cuttings during the time of root formation (Trobec et al., 2005). These results strongly suggest that a relationship exists among red light, phenolic compounds, and root formation. It is likely that red light irradiation inhibited

Table 3. Correlation analysis between phenolic compounds and root growth.

Phenolic compound	Pearson's correlation coefficient ( $r$ )	
	Rooting %	No. of roots
3,4-Dihydroxybenzoic acid	-0.419*	-0.768**
Gallic acid	-0.322	-0.467
Caffeic acid	-0.260	-0.322
Ferulic acid	-0.358	-0.732**

\*Correlation is significant at the 0.05 level.

\*\*Correlation is significant at the 0.01 level.

Table 4. Analysis of variance summary for number of roots, number of leaves, leaf dry weight, and shoot dry weight of *P. cynaroides* plantlets.

Source of variation	df	Mean square	F value	P value
<b>Number of roots<sup>z</sup></b>				
Treatment	3	2.8444	13.04	0.0006
Error	11	0.2181		
Corrected total	14			
<b>Number of leaves</b>				
Treatment	3	186.9408	31.39	<0.0001
Error	56	5.9547		
Corrected total	59			
<b>Leaf dry weight</b>				
Treatment	3	1603.2000	5.49	0.0022
Error	56	292.2167		
Corrected total	59			
<b>Shoot dry weight</b>				
Treatment	3	294.8167	0.79	0.5056
Error	56	374.1095		
Corrected total	59			

<sup>z</sup>Only includes values from rooted plantlets.

Table 5. Analysis of variance summary of phenolic compounds for *P. cynaroides* plantlets.

Source of variation	df	Mean square	F value	P value
<b>3,4-Dihydroxybenzoic acid</b>				
Treatment	3	42.2209	768.32	<0.0001
Error	56	0.0550		
Corrected total	59			
<b>Gallic acid</b>				
Treatment	3	184.5979	1061.92	<0.0001
Error	56	0.1738		
Corrected total	59			
<b>Caffeic acid</b>				
Treatment	3	209.5320	171.13	<0.0001
Error	56	1.2244		
Corrected total	59			
<b>Ferulic acid</b>				
Treatment	3	13.8581	22.09	<0.0001
Error	56	0.6274		
Corrected total	59			



the accumulation of 3,4-dihydroxybenzoic acid, gallic acid, and ferulic acid in *P. cynaroides* plantlets, which resulted in the stimulation of adventitious root formation.

The results of the present study also showed that, compared with monochromatic red light, the accumulation of 3,4-dihydroxybenzoic acid, gallic acid, and ferulic acid in *P. cynaroides* plantlets was enhanced by the presence of blue light, whether individually or in combination with red light (red + blue LEDs) (Table 2). This is in agreement with studies that showed that blue light stimulates the biosynthesis of phenolic compounds in plants (Leal-Costa et al., 2010; Meng et al., 2004; Rao and Ravishankar, 2002; Saleh, 2007). Similarly, chlorogenic acid in lettuce seedlings grown under blue-containing LEDs was also higher than those irradiated by red lights (Johkan et al., 2010). Under white fluorescent light, in which a wide range of wavelengths including high levels of blue light were present (Fig. 1), plantlets were found to contain the highest concentrations of all types of phenolic compounds (Table 2). Findings by Shiga et al. (2009) and Shohael et al. (2006) also showed similar trends in terms of total phenolic content under white light. Furthermore, ultraviolet light has been found to induce the production of phenolic compounds (Bakhshi and Arakawa, 2006). Ultraviolet light (peak at 404 nm) was detected in the white FLs (Fig. 1) and therefore could be a contributory factor toward the high phenolic compound concentrations. In addition, of all the phenolics, caffeic acid in particular is known as a root growth inhibitor of plants (Barkosky et al., 2000; Batish et al., 2008), and thus, its high concentration under white light is likely to be a factor in the inhibition of root formation of *P. cynaroides* plantlets. Based on these findings, it is probable that the unsuccessful induction of root formation in *P. cynaroides* explants in previous studies, in which white FLs were used as a light source (Thillerot et al., 2006), was the result of the high concentrations of endogenous phenolic compounds.

In conclusion, the highest root and leaf formation was obtained in plantlets irradiated by red LEDs, whereas the lowest concentrations of 3,4-dihydroxybenzoic acid, gallic acid, and ferulic acid were detected in plantlets under the same light source. The relationships among light quality, phenolic compound concentration, and root growth were established. Inverse correlations between phenolic compounds and root growth indicate that low endogenous concentrations of 3,4-dihydroxybenzoic acid and ferulic acid under red LEDs stimulate root formation. Our results suggest that red LEDs are suitable as a light source to promote root and leaf formation in *P. cynaroides* plantlets.

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