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Effects of Pulsed Electric Field on Secondary Metabolism of *Vitis vinifera* L. cv. Gamay Fréaux Suspension Culture and Exudates

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Abstract Plant cell cultures provide a large potential for the production of secondary metabolites. Through the application of different physical and chemical cell stress factors, we investigated the production of the secondary metabolites in plant cell cultures. The effects of pulsed electric field (PEF) and ethephon on growth and secondary metabolism, particularly anthocyanins and phenolic acids synthesis, were investigated by using suspension culture of *Vitis vinifera* L. cv. Gamay Fréaux as a model system. Anthocyanins were measured by spectrophotometer and extracellular phenolic acids were determined by high-performance liquid chromatography. The compounds were identified by liquid chromatography–mass spectrometry and nuclear magnetic resonance. After the treatments with PEF and ethephon, the concentrations of anthocyanins and phenolic acids in cell culture were higher than in the control, without loss of biomass. The combination of PEF treatment and ethephon improved secondary metabolites formation. Production levels of extracellular phenolic acids, 3-*O*-glucosyl-resveratrol were increased by PEF and ethephon treatments. The results show that PEF induced a defense response of plant cells and may have altered the cell/membrane's dielectric properties. PEF, an external stimulus or stress, is proposed as a promising new abiotic elicitor for stimulating secondary metabolites biosynthesis in plant cell cultures.

Keywords Pulsed electric field (PEF) · Ethephon, cell culture · *Vitis vinifera* · Phenolic acid · Anthocyanin · Resveratrol · Exudate · Medium

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Introduction

Pulsed electric field (PEF) has been applied to various biological systems, including foods, biopolymers, or microbial populations for more than 40 years and has received renewed and increased attention during the past decades. Most of the concerns are on inactivation of microorganisms [1–5]. Additional publications deal with effects of PEF on real food systems, with special attention focused on engineering aspects, PEF-induced inactivation of enzymes and permeabilization of plant membranes [6, 7]. Studies on the effects of PEF on plant tissue are few, and most of them have been concerned with investigations of whether PEF could be combined with other processes [8]. The use of PEF with very low intensity as an external stress source, induces a stress reaction of the cells with a possible stimulation of secondary metabolite production. Generally, it is known that both biotic and abiotic stresses cause plants to react in this manner. This is of high interest, since many secondary metabolites are proposed to have beneficial effects on human health. PEF has been reported to enhance taxuyunnanine C production in suspension cultures of *Taxus chinensis* [9], to lead to the permeabilization of plant membrane and breakage of cells and tissues of food material [10]. The irreversible permeabilization of cell membranes in plant tissues by PEF has offered interesting process applications, such as permeabilization of plant metabolites and mass/heat transfer of food products, which enhance plant secondary metabolites production [9].

In our study, we focused on anthocyanins and phenolic acids production. Phenolic acids have been proposed to have beneficial effects on human health because of the antioxidant, anti-cardiovascular disease, and anti-coronary heart disease properties [11], which can be used in pharmaceutical, food, and cosmetic industries. Anthocyanins are responsible for colors ranging from pink through red, violet, or dark blue. The anthocyanins found in grape are the glucoside forms of cyanidin, malnidin delphinindin, peonidin, petunidin, and pelargonidin. Anthocyanins are of particular interest to the food colorant industry, due to their ability to impart variant colors of the product. It seems very likely that they can also enhance the health-promoting qualities of foods. Resveratrol (3,4′,5-trihydroxystilbene) is a naturally occuring phytoalexin produced by some spermatophytes, such as *Vitis vinifera* and other members of *Vitaceae* as a response to infection, injury, fungal attack, or exposure to ultraviolet light [12]. Resveratrol has been shown to modulate the metabolism of lipids, and to inhibit the oxidation of low-density lipoproteins and the aggregation of platelets. Moreover, as phytoestrogen, resveratrol may provide cardiovascular protection. This compound also possesses anti-inflammatory and anticancer properties [13].

Plant cell culture has recently received much attention as a useful technology for the production of valuable secondary metabolites. It is not limited by environmental, ecological, or climatic conditions, thus the cells can proliferate at higher growth rates than whole plants in cultivation [14]. Under controlled conditions, plant cell cultures are not limited by biotic and abiotic factors. Use of cell cultures can also secure and assure diversity, especially of endangered plants in the future [15]. In recent time, cell cultures are being used as alternative to traditional agriculture in order to better understand the biochemical pathways of these plants, and ease the industrial production of secondary metabolites [16]. Although natural products have provided a wide variety of complex and chemically diverse pharmaceuticals, modern natural product drug discovery encounters several obstacles. Plant cell culture offers a good alternative to whole plant collection and allows for the production of bioactive secondary metabolites under controlled and reproducible protocols [17]. The accumulation of some metabolites in plant cell cultures

is higher than in parent plants [18]. The comparison of product yield of phenolic compounds from plant cell cultures and parent plants is shown in Table 1 [14, 19].

In this study, the effects of PEF and ethephon on growth and secondary metabolites production by plant cell culture were investigated by using suspension culture of *V. vinifera* as a model system. Anthocyanins and extracellular phenolic acids content were determined. Through the application of PEF and elicitors, we tried to enhance the production of the secondary metabolites from plant suspension cell culture. To better understand the possible mechanism of PEF induction of plant secondary metabolism, the dynamic profiles of cell concentration and medium conductivity were analyzed.

Materials and Methods

Plant Cell Culture

The cell culture of *V. vinifera* L. cv. Gamay Fréaux was originally obtained 15 years ago from Francois Cormier (Food Research and Development Centre, Agriculture Canada). Since then, it has been continuously maintained at the Department of Food Biotechnology of Berlin University of Technology. The cell culture was cultivated on B5 medium [20] with 0.1 mg/L NAA, 0.2 mg/L kinetin, 0.25 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 preferably. 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±2 °C and under 12-h light:12-h dark (LD 12:12) lighting conditions. The inoculum size was about 100 g fresh weight/L medium.

Treatments and Samples

V. vinifera cell suspension cultures were pooled in a sterile suction filter, and the medium passed the filter dropwise (15 min). From this pool, 4 g of calli was transferred to 100-mL flasks containing 25 mL sterile B5 medium. Cell cultures were kept on a rotary shaker at 110 rpm at 25 °C and under LD 12:12. The PEF and ethephon [(2-chloroethyl) phosphonic acid] treatments were made 1 day after the sub-cultivation. Ethephon was added into the suspension cultures as elicitor. It was sterilized through 0.22-μm Milipore filters and added

Table 1 Product yield of phenolic compounds from plant cell cultures compared with parent plants

Product	Plant	Yield (% dry weight)		Culture/plant
		Culture	Plant	
Anthocyanin	*Vitis* sp.	16	10	1.6
	Euphorbia milli	4	0.3	13.3
	Perilla frutescens	24	1.5	16
Caffeic acid	*Vanilla planifolia*	0.2	0.05	4
Rosmarinic acid	*Coleus blumei*	27	3	9

Adapted from Zhong and Smetanska [14, 19]

to cell culture to give a concentration of 28 mg/L suspension culture. Pulsed electric field applied for permeabilization of cell membranes was 1.6 kV/cm and 10 pulses at room temperature. Ethephon addition and PEF treatment to the *V. vinifera* suspension cultures was in triplicate. Cultures without PEF and ethephon treatment were taken as control. Sampling was performed on 1, 4, 7, 9, and 14 days after treatments.

Determination of Cell Growth, pH Value, and Conductivity

The growth of cell cultures was measured by determining fresh weight, dry weight (DW) and pH value. The cell cultures were filtrated under vacuum. After that, the cells were weighed as fresh weight. One gram of fresh cells was dried at 105 °C in a vacuum oven overnight to a constant weight, and then dry weight was determined. Lastly, the cell concentration based on dry cell weight was calculated. The pH value of growing medium was determined by a pH meter (CG811, Schott Geräte GmbH, Hofheim, Germany). The conductivity of growing medium was measured using a conductivity meter (WTW LF323, Weilheim, Germany).

Anthocyanins Content Analysis

Anthocyanins were extracted from 100 mg of *V. vinifera* cell suspension cultures, 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 was added and samples were incubated in a heat block for 20 min at 85 °C. After centrifugation for 5 min at 13,000 rpm, the supernatants were collected and the pellets re-extracted twice more with the extraction solvent twice more. Supernatants were combined and 50 μL of 37% (*v*/*v*) hydrochloric acid for stabilizing of anthocyanins was added. After 10 min incubation in the darkness at room temperature, the sample was diluted with 1:1 (*v*/*v*) with the extraction solvent. Absorbance at 535 nm was recorded against the buffer, and anthocyanins content was calculated by the following equation:

$$\text{Anthocyanin}\,(\text{g/g dry weight}) = \frac{E \times M \times V_s}{d \times \varepsilon \times m \times \text{DW\%}}$$

Where, E=extinction, M=molecular weight of anthocyanin (445.2 g/mol), V_s=sample volume (L), d=distance of the tube (cm), ε=extinction coefficient (98,200 L/mol/cm), m=input weight (g), DW%=percentage of fresh weight to dry weight. For compound identification, the extracts were analyzed with HPLC [21].

Extraction of Phenolic Acids

Two solvents (ethyl acetate and methylene chloride) were used to extract the phenolic acids from growing medium. Firstly, the collected medium was filtrated via suction filter (0.4 μm), and then the pH value was adjusted to 2.0, 100 μL of 3 mmol/L *p*-coumaric acid was added to 50 mL sample medium. The medium was split in 25 mL each.

1. Ethyl acetate extraction. Twenty-five milliliters sample medium was used to extract with ethyl acetate. Firstly, 15 mL ethyl acetate was added into the medium in a separatory funnel. After careful penning, the lower phase was removed and the upper phase was collected in a round-bottom flask. The removed lower phase was re-extracted two times with 5 mL ethyl acetate. The upper phase was collected in the same round-bottom flask.

2. Methylene chloride extraction. The other 25-mL medium was extracted with methylene chloride (dichloromethane). Firstly, 15 mL methylene chloride was added into medium in a separatory funnel. After careful penning, the lower phase was collected in a round-bottom flask. The upper phase was re-extracted two times with 5 mL methylene chloride. The lower phase was collected in the same round-bottom flask.

The residue was re-dissolved in 2 mL HPLC–water, then concentrated to 1 mL in a centrifugation evaporator (SPD 111V Speed Vac. Concentrator, Thermo Scientific, USA; CVC 3000V, Vacuubrand GmbH, Wertheim, Germany) at 25 °C. Lastly, the remaining 1-mL extract was transferred to SPIN-X-filter and centrifuged for 5 min, and then transferred to a 2-mL HPLC vial.

Instrumentation and Chromatographic Conditions

The separation of phenolic compounds was performed on HPLC (Dionex Summit P680A HPLC-System, USA), equipped with P680 pump, ASI-100 automated sample injector, a Narrow-Bore AcclaimPA C16-column (3 μm, 2.1×150 mm, Dionex) and PSA-100 photodiode array detector (Dionex) and software Chromeleon 6.8 (Dionex, USA). The column was operated at a temperature of 35 °C. The mobile phase consisted of 0.1% (v/v) phosphoric acid in water (eluent A) and of 40% (v/v) acetonitrile (eluent B). A multistep gradient was used for all separations with an initial injection volume of 40 μL and a flow rate of 0.4 mL/min. The multistep gradient was as follows: 0–1 min: 0.5% (v/v) B; 1–10 min: 0.5–40% B; 10–12 min: 40% B; 12–18 min: 40–80% B; 18–20 min: 80% B; 20–24 min: 80–99% B; 24–30 min: 99–100% B; 30–34 min: 100–0.5% B; 34–39 min: 0.5% B. Simultaneous monitoring was performed at 290, 330 and 254 nm. Diode array detection was used for the identification of the compounds. Retention times and UV±visible spectra of the peaks were compared with those of the standards. Compounds were identified by using standards and by performing LC-MS and NMR [21]. Phenolic acids quantity was calculated from HPLC peak areas at 290 nm against the internal standard.

Results and Discussion

In this research, the PEF treatment was applied 1 day after the sub-cultivation. Cells at different growth stages showed different sensitivities of their physiology and metabolism to PEF elicitation. PEF was more effective in plant cells in the early exponential phase, as it has been shown by Ye et al. [9]. A previous report on the inactivation of microbial cells by PEF has also showed that cells in the exponential growth phase were more sensitive to PEF treatment than those in the stationary stage [22]. Membrane properties of microorganisms varied in different growth stages, and in the exponential growth phase, the membrane zone between the mother cell and its progeny was very sensitive to PEF [23]. According to these findings, we applied the PEF to the cell culture at an early stage.

Effects of PEF and Ethephon on Cell Growth

To investigate the effects of PEF and ethephon on the cell growth, fresh cell weight, dry cell weight, and medium pH value were measured. As shown in Fig. 1, there were no significant changes in dry weight for the different cases except on day 4. The biggest biomass accumulation has been reached on day 9 after the treatments. Dong et al. have

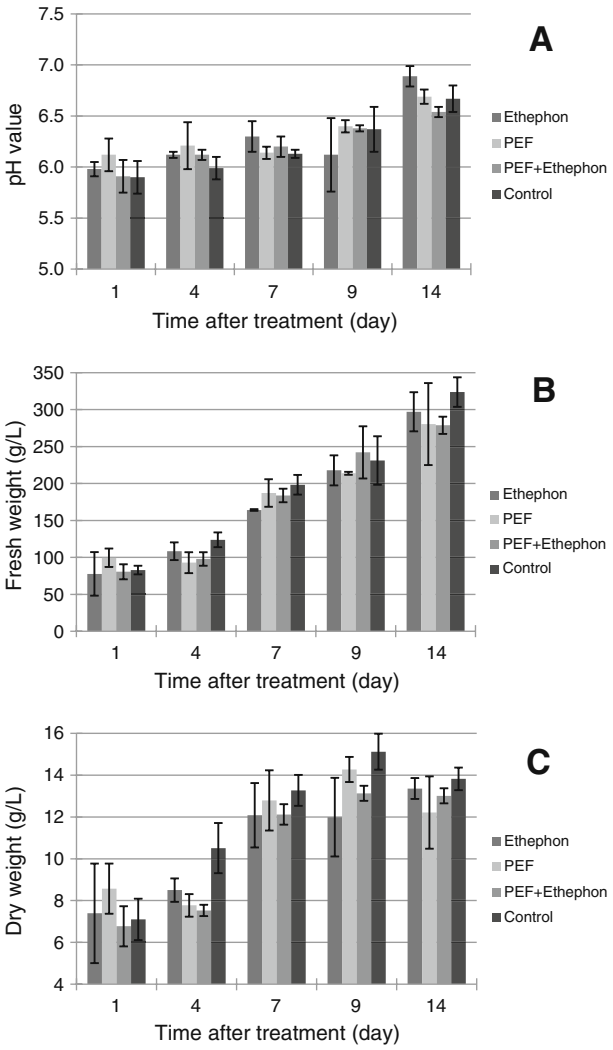

Fig. 1 Dynamic profiles of cell concentration, by **a** pH value; **b** fresh weight; and **c** dry weight

found that the application of elicitation in the lag phase severely inhibited the cell growth, because the cells were adapting themselves to the new environment after the inoculation [24]. But it may vary among different cell lines. Even though Ye et al. [9] used the same cell line, *T. chinensis*, as Dong et al. [24], they found that elicitation in the early exponential phase was more effective than later. Our previous studies have showed that the growth of *V. vinifera* cell culture was not affected by elicitation, precursor feeding, or stress factors [25]. The results of this study also show that PEF, ethephon, and the combination of PEF and ethephon apparently did not affect cell growth. Biddington reported that ethephon was neither inhibitory to growth, nor did it stimulate growth [26]. This was confirmed by our study.

Effects of PEF and Ethephon on Anthocyanins Production and Composition

The treatment of PEF enhanced the total anthocyanins production, especially on days 9 and 14 after the treatment (Fig. 2). The stimulating activity was 30% and 71%,

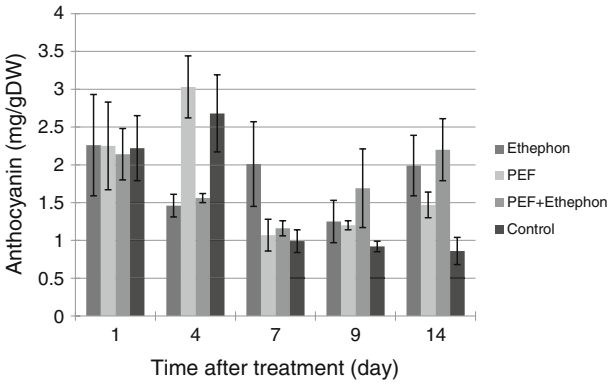

Fig. 2 Anthocyanin content of cell culture

respectively. The stimulating activity here, was defined as the ratio of average accumulation titer under treatment to that of control. Ethephon also had an effective influence on the anthocaynins production. The combination of PEF and ethephon was the most effective treament, with a stimulating activity of 84% and 156% on days 9 and 14, respectively. However, the content of anthocyanins decreased after day 4. Therefore, the biomass was taken into consideration, when we tried to get the final production of anthocyanins.

The anthocyanin compounds present in *V. vinifera* cell culture were identified by Mewis et al. [21]. The major anthocyanin monoglucosides present in cell suspension culture were cyanidin 3-*O*-glucoside and peonidin 3-*O*-glucoside and major cinnamoyl derivatives were cyanidin 3-*O*-*p*-coumarylglucoside 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*-coumarylglucoside. Although the total anthocyanins 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, while the delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside were relatively low. Ethephon had a positive effect on the cyanidin-3-*O*-*p*-coumaryl glucoside production, as well as on peonidin-3-*O*-glucoside and peonidin-3-*O*-p-coumaryl glucoside (data now shown). Gomez-Cordoves et al. reported that the anthocyanin composition of starking apples was altered by ethephon treament [27]. Li et al. reported that ethephon caused an enhancement of red peel color of 'Fuji' apple, and anthocyanin showed different accumulation pattern

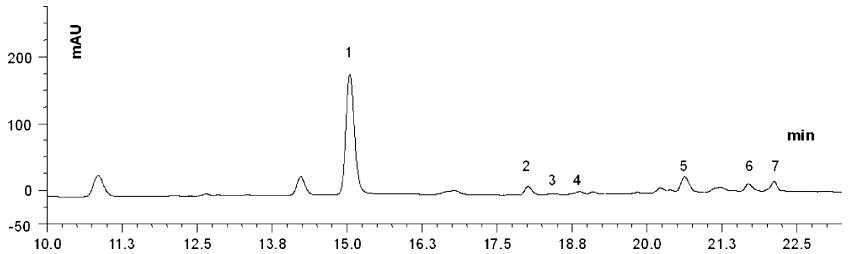

Fig. 3 HPLC chromatogram of cell culture medium. *1* *p*-coumaric acid (15.047 min), *2* 3-*O*-glucosyl-resveratrol (18.017 min), *3* phenolic acid ester 2 (18.447 min), *4* 4-(3,5-dihydroxy-phenyl)-phenol (18.877 min), *5* phenolic acid derivate 2 (20.627 min), *6* phenolic acid derivate 3 (21.690 min), *7* phenolic acid derivate 4 (22.127 min)

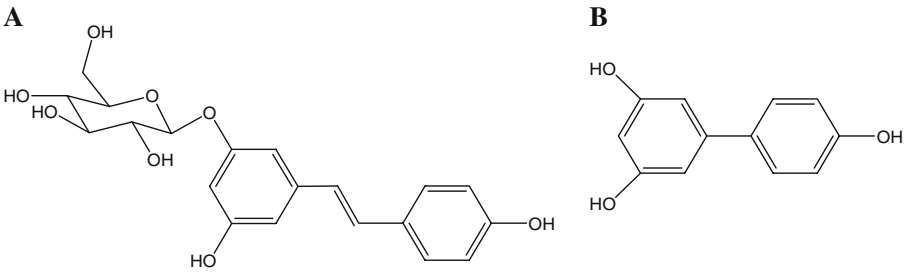

Fig. 4 Chemical structures of **a** 3-*O*-glucosyl-resveratrol; and **b** 4-(3,5-dihydroxy-phenyl)-phenol

within different treatments [28]. Awad et al. found that ethephon application greatly increased anthocyanin accumulation in 'Jonagold' apple skin, but dependence of anthocyanin synthesis on ethylene was complicated [29].

Effects of PEF on Extracellular Phenolic Acids

To better understand the PEF effect on cell membrane properties, the content of extracellular phenolic acids and the conductivity of growing medium were investigated. The first step was to identify the phenolics present in the cell culture, as well as in exudates. The chromatogram of a sample treated with PEF is shown in Fig. 3. Six phenolic compounds were detected in HPLC. According to the results from LC-MS and NMR, 3-*O*-glucosyl-resveratrol (Fig. 4a) and 4-(3,5-dihydroxy-phenyl)-phenol (Fig. 4b) were identified by Mewis et al. [21].

For both the control and PEF-treated samples, the content of total extracellular phenlics reached the highest point on day 9 after the treament, and declined quickly thereafter. After PEF treatment, extracellular phenolics content was found to accumulate quickly after day 7, and reached 11% higher than that of control on the ninth day after PEF elicitation (Fig. 5). The results indicate that PEF caused a release of secondary metabolites into the medium, which may be related to the changes of the cell membrane dielectric properties.

The metabolism of phenolic substances has been intensively studied in cell suspension cultures derived from different plant species. Teguo et al. reported that suspension cultures of *V. vinifera* accumulated high levels of stilbene glycosides, e.g. (*E*)-piceatannol (3,5,3′,4′-tetrahydroxystilbene) 3-*O*-*β*-glucoside and (*Z*)-resveratrol (3,5,4′-trihydroxystilbene) 3-*O*-*β*-glucoside, which can be isolated for biological study of the exact role of each phenolic

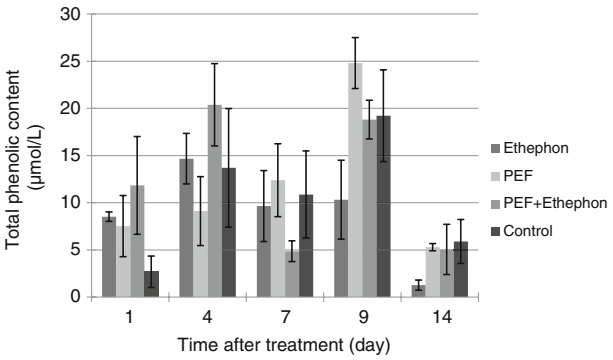

Fig. 5 Total extracellular phenolics content

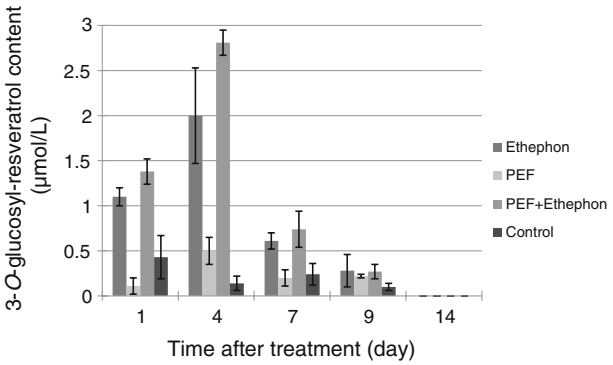

Fig. 6 Extracellular 3-*O*-glucosyl-resveratrol content

compound [30]. Romero-Perez reported the content of *cis*- and *trans*-resveratrol and their glucosides in white and Rose *V. vinifera* wines from spain, was between 0.051 and 2.15 mg/L [31]. But to the best of our knowledge, isolation of resveratrol from *V. vinifera* suspension culture medium has not been previously reported. 3-*O*-glucosyl-resveratrol accumulated to the highest level on day 4 after the treaments. The 3-*O*-glucosyl-resveratrol content of PEF, ethephon, combination of PEF and ethephon treatments was 3.6-, 14.3-, and 20.1-fold of the control on day 4 after the treatments, respectively. However, the content decreased after 4 days in all cases, and none was detected on day 14 after the treatments (Fig. 6). The reason might be that the nutrient sources were decreased. Our data show that ethephon is an effective stimulant for the resveratrol glucoside production, and PEF may enhance the effect of ethephon.

To better understand the potential mechanism of PEF effect on cell growth and the cell membrane properties, the conductivity of medium was investigated (Fig. 7). The medium conductivity 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 [32–34]. In our study, a clear linear correlation between the decrease in conductivity and the increase in dry cell weight was found in the control samples (R^2=0.94, data not shown). However, the mirror-images of the conductivity curve and growth curve did not show in the PEF-treated cultures. Instead, the two curves became incongruous, and there was a slight increase of conductivity after 4 days of the PEF treatment, indicating the cell/membrane's dielectric properties may have changed with the PEF treatment. Fincan et al. studied the effect of PEF on onion tissue, and found the final conductivity increase was directly proportional to the number of

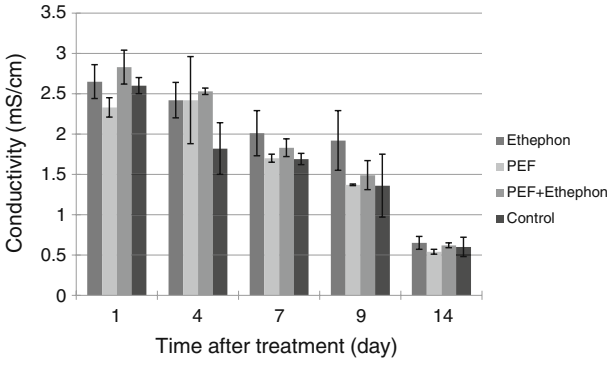

Fig. 7 Conductivity of growing medium

permeabilized cells [8]. In our experiments, the changes of cell/membrane's dielectric properties may relate to the permeability of the cell membrane, and then subsequently enhance the accumulation of extracellular phenolics in medium (Figs. 5 and 6). Increasing the membrane permeability led to the application of PEF-assisted extraction of cellular content and transfer of genetic material across cell membrane [7]. The permeability of the cells lasted for several days, because the maximal amount of extracellular phenolics was reached 9 days after PEF treatment. Similar results were found in the extracellular accumulation of taxuyunnanine C in suspension cell culture of *T. chinensis* [9].

In conclusion, the present work has demonstrated that PEF can elicit defense response and stimulate the accumulation of secondary metabolites in plant cell cultures and exudates. PEF treatment can be optimized to enhance the desired secondary metabolites production, without harmful effects on plant cell growth. The combination of PEF and ethephon treatment has shown to be effective in improving secondary metabolites production. In addition, PEF may change the cell/membrane's properties and induce the release of intracellular metabolites into growing medium. The findings suggest that PEF, as an external stimulus or stress, can be used as a novel abiotic elicitor to improve secondary metabolites production in plant cell cultures. The mechanism on how the cultured cells sense PEF elicitation and transfer the signal to induce secondary metabolism is unclear and still needs further study.

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