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Stimulation of anthocyanin synthesis in grape (*Vitis vinifera*) cell cultures by pulsed electric fields and ethephon

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Abstract Anthocyanin from grape cell cultures can be used as a natural alternative to synthetic dyes; particularly due to their reported health-promoting properties. In this study, production of anthocyanin in cell suspension culture of *Vitis vinifera* was evaluated following treatment with either ethephon and/or pulsed electric fields (PEF). Overall, total production of anthocyanin increased in treated cells compared to untreated cells. Treatment of cell suspension with PEF at day 14 of culture resulted in 1.7-fold increase (1.42 mg/g DW) in anthocyanin content when compared to control cells; while, treatment with ethephon resulted in 2.3-fold increase (1.99 mg/g DW) in anthocyanin content. When cells were treated with both ethephon and PEF, 2.5-fold increase in anthocyanin content (2.2 mg/g DW) was observed. These findings demonstrate that PEF induces a defense response in plant cells, and it may also alter the dielectric properties of cells and/or cell membranes, and would serve as a viable elicitor of secondary metabolites in plant cell cultures.

Keywords Anthocyanins · *Vitis vinifera* · Elicitors · Ethephon · Pulsed electric fields · Cell culture

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Introduction

The use of colour agents as additives in foods and drinks is a significant factor influencing food manufacturers and customers in their choice of food processing methods (Spears 1988). Food colouring agents can be produced from either artificial or natural sources. Anthocyanins are natural pigments responsible for the colour of many fruits and vegetables and range in colour from blue to red. Commercially, these pigments are extracted from materials containing copious amounts of anthocyanins, such as grape skin, red cabbage, and sweet potatoes (Fauconneau et al. 1997). Similar to a variety of fruits, vegetables, and ornamental plants, grapes (*Vitis vinifera*) contain anthocyanins that range in colour from pink through red and violet or dark blue (Margarita et al. 2009).
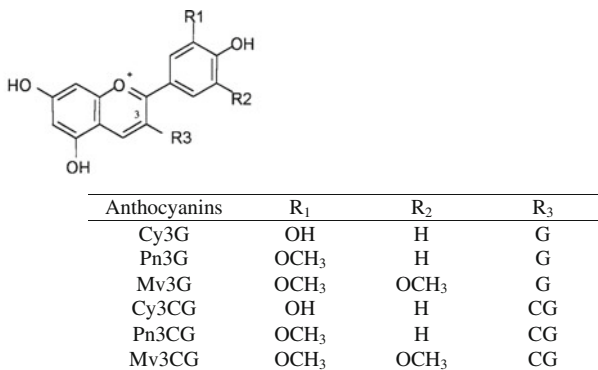
Grapes contain phenolic compounds, including flavonoids and non-flavonoids. The most abundant classes of flavonoids include the flavan-3-ols, anthocyanins, and flavonols, whereas the most abundant class of non-flavonoids include the hydroxycinnamates (Yamakawa et al. 1983). The major anthocyanins (Fig. 1) present in *V. vinifera* cell culture are cyanidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside, and acylated anthocyanins (cyanidin 3-*p*-coumaroyl glucoside, peonidin 3-*p*-coumaroyl glucoside, and malvidin 3-*p*-coumaroyl glucoside) (Curtin et al. 2003).

The quality of the seasonal plant varies depending on the growth conditions and geographic location. Hence, the production of natural colours and pigments using biotechnology has received much attention over the last decade. Plant cell and tissue culture methods have replaced the conventional plant-growing techniques. The accumulation of colours and pigments in cell culture can be facilitated by the direct selection of high-producing cell lines (Sada and

Anthocyanins	R₁	R₂	R₃
Cy3G	OH	H	G
Pn3G	OCH₃	H	G
Mv3G	OCH₃	OCH₃	G
Cy3CG	OH	H	CG
Pn3CG	OCH₃	H	CG
Mv3CG	OCH₃	OCH₃	CG

Fig. 1 Structures of the major anthocyanins in *V. vinifera* suspension culture. Residues: hydrogen (H), hydroxyl (OH), methyl (OCH3), *p*-coumaric acid (C), glucose (G). Full compound names: cyanidin 3-glucoside (Cy3G), peonidin 3-glucoside (Pn3G), malvidin 3-glucoside (Mv3G), cyanidin 3-*p*-coumaroylglucoside (Cy3CG), peonidin 3-*p*-oumaroylglucoside (Pn3CG), malvidin 3-*p*-coumaroylglucoside (Mv3CG)

Shuler 1989). For anthocyanin production, the new sources such as from *Cleome rosea* (Simoes-Gurgel et al. 2011), *Rosa hybrida* L. (Ram et al. 2011), *Crataegus sinaica* (Maharik et al. 2009) and *Melastoma malabathricum* (See 2008), cell cultures have been established in recent years. Several methods have been used to improve the production of secondary metabolites in plant cell culture systems, i.e., elicitation (Knorr et al. 1994), two phase culture (Brodelius and Pedersen 1993), hairy root culture (Choi et al. 1995), immobilization (Toivnen 1993) and DNA methylation (Baranek et al. 2010; Kiselev et al. 2011). Among them, elicitation is the most widely method to improving secondary metabolite production.

Secondary metabolite synthesis in plant cell and tissue culture can be targeted by the application of physical, chemical, or biological elicitors called stress factors. The elicitors mimic the effects of stresses and thereby activate the plant biochemical system, which results in increased secondary metabolite production in plant tissues. The stimulation of anthocyanin accumulation by elicitors has also been induced in *V. vinifera* cell culture by osmotic stress (Do and Cormier 1991), jasmonic acid, and light irradiation (Curtin et al. 2003), as well as in in vitro cultures of other species, such as in *Cocos nucifera* by chitosan (Chakraborty et al. 2009), in *Daucus carota* by methyl jasmonate (Sircar and Mitra 2008), in *Hypericum perforatum* L. by jasmonic acid (Gadzovska et al. 2007) and guggulsterone production in *Commiphora wightti* cell cultures by plant gums (Dass and Ramawat 2009). Using this approach, we have previously demonstrated that ethephon treatment increased the accumulation of anthocyanins in *V. vinifera* cell cultures (Saw et al. 2010). In this study, we explore the effects of ethephon and pulsed electric fields (PEFs) on anthocyanin synthesis and propose a method to improve pigment quality.

Most of the old techniques used in food preservation and juice extraction are based on heating and chemical treatment. A non-thermal food preservation method, PEF processing, uses short bursts of electricity to inactivate microbes. In PEF processing techniques, food is placed between two electrodes, and high-voltage pulses in the order of 20–80 kV are generated (usually for a couple of microseconds). The electric field generated by the applied high voltage inactivates microbes. The applied external electric field induces an electric potential across a cell membrane that results in a charge separation in the membrane. PEFs can be used to enhance the extraction efficiency of intracellular metabolites because of their specific effect on the cell membrane. For this reason, the PEF method replaced older techniques in the food industry (Zimmermann 1986; Brodelius et al. 1988; Knorr 1999; Knorr and Angersbach 1998). When the transmembrane potential exceeds a critical value, the repulsion between charge-carrying molecules results in the formation of pores in the cell membrane (Brodelius et al. 1988). Electropermeabilisation has also been used to release secondary metabolites from plant tissue cultures, while attempting to maintain cell viability (Hunter and Kilby 1988).

PEF has been reported to enhance taxuyunnanine C production in suspension cultures of *Taxus chinensis* (Ye et al. 2004), facilitate the permeabilisation of plant membranes, and induce the breakage of the cells and tissues of food materials (Töpfl 2006). In this study, we investigated the effects of PEFs and ethephon as elicitors of anthocyanin production in *V. vinifera* cell cultures. The dynamic profiles of cell concentration and medium conductivity were also analysed to better understand the possible mechanism of PEF-induced secondary metabolism in plants.

Materials and methods

Plant cells and culture conditions

The cell culture of *V. vinifera* L. cv. Gamay Freaux was originally obtained from Francois Cormier (Food Research and Development Centre, Agriculture Canada), and it has been continuously maintained in the Department of Food Biotechnology and Process Engineering of the Berlin University of Technology. The cell culture was cultivated on B5 medium with 0.1 mg/L 1-Naphthaleneacetic acid(-NAA), 0.2 mg/L kinetin, 0.25 g/L casein hydrolysate, 3% sucrose, and 0.8% agar. Callus cultures were transferred every 28 days to fresh solidified sterile medium. Red-pigmented cell aggregates were selected preferentially. Cell suspension cultures were established by transferring cell aggregates into 50 mL of liquid B5 medium in 200-mL

Erlenmeyer flasks and continuously agitating the flasks on a rotary shaker at 110 rpm. The cell cultures were transferred to new medium every 3 weeks. *V. vinifera* cell cultures were maintained at $25 \pm 2°C$ and under 12-h light: 12-h dark lighting conditions. The inoculum size was approximately 100 g fresh weight/L of the medium.

Chemicals

The chemicals and reagents used in this research were analytical grade and purchased from Sigma (St. Louis, MO, USA).

Preparation for elicitation and sampling

In this study, ethephon was added into the suspension cultures as an elicitor. It was sterilised through 0.22-μm Millipore filters and added at the time of inoculation to give a concentration of 50 μL/25 mL of suspension culture. PEF treatments were performed in treatment chambers that were autoclaved before usage to achieve sterile conditions. The sample was treated in a batch parallel-electrode treatment chamber with a distance of 5 cm between electrodes. The PEF treatment consisted of 10 monopolar exponential-decay pulses at an electric field strength of 1.6 kV/cm and a frequency of 1 Hz, which corresponds to a total specific energy of 0.32 J/kg at room temperature, on the inoculation day. All experiments were performed in triplicate. Sampling was performed 1, 4, 7, 9, and 14 days after the ethephon and PEF treatments. Each treatment of the *V. vinifera* suspension cultures with ethephon and PEF was repeated three times. Untreated cell cultures were included as a control group.

Determination of cell growth, pH, and conductivity

The growth of cell cultures was measured by determining the fresh weight (FW), pH, and conductivity of the medium. The biomass was evaluated as the FW of cells after filtering under vacuum for 1 min at room temperature. The pH of the growth medium was determined using a pH meter (CG811; Schott Geräte GmbH, Hofheim, Germany), and the conductivity of the growth medium was measured using a conductivity meter (WTW LF 323; Weiheim, Germany).

Anthocyanin content analysis

Anthocyanins were extracted from 100 mg fresh cells, after removing the medium with a suction filter. To each sample, 750 μL of 79% (v/v) ethanol with 1% (v/v) glacial acetic acid (extraction solvent) was added, and samples were incubated in a heat block at 85°C for 20 min. After

centrifugation at 13,000 rpm for 5 min, the supernatants were collected, and the pellets were re-extracted with 600 μL of extraction solvent twice. Supernatants were combined, and 50 μL of 37% (v/v) hydrochloric acid was added to stabilise the anthocyanins. After 10-min incubation in the dark at room temperature, the sample was diluted 1:1 (v/v) with the extraction solvent. Total anthocyanin content was determined by the absorbance recorded at 535 nm using $\varepsilon = 98.2$ (dilution factor = 2). For compound identification, the extracts were analysed by HPLC, according to a previously reported method (Mewis et al. 2010).

Statistical analysis

All analyses were performed in triplicate, and data were reported as the mean ± standard deviation (SD). Data were subjected to analysis of variance (ANOVA) ($P < 0.05$) followed by the post hoc test Tukey's honestly significant difference (HSD) in the statistic program Excel (Microsoft Office 2007) and SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Effects of ethephon on cell growth and anthocyanin synthesis

To investigate the effects of ethephon addition on cell growth and anthocyanin production from *V. vinifera* suspension cultures, the medium containing ethephon at a concentration of 50 μL/25 mL of suspension culture was prepared, and the cells were cultivated. The results are shown in Fig. 2. During cultivation, the fresh cell weight (FCW) increased dynamically. Although it increased daily, the FCW of the treated sample was less than that of the control samples.
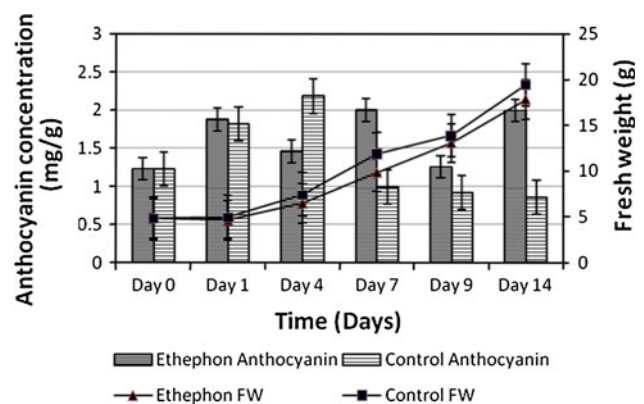
Fig. 2 Effects of ethepon on cell growth and anthocyanin synthesis

The anthocyanin concentration in the ethephon-treated sample was 33% lower than that in the untreated sample on day 4, and the anthocyanin content in the treated sample was approximately twofold higher than that of the control culture on day 7 (Fig. 2). On day 14, anthocyanin production also increased and reached its maximum value (2 mg/g DW), which was 2.3-fold higher than that of the control culture. Over the 2 weeks of cultivation, the average anthocyanin content was 1.5-fold higher in the ethephon-treated samples than in the control samples.
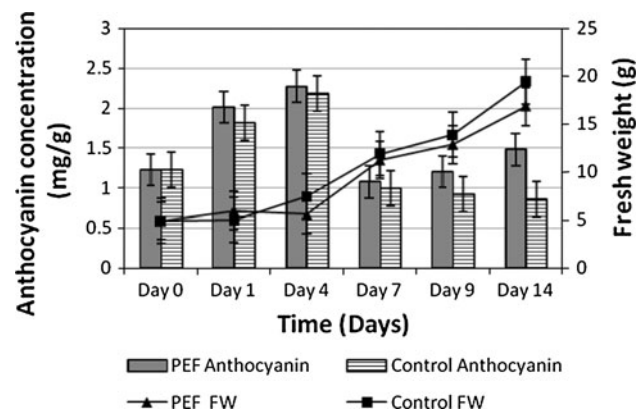
Effects of PEF treatment on cell growth and anthocyanin synthesis

The effect of PEFs on cell growth and anthocyanin synthesis in grape callus culture is shown in Fig. 3. Although the growth rate of the PEF-treated samples was lower than that of the control samples, it increased daily. The growth rate of the cells in the PEF-treated samples was 1.2-fold higher than that in the control sample on day 1. The average growth rate of the PEF-treated samples was 7% lower than that of the untreated samples.

The maximum anthocyanin synthesis in the PEF-treated samples occurred on day 4 (2.3 mg/g DW), and it decreased after day 4 in both the PEF-treated and untreated samples. On average, the anthocyanin content in the PEF-treated samples was 1.25-fold larger than that in the untreated samples. By day 14, the anthocyanin concentration had decreased by approximately 50% in both the treated and untreated samples.

Effects of the combined ethephon and PEF treatment on cell growth and anthocyanin synthesis

The combination effects of ethephon and PEF treatment on cell growth and anthocyanin synthesis in the *V. vinifera* suspension cultures were investigated in this study, and the results are shown in Fig. 4. Compared to the FCWs recorded after the individual treatments, the FCW after the combined treatment was unchanged. Although the FW of the treated samples increased approximately fourfold in 2 weeks, it was slightly lower (10%) than that of the control samples. Thus, the combined PEF and ethephon treatment did not affect cell growth as compared to the growth of the control.

Regarding the anthocyanin concentration, the graph illustrates that the anthocyanin concentration decreased in the treated samples on day 7, after which it increased. In the combination treatment samples, maximum anthocyanin synthesis (2.2 mg/g DW) was observed on day 14. The production was 2.5-fold higher than that in the control samples.

The anthocyanins present in *V. vinifera* cell culture were identified according to the method of Mewis et al. An HPLC chromatogram of the anthocyanins is shown in Fig. 5. The major anthocyanin monoglucosides present in *V. vinifera* cell cultures were cyanidin 3-*O*-glucoside and peonidin 3-*O*-glucoside, and the major cinnamoyl derivatives were cyanidin 3-*O*-*p*-coumaryl glucoside and peonidin 3-*O*-*p*-coumarylglucoside. Three minor anthocyanin compounds were found: delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and delphinidin 3-*O*-*p*-coumaryl glucoside (Mewis et al. 2010).

Effects of ethephon and PEF treatment on the pH and conductivity of the culture medium

To investigate the effects of ethephon and PEF treatment on the *V. vinifera* suspension cultures, the pH and conductivity of the culture medium were measured, and the results are shown in Table 1.

The treatments did not apparently affect the pH value of the medium because there was no significant difference in pH between the treated samples and controls. However,

Fig. 3 Effects of PEF treatment on cell growth and anthocyanin systhesis
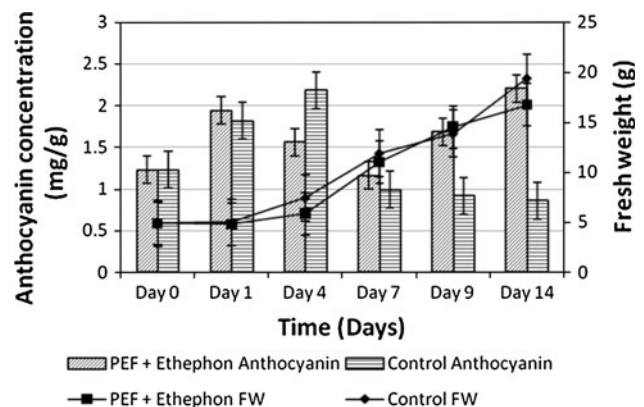
Fig. 4 Effects of the combined ethephon and PEF treatment on cell growth and anthocyanin synthesis

Fig. 5 a HPLC chromatogram of anthocyanins from *V. vinifera* cell culture. *Peak 1*: delphinidin-3-*O*-glucoside (10.5 min), *Peak 2*: cyanidin-3-*O*-glucoside (10.8 min), *Peak 3*: petunidin-3-*O*-glucoside (11.0 min), *Peak 4*: peonidin-3-*O*-glucoside (11.5 min), *Peak 5*: delphinidin-3-*O*-p-coumarylglucoside (13.6 min), *Peak 6*: cyanidin-3-*O*-p-coumarylglucoside (14.3 min), *Peak 7*: peonidin-3-*O*-p-coumarylglucoside (14.9 min). **b** HPLC chromatogram of phenolic acids from *V. vinifera* cell culture. *Peak 1*: *p*-coumaric acid (15.2 min), *Peak 2*: 3-*O*-glucosyl-resveratrol (18.1 min), *Peak 3*: 4-(3,5-dihydroxy-phenyl)-phenol (19.1 min), *Peak 4*: phenolic acid derivate (19.9 min)
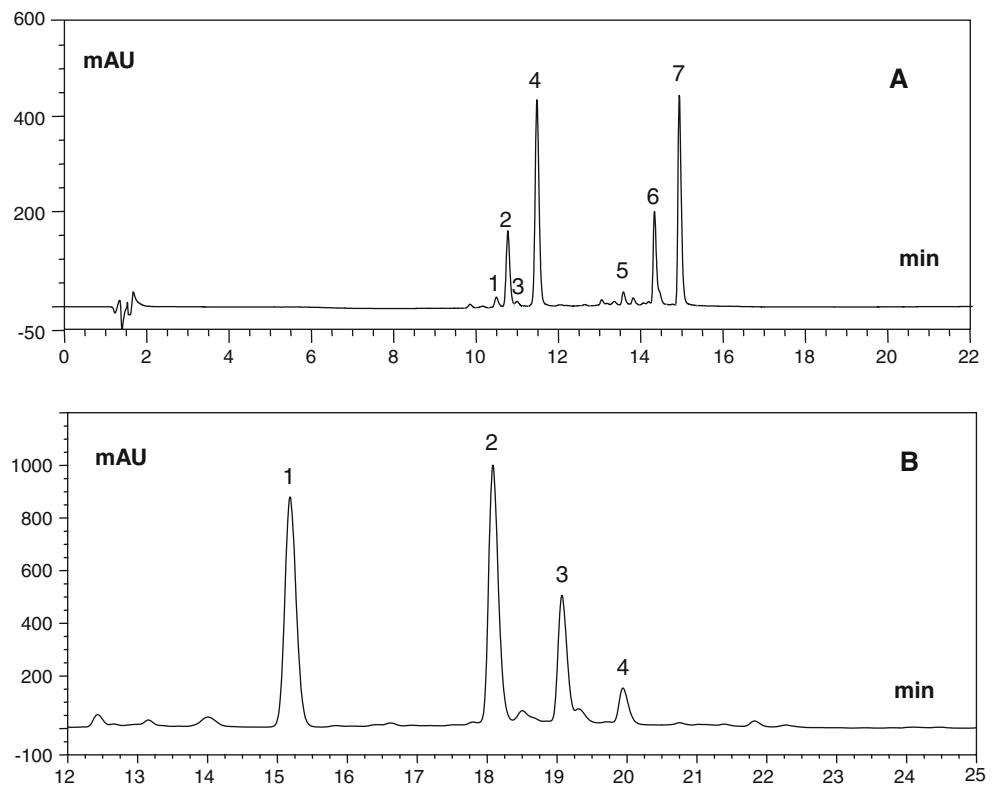
Table 1 The pH and the conductivity of the medium

Sample	Day	pH	Conductivity (mS/cm) 21°C
Ethephon	Day 1	5.98 ± 0.07^a	2.65 ± 0.21^b
	Day 4	6.12 ± 0.03^b	2.42 ± 0.22^b
	Day 7	6.30 ± 0.15^b	$2.01 \pm 0.28^{a,b}$
	Day 9	6.12 ± 0.36^b	1.92 ± 0.37^a
	Day 14	6.89 ± 0.10^c	
	Day 14	6.89 ± 0.10^d	0.65 ± 0.08^a
PEF	Day 1	6.12 ± 0.16^a	2.33 ± 0.12^d
	Day 4	$6.21 \pm 0.23^{a,b}$	2.42 ± 0.54^d
	Day 7	6.14 ± 0.06^a	1.70 ± 0.05^c
	Day 9	6.40 ± 0.06^b	1.37 ± 0.01^b
	Day 14	6.69 ± 0.07^c	0.54 ± 0.03^a
PEF + ethephon	Day 1	5.19 ± 0.16^a	2.83 ± 0.21^e
	Day 4	6.12 ± 0.05^b	2.53 ± 0.04^d
	Day 7	$6.20 \pm 0.10^{b,c}$	1.83 ± 0.11^c
	Day 9	6.38 ± 0.03^c	1.49 ± 0.18^b
	Day 14	6.54 ± 0.05^d	0.62 ± 0.03^a
Control	Day 1	5.90 ± 0.16^a	2.60 ± 0.10^d
	Day 4	5.99 ± 0.11^a	1.82 ± 0.32^c
	Day 7	6.13 ± 0.04^b	$1.69 \pm 0.07^{b,c}$
	Day 9	6.37 ± 0.22^c	1.36 ± 0.39^b
	Day 14	6.67 ± 0.13^c	0.60 ± 0.12^a

The values are mean ± standard deviation. Differences are compared for each treatment and for each sampling day. Mean values followed by different lower-case letter in each column and for each treatment are significantly different ($P \leq 0.05$) by HSD and means with the same letters within a column are not significantly different from each other at $P \leq 0.05$

both the pH values in the ethephon and PEF treated samples were dynamically raised concerning with the increased cell growth.

Discussion

Our study showed that grape suspension cultures exposed to PEF and ethephon exhibited higher anthocyanin synthesis than non-treated cell cultures. In this study, fresh medium was used, and ethephon treatment was performed immediately after sub-cultivation of the cell cultures. Among the precursors and elicitors used in our previous study (Saw et al. 2010), ethephon was found to optimally enhance anthocyanin synthesis. Many studies have demonstrated that anthocyanin composition is related to ethephon treatment. Ethephon caused an enhancement of the red peel colour of 'Fuji' apples and alterations in the anthocyanin composition of 'Starking' apples, and the anthocyanin accumulation pattern was altered by different treatments (Gomez-Cordoves et al. 1996; Li et al. 2002). Additionally, in 'Jonagold' apple skin, ethephon application greatly increased anthocyanin accumulation, but the dependence of anthocyanin synthesis on ethylene was complicated (Awad and Jager 2002). According to our results, anthocyanin content decreased after day 4 in the control samples, whereas it increased in the ethephon-

treated samples on days 7 and 14 (2- and 2.3-fold, respectively). Therefore, our study confirmed that ethephon had a strong effect on the synthesis of anthocyanin in the cells.

In the cell suspensions of *V. vinifera*, anthocyanin synthesis occurred early during the cell cycle because dihydroflavonol reductase (DFR) activity and anthocyanin synthesis were correlated during this period. Therefore, treatments that greatly increase DFR activity, such as phosphate deprivation, enhance anthocyanin synthesis in cell cultures of *V. vinifera* (Dedaldechamp et al. 1995). PEF was more effective in plant cells in the early exponential phase (Ye et al. 2004), and the cells in the exponential growth phase were more sensitive to PEF treatment than those in the stationary stage (Rodrigo et al. 2003). This is because the membrane properties of microorganisms vary in different growth stages, and in the exponential growth phase, the membrane zone between the mother cell and its progeny is very sensitive to PEF (Barbisa-Conovas et al. 1999). In our study, anthocyanin content peaked on day 4 after PEF treatment, but it decreased by 50% of the day 4 amount on day 14. However, PEF stimulated anthocyanin production in the treated cells, especially on days 9 and 14. Although the total anthocyanin concentration was not significantly affected by PEF and ethephon treatments, the percentage composition might be altered. In the control, the concentrations of peonidin-3-*O*-*p*-coumaryl glucoside, peonidin-3-*O*-glucoside, and cyanidin-3-*O*-*p*-coumaryl glucoside were high, whereas those of delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside were relatively low. Ethephon had a positive effect on cyanidin-3-*O*-*p*-coumaryl glucoside, peonidin-3-*O*-glucoside, and peonidin-3-*O*-*p*-coumaryl glucoside production (data now shown).

Cell growth was also measured to investigate the effects of PEF and ethephon treatment. Excluding day 9, no significant changes in cell growth were observed, and the largest biomass accumulation was recorded on day 14. This is because the cells were adapting themselves to the new environment after inoculation, and the application of elicitation in the lag phase severely inhibited cell growth (Dong and Zhong 2001). However, this may vary among different cell lines. Even though Ye et al. used the same *Taxus chinensis* cell line as Dong et al., they found that elicitation in the early exponential phase was more effective than that in later phases. It was previously reported that the growth of *V. vinifera* cell culture was not affected by elicitation and precursor feedings (Saw et al. 2010). According to the results of this study, PEF and ethephon did not affect cell growth either alone or in combination. Our study confirmed the previous findings that ethephon was neither inhibitory nor stimulatory on cell growth (Biddington 1992).

The structure and colours of anthocyanins are affected by pH (Gross 1987). The accumulation of anthocyanins in cell cultures is thus regulated by pH. Zhang and Furusaki (1997) reported that over a pH range of 3.7–8.7, the initial alkaline pH of 8.7 was optimal for anthocyanin production in strawberry cell suspension culture. Therefore, the initial pH value for the *V. vinifera* cell culture in this study was approximately to be 6 of the growth curve. The pH values of all samples tended change in a similar manner. The pH values of the PEF- and ethephon-treated samples on day 9 were slightly reduced. Otherwise, the pH values increased as the cultivation progressed and peaked on day 14. Because the pH value of the growth medium was within the optimal range for *V. vinifera* cell cultures, the treatments did not affect the pH or consequently anthocyanin synthesis.

When the conductivity of the medium was investigated, it was found to be directly proportional to the concentration of ionic components such as nitrates, suggesting that the cellular uptake rate of ionic constituents can be consistently correlated with the cell growth rate (Ryu et al. 1990; Hahlbrock 1975; Hahlbrock et al. 1974). In our study, a clear linear correlation between the decrease in conductivity and the increase in FCW was found in PEF-treated samples. Pavlov et al. (2004) found that the intensive growth of *Beta vulgaris* hairy root culture and biosynthesis of betalains were correlated with a decrease in conductivity, particularly, due to limited amounts of ammonium ions and low levels of nitrate ions in the medium. There was a slight increase in conductivity after 4 days of PEF treatment, indicating that the dielectric properties of the cell membrane may have been altered by PEF treatment. In a previous study, the final increase in conductivity was directly proportional to the number of permeabilised cells of onion tissue after PEF treatment (Dedaldechamp et al. 1995). In our experiments, changes in the dielectric properties of the cell membrane may have been related to the permeability of the cell membrane and transfer of genetic material across it. The data revealed possibilities to optimise the composition of the nutrient medium to enhance anthocyanin biosynthesis in future studies.

Conclusion

In conclusion, our study revealed that the application of PEFs has the potential to stimulate anthocyanin synthesis in *V. vinifera* suspension culture. PEF treatment can be optimised to enhance the production of the desired secondary metabolites without negative effects on plant cell growth. The combination of PEF and ethephon treatment has demonstrated effectiveness in improving secondary metabolite production. The findings suggest that PEF, as an

external stimulus or stress, can be used as a novel abiotic elicitor to improve secondary metabolite production in plant cell cultures. The mechanism by which cultured cells sense PEF and ethephon elicitation and transfer the signal to induce secondary metabolism is unclear, and the expression of genes regulating anthocyanin production should be investigated further.

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