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Effects of Diode and He-Ne Lasers on *In Vitro* Production of Anthocyanin in Apple Cell Suspension Culture

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Abstract
Plant cell cultures could be used as an important tool for biochemical production, ranging from natural pigments to pharmaceutical products. Anthocyanin is affected by a variety of factors. Light, an important plant environmental factor, influences the anthocyanin production in vegetative plant tissues. Here, we have investigated the influence of the blue laser-diode (BLD) and red laser-He Ne (RLHN) irradiation on the ability of apple cells in producing anthocyanin in suspension culture. Callus was induced from young leaf of apple and cell suspension cultures were subsequently formed from calluses. The normal cell suspension into L-shape tube test exposed to RLHN (6.46, 4.82, 1.54 $mWcm^{-2}$ and 666.66 μWcm^{-2}) and BLD (67.09, 32.74, 30.4, 13.73 $mWcm^{-2}$) laser radiation for 20 min. Results showed that the anthocyanin production was enhanced significantly by both RLHN and BRD compared with darkness and fluorescent light cells ($P<0.05$). The cell cultures exposed to 67.09 BLD and 6.46 RLHN indicated highest total anthocyanin (TA) and total monomeric anthocyanin (TMA) contents, while cell cultures exposed to darkness and fluorescent light revealed lowest TA and TAM content, respectively. In addition, we have found that RLHN nearly increased TA content as well as BLD. However, the BLD laser was more effective on TAM content than RLHN laser. At the minimum intensity, the RLHN laser could enhance TA content whereas scarcely affecting TMA content. Nevertheless, the BLD laser improved both TA and TMA contents. These results suggest that TAM production is impressed by quantity more than quality by laser irradiation.

Keywords: Blue Laser-Diode, Callus, Irradiation, Light, Red Laser-He.

Abbreviations: CHS, Chalcone Synthase; **PAL**, Phenylalaninamonia Lyase; **DFR**, Dihydroflavonol 4-reductas; **CHI,** Chalcone Isomerase; **TA**, Total Anthocyanin; **TMA**, Total Monomeric Anthocyanin; **ROS**, Reactive Oxygen Species.

Introduction

Anthocyanins, the largest group of water-soluble pigments in the plant kingdom belonging to the family of compounds known as flavonoids (Mazza and Miniati, 1993), play an important role in attracting insects or animals for pollination and seed dispersal. Anthocyanin pigments are as non-enzyme antioxidants system and protectants of DNA and the photosynthetic apparatus from high radiation fluxes (Gould *et al*., 2004). Other possible functions of

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anthocyanins, such as the protection against cold stress or providing drought resistance, are likely to be associated with activities restricted to particular classes of plants (Chalker-Scott, 1999). Flavonoids, including anthocyanins, are potent antioxidants, raising the possibility they may scavenge reactive oxygen species (ROS) generated during photosynthesis, particularly under conditions of photoinhibition (typically high light and low temperature) (Albert *et al*., 2009).

Plant cell culture is an effective tool in the study and production of secondary metabolites. Anthocyanin pigments can be produced by growing plant cells in tissue culture. Plants having no pigmentation *in vivo* may produce anthocyanin in tissue culture. Production of anthocyanins via plant cell cultures from various plant species has been successful, such as with *Catharanthus roseus* (Filippini *et al*., 2003), strawberry (Mori *et al*., 1993), and purple sweet potato (Terahara *et al*., 2004).

Environmental factors, including light intensity, wavelength and duration, low temperature, water and carbohydrate levels, nutrient elements, and pathogen attack, may affect anthocyanin production in vegetative tissues grown in tissue culture media (Dixon and Paiva, 1995; Chalker-Scott, 1999). Light, an important plant environmental factor, influences the anthocyanin production in vegetative plant tissues. There are many examples in which the biosynthesis of secondary metabolites by plant cell suspensions are enhanced by light irradiation (Bjoerk, 1986). Anthocyanin production can be induced by light, blue being the most effective one. In strawberry, blue light (λ= 460 nm, 4 Wm^{-2}) enhanced anthocyanin biosynthesis, whereas red light (λ= 650 nm, 4 Wm^{-2}) hardly affected it (Kurata *et al*., 2000).

In most studies on effects of light quantity, quality, and duration on the anthocyanin biosynthesis in several organs of diverse plant species in different tissue culture conditions, fluorescent and LED lamps have been used as the light sources for the blue and red light (Stutter *et al*., 2005). Laser rays belonged to unionizing radiation laser is an abbreviation of "light Amplification by Stimulation of Radiation". It is identified by the emitted wavelength and the power. For the last two decades**,** application of lasers in medicinal and biological studies has increased. Laser beam, due to certain properties, such as single wavelength, focus, and intensity, may affect differently than natural light illumination on plant physiological and biochemical processes (Vasilevski *et al*., 2001).

Chen (2009), in a study to determine the response of antioxidant defense system to laser radiation in apical meristem and malondialdahyde (MDA) production of *Isatis indigotica* seedlings, subjected to UV-B radiation (10.08 kJm^{-2}) for 8 h d^{-1} for 8 d (PAR, 220 $\mu molm^{-2}s^{-1}$) and then exposed to He-Ne laser radiation (633 nm; 5.23 $mWmm^{-2}$; radiation diameter: 1.5 mm) for 5 minutes each day without ambient light radiation. Results showed that UV-B treatment alone caused a significant increase in the concentration of MDA compound. In contrast, He-Ne laser radiation alone caused a decrease of MDA concentration ($P<0.05$). These results suggest that laser irradiation has been lead to increased lipid peroxidation of membrane followed by enhanced anthocyanin production in order to overcome the lipid membrane damages.

So far, there have been very few reports regarding the application of the laser radiation as a light source in promoting or inducing *in vitro* production of anthocyanin or any other secondary metabolite (SM) in plant cell suspension culture. In this research, we have investigated the effects of BLD and RLHN irradiation on the *in vitro* production of anthocyanin from cell suspension of apple (*Malus* sp.).

Materials and Methods

Plant Material and Callus Induction

Plant material was prepared from a red-fleshed fruit bearing apple tree (*Malus* sp.) which is postulated to be a hybrid of *Malus*

pumila var. Niedzwetzkyana (Van Nocker *et al*., 2012) located in Shahid Bakery Jahad-Agriculture Educational Center of West Azarbaijan province of Iran. Buds of this tree were decontaminated using the procedure previously described by Dobranszki *et al*., (2010) and shoot tips, including some leaf primordia, were then cultured in the MS medium supplemented with 1.0 mg L^{-1} benzyl adenine (BA), FeEDDHA100 mg L^{-1}, 3 g L^{-1}Polyvinylpyrrolidone (PVP), and 3%, sucrose (w/v). After sufficient growth of buds, the seedlings were proliferated in the same culture medium and their leaves were used as explants for callus production (Mahna and Motallebi-Azar, 2007). The medium for callus induction was modified MS supplemented with 3%, sucrose (w/v), 100 mg L^{-1} *myo*-inositol, 0.5 mg L^{-1} kenetine, 1.5 mg L^{-1} 2,4-D, and 1 mg L^{-1} NAA (naphthalenecetic acid). All cultures were maintained in a box in a completely dark condition at 23±2°C. It was subcultured every four weeks on the modified MS medium.

Establishment of Plant Cell Suspension

Cell suspension cultures were initiated by transferring 2 g of fresh friable callus tissue to 100 ml liquid MS medium supplemented with 3% (w/v) sucrose, 1.2 mM asparagine, 284 µM ascorbic acid, 328 µM thiourea, 555 µM *myo*-inositol, 2 µM Ca pantothenate, 8 µM nicotinic acid, 3 µM thiamin, 4.8 µM pyridoxin HCL, 0.04 µM biotin, and 1 mg L^{-1} 2, 4-D dichlorophenoxyacetic acid in a 250 ml flask (Codron *et al*., 1979). They were incubated on a rotary shaker at 80 rpm under continuous fluorescent light of 24 µmol m^{-2} s^{-1} at 23±2°C for three weeks, during which the medium was changed every week (Kurata *et al*., 2000). Afterwards, this suspended callus tissue was filtered through a mesh with a pore size of 53 µm and obtained cell suspension was transferred into L-shaped test tubes with 2 cm diameter under the same light condition on a shaker and, after 15 days, exposed to the defined laser radiation treatments for 20 min.

Blue Laser-Diode and Red Laser-He-Ne Irradiation Treatments

The lasers were constructed with a RLHN laser; wavelength: 630-640 nm, power intensity: 5 mW, irradiation diameter: 0.5 x 1.5 mm^2, and a BLD laser; wavelength: 437 nm, power intensity: 50 mW, irradiation diameter: 0.5 x 1.5 mm^2. This experiment was carried out with ten irradiation treatments: blue laser-diode at the intensities of 67.09, 32.74, 30.4, and 13.73 $mWcm^{-2}$; red laser-He Ne at the intensities of 6.46, 4.82, 1.54 $mWcm^{-2}$, and 666.66 μWcm^{-2} and two controls including darkness and fluorescent light with an intensity of 24 µmol m^{-2} s^{-1} in a completely randomized design with three replications for each treatment (Fig. 1). Mean comparisons were achieved through Duncan's New Multiple Range Test (DNMRT) at $P<0.05$.

Fig. 1. Treatment of cell suspensions poured in L-shaped test tubes by red laser-He Ne (left) and blue laser-diode (right) lasers on a shaker

Determination of Total Anthocyanin by General Method

For measurement of total anthocyanin (TA) content, two week after treatment, 1 ml of cell suspension cultures were mixed with 1 ml of the methanol-HCl (99:1 v/v) at room temperature. This mixture incubated in the dark at 4°C for 24 hours and they were centrifuged at 1,000 x g for 10 min. The absorbance (supernatant) was recorded at 530 nm and the anthocyanin content was calculated using the following formula: (Wrolstad and Struthers, 1971a; Wrolstad, 1976b; Wrolstad *et al*, 1990c).

$$C\left(mgKg^{-1}\right) = \varepsilon L/A$$

C represents total anthocyanin content, L is the cuvette diameter, and ε is the molar absorptivity of cyanidin-3-galactoside (30,200 $lmol^{-1}$ cm^{-1}). A was estimated by the following equation:

$$A = \text{absorption read by spectrophotometer} \times MV \times DF$$

In the above formula, MV is exhibitor molecular weight of cyanidin-3-galactoside (445.2) and DF is the dilution factor (3).

Determination of Total Monomeric Anthocyanin by pH Differential Method

Total monomeric anthocyanin (TMA) content was determined by the pH differential method described previously by Giusti and Wrolstad (2005). One ml of cell suspension was mixed with one ml of the methanol-HCl (99.9:0.1 v/v) and the mixture was centrifuged at 1,000 x g for 10 min at room temperature and the supernatant incubated in the dark at 4 °C for 24 hours. To measure the absorbance at pH 1.0 and 4.5, the samples were diluted 2 times with pH 1.0 potassium chloride buffer and pH 4.5 sodium acetate buffer, respectively (Fig. 2). The maximum absorption of the sample in pH 1.0 and pH 4.5 buffers was found at 530 nm, which indicated that the major anthocyanin in the extract was likely cyanidin-3-galactoside. Therefore, the total monomeric anthocyanin content of prepared mixture was calculated in terms of cyanidin-3-galactoside. The concentration of monomeric anthocyanin pigment in the original sample was calculated by the following equation:

$$Monomeric\ anthocyanin\ pigment\ \left(mgL^{-1}\right) = \left(A\ diff\ \times MV\ \times DF \times 1000\right)/\left(\varepsilon \times 1\right)$$

where, MW represents molecular weight of cyanidin-3-galactoside (445.2), DF is the dilution factor (3), and ε is molar absorptivity of cyanidin-3-galactoside (34,300 $lmol^{-1}$ cm). A was calculated from the following equation:

$$A_{diff} = \left(A_{530} - A_{700}\right)_{pH\,1.0} - \left(A_{530} - A_{700}\right)_{pH\,4.5}$$

Note that A_{700} was measured and subtracted off in order to eliminate the effect of haze or sediments in the sample (Giusti and Wrolstad, 2005).

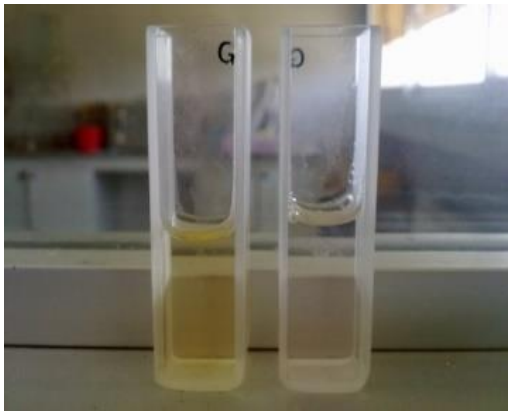

Fig. 2. Diluted samples with pH 1.0 potassium chloride buffer (right) and pH 4.5 sodium acetate buffer (left)

Statistical Analysis

Calculations and statistical analysis were carried out using SAS Portable ver.9.1.3 software (Statistical Analysis System, SAS Institute Inc., 1985). Based on the experimental, completely randomized design (CRD) selected in the study, data were analyzed using Duncan's new multiple range test (DNMRT). Mean values are expressed for comparison of the means, the at 5% level of significance ($P < 0.05$).

Results and Discussion

Anthocyanin accumulation in apple cell may be a general response to electrical stress induced by laser irradiation. Laser

irradiation, due to single wavelength, short pulps, and high power, be generate heat-shock electrical stress in tissue plants. The effects of laser-induced stress on cells and tissue can be quite disparate (Doukas and Flotte, 1996). Results of this study for *in vitro* production of anthocyanin show that normal cell cultures exposed to BLD and RLHN (630-640 nm and 437 nm, respectively) irradiation have a significant difference ($P<0.05$) as compared to the fluorescent light (control) and darkness (Fig. 3). The TA and TMA content increased by both BLD and RLHN irradiation.

For concerning influence of wavelength laser irradiation on anthocyanin biosynthesis, result indicated that it was affected by both BLD (437 nm) and RLHN (630-640 nm). Furthermore, for appointing the best laser, analysis of the two kind of lasers with four intensities to each of the lasers was done to evaluate the effect of laser irradiation. We found that the minimum (666.66 μWcm^{-2}) irradiation intensity of the RLHN enhanced TA whereas hardly affected TMA in apple cells. These results were in concordance with Kurata *et al*. (2000). Kurata *et al*. (2000) stated that blue light enhanced anthocyanin biosynthesis in cell strawberry whereas red light hardly affected it. However, the minimum irradiation intensity of the BLD improved both TA and TMA.

It is reported that the laser irradiation, due to wavelength, intensity, and spatial and temporal coherence, exerts an anisotropic effect on cell, inducing directed changes in cell structures (Anisimov *et al*., 1997). Since the power or intensity of BLD applied in the present study was about 10 time more than RLHN, however, it is acceptable that BLD can be more effective as compared to RLHN irradiation. The higher the intensity of BLD and RLHN irradiation induced, the higher TA and TMA contents are. Thereby, 0.013 mgl^{-1} TMA, 84.8 mg kg^{-1} TA, and 0.0143 mg L^{-1} TMA, 112.94 mg kg^{-1} TA were measured at the maximum light densities of 6.46 $mWcm^{-2}$ and 67.09 $mWcm^{-2}$ for RLHN and BLD, respectively. The level of TMA achieved by maximum intensity of BLD was the same as that by maximum intensity of RLHN. The level of TA in BLD treated samples was higher than in RLHN treated ones.

Furthermore, BLD came up to be more effective for *in vitro* production of TA through cell suspension culture than RLHN (Fig. 3). As it can be seen in Figure 4, there are great differences regarding the effects of two intensity levels (67.09 $mWcm^{-2}$, 32.74 $mWcm^{-2}$) of BLD and one intensity level (6.46 $mWcm^{-2}$) of RLHN. Figure 4 shows the depth color pigment or higher anthocyanin production of 67.09 $mWcm^{-2}$ intensity of BLD irradiation higher than all laser treatments.

Several enzymes, including chalcone synthase (CHS) as the first enzyme for anthocyanin production, Phenylalaninamonia Lyase (PAL), Dihydro Flavanol 4-Reductase (DFR), and Chalcone Isomerase (CHI) involved in anthocyanin biosynthesis are activated by light, hence their encoding genes are up-regulated (Feinbaum and Ausubel, 1988; Ohl *et al*., 1989). For example, the photo-control of CHS expression has been reported in petunia (Katz and Weiss, 1998) and DFR has been shown to be induced by red light in *Arabidopsis* (Kubasek *et al*., 1998). In general, the photoreceptor is a switch for controlling the expression of specific genes involved in growth, developmental processes, and secondary metabolism in plants (Jenkins *et al*., 1995; Ahmad *et al*., 1998).

Phytochromes are among the most extensively researched photoreceptors which sense light, and are known to be involved in anthocyanin biosynthesis (Mancinelli *et al*., 1991; Downey *et al*., 2004). Phytochromes respond to red (660 nm) and far-red (730 nm) light, and direct plant gene expression by switching between the red absorbing form (Pr) and the far-red absorbing form (Pfr) (Zhou and

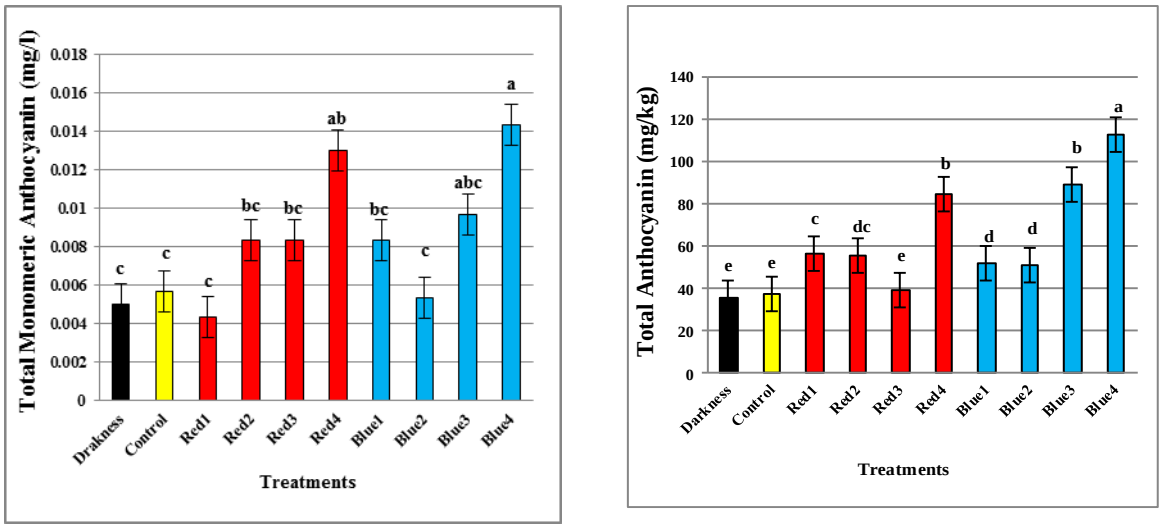

Fig. 3. Effect of blue laser-diode and red laser-He Ne irradiation on the total anthocyanin (TA) and total monomeric anthocyanin (TMA) in cell suspension of apple. TA and TMA anthocyanin measured two weeks after laser irradiation treatments. Darkness and Control represent dark and fluorescent lights, respectively. Red I1 to Red I4 treatments are different levels of irradiation intensity of Red laser-He-Ne and Blue I1 to Blue I4 are different levels of irradiation intensity of blue laser-diode. Means with identical letters do not have a significant difference at P<0.05 based on DNMRT.

Fig. 4. Comparison of anthocyanin production of three treatments including Blue I4 (67.09 mWcm^{-2}) and Blue I3 (32.74 mWcm^{-2}) of blue laser-diode and Red I4 (6.46 mWcm^{-2}) red laser-He Ne with three replications (a, b, c) for each treatment in terms of color intensity or anthocyanin production ratio from cell suspension of apple response to red laser-He Ne and Blue laser-diode irradiation.

Singh, 2004). Kubasek *et al*. (1998) proposed that in *Arabidopsis* seedlings, phytochrome might be involved in red light induced DFR expression. In addition, in *Arabidopsis*, anthocyanin accumulation and gene expression of CHS were enhanced by blue light with cryptochrome identified as the photoreceptor mediating the blue light response (Jackson and Jenkins, 1995; Kubasek *et al*., 1998). The photoreceptors including phytochrome, cryptochrome, and UV-B receptor seem to

function in the photoregulation of flower pigmentation. Meng, *et al*. (2004) has explained that the photoreceptors including phytochrome, cryptochrome, and UV-B receptor appear to play roles in the photoregulation of flower pigmentation of *Gerbera hybrida*.

In our experiment, RLHN and BLD lasers irradiation improved anthocyanin biosynthesis in cell suspension of apple. It can be postulated that BLD and RLHN laser lights have provoked anthocyanin production in apple cells through cryptochrome (for blue irradiation) and phytochrome (for red irradiation) photoreceptors stimulation and lead to higher expression of genes encoding key enzymes, such as CHS, PAL, DFR, and CHI on the anthocyanin biosynthetic pathway. Also, laser irradiation, due to its certain properties, such as single wavelength, focus, and high power, can affect differently than natural light. An alternative mechanism of the effect of laser irradiation on anthocyanin production, therefore, would be acting through stress responsive pathways.

It is plausible that laser irradiation may exert a stress condition and lead to the production of head-shock electrical stress. Laser-induced Stress can fracture tissue, kill cells, decrease cell viability, and increase the permeability of the plasma membrane, since anthocyanin compound induced response to conditional stress comprising high light, lipid peroxidation, and other shock environmental elements. In return, the affected apple cells could promote the synthesis of antioxidants system, such as anthocyanins, to prevent lipid peroxidation damage of cell membrane, reduce the permeability of the plasma membrane, and ultimately overcome the harmful effects of laser-induced stress.

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