中華農業研究(J. Agric. Res. China) 52:291~296(2003)

Effect of Light/Dark Cycling on Growth and Anthocyanin Production[1] of *Ajuga reptens* in Callus Culture

Fong-Wu How[2,4] and Mary Ann Smith[3]

ABSTRACT

How, F. W. and M. A. Smith. 2003. Effect of light/dark cycling on growth and anthocyanin production of a*juga reptens* in callus culture. J. Agric. China 52: 291-296.

The effect of four different light/dark cycling treatments (16 hours light/8 hours dark, 16 hours light/32 hours dark, 16 hours light/56 hours dark, and 8 hours light/16 hours dark) on growth and anthocyanin production of *Ajuga reptens* in callus culture was investigated. The results showed that light/dark cycling has no significant influence on callus production. In contrast with callus production, the level of anthocyanin seems depend on the duration of the irradiance and become proportionally less amount as the length of the duration decrease. These results suggest that maximum anthocyanin production probably can be obtained by subculturing the callus of *A. reptens* in low light level (even in the dark) for full callus production, then transferring to a high irradiance light condition for a few days for anthocyanin synthesis.

Key Words : *Ajuga reptens*, Anthocyanin production, Callus culture.

INTRODUCTION

Ajuga reptens Linn. can produce anthocyanins containing aromatic and aliphatic compounds which make them unusually stable, and confer strong potential for use in food processing. It has been investigated as a potential in vitro pigment source. Although the capability for the formation of anthocyanin is generally determined by hereditary factors, the amount of pigment formation is affected by numerous environmental factors:

1. Contribution No.2170 from Agricultural Research Institute, Council of Agriculture. Accepted : September 1, 2003.

2. Senior Horticulturist and Director, Floriculture Research Center, ARI, Wufeng, Taichung, Taiwan, ROC.

3. Professor, Department of Natural Resource & Environmental Science, University of Illinois at Urbana-Champaign.

4. Corresponding author, e-mail: how@wufeng.tari.gov.tw ; Fax: (05)5820834.

nutrition and water availability, age of stock plant, temperature and light (Mancinelli *et al.* 1979; Mancinelli, 1983; Rabino 1977).

Light, in addition to its effects on in vitro growth and development (Seibert *et al.* 1980), is an important factor affecting the production of plant metabolites, including primary products such as enzymes, carbohydrates, lipids and amino acids, and secondary products such as flavones and flavonols, naphthoquinones and polyphenol, anthocyanins, volatiles and terpenes (Zhong *et al.* 1991). For each plant tissue culture pigment production system, experiments will have to determine the optimal light requirements, including the duration of illumination necessary for maximal color production. However, light requirements and optimal light conditions have never been thoroughly investigated in tissue culture systems (Ilker 1987). The common day length chosen is 16 hours light/8 hours dark in tissue culture.

Little is known about the effect of light/dark cycling on in vitro culture (Pierik 1987). Considering the effect of photoregulation on anthocyanin synthesis, Mancinelli (1983) pointed out that the general characteristics of light on this photoresponse are the same as those found for photoregulation of various aspects of plant growth and development. The general characteristics of the action of light on anthocyanin production are those typical of the High Irradiance Response (HIR) of plant photomorphogensis. In the intact plant, the full expression of anthocyanin synthesis requires prolonged exposure (hours to days) to high flux rates of visible and near visible radiation (Mancinelli 1983). In red cabbage and tomato, Mancinelli *et al.* (1979) found that the spectral sensitivity of light-dependent anthocyanin production, a typical HIR response, is not constant and is markedly affected by the duration and mode of application (continuous, photoperiodic, short cycle) of the light treatments. Differences in the duration and mode of application of light treatments result in large differences in anthocyanin production.

In *Kalanchoe* more anthocyanin production is formed under short day conditions than under long day conditions (Neyland 1963). But the light requirement for anthocyanin production is different in plants, since light receptors interact at various levels which are genetically encoded (Ilker 1987).

The objective of this study was to investigate the effect of light/dark cycling on anthocyanin production of *Ajuga* in callus culture. In addition to the practical aspect for producing food colors, the results will give basic information about the photoregulation of anthocyanin synthesis in callus culture of *Ajuga.*

MATERIALS AND METHODS

Induction of callus

The callus cultures used in this project were induced from leaves of *Aiuga reptans* plants growing in a greenhouse. The induction of callus was conducted in the dark at 25 ± 1℃ . Four pieces of callus approx 0.75g each were placed in 250 ml flask containing 50 ml medium. The medium consisted WPM salt (Sigma, St Louis, Mo.), supplemented with, in mg/l: 0.5 thiamine-HCL ; 0.5 pyridoxine-HCL; 0.5 nicotinic acid; 0.5 glycine,; 100 myoinositol; 50 L-ascorbic acid; 5,000 Fe EDTA; 150 polyvinypyrollidone (PVP-T); 7,000 BBL agar (Becton Dickinson, Cockeysville MD, USA); 30,000 sucrose; 0.5 2,4-D, and 0.75 kinetin. The pH value of the medium was adjusted to pH 5.7 before autoclave.

Effect of Light/Dark cycle on anthocyanin production

Four kinds of different light/dark cycling treatments included 16 hrs light/8 hrs dark (16 L/8 D), 16 hrs light/32 hrs dark (16 L/32 D), 16 hrs light/56 hrs dark (16 L/56 D) and 8 hrs light/ 16 hrs dark (8 L/16 D) were

tested. During light period, cultures were placed under cool white fluorescent tubes (62.0 μmol $s^{-1}m^{-2}$). Cultures were moved into a box which excludes light during the dark period. The box was located in the same shelf and to maintain the same temperature of 26±1℃. Each treatment was composed of 10 cubes. Effect of the treatments was evaluated after a 4-week subculture for biomass and color development.

Growth measurement

Callus growth was measured by determining fresh weight (FW) and dry weight (DW). For dry weight measurement, callus were dried in a forced air oven for 20 hours at 80℃ prior to collection of data on callus dry mass.

Anthocyanin analysis

Anthocyanin was analyzed using the method modified from Francis (1982). Callus of *Ajuga* (5g) was blended for 2 min. with 20 ml of a mixture of 1% hydrochloric acid. The extract was then stored overnight at 4℃. The absorbances were measured after filtration at 535 nm with a spectrophotometer. The amount of anthocyanin was calculated from dividing the absorption value at 535 nm by 98.2 ($E^{1\%}$ value).

Statistical analysis

Callus growth was collected from 20 replicates (5 cubes) per treatment. Anthocyanin analysis was collected from 5 replicates per treatment. All data was analyzed by ANOVA using a completely randomized experimental design.

RESULTS

Effect of Light/Dark cycle on anthocyanin production

The growth and anthocyanin production of *A. reptens* in callus cultures under different light/dark cycling treatments is showing in Table 1. After 4 weeks' subculture, there were no clear differences in callus production (based on FW and DW measurement) among these treatments. This result showed that light and light/dark cycling effect on callus production in *Ajuga* was not significant.

For anthocyanin production, the first visible pigment formation was found about 6 days after subculture under 16 L/8 D, 10 days undes 16 L/32 D, 13 days under 16 L/56 D, and 9 days under 8 L/16 D, respectively. After 4 weeks' subculture, the anthocyanin yields were comparable among these treatments. Under a different amount of irradiance (duration) there were clear differences in anthocyanin production. The highest yield was obtained on 16 L/8 D treatment, the lowest yield was obtained on 16 L/56 D treatmeut. It was not significantly different in the two treatments of 16 L/32 D and 8 L/16 D. Since treatment 16 L/32 D and 8 L/16 D produced the same amount of total irradiance, this preliminary result indicated that different light/dark cycling treatments had no significant effect on the anthocyanin production in callus culture of *Ajuga*.

Table 1. Growth and anthocyanin production in *Ajuga reptens* callus grown in different light/dark cycling conditions

Treatment	Fresh wt (g)	Dry wt (g)	Anthocyanins (mg/g callus)
16 hrs L/ 8 hrs D	4.864 a [z]	0.191 a	0.0153 a
16 hrs L/32 hrs D	5.420 a	0.191 a	0.0093 b
16 hrs L/56 hrs D	5.181 a	0.204 a	0.0065 c
8 hrs L/16 hrs D	4.860 a	0.186 a	0.0095 b

[z] Values followed by the same letter at the same raw are not significantly different (p=0.05) according to Fisher's LSD test .

DISCUSSION

Under normal tissue culture conditions, callus cultures are known to develop chloroplasts in the light, carry out photosynthesis, and evolve oxygen (Bergmann *et al.* 1966; Neumann *et al.* 1973 ; Seibert *et al.* 1980). However, the presence of sucrose in the culture medium inhibits both chlorophyll synthesis and carbon fixation in callus culture (Seibert *et al.* 1980). Thus plant callus cultures are not photosynthetically efficient and generally not autotrophic. The results in this experiment also showed that light or light/dark cycling had little effect on the growth of *Ajuga* callus measured in term of fresh and dry weight. Therefore, when maintaining or producing callus of *Ajuga*, the culture should not be maintained in light to avoid morphogenesis.

As for light and anthocyanin synthesis, various considerations suggest that phytochrome is the photoreceptor active in the photocontrol of HIR anthocyanin synthesis plant (Rabino *et al.* 1977). Triggering of anthocyanin synthesis by light has been demonstrated in callus cultures from several plant species (Strickland *et al.* 1972). Although it was possible to replace the light requirement of anthocyanin biosynthesis with auxin, the time dependence of light induced and auxin-induced anthocyanin synthesis was quite different (Altermann *et al.* 1977; Seibert *et al.* 1980). The effect of cyclic irradiation on anthocyanin formation had been studied (Ku & Mancinelli, 1972), but the results are not easy to understand because the level of light irradiance applied and the plant species are often quite different. Fritsch and Grisebach (1975) mentioned that callus culture of *Haplopappus gracillis* left in the dark for 3 days prior to light exposure accumulate more anthocyanin compare to the cultures kept in the dark only one day prior to light exposure. However in this experiment, the level of anthocyanin under different cyclic irradiation seems depend on the duration of the irradiance and becomes proportionally less amount as the length of the duration decrease. The difference of cyclic irradiance response to anthocyanin synthesis probably due to the difference in plant species or different irradiation level.

Based on the preliminary result, maximum anthocyanin production can be obtained from subculturing the callus of *Ajuga* in the dark for 2 to 3 weeks, then transferring to a high irradiance light condition for a few days. Further experiments are necessary to optimize the optimum dark duration and subsequent light duration and/or the irradiance level for the maximun anthocyanin production of *Ajuga* in vitro.

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明暗週期對筋骨草癒合組織合成花青素之影響[1]

侯鳳舞[2,4] Mary Ann Smith[3]

摘 要

侯鳳舞、M. A. Smith。2003。明暗週期對筋骨草癒合組織合成花青素之影響。中華農業研究 52:291-296。

筋骨草(*Ajuga reptens*)葉之花青素，含極穩定之芳香及脂肪化合物，爲深具開發潛力之食品用天然色素。本研究以筋骨草葉片癒合組織爲材料，於四種不同明暗週期(16 hours light /8 hours dark, 16 hours light/32 hours dark, 16 hours light/56 hours dark 及 8 hours light/16 hours dark)環境下，探討瓶內培養對筋骨草癒合組織增產及花青素合成之影響。試驗結果顯示明暗週期對筋骨草癒合組織之增殖無顯著結果。花青素之合成則明顯地受光期影響，但明暗週期之影響不顯著。高光期環境下之花青素合成量顯著增加。因此，由筋骨草葉片癒合組織合成花青素之光照條件，初期應在低光環境下以加速癒合組織增殖，然後移到高光照環境下培養，較有利於花青素之合成。

關鍵詞：筋骨草、花青素、癒合組織。

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1. 行政院農業委員會農業試驗所研究報告第 2170 號。接受日期:92 年 9 月 1 日。
2. 本所花卉研究中心研究員兼主任。臺灣省 臺中縣 霧峰鄉。
3. 美國伊利諾大學自然資源及環境科學系教授。
4. 通訊作者，電子郵件：how@wufeng.tari.gov.tw；傳眞機：(05)5820834。