Energy Research Journal 1 (2): 189-192, 2010
ISSN 1949-0151

Effect of Elicitors and Precursors on the Synthesis of Anthocyanin in Grape *Vitis vinifera* Cell Cultures

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Abstract: Problem statement: Nowadays, plant cell suspension cultures and immobilized cells are being utilized for the higher yield and quality of the products than extraction of whole plants. Anthocyanins are compounds found in plants that have powerful antioxidant properties. They also provide some of the coloring or pigment of plants, flowers and fruits. Anthocyanin from grape cell cultures can be used as a natural alternative to synthetic dyes, particularly in light of their various health-promoting properties. To enchance the production of anthocyanin in grape (*Vitis vinifera*) cell culture, the effects of elicitation and precursor feeding on the colorant production were investigated. **Approach:** In this study, salicylic acid and ethephon were used as elicitors and, phenylalanine and skhikimic acids were used as precursors to improve the productivity of useful metabolites for archiving high concentration in *Vitis vinifera* suspension cultures. When the cells were elicitated with 50 µL/25 mL suspension salyclic acid, the anthocyanin concentration was increased to 0.03 µg mg^{-1} in 18 days culture as compared to that in the unelicitated cells. **Results:** As for precursors, both of the shikimic acid and phenylalanine could promote the synthesis of anthocyanin in the grape cell cultures. After 18 days of the treatment with shikimic acid, it had shown that the anthocyanin concentration in the treatment was 0.05 µg mg^{-1} more than the control in the cell cultures. **Conclusion:** In the cell cultures with phenylalanine showed that anthocyanin synthesis was 0.03 µg mg^{-1} higher than that of without phenylalanine.

Key words: Anthocyanin, elicitors, precursors, *Vitis vinifera*

INTRODUCTION

Most important food colors and flavors are of plant origins and these compounds can be obtained from field grown plants. The four groups of compounds comprising the food colors include benzopyrans (anthocyanins, flavones and flavonones), betalains (betacyanin and betaxanthines), carotenoides (carotenones and xanthophylls) and chlorophylls (Bridle and Timberlake, 1997). Among them, anthocyanins are the most prominent among the flavonoids and they are a class of secondary metabolite that occur across the plant kingdom and are responsible for the variety of red to purples that are seen in flowers, fruits and autumn leaves. These pigments are extracted for commercial use from material such as grape pomace, red cabbage and sweet potato (Fauconneau *et al*., 1997) and are used to color foods and beverages. They are desirable as a natural alternative to synthetic dyes, particularly in light of their various health promoting properties (Dörnenburg and Knorr, 1996).

Plant cell and tissue cultures can be used for the synthesis and production of secondary metabolites like colors, flavors and sweeteners (Kieran *et al*., 1997). However, the industrial application of plant cell suspension cultures has, to date, been limited (Hirasuna *et al*., 1991). Plant tissue culture is an expensive methodology and its application may only be warranted for products of high value. One of the difficulties of industrial production by plant cell culture is the low productivity caused by low concentration of the secondary metabolites. To enhance the productivity, optimization of medium composition or addition of enhancer such as elicitors and precursors has been investigated (Amakawa *et al*., 1983; Sada and Shuler, 1989; Aoyagi *et al*., 1996).

Secondary metabolite synthesis in the plant cell tissue cultures can be targeted by the application of physical, chemical and biological elicitors. The elicitors mimic the effects of stresses and thereby activate the plant biochemical system, which results in increased contents of secondary metabolites in plant tissues. The

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other possibility for increasing the secondary metabolite synthesis is the supplying of plants or their cultures with the precursors.

Taking this approach, we have already known that jasmonic acid and light irradiation elicit increased accumulation of anthocyanins in *Vitis vinifera* cell cultures (Zhang *et al*., 2002). This study is concerning with the technology of receiving anthocyanin from *Vitis vinifera* cell cultures by using precursors and elicitors. We have to investigate the optimum precursor or elicitor that can enhance the anthocyanin accumulation in this *V. vinifera* cell cultures.

MATERIALS AND METHODS

Plant material: The cells cultures of *Vitis vinifera* are maintained in the suspension of B5 medium supplemented with phytohormones (NAA 0.1 mg L^{-1}, kinetin 0.2 mg L^{-1} and casein hydrolysate 0.25 g L^{-1}). The suspension cultures were initiated by transfer of callus tissues into the liquid medium. Cells were subcultured in the same medium every 2 weeks and the cultures are maintained at 25±2°C on a rotary shaker under light irradiation.

Preparation for elicitation: In this research, salicylic acid and ethephon were added into the suspension cultures as elicitors. Phenylalanine and shikimic acid were used as precursors for the cell cultures. They were sterilized through 0.22 µm milipore filters and added at the time of inoculation to give the concentration of 50 µL/25 mL suspension culture. The cultures were grown at 25±2°C under continuous white fluorescent light irradiation on a rotary shaker.

Sample collection: Each of elicitors and precursors is added to the *Vitis vinifera* suspension cultures in triplicate. Cultures without elicitors and precursors were also included as control group. Biomass was evaluated as Dry Cell Weight (DCW) of cells in vacuum at room temperature. Sampling was performed on 2, 4, 7, 10, 15 and 18 days after addition of the elicitors and precursors.

Cell growth and analysis of anthocyanin content: The growth of cell cultures was measured by determining Dry Cell Weight (DCW). Anthocyanins were extracted quantitatively from fresh filter dried suspension cells. Each 100 mg of sample is dissolved with 750 µL of solution (70% Ethanol and 1% CH_3COOH) at 85°C for 20 min. Extracts were clarified by centrifugation at 13000 rpm for 5 min. The supernatants are collected and the pellets were dissolved with the above solution and repeated these steps for next two times. The collected supernatant are added with 50 µL of Hydrochloric acid (37% v/v) and placed in the dark incubator for 10 min. The sample is diluted with 1:1 (v/v) with the solution (70% Ethanol and 1% CH_3COOH). Absorbance at 535 nm was recorded against the buffer and the anthocyanin content was calculated by:

$$\left[\frac{E\times MG\times VP}{\varepsilon\times 10\times d}\right]\times\left[\frac{\text{Re rence weight(g)}}{\text{Input(g)}}\right]\times\left[\frac{100\%}{TG\%}\right] \quad (1)$$

Where:

E = Extinction
MG = Molecular weight of anthocyanin (445.2 g moL^{-1})
VP = Dilution factor
ε = Extinction coefficient (98.2)
d = Distance (cm) of the tube
TG% = Percentage of fresh weight to dry weight

RESULTS AND DISCUSSION

Effect of salicylic acid on the growth of fresh cell weight and anthocyanin content in *Vitis vinifera* suspension culture: By addition of salicylic acid to the cell cultures, the Fresh Cell Weight (FCW) went up dynamically. Although it increases day by day, the FCW of the treated sample was less than that of the control one. The maximum fresh cell weight reached at day 15 (Fig. 1). The anthocyanin concentration in salicylic acid treated sample is not different from the control one during the first 7 days and it is lower on day 10, but anthocyanin synthesis increase again and day 18 is the optimum (Fig. 2). It seems anthocyain synthesis increase when there is no more cell growth.

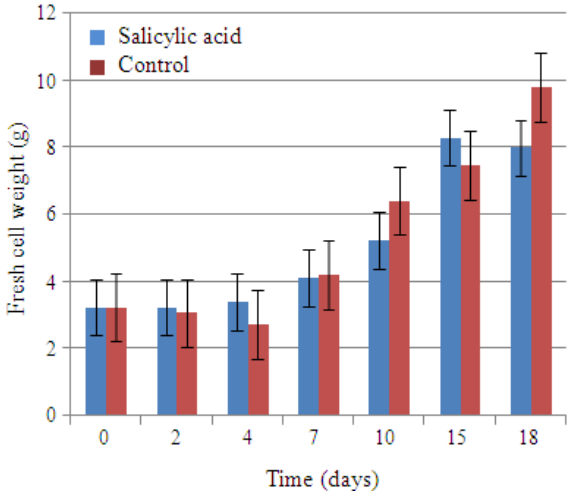

Fig. 1: Effect of salicylic acid on the fresh cell weight of *Vitis vinifera*

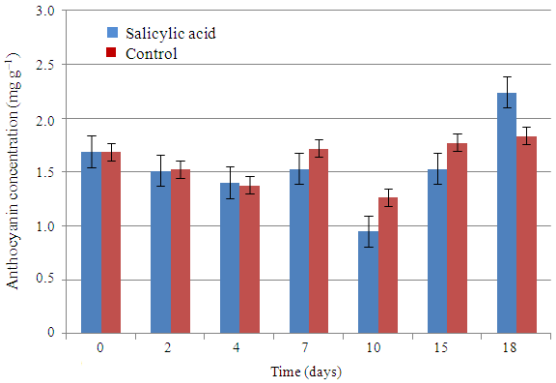

Fig. 2: Effect of salycylic acid on the synthesis of anthocyanin in *Vitis vinifera* suspension culture

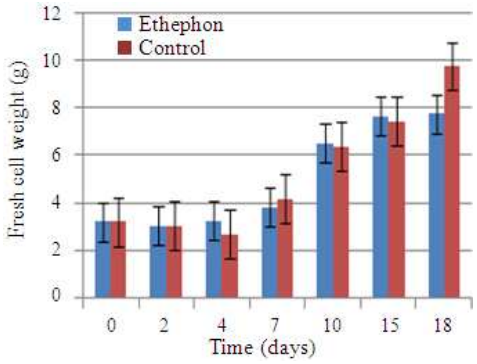

Fig. 3: Effect of ethephon on the fresh cell weight of *Vitis vinifera*

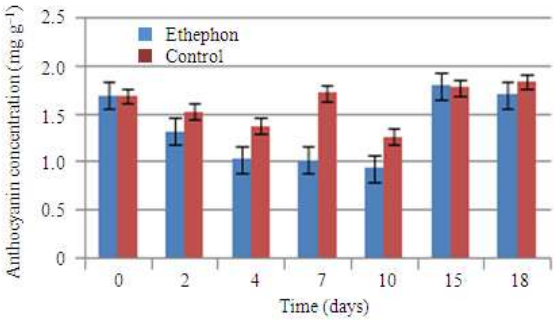

Fig. 4: Effect of ethephon on the synthesis of anthocyanin in *Vitis vinifera* suspension culture

Effect of ethephon on the growth of fresh cell weight and anthocyanin content in *Vitis vinifera* suspension culture: The effect of ethephon on the fresh cell weight in grape callus culture is shown in Fig. 3. Although the growth rate of ethephon treated sample is lower than that of the control one, it increases day by day. The anthocyanin concentration increased gradually and it reached to the maximum level on day 18 (Fig. 4).

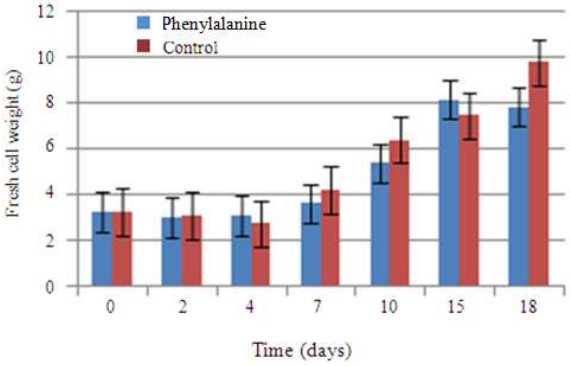

Fig. 5: Effect of Phenylalanine on the fresh cell weight of *Vitis vinifera*

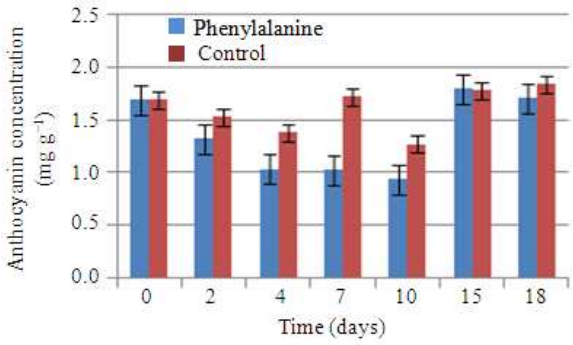

Fig. 6: Effect of phenylalanine on the synthesis of anthocyanin in *Vitis vinifera* suspension culture

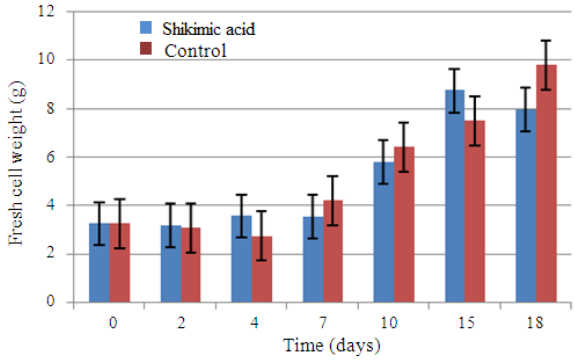

Fig. 7: Effect of shikimic acid on the fresh cell weight of *Vitis vinifera*

Effect of phenylalanine on the growth of fresh cell weight and anthocyanin content in *Vitis vinifera* suspension culture: Figure 5 shows the effect of phenylalanine on the fresh cell weight of *Vitis vinifera* during 18 days. The addition of phenylalanine could not affect the fresh cell weight significantly. The anthocyanin synthesis even decrease during first 10 days but it is increased again between 15-18 days (Fig. 6).

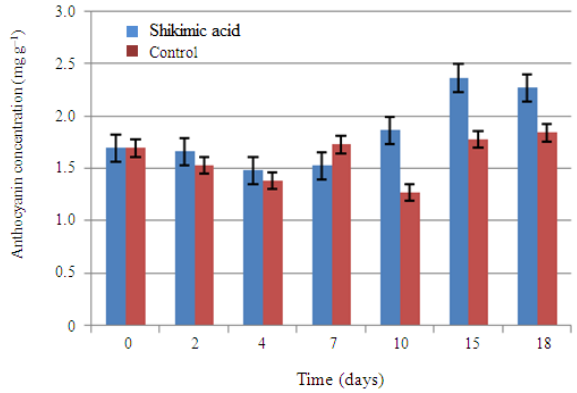

Fig. 8: Effect of shikimic acid on the synthesis of anthocyanin in *Vitis vinifera* suspension culture

Effect of shikimic acid on the growth of fresh cell weight and anthocyanin content in *Vitis vinifera* suspension culture: The growth of fresh cell weight in the samples of shikimic acid treatment was not much different from the above treatments. It increased day by day until Day 15 and the starting of death phase on Day 18 (Fig. 7). The results in Fig. 8 showed that shikimic acid could enhance the synthesis of anthocyanin in *Vitis vinifera* suspension cultures.

CONCLUSION

This study was focused on the effect of elicitors and precursors on the growth of fresh cells and the synthesis of anthoyanin in grape (*Vitis vinifera*) suspension culture. During the growth of 18 days, the optimum cell weigh was on day 15 in every treatment but the growth from the control one continues until day 18. By using elicitors, the results showed that salyclic acid and ethephon could enhance the anthocyanin synthesis in the cells. Althogh phenylalanin had less effect on the anthocyanin concentration, shikimic acid has potential effect on the synthesis of anthocyanin.

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