

行政院國家科學委員會專題研究計畫 成果報告

發光二極體(LED)和酚類化合物對帝王花(*Protea cynaroides* L.)組織培養之生長及形態發生的影響 研究成果報告(精簡版)

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The influence of light-emitting diode (LED) and phenolic compounds on the growth and morphogenesis of *Protea cynaroides* L. *in vitro*

發光二極體(LED)和酚類化合物對帝王花(*Protea cynaroides* L.)組織培養之生長及形態發生的影響

INTRODUCTION

Light is an essential energy source for plants. It is also an important stimulus for plant development, and a key factor in morphogenesis (Okamoto *et al.*, 1997). The 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). Fluorescent tubes, 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 problems of fluorescent tubes is the difficulty to control light quality, which has been widely reported to have significant influences on plant morphology. The advantages that LEDs have over fluorescent tubes are their high energy-conversion efficiency, wavelength specificity, light intensity adjustability, low thermal energy output and long life span (Bula *et al.*, 1991; Hoenecke *et al.*, 1992; Okamoto *et al.*, 1997).

With technological advances in recent years, numerous studies have been carried out on many horticultural crops to investigate the potential of light-emitting diodes (LEDs) as an alternative light source for growing tissue-cultured plants. According to research results, plants have shown significant improvements in growth and morphogenesis when exposed to LEDs. 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 application of red light (660 nm) to *Pelargonium* plantlets *in vitro* significantly stimulated stem elongation, while blue light (450 nm) strongly inhibited stem elongation. Red light also promoted starch accumulation by inhibiting the translocation of photosynthates out of leaves in several plant species (Saebo *et al.*, 1995), while blue light increased chloroplast development, chlorophyll formation and stomata opening (Senger, 1982). The stimulation of root formation by LEDs has been shown by Poudel *et al.* (2008), where a higher rooting percentage and higher root numbers of grape explants were obtained when cultured under red LEDs.

Besides the influence of LEDs on plant morphology, the production of phenolic compounds and other secondary metabolites in plants have also been shown to be affected by LED radiation. Phenolic content of buckwheat leaves was significantly increased when the plants received a combination of red, green (510 nm) and blue LED lights (Hossen, 2007). In *Saussurea medusa*, a positive correlation was found between blue LEDs and the biosynthesis of flavonoids, while red LEDs inhibited flavonoid biosynthesis (Guo *et al.*, 2007). Moreover, phenolic compounds are known to be involved in the regulation of plant growth and development (Rice, 1984). Confirmation of phenolic compounds as regulators of morphogenesis has been shown by their use as additives in tissue culture media. These include, among others, chlorogenic acid (Hammerschlag 1982), phloroglucinol (James and Thurbon 1981; Zimmerman 1984), phloretic acid (Jones and Hatfield 1976) and 3,4-dihydroxybenzoic acid (Mucciarelli *et al.*, 2000, Wu *et al.* 2007b). Mucciarelli *et al.* (2000) reported auxin-like activity of 3,4-dihydroxybenzoic acid in tobacco callus and tissue cultures. Their study showed that the presence of 3,4-dihydroxybenzoic acid at low concentrations resulted in stimulation of cell dedifferentiation, callus induction and rooting of leaf tissues. On the contrary, high concentrations of 3,4-dihydroxybenzoic acid inhibited the growth of leaves, shoots and roots.

This study was divided into two experiments. In Experiment 1, the objective was to investigate the effects of red LED, blue LED and a combination of red and blue LEDs on the growth and morphogenesis of whole *P. cynaroides* plantlets germinated *in vitro*, as well as cotyledons excised from seedlings. In addition, phenolic analyses were carried out on all explants to establish the correlation among light sources, explant morphogenesis and endogenous phenolic compound concentration. In Experiment 2, 3,4-dihydroxybenzoic acid, salicylic acid and phloroglucinol were added to the growth medium of adventitious bud cultures to study their effects on growth and morphogenesis.

MATERIALS AND METHODS

Explant and growth media (Experiment 1 and Experiment 2)

In both experiments, *P. cynaroides* seedlings were established using mature embryos excised from seeds. Surface-sterilization, growth medium and culture conditions used were done according to Wu *et al.* (2007a). For Experiment 1, cotyledons (50 mm x 50 mm) and whole plantlets (with two true leaves, roots removed) were cultured in 90-mm petri-dishes containing full-strength MS

medium, supplemented with sucrose (3%) and agar (9 g L⁻¹). Cotyledons were placed onto the growth medium with their abaxial surface facing down, while whole plantlets were placed horizontally, laying on the medium. Petri-dishes containing the plantlets were positioned vertically on transparent plastic stands. For Experiment 2, germinated seedlings were subcultured to medium containing half-strength MS medium supplemented with 2.69 μM NAA and 8.87 μM BAP for four weeks to induce adventitious bud formation. Adventitious buds that formed on the seedlings were removed and cultured on quarter-strength MS media containing 3,4-dihydroxybenzoic acid (1, 100, 1000 μM), salicylic acid (1, 100, 1000 μM) or phloroglucinol (1, 100, 1000 μM). In addition, all media treatments were prepared with or without growth regulators (4.90 μM IBA and 8.87 μM BAP). The pH of all media was adjusted to 5.7 prior to autoclaving. All media were sterilized in an autoclave at 104 KPa at 121°C for 20 minutes.

Light treatment (Experiment 1)

Five light treatments were used: white fluorescent tubes (control), red (660 nm) LED, blue (450 nm) LED, red + blue (50:50) (R/B) LEDs and darkness. The LEDs were purchased from Ryh Dah Inc. (Taiwan). The wavelengths of the LEDs were confirmed using a spectroradiometer (International Light Technologies, ILT900). A customized LED lighting system for the three types of LED light sources was developed and constructed inside an aluminum box (550 mm x 550 mm x 250 mm) with a lid. Twenty strips of LEDs (500 mm in length), 15 mm apart, were secured onto the inside of the lid. Alternative strips of red and blue LEDs were used in the R/B treatment. Each set of LED system (comprised of 20 strips) was connected to a timer, with the photoperiod adjusted to 16 h/8 h (light/dark). A thermometer was mounted on the side of the interior of the box, with a digital display on the outside. A standard computer cooling fan (100 mm diameter) was placed on one side wall and at the rear end of the box. The entire LED lighting system was placed inside a growth room where the temperature was maintained at 25±2°C. For the control, cool, white fluorescent tubes in the growth room were used. The photosynthetic photon flux (PPF) for all the light sources was adjusted to 50 μmol.m⁻².sec⁻¹. The PPF (LI-1800, LI-COR Inc.) was measured when the culture bench was empty.

Analysis of phenolic compounds (Experiment 1)

Cotyledons and whole plantlets (composed of leaves and shoots) 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 an HPLC system (Hitachi L-2130 pump and a LiChroCART[®] column packed with Purospher[®] STAR RP-18e resin). Elution was performed in a step-wise gradient with a water (pH 2.6 adjusted with H₃PO₄)/acetonitrile volumetric ratio of 0 min, 7% ACN; 0–20 min, 20% ACN; 20–28 min, 23% ACN; 28–40 min, 27%, ACN; 40–45 min, 29%, ACN; 45–47 min, 33%, ACN. The flow rate was 1 mL min⁻¹, and the injection volume was 20 μ L. In this study, the separated compounds (including gallic, 3,4-dihydroxybenzoic, ferulic and caffeic acids) were directly monitored at 270 nm by an UV spectrophotometer (Hitachi L2400) coupled in the HPLC analyzing system, and then determined by a calibration curve prepared from standards.

Experimental design and statistical analysis

For cotyledons in Experiment 1, sixteen explants were placed in a petri-dish, with 15 replications. Data for the number of roots, root length and root dry weight were recorded. For whole plantlets, three plantlets per petri-dish was used with 15 replications. Data for the number of leaves, leaf dry weight and shoot dry weight were collected. In Experiment 2, one explant per test tube was used, with ten replications. All data were collected 45 days after culture. A completely randomized design as used in all experiments. Data were analyzed using Duncan's Multiple Range test to compare treatment means. Statistical analyses were done using the Statistical Analysis System (SAS) program (SAS Institute Inc., 1996).

RESULTS AND DISCUSSION

Experiment 1 – Explant growth and morphogenesis

Overall results demonstrated that exposure to red LEDs had the most effect on cotyledon explants. Although results showed that explants grown in the dark formed significantly more roots than those placed under a light source (Table 1), roots produced by cotyledons grown under red LED were

significantly longer. No significant differences were observed in the number of roots formed between explants grown under red LEDs and blue LEDs, however, significantly fewer roots formed on cotyledons cultured under the combination of red and blue LEDs. Similar results were observed in the dry weight of roots, where roots produced by cotyledons grown under red LEDs produced significantly higher dry weight than all other treatments (Table 1). In comparison to LEDs, the root length and root dry weight produced by cotyledons cultured under fluorescent tubes were significantly lower than those cultured under red LED, while the number of roots were significantly lower than those grown under blue LED. Explants grown under a combination of red and blue LEDs (R/B) performed the poorest of all treatments in all growth parameters (Table 1).

Table 2 illustrates the effects of light sources on the growth of *P. cynaroides* whole plantlets. Compared to fluorescent tubes, the use of LEDs significantly improved the formation of new leaves on plantlets. Red LEDs in particular induced significantly more new leaves to form than any other LEDs. However, the leaf dry weight of cultures grown under the combination of red and blue LEDs were significantly higher than those grown under other LEDs or fluorescent tubes, while the shoot dry weight of explants cultured under different light sources was similar. In comparison to plantlets grown under a light source, significantly lower number of leaves, leaf dry weight and shoot dry weight of plantlets grown in the dark were observed (Table 2). This may be due to the fact that leaves and shoots formed in the dark are usually thinner and less developed.

Experiment 1 – Phenolic analysis

Results of the phenolic analyses of cotyledons are shown in Table 3. Results showed that caffeic acid and gallic acid played an important in root initiation and root elongation, respectively. It has been recently reported that caffeic acid strongly inhibits root initiation (Batish *et al.* 2008, Singh *et al.* 2009). This tendency was confirmed by the significantly low number of roots formed by cotyledons exposed to the combination of red and blue LEDs (R/B) (Table 1), in which a high concentration of caffeic acid was found. In contrast, a relatively low concentration of caffeic acid was detected in dark-grown cotyledons, which resulted in the formation of a high number of roots. Furthermore, analysis results indicate that root elongation was promoted by the presence of gallic acid. This was shown by the lack of root elongation observed in cotyledons cultured in the dark, in which no gallic acid was detected. Moreover, confirmation that gallic acid promoted root elongation was demonstrated in cotyledons cultured under red LEDs where the presence of high concentrations of gallic acid resulted in the production of significantly longer roots.

Results of the effects of LEDs on the endogenous concentrations of phenolic compounds in whole plantlets are shown in Table 4. Overall, explants grown under fluorescent tubes contained higher concentrations of 3,4-dihydroxybenzoic acid, gallic acid and caffeic acid. Results indicate that the endogenous levels of 3,4-dihydroxybenzoic acid had the most effect on leaf formation in plantlets. Plantlets grown under red LEDs contained the lowest concentration of 3,4-dihydroxybenzoic acid, which resulted in the formation of the highest number of new leaves. In contrast, the highest concentrations of 3,4-dihydroxybenzoic acid were found in explants grown under fluorescent tubes and in the dark, which significantly inhibited the formation of new leaves.

Experiment 2 – Explant growth and morphogenesis

The effects of exogenously applied phenolic compounds on the growth and morphogenesis of adventitious buds are shown in Table 5. Results show that 3,4-dihydroxybenzoic acid increased the growth of adventitious buds irrespective of the concentration (1, 100, 1000 μM), as shown by their high bud weight. In addition, the presence of 3,4-dihydroxybenzoic acid in the medium also increased the number of new buds formed. Of particular importance is that the increases in bud weight and bud number were observed in growth media with 3,4-dihydroxybenzoic acid only, while bud growth and the formation of new buds were inhibited in growth medium containing both 3,4-dihydroxybenzoic acid and growth regulators (4.90 μM IBA and 8.87 μM BAP). Furthermore, the use of 1 μM salicylic acid stimulated the growth of buds, and increased the number of buds and leaves formed compared to those cultured on medium containing 100 μM and 1000 μM , irrespective of whether other growth regulators were present or not. The addition of phloroglucinol, which is one of the most commonly used phenolics in tissue culture, in the growth medium did not improve the growth of adventitious buds. Compared to the control treatment, similar values were collected for all growth parameters.

CONCLUSION

In experiment one, of all the light treatments, red LEDs significantly improved the root length and root dry weight of *P. cynaroides* cotyledon explants, and increased the formation of new leaves in whole plantlets. Results indicate that red LEDs could be used as an alternative light source to conventional fluorescent tubes in the *in vitro* propagation of *P. cynaroides* explants. In experiment 2, results showed that 3,4-dihydroxybenzoic acid and salicylic acid at low concentrations stimulated the growth of *P. cynaroides* adventitious buds. These results strongly suggest that 3,4-

dihydroxybenzoic acid could be used as a growth promoter for the *in vitro* propagation of *P. cynaroides* explants.

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Table 1. Effects of different light sources on root formation of *P. cynaroides* cotyledons after 45 days in culture.

Treatment	No. of roots	Root length (mm)	Root dry weight (mg)
Fluorescent tube	9.5±4.6 c	6.7±1.8 b	5.0±1.2 b
Red LED	11.8±4.1 bc	12.4±4.8 a	12.7±2.4 a
Blue LED	12.9±3.6 b	6.1±1.8 bc	6.6±1.3 b
R/B LED	2.3±1.2 d	4.6±1.9 cd	1.1±1.3 c
Dark	17.9±5.1 a	4.0±1.2 d	4.9±2.3 b

Table 2. Effects of different light sources on growth and morphogenesis of whole *P. cynaroides* plantlets after 45 days in culture.

Treatment	No. of leaves	Leaf dry weight (mg)	Shoot dry weight (mg)
Fluorescent tube	5.6±2.1 d	29.0±7.6 b	64.8±6.1 a
Red LED	13.8±2.3 a	40.5±8.4 b	58.1±5.1 a
Blue LED	11.7±2.4 b	37.0±5.0 b	55.6±5.3 a
R/B LED	9.5±2.9 c	53.8±7.2 a	63.8±6.4 a
Dark	3.0±0.5 e	4.3±2.6 c	21.4±3.0 b

Means in the same column with different letters are significantly different according to Duncan's Multiple Range Test at $P < 0.001$.

Table 3. Endogenous concentrations of phenolic compounds in *P. cynaroides* cotyledons grown under different light sources after 45 day in culture.

Treatment	3,4-Dihydroxybenzoic acid (%)	Gallic acid (%)	Caffeic acid (%)	Ferulic acid (%)
Fluorescent tube	4.64	0.48	4.33	3.28
Red LED	4.35	0.68	4.30	3.73
Blue LED	4.46	0.48	4.31	3.11
R/B LED	5.03	0.40	4.66	3.86
Dark	4.88	0	2.16	3.19

Table 4. Endogenous concentrations of phenolic compounds in *P. cynaroides* plantlets grown under different light sources after 45 days in culture.

Treatment	3,4-Dihydroxybenzoic acid (%)	Gallic acid (%)	Caffeic acid (%)	Ferulic acid (%)
Fluorescent tube	0.84	1.45	1.59	0.97
Red LED	0.43	0.70	0.84	0.74
Blue LED	0.60	0.80	0.80	0.81
R/B LED	0.61	0.79	0.90	0.87
Dark	0.70	0.71	0.76	1.18

Table 5. Effects of phenolic compounds on morphogenesis of *P. cynaroides* adventitious bud explants cultured on quarter-strength MS medium with (PGR +) or without (PGR -) growth regulators. Data collected after 45 days in culture.

	Bud fresh weight (mg)		Number of new buds formed		Number of new leaves formed	
	PGR +	PGR -	PGR +	PGR -	PGR +	PGR -
Quarter-strength MS only	36.6	21.4	1.9	2.1	12.6	8.9
Phloroglucinol (μM)						
1	29.4	19.5	2.5	2.5	12.2	8.2
100	27.1	13.6	1.9	2.4	11.3	9.7
1000	23.4	19.5	1.8	2.7	11.1	11.0
3,4-Dihydroxybenzoic acid (μM)						
1	29.9	72.2	2.0	4.0	9.4	9.2
100	42.9	112.3	2.7	3.7	12.2	10.6
1000	35.5	78.8	3.8	3.5	11.7	7.6
Salicylic acid (μM)						
1	48.6	22.3	3.9	1.4	13.5	11.3
100	17.1	14.6	2.1	1.2	7.8	6.2
1000	18.9	17.1	1	1	6.7	3.5

無衍生研發成果推廣資料

98 年度專題研究計畫研究成果彙整表

計畫主持人：吳浩群		計畫編號：98-2313-B-343-001-					
計畫名稱：發光二極體(LED)和酚類化合物對帝王花(Protea cynaroides L.)組織培養之生長及形態發生的影響							
成果項目		量化			單位	備註(質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等)	
		實際已達成數(被接受或已發表)	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比			
國內	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	1	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力(本國籍)	碩士生	0	2	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	2	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力(外國籍)	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

<p>其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)</p>	<p>The findings of this research could be used in collaboration with other plant tissue culture laboratories and applied in the agricultural industry.</p>
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	成果項目	量化	名稱或內容性質簡述
科 教 處 計 畫 加 填 項 目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與(閱聽)人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

達成目標

未達成目標（請說明，以 100 字為限）

實驗失敗

因故實驗中斷

其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文： 已發表 未發表之文稿 撰寫中 無

專利： 已獲得 申請中 無

技轉： 已技轉 洽談中 無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

Results of this investigation demonstrate that red light provided by LEDs is the most important wavelength for *P. cynaroides* explants, and could be used as an alternative light source in tissue culture production. Results show that there is a high potential of LEDs to be used to increase active compounds in medicinal plants, or to improve the growth of economically important plants such as Orchid and herbal plants.