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Effect of LED light radiation on antioxidant activity and *Agrobacterium*-mediate transformation of azuki bean epicotyls

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Abstract - Influence of LED (light-emitting diode) light on antioxidant activity of etiolated azuki bean seedlings was studied. Free radical scavenging activity was measured with the standard DPPH (1, 1-diphenyl-2- picryl hydrazyl) method. Red (625-630 nm) light radiated seedlings expressed significantly higher scavenging activity than other treatments, such as blue (465-470 nm) and white light. In order to testing the possible influence of LED radiation to azuki bean epicotyls on Agrobacterium-mediated gene transfer efficiency, LED light (red, blue, or white) was applied simultaneously to plant segments with Agrobacterium tumefaciens strain EHA105 in which contains the binary plasmid pCAMBIA1201. Evidences of Agrobacterium- induced necrosis in target plant tissues and its link to reactive oxygen species (ROS) were investigated. The degrees of hypersensitive necrotic reaction in plant cells were affected by LED treatments. Irradiation with blue light resulted in increasing GUS gene expression in azuki bean explants and the increase in gene expression is possibly correlated with moderates DPPH-radical scavenging activity and low H_2O_2 accumulation in blue light-treated plant segments. Our data revealed that blue or red LED light treatment promotes Agrobacterium infection and blue light treatment improves transformation efficiency with higher cell viability. A possible application of our findings to the Agrobacterium-mediated azuki bean transformation was presented in the discussion.

Index Terms - light-emitting diode (LED), antioxidant activity, epicotyl, azuki bean

I. INTRODUCTION

Phaseoleae includes grain legume crops such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), mungbean (*Vigna radiata*), and cowpea (*V. unguiculata*) are major oil or protein sources for human beings and animals. Azuki bean (*V. angularis*) is an important grain legume in Asia. Regeneration of azuki bean plants from epicotyls in Murashige and Skoog (MS) medium with high concentration of 6-benzylaminopurine (6-BA) has been reported by Ishimoto et al. [1]. The development of routine azuki bean transformation using this regeneration system that coupled with the inducer (acetosyringone, AS) during co-cultivation with *Agrobacterium tumefaciens* was accomplished by Yamada et al. [2]. Despite their great efforts in azuki bean gene transfer works, the difficulties of transformation remains in the time-consuming and labor-intensive procedures. In addition, browning and necrosis of transformed cells or tissues are very common in *Agrobacterium*-mediated gene transfer in azuki bean epicotyls [3].

Tissue browning and/or necrosis leading to poor cell growth and plant regeneration *in vitro* and successful use of antioxidants solving the problems in the tissue culture systems were not new. *Agrobacterium*-mediated gene transformation associated with plant tissues browning and necrosis have been reported in many types of explants species. Since 1996, three groups of antioxidants that used in plant tissue culture were commonly applied to plant gene transfer system according to their *in vitro* functions to control of tissue browning and necrosis [4]. *Agrobacterium*-mediated gene transfer is a process of pathogen infection. In the early infection stage, the initial response of plants to bacterium attacks is an oxidative burst with mass production of reactive oxygen species (ROS) [5]. This oxidative burst response in *Agrobacterium*-mediated gene transfer in azuki bean epicotyls was observed by Cheng et al. [3]. H_2O_2 accumulation is followed by the hypersensitive reaction (HR) to bacterium leading to cell death. This defense response indeed is a common mechanism as H_2O_2 not only can kill pathogens and inhibit their growth but also can reduce the survival rate of target plant cells/tissues following *Agrobacterium*-mediated transformation [3, 4]. There are two potential problems in use of antioxidants to control tissue browning in plant transformation. First, ROSs (e.g. H_2O_2) play dual role in the regeneration of various plant cells. Accumulation of H_2O_2 to a certain level may have positive effect on plant cell regeneration [6] in one hand, but high levels of H_2O_2 can inhibit the expression of totipotency in plant tissues [7, 8]. Second, the antioxidant itself may cause cell death and reduce the survival rate of target plant cells [3, 9].

Light-emitting diodes (LEDs), a relatively new development of semiconductor solid light sources technology, have been gradually and extensively conducted worldwide by taking advantage of LED providing precise

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light spectrum and close illumination on light quality physiology and light formula in plant production systems [10, 11]. An optimized light condition regulation means environmental friendly and can be a guarantee for high-quality production in agriculture too [10]. Generally, light conditions including light quality, light intensity, and photoperiod not only enhanced yield but also increased the accumulation of health-beneficial phytochemicals in plants. Blue light is important for chloroplast development, chlorophyll biosynthesis, and stomata opening [12]. Blue LED promoted the growth of lettuce plants after transplanting by increase in shoot and root biomasses and antioxidant activities in lettuce seedlings before transplanting [13]. Red light is important in seedling growth, photosynthetic complexes assembling, and enzyme induction [14, 15]. Supplement red light could increase phenolic contents and potentially alter antioxidant activity in plant tissues. The objective of this investigation was to study the biochemical and physiological responses of epicotyls from azuki bean under LED irradiation. Supplement of LED light (blue, red, or white) with *A. tumefaciens* infection simultaneously to plant segments that improves gene transfer efficiency and reduces browning/necrosis in the target tissues was studied.

II. MATERIALS AND METHODS

A. Plant material, growing conditions, and explant preparation

Seeds of azuki bean (*V. angularis* Willd.) cultivar KS8 were obtained from the Kaohsiung District Agricultural Research and Extension Station, Kaohsiung 90846, Taiwan, Republic of China. Seeds were sterilized by placing them into tightly sealed desiccators containing chlorine gas, made by mixing 3.5 ml of 12N HCl and 100 ml bleach (5.25% sodium hypochlorite) for 16 h [16]. Sterilized seeds were soaked in a Magenta TM GA7 Vessel (Magenta Corporation, Inc., Chicago, IL) containing 250 ml of sterile deionized water, overnight at room temperature to soften the seed coat. Prior to germination, seed coats were carefully bruised off with a scalpel without damaging the embryonic axis and germinated on a basal Murashige and Skoog (MS) medium [17] containing 30 g l^{-1} sucrose and 8 g l^{-1} agar (Wako Pure Chemical Industries) (pH 5.8).

Plant seeds were placed in a growth chamber averaging 25°C in the dark (D). 7 days after plating, epicotyls of etiolated azuki bean seedlings were harvested and cut into pieces of 10 mm long with a scalpel blade [2]. 20-25 epicotyl segments were plated side-by-side on co-cultivation medium (MS basal medium, 30 g l^{-1} sucrose, 10 mg l^{-1} 6-BA, and 8 g l^{-1} agar) for 48 h. For the study of LED light influence on plant seedling growth, treatments of white fluorescent lighting (35-55 μmol $m^{-2}s^{-1}$), red LED (625-630 nm), or blue LED (465-470 nm) in a 16/8 h (light/dark) photoperiod were applied. In transformation experiments, LED light (red, blue, or white) was applied simultaneously to plant segments with *A. tumefaciens* strain EHA105 (Fig. 1).

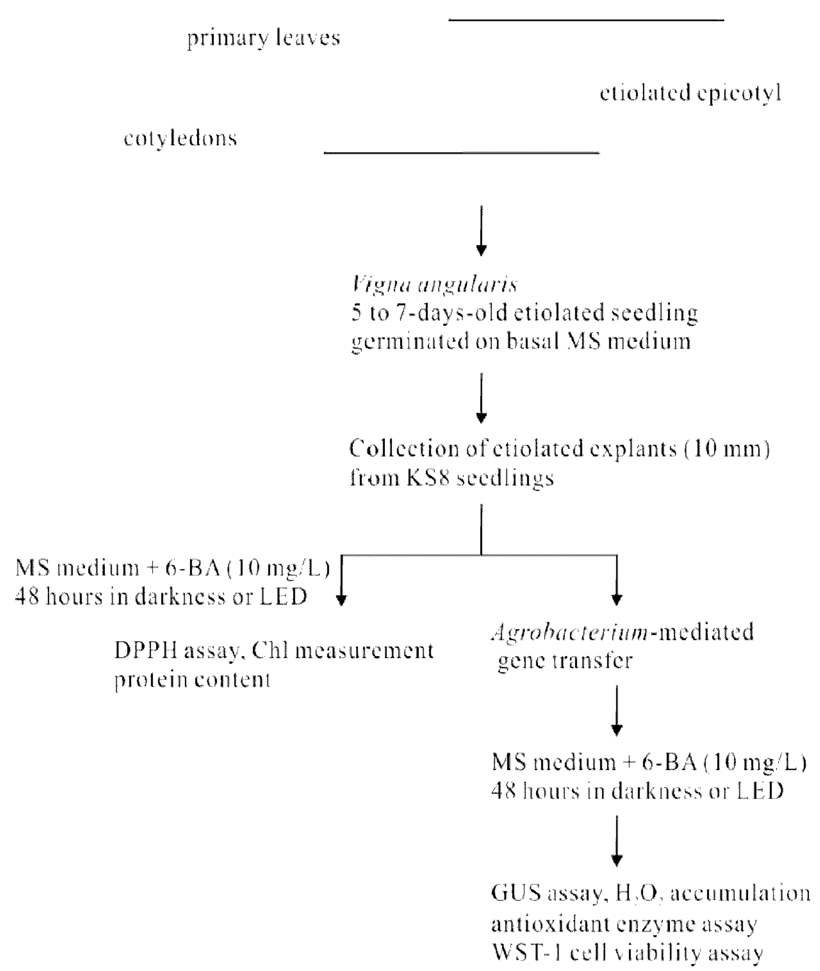


Fig. 1 Step-wise procedure for plant material preparation and experimental methods.

B. Determination of photosynthetic pigments and DPPH assay

Azuki bean epicotyl segments were homogenized on ice with a Polytron homogenizer (Art-Miccra D-8, Mullhelm, Germany) in 2 ml of cold 80% acetone. The homogenate was centrifuged at 10,000 xg (Hettich Centrifuge Universal 16R, Tuttlingen, Germany) at 2℃ for 5 min and the absorbance of the supernatant was measured at 470, 626, 645, 663, and 730 nm using a UV-VIS spectrophotometer (Unicam Helios β, Cambridge, United Kingdom). The chlorophyll *a* and *b* contents and *a*/*b* were calculated as described by Lichtenthaler [18].

Plant DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical-scavenging activity was measured according to Abe et al. [19]. Azuki bean segments (~0.2 g) were homogenized on ice in 2 ml of 100% ethanol. The ethanol extracts (0.5 ml) were mixed with 0.25 ml DPPH (0.5 mM) ethanol solution and 0.5 ml of 100 mM acetate buffer (pH 5.5). After 30 min incubation at room temperature, read the absorbance at 517 nm. The inhibitory percentage of DPPH was calculated according to the following equation:

$$\%\text{Inhibition} = \frac{Abs_{CK} \quad Abs_{S}}{Abs_{CK}} \times 100\%$$

Abs_{ck}: OD_{517} of control; Abs_s: OD_{517} of sample

C. Estimation of H_2O_2

H_2O_2 content was measured according to Velikova et al. [20] and Sumithra et al. [21]. Plant segments were homogenized with a mortar and pestle under liquid nitrogen conditions in cold 0.1% TCA and the homogenate was centrifuged at 10,000 xg at 2℃ for 15 min. The reaction mixture contained 0.5 ml supernatant frond extract, 0.5 ml potassium phosphate buffer (10 mM, pH 7.0) and 1 ml KI (1 M). The reaction mix was allowed to stand in the dark for 1 h and the absorbance was recorded at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve that prepared with known concentrations of H_2O_2.

D. Enzyme assays

Plant material (~0.1 g) was homogenized with a mortar and pestle under liquid nitrogen conditions in 0.4 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF), with addition of ascorbic acid (ASA, 5 mM final concentration) for the ascorbate peroxidase (APX, EC 1.11.1.11) assay [22]. The homogenate was centrifuged at 15,000 xg for 30 min at 2°C, and the supernatant was used for the enzyme assays. Protein content was quantified according to the method of Bradford [23], using a standard curve generated with bovine serum albumin.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich [24] and Dhindsa et al. [25]. The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 63 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 5-10 μl of enzyme extract. The reaction mixtures were illuminated at an intensity of 70 μE $m^{-2}s^{-1}$ for 20 min. The absorbance of the supernatant at 560 nm was measured. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT.

Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the change of absorbance at 240 nm that accompanied the consumption of H_2O_2 [26, 27]. 0.2 ml of diluted enzyme extract (100X) was added to 3.0 ml of 40 mM buffered hydrogen peroxide (in potassium phosphate buffer; 50 mM pH 7.0). The decrease of absorbance at 240 nm ($\varepsilon = 40\ mM^{-1}cm^{-1}$) was measured.

Guaiacol Peroxidase (GPX, EC 1.11.1.7) activity was determined as oxidation of guaiacol by H_2O_2 [28]. The reaction mixture was 1.0 ml of 100 mM potassium phosphate buffer (pH 6.5), 0.2 ml of 10 mM buffered H_2O_2 (in potassium phosphate buffer; pH 6.5), 0.2 ml of 2.5% guaiacol, 0.5 ml of dH_2O and 0.1 ml of diluted enzyme extract (20X). The increase in absorbance at 420 nm was measured.

APX activity was determined from the decrease in absorbance at 240 nm as described by Chen and Asada [29] with modifications. The reaction solution contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ASA, and 5 mM H_2O_2. The reaction was started by adding 20 μl of enzyme extract.

Glutathione reductase (GR, EC 1.6.4.2) activity was assayed as the increase of absorbance at 412 nm ($\varepsilon = 13.6\ mM^{-1}cm^{-1}$) resulting from the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to 2-nitro-5-thiobenzoic acid (TNB) [30, 31]. The final reaction volume contained 1 ml 0.2 M potassium phosphate (pH 7.5) containing 1 mM EDTA, 0.5 ml 3 mM DTNB in 0.01 M phosphate buffer, 0.29 ml H_2O, 0.1 ml 2 mM NADPH, 0.01 ml plant crude extract, and 0.1 ml 20 mM oxidized glutathione (GSSG).

SOD, CAT, and GR activity was calculated per milligram of protein per minute and expressed as a percentage of the control. APX and GPX activity was expressed as an increase in absorbance min^{-1} that caused by the enzyme sample.

E. Agrobacterium strain and plasmid

A. tumefaciens strain EHA105 containing the disarmed and a dual marker binary plasmid pCAMBIA1201 (Fig. 2) comprising the β-glucuronidase (GUS) reporter gene and hpt gene (hygromycin phosphotransferase) as a selective marker with a multiple cloning site was used for transformation (pCAMBIA vector, http://www.cambia.org/daisy/cambia/585). The pCAMBIA1201 (11.98 kb) was transferred to *A. tumefaciens* by electroporation. Azuki bean transformation was mainly based on the standard *Agrobacterium*-mediated epicotyl gene transfer method as described by Yamada et al. [2].

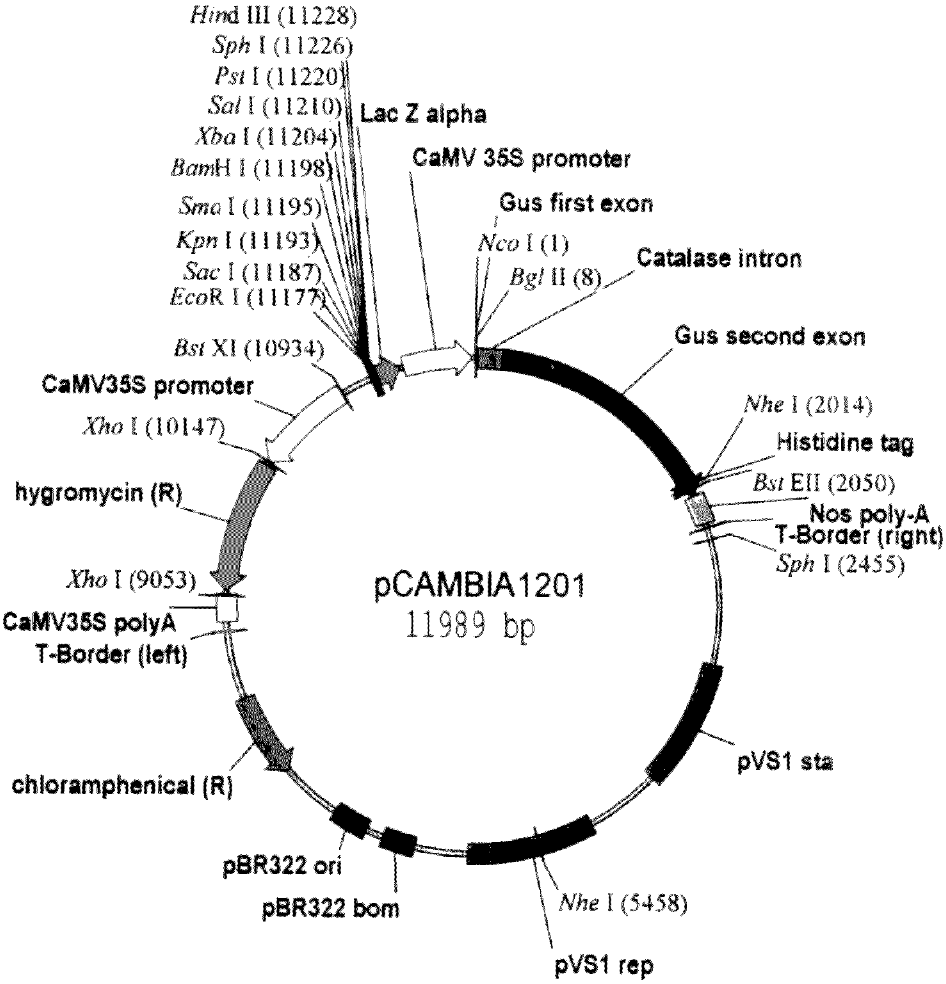


Fig. 2 The pCAMBIN1201 vector consisted of the cauliflower mosaic virus (*CaMV*) 35S promoter-derived bi-directional promoters.

F. Histochemical GUS assay and image analysis

Transient GUS expression was monitored on epicotyl explants that placed on co-cultivation medium for 48 hours. Tissue slices (~0.5 mm thickness) were incubated in a staining solution (0.1 M $NaHPO_4$ buffer (pH 7.0), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA, 800 mg l-1 X-Gluc (5-bromo-4-chloro-3-indolyl-β-D- glucuronic acid cyclohexyl-ammonium), 0.06% (v/v) Triton X-100) following standard procedure as described by Jefferson et al. [32].

Cross-sections of epicotyl explants (0.5 mm) were examined under microscope (Nikon SMZ-10A) and the area of GUS blue stains or degree of necrosis was monitored and photographed (Nikon Coolpix 995 digital camera). Digital images of each segment (n = 6) were obtained and processed with Image J software [33]. Necrosis or GUS expression was expressed as percentage of areas/total area.

G. WST-1 assay

WST-1 (4-[3-4-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate, Wako Pure Chemical Industries, Osaka, Japan) assay according to Chucheep et al. [34] was applied to evaluate effects of *Agrobaterium* infection on the LED-treated azuki bean seedlings. Azuki bean samples (0.5 mm, 10 slides per assay) that excised every hour (for 6-hour) from the Agrobacterium co-cultivation epicotyl segments at 25°C were used for WST-1 reduction assay. The typical 3 ml reaction medium for WST-1 assays contained 50 mM Hepes phosphate buffer (pH 7.4) and 0.1% Tween 20 and a final concentration of 0.2 mM WST-1 and 5 μM PMS. The excised epicotyl segments (explants) were washed 3 times with distilled water before addition into 3 ml of reaction medium. Then, the samples were vacuum-infiltrated for 10 min (10 bar) and incubated at 30°C for 6 h. Cell viability of the explants was determined by measuring the absorbance of the yellowish compound produced by UV-VIS spectrophotometer (Unicam Helios β, Cambridge, United Kingdom) at 450 nm at 1 h intervals up to 6 h after the start of incubation. The values were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. Background absorbance in the absence of epicotyl segments was subtracted in all assays. The data are usually expressed as transient absorbance (ΔOD = OD at 450 nm - OD at 600 nm).

H. Statistical analyses

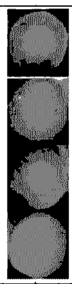
Data presented here are the mean values ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed by computer software SPSS (ver.20: SPSS Inc., Chicago, IL, USA). All data were subjected to a one-way analysis of variance and the mean differences were compared by lowest standard deviations (LSD) test and P-values < 0.05 were considered significant.

III. RESULTS

A. Effect of LED on seedling growth and photosynthetic pigment contents

Growth of azuki bean seedlings was significantly affected by LED treatments (Table 1). When compared to the controls, there was an increase in fresh weight (FW) of LED treated plants. FW increased about 2.60- to 2.78-fold in explants that treated for 2 days with LED light. The photosynthetic pigments were quantitatively estimated in acetone extracts from dark and LED-treated azuki bean plants. As shown in Table 1, irradiation with LED light resulted in increasing pigment content in plant tissues. This pigment accumulation was profound by the cultivation under various LED-treated seedlings. In the experiments, accelerated anthocyanin accumulation was not found in plants treated with LED light (Data not shown). A comparison of photosynthetic pigment ratios showed that the changes in the chlorophyll a/b ratio after LED treatments (Table 1) is due to a significantly reduction of chlorophyll a in the blue LED-treated plants. This is confirmed by the absorbance spectrum of the total chlorophyll (Fig. 3). The absorbance spectrum of the blue light irradiation revealed much lower absorption at 400-500 nm and 600-700 nm wavelength compare to red or white LED treatment.

Table 1. Effects of LED irradiation on azuki bean seedling growth

LED radiation		Chl content (mg g^{-1} FW[a])	Chl *a/b*	Protein content (mg g^{-1} FW)
White		36.53	2.28	8.67 ± 0.76
Red		13.80	2.16	8.28 ± 1.28
Blue		9.82	1.77	8.12 ± 0.85
Darkness		-	-	3.12 ± 0.72

[a]FW: fresh weight

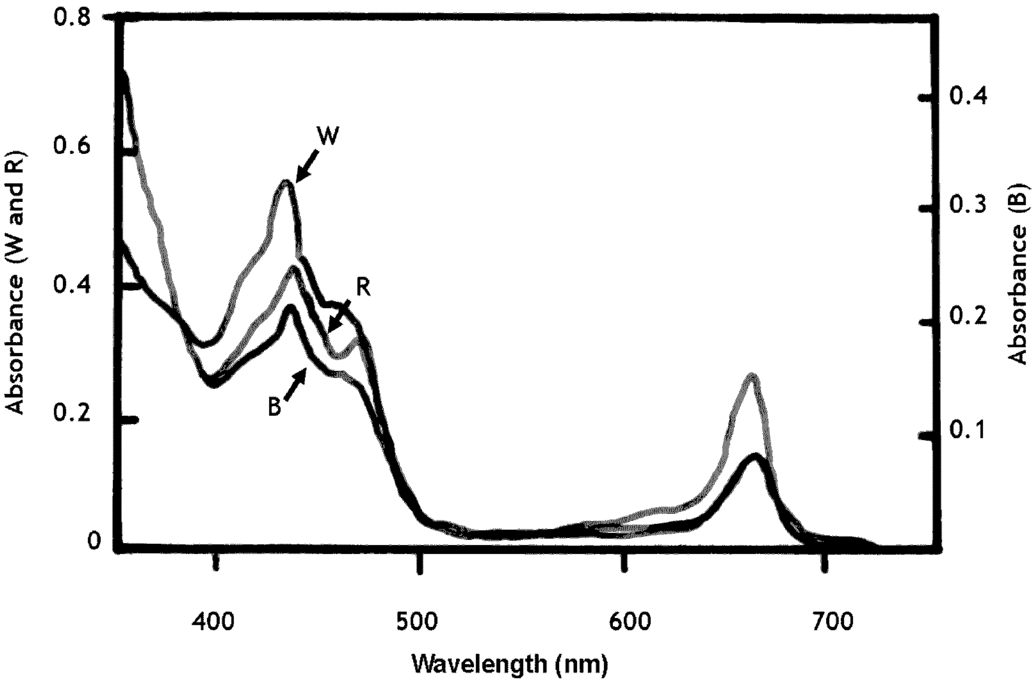


Fig. 3 Absorbance spectrum of acetone-soluble pigments from LED light radiated azuki bean seedling. Pigments were extracted from freshly prepared blue (B), red (R), and white (W) LED light treated epicotyls of azuki bean. The absorbance spectrum from scans of equal amounts of tissues (0.1 g F.W.) from each extracts are shown. Vertical scale represents scanning wavelength in nanometers (nm).

B. Effect of LED on DPPH radical-scavenging activity

Changes of the DPPH radical-scavenging activity in the seedling segments of azuki bean were monitored for 48 hours during incubation (Fig. 4). Significant enhancement of free radical scavenging activity was shown at red LED-treated seedlings ($P < 0.05$). The free radical scavenging activity of azuki bean plants was also increased significantly by dark treatment for 12 hours and peaked at 48 hours after incubation (Fig. 4).

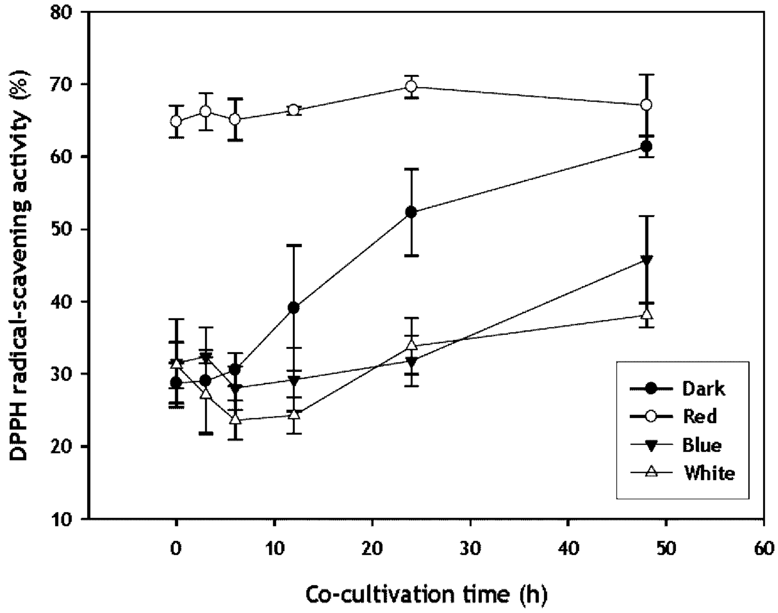


Fig. 4 Effect of LED irradiation on DPPH-radical scavenging activity of azuki bean seedling. Seedlings were treated with LED light for 7 days then infected with Agrobacterium and co-cultivated for 48 h. DPPH-radical scavenging activity was measured at 0, 3, 6, 12, 24, and 48 h during co-cultivation from epicotyl segments. Vertical bars represent mean ± standard deviation (n = 3).

C. Effect of LED on SOD, POD, CAT, APX, and GR activity

The effect of LED exposure on the enzymatic resistance to *Agrobacterium*-mediated oxidative stress of azuki bean seedling segments was also investigated. As shown in Fig. 5a, the activity of SOD decreased significantly ($P < 0.05$) in azuki bean plants after 48 hours co-cultivation with LED. After illumination with LED light, activities of SOD were decreased in the range from 37.4 (blue) to 52% (red and white) in LED-treated plant segments as compared to control (73.5%). CAT activity was measured and showed no significant difference among treatments (Fig. 5b). However, APX activity was reduced at red light treated samples ($P < 0.05$) (Fig. 5c).

Similar phenomenon was also found in GPX activity of azuki bean seedling segments cultivated at different LED light. After 48 hours of exposure to LED, GPX activity was 3.63- (white) and 3.16-fold (red) increased than the control value ($P < 0.05$) (Fig. 5d). Although, GPX activity showed recovery at red LED treatments at 12 hours of co-cultivation, it was only 21% higher than the dark control plants. After exposure to LED light, activities of GR were decreased as compared to control (Fig. 5e). Although GR activities in the range from 55.7% to 66.3% in red LED-treated plant were determined (Fig. 5e). It was still 15.4% less than the unstressed control plants.

D. Effect of LED on GUS gene expression and H2O2 accumulation

Histochemical staining method was applied to determine effect of LED irradiation on GUS gene expression. As shown in Table 2, irradiation with red light resulted in increasing GUS gene expression in plant segments. However, either blue or white light treatment has no profound effect on GUS gene expression. *Agrobacterium*-mediated tissue necrosis on azuki bean epicotyls was observed (Table 2). After co-cultivation for 12 hours, a reversed U-shaped response curve of H_2O_2 content was observed in the treatments (Table 3). Increase in H_2O_2 content with LED light treatments in epicotyl segments was correlated to the GUS gene expression. In response to the lowest GUS gene expression and tissue necrosis, H_2O_2 content in control plant samples (darkness) was 8.5% to 27.1% lower than that of LED-treated samples.

E. Effect of LED on cell viability

WST-1 assay for cell viability was applied to evaluate agroinfection-induced damage in three kinds of LED-treated azuki bean seedlings. As shown in Fig. 6, higher OD values (yellowish color development) were found in blue light-irradiated azuki bean seedlings, indicating the plant segments had higher cell viability than the others. After co-cultivation for 12 hours, irradiation with red light resulted in the lowest cell viability in the epicotyl segments.

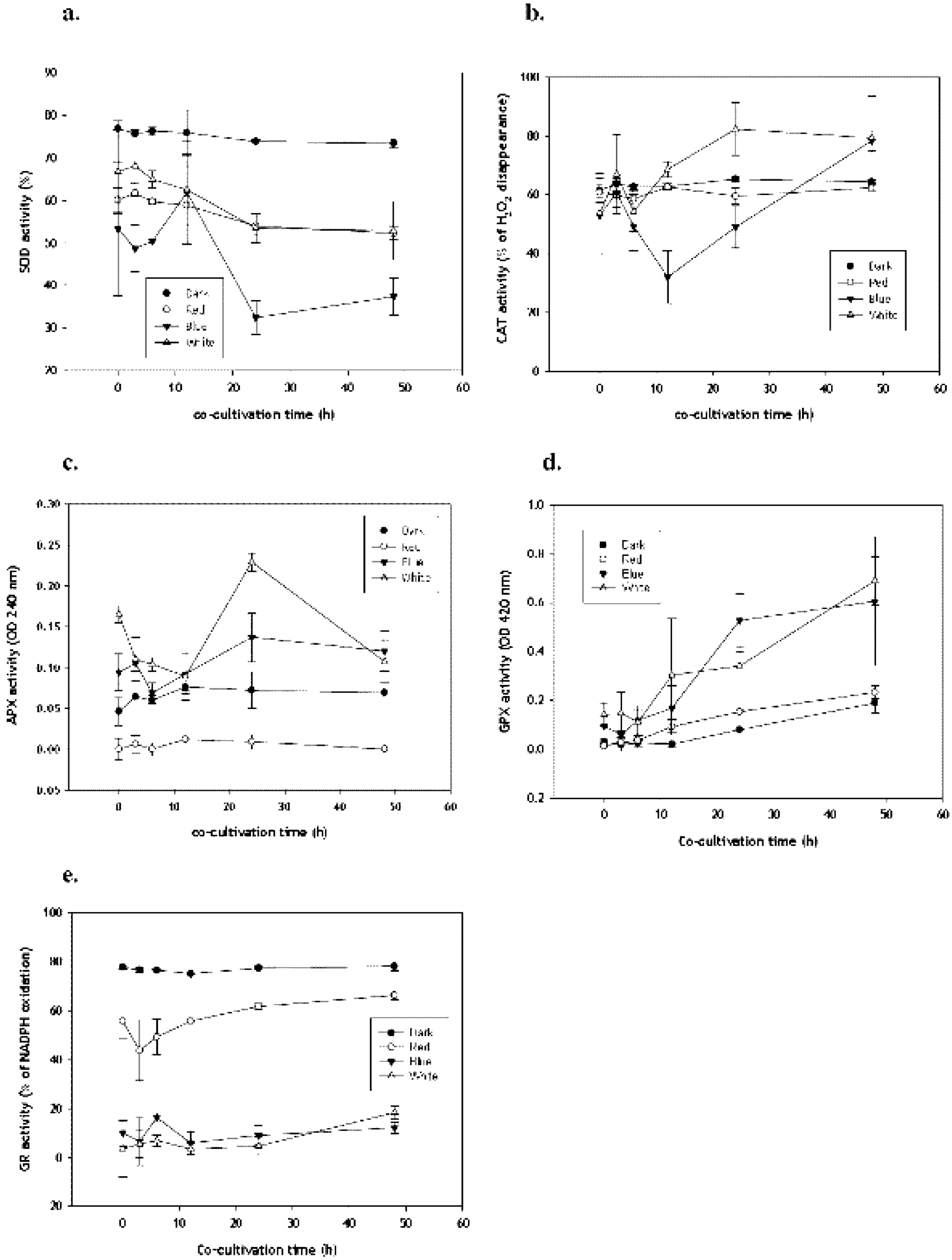


Fig. 5 Effect of LED irradiation on antioxidant enzyme activity of azuki bean seedlings. SOD (a), CAT (b), APX (c), GPX (d), and GR (e) activity were measured at 0, 3, 6, 12, 24, and 48 h during co-cultivation from epicotyls segments. Vertical bars represent mean ± standard deviation (n = 3).

Table 2. Effect of LED irradiation to the degree of *GUS* expression and tissue necrosis on azuki bean epicotyls.

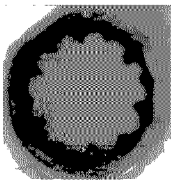
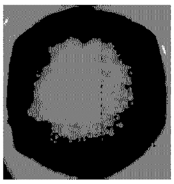
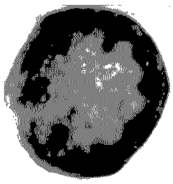
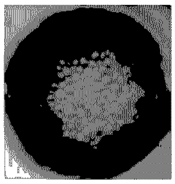
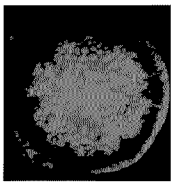
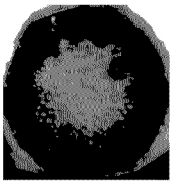
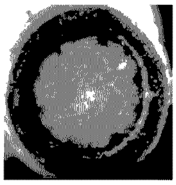
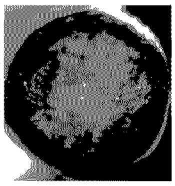
	Dark	Red	Blue	White
	32.65±25.40	47.69±11.61	39.78±18.58	34.90±18.79
GUS staining				
Necrotic response				

Table 3. Effect of LED irradiation to H_2O_2 accumulation (%) in azuki bean explants

LED light	Co-cultivation time (hour)				
	0	3	6	9	12
Red	21.04±20.72	44.59±14.25	62.57±14.76	51.79±30.03	59.37±10.38
Blue	15.97±13.84	28.11±9.58	39.47±16.41	49.86±8.22	47.24±19.83
White	14.25±9.60	40.49±25.11	54.73±10.50	86.00±60.56	51.74±23.50
Darkness	4.29±2.32	15.23±5.74	25.49±5.23	46.34±8.80	43.13±8.06

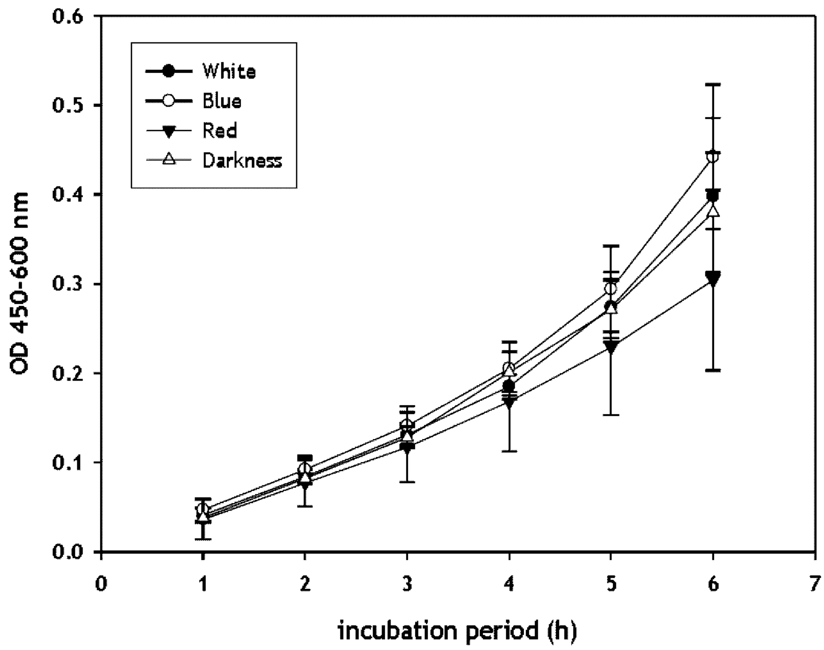


Fig. 6 Evaluation of tissue injury of azuki bean epicotyls infected with Agrobacterium. Three LED irradiated epicotyl segments from co-cultivation of Agrobacterium-mediated gene transfer were washed and incubated with 3 mL of reaction medium (50mM Hepes phosphate buffer at pH 7.4 and 0.1% Tween 20, containing a final concentration of 0.2mM WST-1 and 5μM PMS). The samples were vacuum-infiltrated for 10 min and incubated at 30℃ for 6 h. The absorbance at 450 nm was measured at 1 h intervals up to 6 h after the start of incubation. The data expressed as transient absorbance (△OD = OD at 450 nm - OD at 600 nm) in order to correct for non-specific turbidity in the samples.

IV. DISCUSSION

LED light irradiation improves seedling quality and growth has been reported by Johkan et al. [13], Liu [10], and Wu et al. [35]. In the experiment, influence of LED light on azuki bean seedling growth was studied. After irradiated for 2 days, comparing to dark control plants, LED light irradiated explants displayed high chlorophyll accumulation and protein content. Red light treated plants showed the highest DPPH-radical scavenging activity. However, chlorophyll *a* reduction leading to the total chlorophyll content decrease in blue light-treated seedlings was observed.

Agrobacterium-mediated gene transformation associated with plant tissues browning and necrosis has been determined as a serious problem in azuki bean epicotyl transformation by Cheng et al. [3]. H_2O_2 accumulation is followed by the hypersensitive reaction to *Agrobacterium* infection leading to target cell death [9]. Antioxidants such as ascorbic acid, dithiothreitol (DTT), glutathione (GSH), selenite (Se), and cysteine (cys) were introduced to control tissue browning and necrosis in azuki bean transformation [9]. Here, LED light was applied to azuki bean epicotyl explants simultaneously with *Agrobacterium* for 48 h (Fig. 1, co-cultivation). Irradiation with blue LED light resulted in increasing GUS gene expression in azuki bean explants (Table 2). This increase in gene expression is possibly correlated with moderates DPPH-radical scavenging activity and low H_2O_2 accumulation in blue light-treated plant segments (Fig. 4 and Table 3). Similar results were observed from red LED light treatments. However, high DPPH-radical scavenging activity and H_2O_2 accumulation with tissue browning and cell necrosis in red light-treated plant segments led to poor cell growth and development (Table 2). Red light contributes β-carotene expression and antioxidant activity for nutrition benefits and blue light enhance seedling weight and chlorophyll induction of radiated pea seedlings were determined by Wu et al. [36].

Increase in *Agrobacterium*-mediated gene transfer efficiency in grain legume such as azuki bean with high concentration 6-BA (10 mg l^{-1}) in the co-cultivation medium were reported by Pigeaire et al. [37] and Yamada et al. [2]. Recently, high levels of 6-BA induce programmed cell death (PCD) in cultured cells of *Arabidopsis thaliana* was characterized by DNA laddering and expression of a specific senescence marker [38]. Therefore, cell viability of the target cells was determined by WST-1 cell viability assay [34]. WST-1, which produces a highly water-soluble formazan dye of a yellowish color after reduction by metabolically active cells was applied to measure cell viability after *Agrobacterium* transformation in azuki bean epicotyl. Higher OD values (yellowish color development) were found in blue light-irradiated azuki bean explants, indicating the blue light-treated epicotyl explants had higher cell viability than the others. Our data revealed that blue or red LED light treatment promotes *Agrobacterium* infection and blue light treatment may improve transformation efficiency with higher cell viability.

ROS production associated with *Agrobacterium*-mediated transformation process in many plant species playing an important role in target tissue browning and necrosis. To control of tissue browning and necrosis in plant tissue culture and plant transformation, antioxidants such as DTT, ascorbate, GSH, cysteine, and many others were used *in vitro* to solve the problems. Adding antioxidants to co-cultivation medium reduced tissue death and increased the frequency of transformation. In this primary study, LED showed effect on azuki bean seedling growth and ROS scavenging activity of epicotyl explants. Both blue and red light promoted the frequency of transformation and blue light increased target cell viability in azuki bean transformation. In the future, the improvement of azuki bean epicotyl transformation and increase in transformed cells survival and regeneration are the target of light regulation. Light formula that defined as an optimized light quality component aiming at proper ROS scavenging activity or lower recalcitrance of epicotyl explants under co-cultivation in azuki bean transformation should be studied further.

V. CONCLUSION

Influence of LED light on antioxidant activity of etiolated azuki bean seedlings was studied. Red light radiated seedlings expressed significantly higher DPPH-radical scavenging activity than other treatments, such as blue and white light. A positive influence of LED radiation to azuki bean epicotyls on *Agrobacterium*-mediated gene transfer efficiency was observed in red and blue light treatment. However, *Agrobacterium*-induced necrosis, H_2O_2 accumulation, and WST-1 cell viability assay in target plant tissues revealed that oxidative responses were induced and the degrees of hypersensitive necrotic reaction in plant cells were affected by LED treatments. The antioxidative enzymes (e.g. APX) play important roles in the defense mechanism against *Agrobacterium*-induced stress.

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VIII. BIOGRAPHIES

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