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Increasing of Phenolic Compounds by Brassinosteroid Applications in Immobilized Cell Suspension Cultures of *Vitis vinifera* L. cv. Cinsault

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ABSTRACT
In this paper, the effects on secondary metabolite accumulation of brassinosteroid (BR) (24-epibrassinolide (24-eBL) on immobilized cells that were obtained from *Vitis vinifera* cv. Cinsault was investigated. 24-eBL was treated to immobilized cells covered calcium alginate beads at concentrations of 0, 0.25, 0.50, 0.75 and 1.0 mg L^{-1} for one month. As a result of this study, it was found that 24-eBL applications modified secondary metabolite accumulation and had positive effects on secondary metabolite production when the suitable concentration was used. While the highest total phenolic, catechin, *p*-coumaric acid and chlorogenic acid contents were found in immobilized cells treated 0.75 mg L^{-1} 24-eBL, the highest epicatechin, quercetin, *trans*-resveratrol contents were obtained in immobilized cells treated 0.50 mg L^{-1} 24-eBL and the highest gallic acid content was determined in immobilized cells treated 0.25 mg L^{-1} 24-eBL. Moreover, the highest 24-eBL concentration (1 mg L^{-1}) decreased the content of secondary metabolite compared to the control (0 mg L^{-1} 24-eBL) except total phenolic and catechin content. To conclude, 0.50 and 0.75 mg L^{-1} 24-eBL concentrations were the most suitable concentrations for immobilized cell culture to provide the highest secondary metabolite accumulation.

Keywords: 24-eBL, Immobilization, Grapevine, Phenolic compounds

1. Introduction

Phenolic compounds ranging from simple phenolic molecules to highly polymerized compounds, constitute major class of plant secondary metabolites (Dai & Mumper 2010). Secondary metabolites are not correlated with photosynthesis, reproduction, respiration or other primary functions required for growth and development of the plants. Therefore, they have been considered waste products without significant use for plants for many years (Verpoorte 2013). However, nowadays, much attention has been devoted to secondary metabolites, due to they are valuable and important raw materials for many areas such as cosmetics, pharmaceuticals, antioxidants, insecticides and organoleptic properties of food industries (Keskin & Kunter 2010; Pehlivan et al. 2016). Plants are grown at certain development stages under natural conditions in which they occupy small quantities and agricultural areas and the material required for obtaining secondary products is mostly achieved from plants collected from natural cultivation areas (Keskin & Kunter 2008). The constant collection of some plant species from nature may come to the threat of extinction, it is difficult and expensive to collect some of them (Gonçalves & Romano 2018; Karaboyacı & Kılıç 2018). Furthermore, the amount and quality of secondary metabolites are influenced by climate conditions and the amount of the pure matters varies according to the quantity and quality of the plant material (Keskin & Kunter 2010).

In recent years, several strategies have been developed to increase secondary metabolite production such as use of cell culture, selection of high productivity cell lines, media modification, permeabilization, nutrient and precursor feeding, plant cell immobilization, elicitation and biotransformation methods (Zhang et al. 2002; Murthy et al. 2014). By immobilization technique, plant cells are fixed to a suitable matrix such as agar, agarose, algae, calcium alginate, gelatine, and polyacrylamide in cell cultures (Pras & Woenderbag 1999). However, alginate gels have received much attention due to their simplicity and relative lack of toxicity (Smetanska 2008; Nielsen et al. 2019). Since plant cells are very sensitive to chemical and physical stress, some biological and technological factors must be considered (Guardiola et al. 1996). In order to improve such processes and overcome the limitations of plant cell culture, immobilization has been considered as a tool for protecting cells against stress factors and enhancing the production of secondary metabolites (Choi et al. 1995). Elicitor applications are another effective strategy for secondary metabolite production in plant cell cultures (Shumakova et al. 2011). Elicitors affect the production of most commonly used secondary metabolites for stress tolerance and plant defense (Zhao et al. 2005). For this purpose Brassinosteroids (BRs), the sixth class of phytohormones could be used. BRs play diverse roles for physiological and developmental processes in plant growth and also respond to various biotic and abiotic stresses (Bajguz & Hayat 2009, Ahammed et al. 2012). 24-eBL is the most

effective and stable BR analog and has been found to high stimulatory effect in enzymatic activity and antioxidant systems in the majority of studies (Hayat et al. 2010). Moreover, 24-eBL also promote the production of secondary metabolites in several plants (Çoban & Baydar 2017; Asci et al. 2019) and the exogenous application of BRs has been determined to increase antioxidant capacity and phenolic content in grapes (Luan et al. 2013; Xi et al. 2013; Ghorbani et al. 2017; Wang et al. 2019; Babalık et al. 2020). Although there are few studies conducted the impact of immobilization on the increased accumulation of secondary metabolites in several plants (Choi et al. 1995; Gillet et al. 2000; Dornenburg 2004) and grapevine (Iborra et al. 1994; Guardiola et al. 1996), there is no study to determine on the effect of brassinosteroid applications on phenolic contents of grapes in immobilized cultures. For this reason, more detailed studies are needed to investigate. This study was performed to provide a better understanding the effects of 24-eBL and immobilized cells on the accumulation of phenolic compounds. This is also the first report to our knowledge of the use of 24-eBL in immobilized cell culture in grapes.

2. Material and Methods

In the research, petioles belonging to *Vitis vinifera* cv. Cinsault preferred in red wine making due to its low tannin and rich aromatic components, were used as plant material. Cuttings were provided from Tekirdağ Viticultural Research Institute. Single node cuttings were planted in pots containing sand, perlite and torf (1:1:1) and then incubated to a controlled environment chamber at 25 °C with cool fluorescent daylight (16 h photoperiod). When 8-10 leaves were formed on the shoots, petioles were taken. Petioles were washed with tap water 3-5 times and then petioles were treated with 70% ethanol for 70 s under laminar airflow cabinet. After pretreatment with ethanol, petioles were rinsed with sterile distilled water for three times and surface-sterilized with commercial bleach (22.5%) for 18 min and last rinsed with sterile distilled water again. Then, petioles were cut into 1 cm pieces and inoculated on MS medium (Murashige & Skoog 1962) containing 1 mg L^{-1} benzylaminopurine (BAP) and 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g L^{-1} sucrose and 7 g L^{-1} agar. The pH of the medium was adjusted to 5.85 prior to autoclaving. Cultures were incubated at 25±1 °C under dark conditions. Calli were transferred to the same fresh media every 40-45 days in order to maintain adequate stock cultures.

2.1. Immobilization of cells

Approximately 1 g of cells from 25 days of callus cultures were mixed with 50 mL of 2% sodium alginate and 25 mL of liquid MS media containing 1 mg L^{-1} BAP, 0.1 mg L^{-1} 2,4-D and 30 g L^{-1} sucrose. The cell/alginate suspension was then added drop by drop through a 5 mL pipette tip into 0.2 M $CaC1_2$ solution, and almost homogeneous size beads (~4 mm in diameter) were formed. The beads were left to harden in $CaCl_2$ solution for 30 min (Sajc et al. 1995), rinsed with sterile water and transferred into MS medium containing different concentrations of 24-eBL.

2.2. Application of 24-eBL to immobilized cells

Immobilized cells were added to 50 mL liquid MS nutrient media containing 1 mg L^{-1} BAP, 0.1 mg L^{-1} 2,4-D and 30 g L^{-1} sucrose with different concentrations of 24-eBL (0, 0.25, 0.50, 0.75 and 1 mg L^{-1}). While choosing these concentrations, the previous study we made on vineyards (Babalık et al. 2020) is taken as reference. The 24-eBL stock solution was prepared by dissolving in dimethyl sulfoxide (DMSO). The immobilized cell cultures were incubated under dark conditions at 25±1 °C on the rotary shaker at 100 rpm for one month. There were 3 replicates per treatment and 5 culture flasks (eg. erlenmayer) per replicates. At the end of the incubation, 1% sodium citric acid solution was added to the medium to dissolving calcium-alginate and they were incubated at 25 °C for 30 min. Then, free cells were washed with sterile water and dried in a 45 °C oven until the constant mass is obtained.

2.3. Extraction of phenolics compounds

For extraction of phenolic compounds 0.1 g of powdered callus was extracted with 10 ml 0.1% HCl in 70% methanol. Extraction was ensured in the ultrasonic bath for 30 min and then centrifuged at 4000 rpm for 15 min. The residue left over from the first extract was washed with HCl:methanol solution once again and the supernatant was collected (it was done for three times total) and evaporated in a rotary evaporator at 45 °C until dry.

2.4. Determination of phenolic compounds

Folin-Ciocalteu reagent assay was used to determine phenolic compounds (Singleton & Rossi 1965). The absorbance was determined by spectrophotometer at 765 nm. Results were expressed as mg gallic acid 100 g^{-1} dry weight (DW).

High-performance liquid chromatography (HPLC) analyses were performed according to the modified procedure of Caponio et al. (1999). Phenolic compounds were determined using the HPLC system including a pump (LC-10 ADvp), auto-sampler (SIL-10 ADvp), column oven (CTO 10Avp) and diode-array UV/VIS detector (DAD λ_{max}:278). The separation was performed on an Agilent Eclipse XDB-C18 (5 µm, 250 x 4.60 mm). Mobile phase A contained 3% acetic acid in water; solvent B contained methanol. The gradient was: 93% A and 7% B for 0.01-0.10 min, 72% A and 28% B for 0.10-20 min, 75% A and 25% B for 20-28 min, 70% A and 30% B for 28-35 min, 70% A and 30% B for 35-50 min, 67% A and 33% B for 50-60 min, 58% A and 42%

B for 60-62 min, 50% A and 50% B for 70-73 min, 30% A and 70% B for 73-75 min, 20% A and 80% B for 75-80 min, 0% A and 100% B for 80-81 min, 93% A and 7% B for 81-90 min. The flow rate was 0.8 mL min^{-1} and the injection volume was 20 µL. Standard solutions, mobile phases, and samples were filtered through a 0.45 µm pore size membrane filter. The detection UV wavelength was 278 nm. The temperature of the column oven was 30 °C. Catechin, chlorogenic acid, epicatechin, gallic acid, *p*-coumaric acid, quercetin, and *trans*-resveratrol contents were expressed as µg g^{-1} DW.

2.5. Statistical analysis

Descriptive statistics were presented as mean and standard deviation. Treatment effects were determined using one-way ANOVA. Duncan's multiple range test (significance level $p<0.05$) was used to compare mean. All statistical analyses were conducted using the software package SPSS (ver:18).

3. Results and Discussion

Phenolic compounds changed significantly according to different concentrations of 24-eBL applications ($p\leq0.05$). The results pointed out that the 24-eBL application caused an increase in phenolic compounds compared to control (Figure 1). As shown in Figure 1A, the highest total phenolic content was observed from the immobilized cells treated 0.75 mg L^{-1} 24-eBL, while the lowest values were recorded from the immobilized cells treated 1 mg L^{-1} 24-eBL. The amount of total phenolic compounds increased 1.9-fold compared to the control. Ahammed et al. (2013) stated that BR applications regulate secondary metabolism. 24-eBL has been reported to increase the activity of secondary metabolism-related enzymes such as phenylalanine ammonialyasethe first enzyme involved in flavonoid biosynthesis) and flavonoid 3-O-glucosyltransferase, which modulate the phenylpropanoid metabolism. Thus, it promotes the synthesis of phenolic compounds (Xi et al. 2013; Li et al. 2016). Asci et al. (2019) reported that exogenous applied BR acts as a signalling molecule to increase phenolic biosynthesis and enhances metabolite accumulation by affecting enzymes and genes involved in biosynthesis. In previous studies, it was found that BR applications increased total phenol and antioxidant capacity compared to control treatment (Xi et al. 2013; Ghorbani et al. 2017).

In our study, chlorogenic acid contents significantly changed depending on the applications. Based on the results, the highest chlorogenic acid was obtained the immobilized cells treated 0.5 mg L^{-1} 24-eBL, while the immobilized cells that treated 1 mg L^{-1} 24-eBL application was the lowest (Figure 1C). Compared to the control, chlorogenic acid contents of immobilized cells treated 0.5 mg L^{-1} 24-eBL application increased 1.45-fold. 24-eBL applications had significant effects on the amount of catechin compared to control cells. The highest catechin accumulation was found in immobilized cells that treated 0.75 mg L^{-1} 24-eBL. However, control cells had the lowest values. Catechin content increased 4.76-fold compared to the control group (Figure 1B). Epicatechin content was ranged from 36.33 to 369.52 µg g^{-1}. The concentration of 0.5 mg L^{-1} of 24-eBL was given the highest epicatechin value. However, 24-eBL at higher concentration (1 mg L^{-1}) caused a significant decline in epicatechin content. Epicatechin contents of immobilized cells treated with 0.5 mg L^{-1} 24-eBL application increased 6.24-fold compared to the non treated 24-eBL cells (Figure 1D). The changes of gallic acid content in *V. vinifera* cv. Cinsault immobilized cell culture subjected 24-eBL application were also shown in Figure 1E. Gallic acid content was low in immobilized cells treated with 1 mg L^{-1} 24-eBL. The highest gallic acid content was found in the immobilized cells treated with 0.25 mg L^{-1} 24-eBL. In this medium, the amount of gallic acid was 2.85-fold higher than the control. The content of *p*-coumaric acid ranged from 2.23 to 15.30 µg g^{-1}. According to data in Figure 1F, the highest amount of *p*-coumaric acid was obtained from the application of 0.75 mg L^{-1} 24-eBL. However, the lowest values were detected in 1 mg L^{-1} 24-eBL application. The amount of *p*-coumaric acid increased 3.57-fold over control. Quercetin contents significantly changed depending on the 24-eBL concentrations. The highest quercetin accumulation (68.38 µg g^{-1}) was observed from 0.5 mg L^{-1} 24-eBL application (Figure 1G). There is currently no literature on the effects of BRs on catechin, chlorogenic acid, epicatechin, gallic acid and *p*-coumaric acid metabolism in grapevines, and therefore it is important to learn more to investigate the mechanism of action of BRs on grapes. This study was performed to eliminate the deficiency in the literature and to understand better the effects of BRs. It was also the first report determining the effects of BRs on catechin, chlorogenic acid, epicatechin, gallic acid and *p*-coumaric acid contents of grapes.

Immobilization is an effective method for enhancing secondary metabolite production in plant cell cultures. In this method agar, agarose, calcium alginate, gelatine, carrageenan or polyacrylamide are used as a matrix to fix plant cells (Pras & Woenderbag 1999; Smetanska 2008). Gillet et al. (2000) stated that immobilized cells have created the formation of aggregate during the growth of cells, moreover, the presence of aggregates can modify cell to cell and cell to matrix interactions. Researchers also reported that synthesis of secondary metabolite could be stimulated as a result of increased gene expression due to cell-cell interaction in cells maintained in the culture medium.

Resveratrol is a phytoalexin that a stilbene derivative and has a potential protective role against cardiovascular disease. It is produced by plants and notably present in grapes (Bonnefont-Rousselot 2016). In the current research, the accumulation of the *trans*-resveratrol in immobilized cell cultures was changed according to the 24-eBL concentrations. The highest *trans*-resveratrol content (16.37 µg g^{-1}) was recorded from the immobilized cells treated with 0.5 µg g^{-1} 24-eBL. In this medium, the amount of *trans*-resveratrol was 1.1-fold higher than the control (Figure 1H). There is only one study conducted on the effects of exogenous brassinosteroid applications on *trans*-resveratrol in grapevine (Babalık et al. 2020). Researchers indicated that *trans*-resveratrol content increased 2.67-fold compared with control vines. The increase in *trans*-resveratrol accumulation through BR applications

was thought to be result from the stimulating effects of BRs on the expression of genes encoding enzymes such as stilbene synthase that function in *trans*-resveratrol synthesis (Babalık et al. 2020). Keskin & Kunter (2009) stated that *in vitro* techniques in grapevine, especially callus and cell suspension cultures, have several advantages to increase productivity in the production of *trans*-resveratrol. In this study, it was also deduced that callus cultures can be used as model systems for the stimulation and determination of trans-resveratrol production in grapevines.

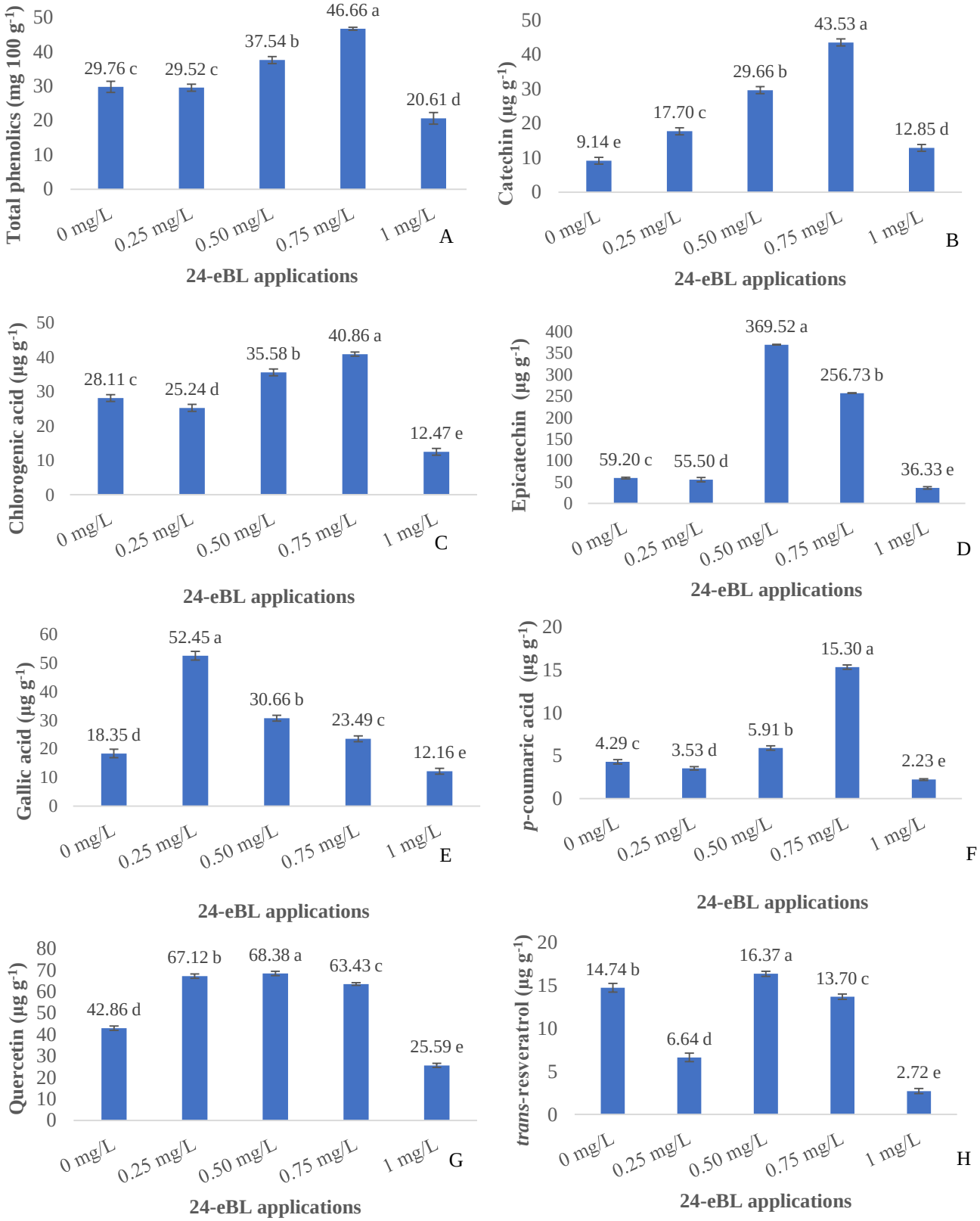

Figure-1. Effects of 24-eBL applications on the total phenolic content (A), catechin (B), chlorogenic acid (C), epicatechin (D), gallic acid (E), *p*-coumaric acid (F), quercetin (G) and *trans*-resveratrol (H) in Cinsault immobilized cells. Different letters indicate statistically significant differences among the applications (p≤0.05)

4. Conclusions

The present study demonstrated that 24-eBL application to immobilized cells has an advantageous effect on secondary metabolite production of cv. Cinsault, effectively. The best results were obtained from 24-eBL applications performed at concentrations of 0.5 or 0.75 mg L^{-1}. However, it has been determined that the application of high concentrations of 24-eBL (1 mg L^{-1}) reduced the secondary metabolite production even compared to control, because of this reason; higher doses of BR have been evaluated not effective.

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