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Elicitor Applications to Cell Suspension Culture for Production of Phenolic Compounds in Grapevine

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ABSTRACT

In this study, the effects of cadmium sulphate ($CdSO_4$), fleuresans irradiation, methyl jasmonate (MeJA) and sucrose treatments on the production of phenolic compounds in grapevine cell suspension cultures initiated from callus from petiole tissues of *Vitis vinifera L. cvs*. Gamay, Kalecik karası and Öküzgözü were investigated. As the elicitors of $CdSO_4$ (0, 1 and 1.5 mM), MeJA (0 and 10 µM) and sucrose (0, 0.20 and 0.25 M) were applied. Cell suspensions were exposed to visible light (10,000 lux) for fleuresans irradiation or cultured in dark constantly. Total phenolics, total flavanols, total flavonols and anthocyanin were determined spectrophotometrically while *trans-r*esveratrol was quantified by HPLC. $CdSO_4$ at 1.5 mM concentration and MeJA at 10 µM concentration yielded the highest phenolic productions in all cultivars. Especially, Kalecik Karası treated with $CdSO_4$ at 1.5 mM had the highest total phenolic (3.144 mg g^{-1}), anthocyanin (1.672 CV g^{-1}) and *trans*-resveratrol (3.650 µg g^{-1}) contents. MeJA application at 10 µM provided the *trans*-resveratrol accumulation as high as 11.681 µg g^{-1} in Öküzgözü. 0.20 M sucrose concentration resulted in the highest total phenolics (4.215 mg g^{-1}) and *trans*-resveratrol (7.550 µg g^{-1}) in Kalecik Karası cultures while the most anthocyanin accumulation (2.024 CV g^{-1}) was achieved from Gamay. Darkness had strongly increased *trans*-resveratrol content in all cultivars, whereas total phenolics and anthocyanin synthesis were induced by light. Elicitor applications of $CdSO_4$, MeJA, sucrose and fleuresans irradiation can be an efficient approach for the production of phenolics in grapevines.

Keywords: Grapevine; Cell suspension; Cadmium; Methyl jasmonate; Anthocyanin, *trans*-resveratrol

Asmada Hücre Süspansiyon Kültürlerine Elisitör Uygulamaları ile Fenolik Bileşiklerin Üretilmesi

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ÖZET

Bu araştırmada asmada hücre süspansiyon kültürlerinde fenolik bileşiklerin üretiminde kadmiyum sülfat ($CdSO_4$) floresan radyasyonu, metil jasmonat (MeJA) ve sukroz uygulamalarının etkileri incelenmiştir. Gamay, Kalecik karası ve

Öküzgözü üzüm çeşitlerine ait yaprak saplarından elde edilen kallus hücre süspansiyon kültürlerine $CdSO_4$ (0, 1 ve 1.5 mM), MeJA (0 ve 10 µM) ve sukroz (0, 0.20 ve 0.25 M) uygulanmıştır. Floresan radyasyonu için hücre süspansiyonları tümüyle karanlıkta ya da 10000 lux ışık altında tutulmuştur. Toplam fenolik, toplam flavanol, toplam flavonol ve antosiyanin içerikleri spektrofotometrik olarak; *trans-r*esveratrol içeriği ise HPLC ile belirlenmiştir. 1.5 mM konsantrasyonundaki $CdSO_4$ ve 10 µM konsantrasyonundaki MeJA bütün çeşitlerde en yüksek fenolik bileşik üretimini sağlamıştır. Özellikle 1.5 mM $CdSO_4$ uygulanmış Kalecik karası en yüksek toplam fenolik madde (3.144 mg g^{-1}), antosiyanin (1.672 CV g^{-1}) ve *trans*-resveratrol (3.650 µg g^{-1}) içeriğine sahip olmuştur. 10 µM konsantrasyonundaki MeJA uygulaması ise Öküzgözü çeşidinde *trans*-resveratrol miktarının 11.681 µg g^{-1} gibi yüksek bir değere çıkmasını sağlamıştır. Sukroz uygulamaları içinde 0.20 M dozu en yüksek toplam fenolik (4.215 mg g^{-1}) ve *trans*-resveratrol (7.550 µg g^{-1}) miktarını Kalecik karasında, en yüksek antosiyanin birikimini ise Gamay çeşidinde sağlamıştır. Karanlık uygulaması bütün çeşitlerde *trans*-resveratrol birikimini kuvvetli bir şekilde artırmıştır. Toplam fenolik ve antosiyanin sentezinin ışık tarafından uyarıldığı belirlenmiştir. Sonuçlar $CdSO_4$, MeJA, sukroz ve floresan radyasyonu gibi elisitör uygulamalarının üzümde fenolik bileşiklerin üretiminde önemli bir yaklaşım olabileceğini göstermiştir.

Anahtar Kelimeler: Asma; Hücre süspansiyonu; Kadmiyum; Metil jasmonat; Antosiyanin, *trans*-resveratrol

1. Introduction

Secondary metabolites are chemical compounds produced by plants. These compounds are not essential for cell structure, photosynthesis, respiratory metabolism or other primary functions. The main role of them is natural defense system against biotic and abiotic stresses (Rispail et al 2005). The interest in these metabolites has increased in recent years since many researchers reported that certain compounds will have a positive impact on preventing cancer and age-related disorders, such as certain neurological diseases and metabolic disorders (Dzhambazova et al 2011). In addition, some compounds were implicated in important biological functions in the body such as antioxidant defense system, immunological regulation and anti-inflammatory processes. Among the secondary metabolites, phenolic compounds characterized by having at least one aromatic ring and one or more hydroxyl groups attached to an aromatic ring, are one of the most important secondary metabolites (Cartea et al 2011). Plant phenolics comprise simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, up to hydrolysable and condensed tannins, lignans and lignins.

Anthocyanins are an important group of natural pigments within the flavonoid family. They play a major role in plants by attracting insects for the purpose of pollination, and they serve as a UV screen for protecting the plant's DNA from damage by sunlight (Isley 1987). Many environmental factors (light, temperature, nutrition, drought and infection) have an effect on the synthesis of anthocyanins (Ismail & Mohamed 2010). Anthocyanins exhibit antioxidant properties, free radical scavenging properties and suppression of proliferation of human cancer cells (Dai et al 2007). Thus, they are widely used in food, beverages, cosmetics, pharmaceuticals etc. Resveratrol is a group of polyphenolic secondary metabolites within the stilbene family which is produced as a defensive reaction in response to biotic and abiotic stresses (Jeandet et al 2002). Resveratrols were detected approximately in 72 plants species (Jang et al 1997). They possess many functions including antioxidant and antimicrobial activities (Daroch et al 2001). Thus, *trans*-resveratrol has great potential in various industries such as medical, pharmaceutical, food and cosmetics.

Secondary metabolites can be obtained by direct extraction from plant organs (leaf, root, flower, fruit etc.) using traditional methods. On the other hand, cell cultures are potential sources in secondary metabolite production (Ramachandra & Ravishankar 2002). These are reliable and continuous techniques but the desired end metabolite content is often low. It is possible to increase the secondary metabolite accumulation in

the cell cultures by application of elicitors (Qu et al 2006; Ahmed & Baig 2014). Cadmium sulphate ($CdSO_4$), a heavy metal, is one of the elicitors. Heavy metals inhibit many physiological processes in plants (Zornoza et al 2002). Cadmium causes oxidative stress by disruption of the electron transport chain or induction of lipid peroxidation. Another elicitor is fleuresans irradiation which induces a photooxidative stress. There is no study on the effect of these two elicitors on production of phenolic compounds in grape cell cultures. Methyl jasmonate (MeJA) is another compound used as an elicitor source in order to increase the secondary metabolite synthesis. Jasmonic acid and MeJA are key compounds of the signal transduction system of plant defense reactions (Krisa et al 1999). Sucrose is a general source of carbohydrates and it is used for creation of osmotic stress.

The aim of this study was to investigate the effect of elicitors $CdSO_4$, fleuresans irradiation, MeJA, and sucrose on phenolic (total phenolic, total flavanols, total flavonols, anthocyanin and *trans*-resveratrol) accumulation in cultured cells of Gamay, Kalecik karası and Öküzgözü grape *(Vitis vinifera L.)* cultivars.

2. Material and Methods

Kalecik karası and Öküzgözü grapevine cultivars were chosen as plant material because they are among red wine cultivars grown widely in Turkey. Gamay, mostly used in studies for production of secondary metabolites in cell suspension cultures (Do & Cormier 1990; Larronde et al 1998; Krisa et al 1999; Zhang et al 2002), was also selected in order to compare our findings with previous studies and to allow ranking the cultivars in terms of secondary metabolite production capacities.

2.1. Callus and cell suspension cultures

Callus tissues were obtained from leaf petioles of Gamay, Kalecik karası and Öküzgözü cultivars by following procedures. The petioles were surface sterilized with commercial bleach (15%) for 15 min and rinsed with sterile distilled water. Petioles were cut into 1 cm pieces and placed onto a B5 culture medium (Gamborg et al 1968) with 30 g L^{-1} sucrose and 8 g L^{-1} bacto agar supplemented with 0.5 mg L^{-1} benzylaminopurine and 0.5 mg L^{-1} indole acetic acid (Shure & Acree 1994). The pH was adjusted to 5.75 before autoclaving. Explants were incubated at 25 °C under dark conditions. Induced calli were subcultured on the same media in order to maintain sufficient stock cultures. Cell suspensions were initiated by inoculating fresh friable fragments of calli (2.5 g each) into 50 mL of liquid media in 250 mL Erlenmeyer flasks. Media were supplemented with macro elements (B5 medium), micro elements (Murashige & Skoog 1962), vitamins (Morel 1970), 0.1 mg L^{-1} naphthalene acetic acid, 0.2 mg L^{-1} kinetin, 250 mg L^{-1} casein hydrolizate and 20 g L^{-1} sucrose. Then, they were placed in a rotary shaker (100 rpm). Incubation conditions were 16/8 h light/dark cycle and 6000 lux light intensity except from fleuresans irradiation. Then, these cultures were used for elicitor applications.

2.2. Elicitor applications

$CdSO_4$, dissolved in water, were applied at 1.0 and 1.5 mM concentrations to cell cultures in exponential growth phase at day 7. Studies show that the amount of metabolite production varied with the duration of incubation time with elicitors. Because of the differences in metabolite levels the cells were harvested at every 2 days until day 6. For fleuresans irradiation, cell cultures at day 7 were placed under continuous fluorescent light at 10,000 lux or cultured in dark (control) on shaker. Cells were harvested to determine metabolite levels at every 3 days until day 15. For MeJA treatments, MeJA was dissolved in 99% ethanol and added into autoclaved media after filter-sterilization. MeJA was added to cell culture media at day 7 of incubation at 10 µM concentration. Cells were harvested at every 3 days until day 15. For sucrose treatment, 0.20 M and 0.25 M sucrose concentrations were applied to cultures at day 5. Control treatment contained only autoclaved distilled water. The cells were harvested at every 3 days until day 15. After elicitor applications, harvested cells were weighed and kept at -20 °C

until the extraction and analysis. For each treatment three replicates and three 250 mL Erlenmeyer flasks for each replication were used in the experiments that 54 flasks for sucrose (3 concentrations and 6 sampling dates) and 36 flasks for each $CdSO_4$ (3 concentrations and 4 sampling dates), fleuresans irradiation (2 different irradiation regimes and 6 sampling dates) and MeJA (2 concentrations and 6 sampling dates) treatments were used and samples were taken from the separate flasks per treatment.

2.3. Extraction of phenolic compounds from harvested cells

Cell samples (2 g) were dried and powdered by liquid nitrogen and were extracted with 10 mL of 96% EtOH for 24 h at 40-45 °C. The incubated mixture is centrifuged at 4.000 rpm. Supernatant was concentrated with the rotary evaporator until dryness and resuspended in a methanol (Kiselev et al 2007). Amounts of total phenolics, total flavanols, total flavonols and anthocyanin were determined spectrophotometrically, and *trans*-resveratrol content was quantified by HPLC. Spectrophotometric readings were performed by a PG Instruments spectrophotometer (T70 Plus Dual Beam/Arlington, USA) and conducted with five repetitions. Folin-Ciocalteu reagent was used to estimate total phenolic content (Singleton & Rossi 1965) which was expressed as gallic acid equivalents (mg GAE g^{-1} fresh cell weight, FCW). Total flavanols were determined by the method of Arnous et al (2001) and expressed as catechin equivalents (mg CE g^{-1} FCW). Total flavonols were determined with Neu's reagent solution by the method of Dai et al (1995). The flavonol contents were expressed as rutin equivalent (mg RE g^{-1} FCW). Anthocyanin accumulation was determined by the method of Qu et al (2006), and it was represented as color value (CV) which was calculated with the Equation 1.

$$CV\ (CV\ g^{-1}\ FCW) = 0.1 \times Absorbance \times Dilution\ factor \quad (1)$$

Separation of *trans*-resveratrol was performed by the modified method of Caponio et al (1999). Reversed phase (RP)-HPLC analysis was done using a SCL-10Avp system controller, a SIL-10AD vp autosampler, a LC-10AD vp pump, a DGU-14 A degasser, a CTO-10 A vp column heater, and a Diode Array Detector set at 278 nm. The 250 x 4.6 mm i.d. 5 µm column was filled with Agilent Eclipse XDB-C18. The flow rate was 0.8 mL min^{-1}, the injection volume was 20 µL, and the column temperature was set at 30 °C. For gradient elution, mobile phase A contained 2% acetic acid; solvent B contained methanol. The gradient program reported by Göktürk-Baydar et al (2011) was used. The data were analyzed using the Shimadzu Class-VP Chromatography Laboratory Automated Software system. The amount of *trans*-resveratrol content in the cells were calculated as µg g^{-1} FCW using external calibration curves obtained for *trans*-resveratrol standard. HPLC determinations were done in triplicate. Data were analyzed by using analysis of variance (ANOVA) using SPSS 16.0 for Windows Software Package and the means were separated by Duncan's multiple range tests.

3. Results and Discussion

In this study, the effects of elicitor applications of $CdSO_4$, fleuresans irradiation, MeJA and sucrose on phenolic accumulation in cell suspension cultures of Gamay, Kalecik karası and Öküzgözü cultivars were determined. $CdSO_4$ treatments positively influenced ($P \leq 0.05$) the syntheses of all of the phenolics depending on its concentrations and sampling dates (Table 1). $CdSO_4$ treatment of 1.5 mM gave the highest phenolic contents in Gamay (2.044 mg g^{-1}) and Kalecik karası (3.144 mg g^{-1}) while both 1.0 and 1.5 mM treatments were suitable concentrations for total flavanols and total flavonols in Öküzgözü. $CdSO_4$ treatment of 1.5 mM resulted in the highest anthocyanin (1.672 CV g^{-1}) and *trans*-resveratrol (3.650 µg g^{-1}) in Kalecik karası cultures. There is no study about the effects of $CdSO_4$ applications on secondary metabolite production of grape cell cultures. On the other hand $CdCl_2$, another compound of cadmium, can be used for enhancing phenolic compounds and tocopherols in grape cell cultures depending on the $CdCl_2$ concentrations and exposure times (Çetin et al 2014) that $CdCl_2$ at 1.0 mM concentration was increased phenolics

Table 1- The effects of $CdSO_4$ application on production of phenolic compounds in grapevine cell cultures

Çizelge 1- $CdSO_4$ uygulamasının asma hücre kültürlerinde fenolik bileşiklerin üretimine etkileri

		Sampling Date (day)	*Total phenolic (mg g^{-1})*	*Total flavanols (mg g^{-1})*	*Total flavonols (mg g^{-1})*	*Anthocyanin (CV g^{-1})*	*Trans-resveratrol (µg g^{-1})*
					GAMAY		
Control		0	0.925 g*	0.064 d	0.045 d	0.624 i	0.721 e
		2	0.677 j	0.051 e	0.087 b	0.699 h	0.703 e
		4	0.884 h	0.048 e	0.044 d	0.787 g	0.862 d
		6	1.505 e	0.071 c	0.064 c	0.685 hi	0.700 e
1.0 mM		0	0.946 g	0.085 b	0.088 b	0.925 f	0.990 c
		2	1.727 b	0.060 d	0.090 b	0.971 ef	1.236 b
		4	1.598 d	0.037 f	0.050 d	1.125 b	0.872 d
		6	0.801 i	0.031 g	0.025 e	1.037 cd	1.613 a
1.5 mM		0	0.990 f	0.074 c	0.091 b	1.013 de	1.194 b
		2	2.044 a	0.093 a	0.107 a	1.099 bc	0.961 c
		4	1.672 c	0.060 d	0.083 b	1.291 a	1.642 a
		6	0.643 k	0.031 g	0.025 e	1.061 bcd	1.261 b
					KALECİK KARASI		
Control		0	1.330 h	0.101 c	0.022 g	0.763 g	0.873 f
		2	1.441 g	0.107 c	0.029 fg	0.776 g	0.850 f
		4	1.474 g	0.110 c	0.040 e	0.773 g	0.851 f
		6	1.302 h	0.099 c	0.033 ef	1.043 e	1.827 e
1.0 mM		0	1.616 f	0.076 de	0.062 d	1.152 d	1.672 e
		2	1.284 h	0.074 de	0.101 c	0.928 f	1.823 e
		4	2.226 c	0.082 d	0.098 c	1.224 d	1.856 e
		6	2.468 b	0.147 b	0.107 c	1.376 c	1.852 e
1.5mM		0	1.696 e	0.104 c	0.032 ef	1.336 c	2.331 c
		2	2.017 d	0.105 c	0.106 c	1.525 b	2.483 c
		4	2.234 c	0.110 c	0.138 b	1.672 a	3.650 a
		6	3.144 a	0.213 a	0.219 a	1.579 b	3.165 b
					ÖKÜZGÖZÜ		
Control		0	1.028 f	0.045 f	0.057 de	0.653 f	1.382 g
		2	1.059 f	0.054 ef	0.063 cd	0.869 d	1.393 g
		4	1.283 de	0.079 d	0.065 cd	0.824 d	2.10 cd
		6	1.221 e	0.094 c	0.066 cd	0.736 e	2.160 cd
1.0 mM		0	1.263 e	0.057 e	0.049 e	0.651 f	1.342 g
		2	1.503 c	0.103 bc	0.078 ab	1.269 b	1.664 e
		4	1.582 b	0.135 a	0.082 a	0.835 d	2.220 bc
		6	1.330 d	0.072 d	0.058 de	1.021 c	2.046 d
1.5 mM		0	1.247 e	0.071 d	0.071 bc	1.077 c	1.512 f
		2	1.524 bc	0.110 b	0.067 c	1.317 ab	2.843 a
		4	1.585 b	0.106 b	0.080 ab	1.360 a	2.962 a
		6	1.754 a	0.136 a	0.079 ab	1.059 c	2.284 b

*, differences between means indicated by the same letters are not statistically significant (P≤0.05)

and tocopherols when cells were harvested at day 2 and 4, respectively. Cadmium toxicity can promote altered metabolism which can include the formation of reactive oxygen species (ROS) in plants under stress conditions (Bergmann et al 2001). Metal ions act as abiotic elicitors and induce biosynthesis of phytoalexins in plant cell cultures (Radman et al 2003). Kidd et al (2001) reported that maize roots exposed to aluminium were exuded high levels of phenolics.

The effects of fleuresans irradiation on phenolic accumulation varied according to types of phenolics (Table 2). Anthocyanin biosynthesis was stimulated considerably by light irradiation and the maximum anthocyanin productions in Gamay (1.584 CV g^{-1}) Kalecik karası (1.893 CV g^{-1}) and Öküzgözü (2.667 CV g^{-1}) were obtained from the cells harvested at day 9. Light induced anthocyanin biosynthesis in cell cultures were reported in *Vitis vinifera* (Zhang et al 2002), *Daucus carota* (Takeda 1990) and *Perilla frutescens* (Zhong et al 1993). Fleuresans irradiation induces photooxidative stress and anthocyanin production is expressed in response to light treatment (Song & Lee 1998). Whereas, the contents of total phenolics, total flavanols, total flavonols and *trans*-resveratrol were the highest on the cells incubated at dark conditions ($P \leq 0.05$). Accordingly, the maximum total phenolic, total flavanol and total flavonol contents of cells cultured in darkness were found as 2.448 mg g^{-1}, 0.106 mg g^{-1} and 0.148 mg g^{-1} respectively in Öküzgözü. The results showed that the dark condition induced *trans*-resveratrol accumulation at all genotypes. The greatest *trans*-resveratrol contents were detected on Gamay (2.473 µg g^{-1}) and Kalecik karası (3.165 µg g^{-1}) cells at days 9 and Öküzgözü cells (9.483 µg g^{-1}) at day 6 (Table 2). Resveratrol can be found in the *cis* or *trans* configurations. Its *trans* form exists in plants, but in red wines a small amount of *cis* form has been detected. The *trans f*orm may change to *cis* form which it's isomer, when after exposure to the UV light (Lopez-Hernandez et al 2007) and high white light (Burns et al 2002).

MeJA positively influenced syntheses of all phenolic compounds on the grape cells (Table 3). Generally, higher concentrations of phenolic compounds were detected at towards the end of the culture. Total phenolic compounds were the highest on the cells applied MeJA and harvested at day 15 in all genotypes and their amounts changed between 1.972 mg g^{-1} (Kalecik karası) and 2.808 mg g^{-1} (Öküzgözü). Total flavanols, total flavonols and anthocyanin contents of MeJA applied cells were higher than those of the controls. MeJA also significantly enhanced *trans*-resveratrol content depending on the sampling date ($P \leq 0.05$) and it was the greatest on Kalecik karası (2.872 µg g^{-1}) and Öküzgözü (11.681 µg g^{-1}) cells harvested at day 9. Maximum anthocyanin accumulation reported after 20 µM jasmonic acid addition to Gamay cells (Zhang et al 2002). In Vinhao grapevine cell cultures, MeJA treatment increased stilbenic production 9-fold compared to the control (Lima et al 2012). MeJA induced anthocyanin accumulation in soybean seedlings as a result of the over-expression of chalcone synthase (Creelman et al 1992). Several elicitors could be used in cell suspension cultures as signaling molecules for *trans*-resveratrol production such as MeJA, cyclodextrins or chitosan (Donnez et al 2009). Jasmonic acid and MeJA are key compounds of the signal transduction system of plant defense reactions (Krisa et al 1999).

Sucrose treatments were applied to create osmotic stress (Do & Cormier 1990) hence to produce secondary metabolites. Sucrose is also essential to induce the expression of the chalcone-synthase gene, one of the genes in the anthocyanin's biosynthetic pathway (Takeuchi et al 1994). The effects of sucrose treatments on the phenolic compounds of grape cells were given in Table 4. Total phenolic, total flavanol, total flavonol and *trans*-resveratrol biosynthesis in Gamay cells increased when they treated with 0.25 M sucrose and harvested at day 9. Otherwise 0.20 M sucrose concentration gave the highest anthocyanin content (2.024 CV g^{-1}) in Gamay. In Kalecik karası, the most abundant total phenolic (4.215 mg g^{-1}), total flavanol (0.164 mg g^{-1}) and total flavonol (0.418 mg g^{-1}) contents were obtained from the cells cultured at 0.20 M sucrose treatment and harvested at day

Table 2- The effects of fleuresans irradiation on production of phenolic compounds in grapevine cell cultures

Çizelge 2- Işık radyasyonu uygulamasının asma hücre kültürlerinde fenolik bileşiklerin üretimine etkileri

	Sampling Date (day)	*Total phenolic (mg g^{-1})*	*Total flavanols (mg g^{-1})*	*Total flavonols (mg g^{-1})*	*Anthocyanin (CV g^{-1})*	*Trans-resveratrol (µg g^{-1})*
				GAMAY		
Dark	0	1.638 c*	0.045 h	0.097 bc	0.771 g	1.791 c
	3	1.637 c	0.055 fg	0.071 e	0.552 h	1.263 d
	6	1.081 i	0.056 f	0.092 c	0.435 i	2.104 b
	9	1.108 h	0.086 c	0.059 f	0.944 f	2.473 a
	12	1.018 j	0.042 i	0.074 e	1.067 e	2.390 a
	15	1.203 g	0.080 d	0.069 e	0.973 f	1.976 b
Light	0	1.262 f	0.053 g	0.088 c	1.075 e	0.974 f
	3	1.480 e	0.075 e	0.098 bc	1.213 d	1.023 ef
	6	1.556 d	0.091 b	0.082 d	1.384 c	0.622 g
	9	1.627 c	0.096 a	0.090 c	1.584 a	0.248 h
	12	1.718 b	0.090 b	0.103 ab	1.347 c	1.023 ef
	15	2.019 a	0.094 a	0.106 a	1.477 b	1.182 de
				KALECİK KARASI		
Dark	0	1.609 c	0.042 e	0.078 c	1.251 fg	1.971 c
	3	1.161 i	0.036 h	0.053 e	1.267 efg	1.633 de
	6	1.192 h	0.026 j	0.051 e	1.235 fg	1.474 e
	9	1.359 g	0.039 f	0.054 e	1.355 d	3.165 a
	12	1.470 e	0.038 g	0.067 d	1.320 de	2.442 b
	15	0.806 j	0.028 i	0.030 f	1.109 h	1.632 de
Light	0	1.741 b	0.060 d	0.080 c	1.208 g	2.411 b
	3	1.486 e	0.090 a	0.076 c	1.360 d	2.483 b
	6	1.416 f	0.083 c	0.066 d	1.736 b	1.662 d
	9	1.510 d	0.090 a	0.088 b	1.893 a	2.011 c
	12	1.793 a	0.090 a	0.111 a	1.501 c	1.590 de
	15	1.737 b	0.086 b	0.063 d	1.293 ef	1.463 e
				ÖKÜZGÖZÜ		
Dark	0	1.666 g	0.072 g	0.113 d	1.981 e	7.890 b
	3	1.785 ef	0.094 d	0.121 c	1.707 g	6.422 d
	6	1.179 i	0.063 h	0.103 e	2.493 b	9.483 a
	9	1.019 j	0.081 f	0.057 h	1.725 g	7.826 b
	12	1.752 f	0.072 g	0.081 g	1.637 h	7.152 c
	15	1.630 h	0.045 i	0.077 g	1.275 i	7.750 b
Light	0	1.822 de	0.090 e	0.107 e	2.179 d	3.192 f
	3	1.800 e	0.091 d	0.095 f	2.149 d	2.384 g
	6	2.232 c	0.100 c	0.138 b	2.248 c	1.665 h
	9	2.407 b	0.106 a	0.104 e	2.667 a	2.583 g
	12	2.448 a	0.104 b	0.148 a	1.952 e	2.742 g
	15	1.835 d	0.080 f	0.104 e	1.859 f	4.351 e

*, differences between means indicated by the same letters are not statistically significant (P≤0.05)

Table 3- The effects of MeJA application on production of phenolic compounds in grapevine cell cultures

Çizelge 3- MeJA uygulamasının asma hücre kültürlerinde fenolik bileşiklerin üretimine etkileri

	Sampling Date (day)	*Total phenolic (mg g^{-1})*	*Total flavanols (mg g^{-1})*	*Total flavonols (mg g^{-1})*	*Anthocyanin (CV g^{-1})*	*Trans-resveratrol (µg g^{-1})*
				GAMAY		
Control	0	1.738 g*	0.036 h	0.098 c	0.587 gh	1.198 f
	3	1.804 f	0.082 f	0.090 cd	0.531 hi	0.862 g
	6	1.527 j	0.066 g	0.049 h	0.491 i	1.561 d
	9	1.769 fg	0.083 ef	0.067 g	0.720 e	1.234 ef
	12	1.584 i	0.091 d	0.097 c	0.667 ef	1.485 d
	15	1.675 h	0.087 e	0.074 fg	0.851 d	1.232 ef
10 µM	0	1.782 f	0.098 c	0.115 b	0.648 fg	1.341 e
	3	2.138 c	0.080 f	0.082 ef	0.637 fg	1.530 d
	6	2.042 e	0.099 c	0.074 fg	0.968 c	1.962 bc
	9	2.086 d	0.110 b	0.085 de	1.163 a	1.900 c
	12	2.321 b	0.126 a	0.112 b	1.069 b	2.083 ab
	15	2.478 a	0.126 a	0.155 a	0.805 d	2.201 a
				KALECİK KARASI		
Control	0	1.506 f	0.088 c	0.059 c	0.541 f	1.914 cd
	3	1.108 h	0.089 c	0.058 c	0.427 g	1.291 f
	6	0.967 i	0.080 de	0.044 g	0.568 ef	0.382 i
	9	1.242 g	0.091 c	0.050 e	0.725 c	0.473 hi
	12	0.642 k	0.082 d	0.052 de	0.637 d	0.562 h
	15	0.697 j	0.067 f	0.040 g	0.595 de	1.060 g
10 µM	0	1.766 b	0.078 e	0.057 cd	0.627 d	2.563 b
	3	1.697 c	0.082 d	0.074 b	0.571 ef	1.752 e
	6	1.665 d	0.097 b	0.049 ef	0.728 c	1.880 cd
	9	1.556 e	0.101 a	0.060 c	1.056 b	2.872 a
	12	1.690 c	0.095 b	0.064 c	1.123 a	1.860 de
	15	1.972 a	0.100 a	0.098 a	0.709 c	2.000 c
				ÖKÜZGÖZÜ		
Control	0	1.330 i	0.050 g	0.057 ef	0.504 f	1.492 ef
	3	1.345 hi	0.030 j	0.058 def	0.520 f	1.681 ef
	6	1.371 gh	0.051 fg	0.053 f	0.552 f	2.860 d
	9	1.398 g	0.065 d	0.065 bc	0.616 e	4.095 c
	12	1.211 j	0.073 c	0.085 a	0.549 f	2.134 e
	15	1.647 f	0.053 ef	0.059 cdef	0.627 e	1.106 f
10 µM	0	1.821 d	0.047 h	0.060 cde	0.608 e	1.854 e
	3	1.951 c	0.055 e	0.066 bc	0.781 d	2.963 d
	6	1.744 e	0.056 e	0.068 b	0.856 bc	6.822 b
	9	1.622 f	0.056 e	0.081 a	0.888 b	11.681 a
	12	2.453 b	0.093 b	0.083 a	1.077 a	3.432 d
	15	2.808 a	0.101 a	0.080 a	0.821 cd	3.060 d

*, differences between means indicated by the same letters are not statistically significant (P≤0.05)

Table 4- The effects of sucrose application on production of phenolic compounds in grapevine cell cultures

Çizelge 4- Asmada hücre kültürlerinde fenolik bileşiklerin üretiminde sukroz uygulamasının etkileri

	Sampling Date (day)	*Total phenolic (mg g^{-1})*	*Total flavanols (mg g^{-1})*	*Total flavonols (mg g^{-1})*	*Anthocyanin (CV g^{-1})*	*Trans-resveratrol (µg g^{-1})*
				GAMAY		
Control	0	0.740 fg*	0.024 f	0.044 e	0.904 de	0.964 f
	3	1.189 e	0.051 d	0.066 d	0.851 de	1.235 e
	6	1.234 e	0.068 d	0.076 cd	1.029 d	1.413 d
	9	1.000 ef	0.031 e	0.022 fg	0.757 f	0.668 h
	12	1.010 ef	0.054 d	0.061 d	0.693 f	1.114 ef
	15	0.808 f	0.026 ef	0.045 e	0.467 g	0.553 i
0.20 M	0	0.650 g	0.028 ef	0.032 f	0.771 ef	0.802 g
	3	2.074 b	0.094 bc	0.103 b	2.024 a	1.981 c
	6	1.800 c	0.095 bc	0.103 b	1.493 b	1.934 c
	9	1.633 cd	0.066 d	0.084 c	1.101 cd	1.450 d
	12	1.605 cd	0.059 d	0.080 c	0.741 ef	1.022 f
	15	1.900 c	0.083 bc	0.115 b	0.765 ef	1.171 e
0.25 M	0	0.731 fg	0.023 f	0.023 fg	1.328 bc	0.973 f
	3	0.727 fg	0.032 e	0.021 fg	1.056 d	1.000 f
	6	1.234 e	0.054 d	0.050 e	0.752 ef	0.990 f
	9	3.510 a	0.164 a	0.361 a	1.480 b	3.740 a
	12	2.457 b	0.158 a	0.089 c	0.765 ef	1.262 e
	15	1.520 d	0.117 b	0.084 c	1.288 c	2.351 b
				KALECİK KARASI		
Control	0	1.683 c	0.058 d	0.023 f	0.925 f	1.793 fg
	3	1.879 c	0.067 c	0.063d	1.128 e	2.491 e
	6	1.474 cd	0.075 c	0.052 de	0.925 f	1.616 fg
	9	1.723 c	0.071 c	0.065 d	0.760 g	1.894 f
	12	1.417 cde	0.058 d	0.044 e	0.875 f	1.040 hi
	15	1.545 cd	0.061 cd	0.052 de	0.992 ef	1.990 f
0.20 M	0	1.588 cd	0.041 de	0.025 f	0.741 g	3.891 b
	3	1.088 e	0.064 cd	0.055 de	1.909 a	7.550 a
	6	2.064 b	0.095 bc	0.082 bc	1.728 ab	1.662 fg
	9	2.397 b	0.099 bc	0.097 b	1.096 e	3.973 b
	12	4.215 a	0.164 a	0.418 a	0.875 f	3.454 c
	15	1.524 cd	0.041 de	0.064 d	0.925 f	3.200 cd
0.25 M	0	1.338 de	0.043 de	0.023 f	0.832 f	0.881 i
	3	1.281 de	0.052 d	0.054 de	1.459 c	1.376 h
	6	1.497 cd	0.057 d	0.039 e	1.819 a	1.372 h
	9	1.682 c	0.112 b	0.083 bc	1.301 d	1.880 f
	12	1.192 e	0.057 cd	0.061 d	1.213 de	1.711 fg
	15	0.742 f	0.039 e	0.034 e	1.088 e	0.733 j
				ÖKÜZGÖZÜ		
Control	0	2.004 ef	0.117 cd	0.083 de	0.877 f	3.472 e
	3	2.366 cd	0.109 d	0.087 de	1.165 d	4.855 b
	6	1.992 ef	0.096 d	0.086 de	1.013 ef	3.476 e
	9	1.320 h	0.075 e	0.027 g	0.875 f	3.030 fg
	12	1.051 i	0.030 g	0.034 fg	0.869 f	2.283 j
	15	1.113 i	0.057 f	0.047 f	0.979 ef	3.190 f
0.20 M	0	2.073 e	0.117 cd	0.083 de	1.061 e	2.692 h
	3	2.463 c	0.098 d	0.091 de	1.496 b	2.270 j
	6	1.545 g	0.103 d	0.097 de	0. 912 ef	2.613 h
	9	2.067 e	0.116 cd	0.132 c	0.712 g	2.940 g
	12	2.151 de	0.140 b	0.112 d	1.104 e	4.042 cd
	15	2.462 c	0.147 b	0.163 b	1.192 c	3.843 d
0.25 M	0	1.869 f	0.094 d	0.086 de	0.979 ef	2.514 hi
	3	2.259 d	0.105 d	0.121 c	1.192 cd	2.435 i
	6	2.275 d	0.098 d	0.106 d	1.093 e	3.254 ef
	9	2.686 bc	0.125 c	0.169 b	1.245 c	4.172 c
	12	2.802 a	0.137 bc	0.217 a	1.629 a	4.91 b
	15	2.706 ab	0.187 a	0.204 a	1.088 e	5.531 a

12 while the greatest anthocyanin contents were obtained from both at 0.20 M and 0.25 M sucrose treatments as 1.909 CV g^{-1} and 1.819 CV g^{-1}, respectively. For Öküzgözü cell cultures, 0.25 M sucrose concentration was found as the most suitable sucrose concentration in terms of all secondary metabolites. The maximum *trans*-resveratrol content (7.550 µg g^{-1}) was obtained from Kalecik karası cell cultures treated with 0.20 M sucrose and harvested at day 3, which represent a 3 fold increase compared with the control cultures (2.491 µg g^{-1}) harvested on the same date. Similarly Larronde et al (1998) reported that total stilbene content was 1.5 times greater at 0.10 M sucrose than that of cells grown without added-sucrose while anthocyanin contents increased 12-fold from control to 0.15 M added sucrose. The production of secondary metabolites by increasing the concentration of carbohydrates has generally attributed to increased precursors available for secondary metabolite. On the other hand, it was also demonstrated that sucrose concentration of 4% decreased cell growth and hence may stimulate metabolite biosynthesis through an osmotic stress phenomenon biosynthesis (Knobloch & Berlin 1983). The synthesis of anthocyanins has been shown to be stimulated by sucrose in cells (Hirasuna et al 1991) of grapevine, and seems to result from an osmotic stress (Do & Cormier 1990). The mechanisms by which plant cells detect and respond to sucrose are very poorly understood. It is known that sucrose was found to modulate other metabolites such as polyphenol accumulation in *Vitis vinifera* cell cultures. Ferri et al (2011) reported the high levels of many flavonoids and stilbenes in Barbera cell suspensions treated with increased sucrose concentrations. The effect of sugars on plant cells seems to be due to the coupling of two mechanisms: osmotic stress and disturbed cellular metabolism (Do & Cormier 1990). *Trans*-resveratrol contents were affected by all sucrose concentrations and sampling dates. Donnez et al (2009) also reported that the amount of *trans*-resveratrol fluctuates widely according to plant species, elicitor and culture conditions.

4. Conclusions

The results showed that fleuresans irradiation, $CdSO_4$, MeJA and sucrose are potent elicitors in cell suspension cultures of Gamay, Kalecik karası and Öküzgözü grapevine cultivars. $CdSO_4$ at 1.5 mM concentration and MeJA at 10 µM concentration compared to controls yielded the highest total phenolics, anthocyanin and *trans*-resveratrol productions in all cultivars while 0.20 and 0.25 M sucrose concentrations were found as the most suitable concentrations depending on the cultivars. Light irradiation resulted in a significant synergistic enhancement of anthocyanin accumulation whereas dark conditions stimulated the total phenolic, total flavanol, total flavonol and *trans*-resveratrol synthesis in all cultivars tested. Further experiments should be studied to examine the relationship between elicitor and metabolite production in cell cultures. The results also demonstrate an efficient avenue for the development of similar strategies to advance the plant cell culture process for commercial production.

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