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Effects of elicitors on secondary metabolite (SM) production and antioxidant activity in sweet basil (*Ocimum basilicum* L.) cell suspension cultures

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Abstract

Elicitor treatments play an important role in inducing some protective signal transmitter enzymes in cells and regulating phenylalanine ammonia-lyase (PAL) activity. The aim of this study was to determine the effects of elicitors [silver nitrate ($AgNO_3$), salicylic acid (SA) and yeast extract (YE)], which were added individually or in combination to *Ocimum basilicum* L. cell suspension cultures, on the production and antioxidant activity of secondary metabolites (SMs). Calluses were obtained from the leaves of *O. basilicum* kept on Murashige and Skoog (MS) medium containing 0.5 mg/l kinetin (KIN) + 2.5 mg/l naphthalene-acetic acid (NAA) and cell suspension cultures were initiated. Then elicitors were applied to the cell suspension cultures individually or in combination, and cells were harvested at the end of the second, fourth, and eighth days. Compared with the control culture, the maximum rosmarinic and chicoric acid production was obtained at the end of the 4th day from SA (24 µM) + YE (80 mg/l) treatment as 20.19 mg/g DW (118%) and 7.55 mg/g DW (123%), respectively. The maximum biosynthesis of isoquercetin and rutin compared with the control culture was 3.88 mg/g DW [YE (80 mg/l)] with a 1.6-fold increase and 6.35 mg/g DW [YE (80 mg/l) + $AgNO_3$ (6 µM)] with a 1.76-fold increase, respectively. Estragole and linalool's highest values compared with the control culture were 4.50 µg/g DW [$AgNO_3$ (6µM) + SA (24 µM)] and 3.02 µg/g DW [SA (24 µM)], respectively. Results clearly show that the elicitor treatment could enhance the biosynthesis of phenolic compounds and terpenoid content in cell suspension cultures of *O. basilicum* and may be used for commercial supply in the future for therapeutic applications.

Keywords: elicitor treatments; *in vitro* culture systems; phenolic; terpenoid

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Introduction

Phenolic compounds and terpenoids are biologically active ingredients that are very important for the food, pharmaceutical, and cosmetic industries. Secondary metabolites derived from medicinal and aromatic plants grown in natural habitats are produced in small amounts but in large numbers of varieties of plants. Herbal production of these compounds, which are weightless but precious and vary depending on the variety, cultivar, organs (Açıkgöz, 2019; Açıkgöz, 2020a), environmental factors (Ahmed *et al.*, 2019), climate (Baldim *et al.*, 2018), season (Hussain *et al.*, 2008), growth and development periods (Açıkgöz *et al.*, 2017; Açıkgöz, 2019), pre- and post-harvest cultural processes (Talebi *et al.*, 2018; Alkuwayti *et al.*, 2019) and extraction methods (Coelho *et al.*, 2018), is time-consuming and cost-effective under natural conditions. Moreover, all these factors make it difficult for these compounds to be produced homogeneously and highly pure under natural conditions. However, these features are very important in the pharmaceutical, food and cosmetic industries (Rao and Ravishankar, 2002). Therefore, callus, hairy root and organ cultures, especially *in vitro* cell suspension cultures, are preferred in obtaining the valuable secondary metabolites for reasons such as the elimination of undesirable characteristics, modification of desired characteristics, obtainment of products with certain quality and standards, being predictable and reliable, and most importantly, being independent of seasonal, environmental and geographic factors (Karuppusamy, 2009; El-Salam *et al.*, 2015).

In addition, the cell suspension culture in plant tissue culture systems is reported to be more effective for interacting easily and quickly with the nutrients of cells, plant growth regulators (PGRs) and elicitors, for its quickly divided cells, and for their practical advantages of applicability compared with callus and other cultures (Kintzios *et al.*, 2003; Georgiev *et al.*, 2007; Mathew and Sankar, 2011; Mathew and Sankar, 2014).

To date, many elicitors (abiotic and biotic) have been successfully used in the synthesis of secondary metabolites in plant tissue culture systems (Mathew and Sankar, 2012; Mathew and Sankar, 2014; Gupta *et al.*, 2018; Krishnan and Siril, 2018; Açıkgöz *et al.*, 2019; Nadeem *et al.*, 2019). Among the elicitors, ethylene inhibitor silver nitrate ($AgNO_3$) (Hegde *et al.*, 2017; Yang *et al.*, 2017; Açıkgöz, 2020b), yeast extract that is one of the endophytic fungi (YE) (Dowom *et al.*, 2017; Sing *et al.*, 2017; Açıkgöz, 2020a), and salicylic acid (SA), which plays a key role in the growth-development and defense systems of plants and having an important function in inducing some protective signal transmission enzymes in cells and regulating the activity of phenylalanine ammonia-lyase (PAL) (Ptak *et al.*, 2017; Yang *et al.*, 2017), are frequently used in plant tissue culture systems, either individually or in combination.

The aerial parts of *Ocimum basilicum* (Lamiaceae) are valuable annual herb used in the food industry and folk medicine (antispasmodic, carminative, digestive, galactagogue, stomachic and tonic agent) (Marwat *et al.*, 2011). In addition to these features, *O. basilicum* has been reported to have antituberculosis, antioxidant, antimicrobial, antifungal and anti-inflammatory potentials (Siddiqui *et al.*, 2012; Koroch *et al.*, 2017; Piras *et al.*, 2018; Aye *et al.*, 2019). These pharmaceutical potentials are caused by phenolic compounds such as rosmarinic acid, chicoric acid, isoquercetin and rutin, and terpenoids such as estragole (methyl chavicol) and linalool, which are dominantly found in sweet basil (Lee and Scagel, 2009; Nguyen *et al.*, 2010; Kwee *et al.*, 2011; Vlase *et al.*, 2014; Piras *et al.*, 2018; Talebi *et al.*, 2018; Alkuwayti *et al.*, 2019).

In the studies conducted in plant tissue culture systems of *O. basilicum* to date, researchers have used light-emitting diodes (LEDs), methyl jasmonate, yeast extract, silver nitrate, cadmium chloride, chitosan, melatonin, salicylic acid and PGRs in hairy root and callus cultures and successfully biosynthesised a number of bioactive compounds such as 1,8-cineole, berberine ursolic acid, betulinic acid, oleanolic acid, rosmarinic acid, caffeic acid, chicoric acid, eugenol, rutin, linalool, isoquercetin, estragole, peonidin and cyanidin (Hakkim *et al.*, 2007; El-Salam *et al.*, 2015; Pandey *et al.*, 2015; Duran *et al.*, 2019; Nadeem *et al.*, 2019; Nazir *et al.*, 2019; Açıkgöz, 2020a; Açıkgöz, 2021; Dağlioğlu *et al.*, 2022). The studies conducted in cell suspension cultures of *O. basilicum* have reported that PGRs of elicitor applications are limited to methyl jasmonate, chitosan, and

mechanical stress (Mathew and Sankar, 2011; Strazzer *et al*., 2011; Mathew and Sankar, 2012; Mathew and Sankar, 2014; Pandey *et al*., 2019), and we have determined that there are limited number of studies of $AgNO_3$, SA and YE elicitors that have been successfully used in other species" of cell suspension cultures before. Considering this information, the aim of the current research is to determine the effects of $AgNO_3$, SA and YE elicitors on the production of SMs and antioxidant activity, which are added individually or in combination to *O. basilicum* L. cell suspension cultures.

Materials and Methods

Plant material and culture conditions

O. basilicum seeds, after being left in 70% ethyl alcohol for 30 seconds, were washed for 120 seconds under running tap water. Then they were kept in 1.95% sodium hypochlorite (NaClO) solution for 30 min and then rinsed in the sterile cabinet with sterile distilled water until the foaming is eliminated. They were then planted in Murashige and Skoog (MS) mediums containing 1.0 mg/l GA_3, 3% (w/v) sucrose and 0.8% (W/V) agar, whose pH was adjusted to 5.6-5.8. Magenta plates were maintained at 25 ± 2 °C in 16/8h light/dark photoperiod (135 µmol m^{-2} s^{-1}) provided by cool white fluorescent tube lights.

Establishment callus culture

About two months after the seeds were planted, the leaves of aseptic seedlings were cut with scalpels and used as an explant source for the creation of callus cultures (Figure 1). The leaf explants were then planted in basic nutrient media [combinations of kinetin (KIN) with 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-acetic acid (NAA)]. The results of combinations of these PGRs are not presented here. However, PGRs to be added to the basic nutrient media were determined by considering criteria such as vital explant rate (%), callus-forming explant rate (%) and callus weight (g). Accordingly, explants were planted in Murashige and Skoog (MS) nutrient media containing 0.5 mg/l KIN + 2.5 mg/l NAA + 3% (w/v) sucrose and 0.8% (w/v) agar. Then, Petri dishes (12 cm) were maintained at 25 ± 2 °C and 16/8h light/dark photoperiod (135 µmol m^{-2} s^{-1}) provided by cool white fluorescent tube lights. The calluses were then brought into subcultures with the same PGRs and nutrient media two times every three weeks.

Establishment cell suspension culture

After six weeks, the calluses were placed in 250 ml conical flasks containing 100 ml of broth medium in the sterile cabinet. The samples were kept on a shaker with an average speed of 105 rpm in 16/8h light/dark photoperiod (135 µmol m^{-2} s^{-1}) conditions at 25 ± 2 °C for 4 weeks. In this process, samples were taken into subcultures twice every two weeks. Fresh cells were then weighed to be 2.0 g in the sterile cabinet and placed in Murashige and Skoog (MS) nutrient media containing 0.5 mg/l KIN (kinetin) + 2.5 mg/l NAA (naphthalene-acetic acid) and 3% (w/v) sucrose. Finally, elicitors [silver nitrate ($AgNO_3$), salicylic acid (SA) and yeast extract (YE)] were added to the cell suspension cultures in different concentrations. Then, cell suspension cultures were kept under 16/8h light/dark photoperiod (135 µmol m^{-2} s^{-1}) conditions at 25 ± 2 °C in the climate chamber on the shaker with an average speed of 105 rpm (Figure 1).

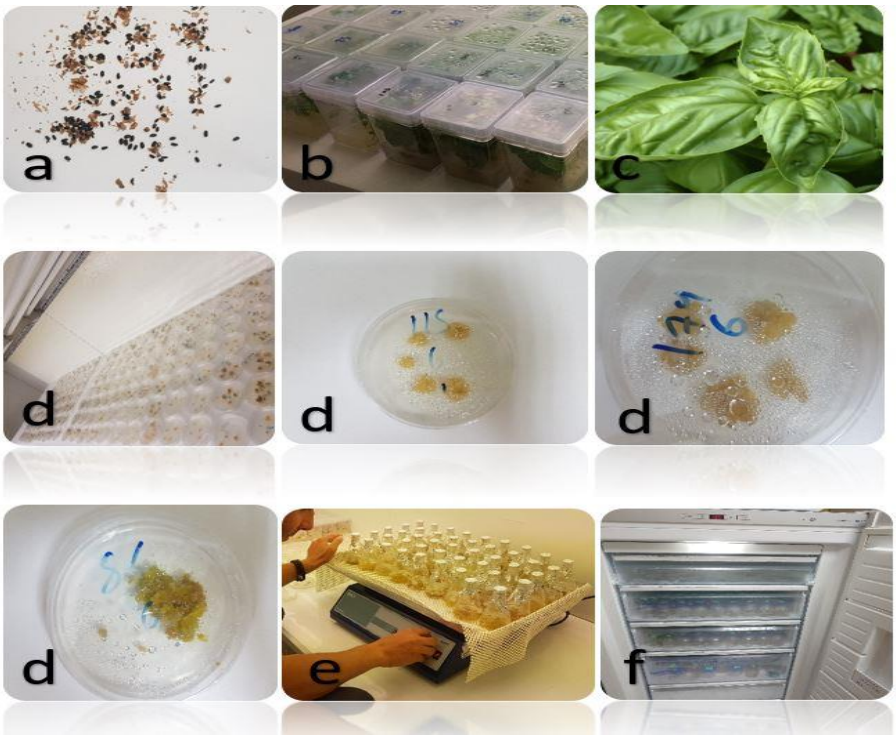

Figure 1. a) Seeds of *Ocimum basilicum* L., b) aseptic seedlings, c) leaves of aseptic seedlings, d) callus induction, e) cell suspension cultures, f) the samples of kept at -20 °C until extraction

Elicitor preparation and treatments

The stock solution was prepared for the elicitors used in the study. After preparing stock solution for silver nitrate ($AgNO_3$), salicylic acid (SA) and yeast extract (YE) elicitors; $AgNO_3$ was prepared at 2 different concentrations (6 and 12 µM) and SA at 2 different concentrations (12 and 24 µM). The stock solution for the yeast elicitor was prepared according to Hahn and Albersheim (1978). Briefly, the yeast was dissolved in distilled water as 20 g/100 mL, then mixed with 400 ml of ethanol and kept in the fridge for four days (+4 °C). The precipitate was again thawed in 100 ml distilled water and mixed with 400 ml ethanol. Then the adhesive precipitate was thawed in 50 ml distilled water and adjusted in 2 different concentrations (40 and 80 mg/l). The pH of the elicitor solutions was adjusted to 5.8 before the cell was added to the suspension cultures and filter sterilization was performed by means of a microfilter of 0.2 µm. Elicitor treatments were then applied individually or in combination to cell suspension cultures. Three samples from each elicitor application were taken at the end of the 2nd, 4th and 8th day. After each treatment, 5 ml was allocated to determine cell count, cell dry weight and cell viability, and then the samples were kept at -20 °C until they were analyzed (Figure 1).

Identification of the cell number, cell dry weight (g/l), and cell viability (%)

Cell growth was determined by measuring the average number of cells (N^C) and cell dry weight (g/l). The cell count was determined by the following formula using the Nageotte counting circle according to Moscatiello et al. (2013). $N^C = \sum_{i=1}^{40} C \cdot 20 \cdot F^D$, where: N^C is the average number of cells, C is the number of cells counted in each rectangle, the absorbance of sample, F^D is the dilution factor.

The cell dry weight was obtained by weighing the filtered cells after they were kept in a drying oven (60 °C at 24 h). Cell viability determination was made according to Laloue *et al.* (1980) using the technique of Trypan blue staining. Briefly, 50 µl of cell suspension culture, 125 µl of Trypan blue and 75 µl of phosphate buffer that keep the cells alive in the counting phase and increase staining efficiency were added to the Eppendorf tubes. After vortexing (20 min), the counting process was made in the light microscope (200 x magnification) and the cell viability rate was demonstrated as percentage (%).

Sample extraction

Elicitor-treated cell samples were individually weighed at 100 mg and 10 ml of methanol was added on them. It is then kept in the ultrasonic-assisted bath (ULT AG 440, 35 kHz fixed frequency, 180 W, Germany) at 45 °C for 120 minutes. Then, the solution was centrifuged for 10 min at 8.000 rpm 3-times at 30 min intervals. The supernatants were collected and used in the required analysis.

Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis

The phenolic contents of the samples were determined by RP-HPLC analysis that was carried out using the Shimadzu Prominence Modular LC20A system via C18 column 250 x 4.6 mm id, 5 µm. The mobile phase includes 1% aq. The column was controlled at 28 °C, the injection was adjusted to 20 µl. The solution was prepared by changing the proportion of acetonitrile (solvent B) to acetic acid (solvent A). The solvent B proportion in the solution was changed from 10% to 40% gradually during the first 28 min, from 40% to 60% up to 39 min, and from 60% to 90% up to 50 min. The mobile phase composition was altered compared with the first condition in 55 min and kept for 10 min, the same procedure was done for the other samples. The analysis time for each sample was 65 min and HPLC chromatograms were formed using photodiode array UV detector at the wavelengths of 272, 280 and 310 nm according to the maximum absorption of analyzed compounds. Each phenolic compound was defined by the addition of standards under the same conditions and by the comparison of retention times. Rosmarinic acid (RA), chicoric acid (CA), isoquercetin (I) and rutin (R) contents in each extract were quantified based on the equation of the external standard calibration curves (R^2 = 0.9998, y = x365706 + 280.17, RA; R^2 = 0.9998, y = x105846 + 102.38, CA; R^2 = 0.9998, y = x124652 + 9862, I and R^2 = 0.9998, y = x520849 + 3642, R) obtained using stock standards methanolic solutions of phenolic compounds at different concentrations (12.5 to 400 µg/ml). All analyzes were performed in triplicate, and data were reported as mean ± standard deviation (SD).

Headspace solid-phase microextraction-gas-chromatography-mass-spectrometry (HS-SPME–GC/MS) analysis

The estragole and linalool contents were measured with HS-SPME–GC/MS (Innovatech Labs, LLC, USA) using a Shimadzu QP2010 Ultra integrated with a Shimadzu AOC-5000 plus autosampler (Shimadzu Scientific Inst., USA). The column was an RTX-5MS capillary column (30 m × 0.32 mm ID, 0.50 µm with integra-guard column). Helium was used as the carrier gas with a constant flow mode at a flow rate of 1 ml/min. Injection temperature of 250 °C, injection volume of 0.5 ml, ionization voltage of 70 eV, the temperature of 100 °C, and heating period of 10 min were used as standard. For the analysis, linalool and estragole stock solutions were prepared in ethyl acetate. Then, 9 calibration points ranging from 0.75–100 µg/ml were prepared from the stock standard solutions and n-tridecane (100 µg/ml). These solutions were used to construct individual terpene calibration curves, and by using these curves, the linalool and estragole contents were expressed as µg/g DW. All analyzes were performed in triplicate, and data were reported as mean ± standard deviation (SD).

Determination of total flavonoid (TFC) and total phenolic content (TPC)

Total flavonoid content (TFC) was determined using aluminum chloride ($AlCl_3$) colorimetric method as described by Fazal *et al.* (2016) with little modifications. In short, 10 µl of $AlCl_3$ and potassium acetate was mixed with 20 µl of the sample. Then, each sample was diluted with 160 µl of distilled water and incubated for 30 min. Spectrophotometrical readings were achieved at 415 nm using a microplate reader. Quercetin standard calibration curve was used for TFC and the values were shown as quercetin equivalents (QE)/g of DW.

Total phenolic content (TPC) was measured using the Folin-Ciocalteu method as described by Velioglu *et al.* (1998) with slight modifications. Shortly, 20 µl of the sample was mixed with 90 µl of Folin-Ciocalteu reagent and incubated for 60 min at room conditions and the mixture was diluted with 90 µl of sodium

carbonate and the absorbance was measured at 725 nm. Gallic acid served as the standard for plotting calibration curve, and the results were shown as gallic acid equivalents (GAE)/g of DW.

Determination of antioxidant activity

Antioxidant activity was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Amarowicz *et al.* (2004). Shortly, 10 mg of the cell sample was dissolved in 4 ml of methanol then added to a methanol solution of DPPH (1 mM, 0.5 ml). The mixture was vortexed and kept in dark condition (60 min). Then the absorbance values of reaction mixtures were measured at 517 nm using UV–vis spectrophotometer. The antioxidant activities of the samples were calculated according to that formula: Antioxidant activity (%) = $(1-S^A/C^A)$ x 100, where S^A is the sample absorbance and C^A is the control absorbance.

Experimental design and data analysis

The data were indicated as the mean ± standard deviation (SD) of three replicates of each experiment. The data were subjected to analysis of variance (ANOVA) using statistical analysis system software (SAS-JMP) version 10. The significant differences were calculated using the least significance difference (Tukey) and the differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Identification of the cell number, cell dry weight (g/l), and cell viability (%)

The effect of elicitor treatments on cell number, cell dry weight (g/l), and cell viability (%) is given in Figure 2, Figure 3, and Figure 4. Cell growth (cell count and cell weight) and cell viability were reduced in all treatments except for the 12 µM and 24 µM concentration of SA. The highest number of cells was 83.800 for the 12 µM treatment of SA, followed by 24 µM treatment of SA with 83.200. The lowest number of cells was 70.940 in the 12 µM treatment of $AgNO_3$ (Figure 2). When the cell's dry weight values were examined (Figure 3), it was observed that the highest cell dry weight was 7.31 g/l in cells harvested at the end of the 8th day (SA, 12 µM), followed by another cells (SA, 24 µM) with 7.26 g/l harvested at the end of 4th day. Among elicitor treatments, the highest decrease in cell viability was found in the combined treatment of YE (80 mg/l) + $AgNO_3$ (12 µM) (81.8%, at the end of 8th day) (Figure 4). Among all these treatments, where elicitors were administered individually or in combination, the only treatment that increased cell viability was the concentration of 12 µM (cells harvested at the end of the 2nd day) and 24 µM (cells harvested at the end of 2nd, 4th and 8th days).

In plant tissue culture systems, the cell growth, cell viability, and biosynthesis of SMs vary depending on the age of the culture (Namdeo, 2007; Kang *et al.*, 2009), the type of elicitor (Kubes *et al.*, 2014; Nazir *et al.*, 2019), and the length of time the cells are exposed to elicitor. Therefore, it is very important to develop elicitor treatments by researchers based on these criteria according to plant cultivars. Literature search has shown that there has been no study on the effects of individual or combined treatment of $AgNO_3$, SA and YE elicitors on cell growth and cell viability in *O. basilicum* cell suspension cultures. However, our literature scan has cleared that $AgNO_3$ and YE treatments applied alone in other species inhibit cell growth and reduce cell viability, as in the present study (Zhao *et al.*, 2010; Cai *et al.*, 2013; Sivanandhan *et al.*, 2014; Zaker *et al.*, 2015; Gonçalves *et al.*, 2019). However, contrary to our results, some research has reported that cell growth can be positively stimulated if $AgNO_3$ and YE elicitors are administered individually or in combination at the appropriate concentrations (Yan *et al.*, 2006; Deepthi and Satheeshkumar, 2016; Sing *et al.*, 2017; Nadeem *et al.*, 2018; Roy and Bharadvaja, 2019). On the other hand, there are studies reporting that the SA treatments alone and when used in combination with other elicitors prevent cell growth (Gadzovska *et al.*, 2013; Xu *et al.*, 2015),

although some studies report that SA treatments promote cell growth in a positive way, as in the present research (Nadeem *et al.*, 2018; Roy and Bharadvaja, 2019).

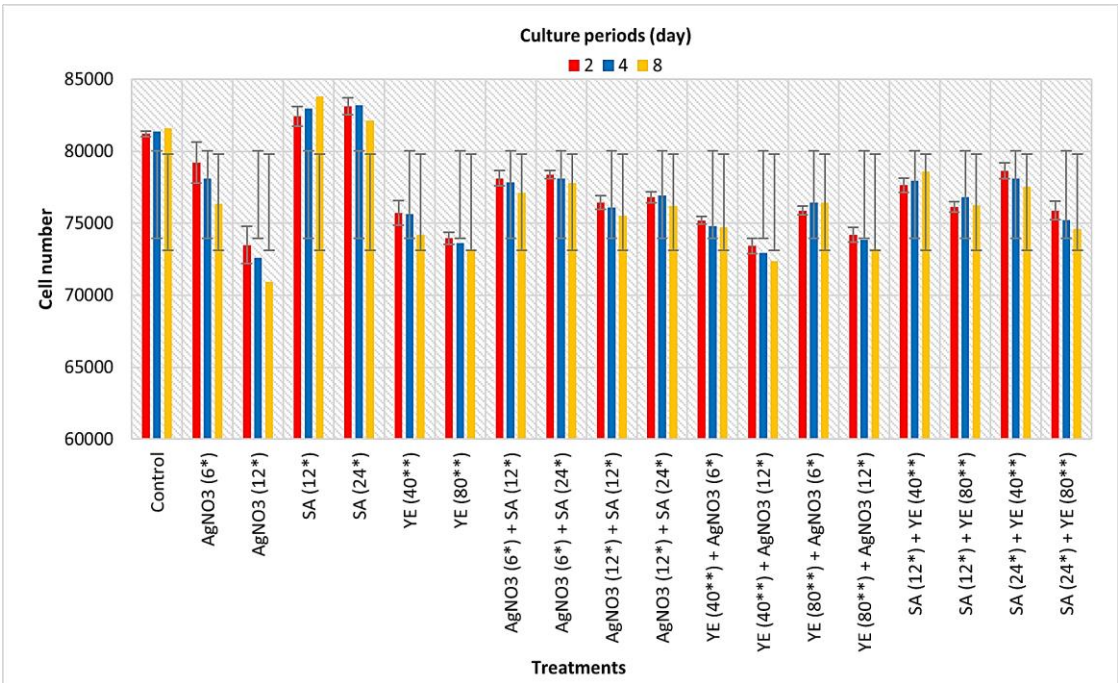

Figure 2. The effect of abiotic and biotic elicitor treatments on cell number
The result are expressed as means ± standard deviation (n=3). Error bars show standard deviation values. $AgNO_3$ = silver nitrate, SA = salicylic acid, YE = yeast extract,* = µM, ** = mg/l

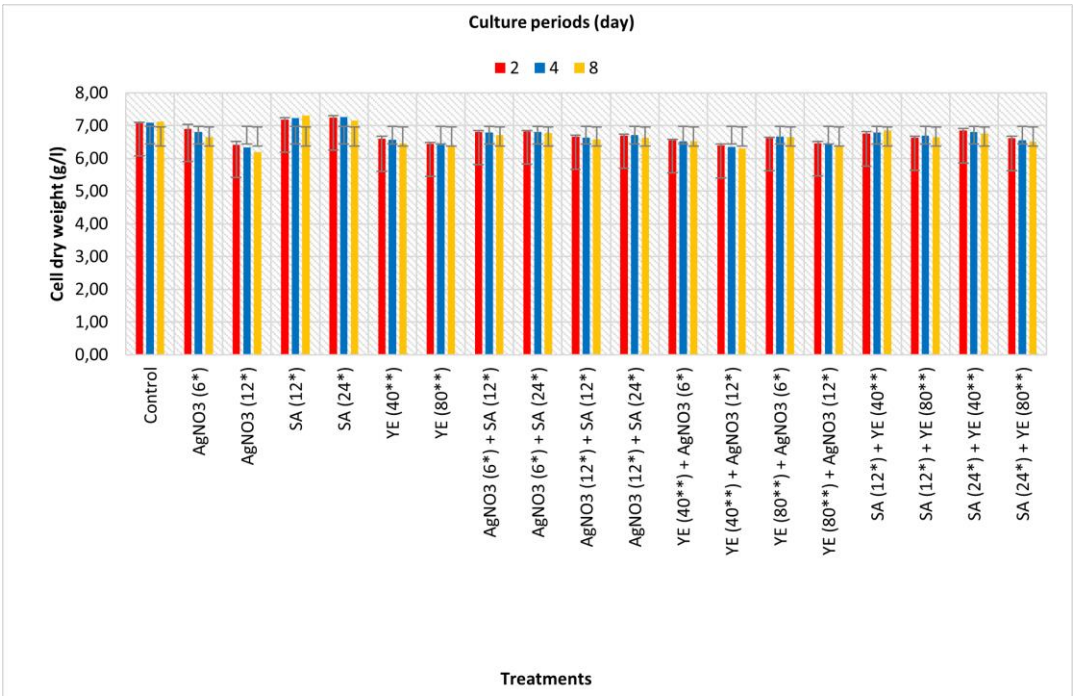

Figure 3. The effect of abiotic and biotic elicitor treatments on cell dry weight (g/l)
The result are expressed as means ± standard deviation (n=3). Error bars show standard deviation values. $AgNO_3$ = silver nitrate, SA = salicylic acid, YE = yeast extract, * = µM, ** = mg/l

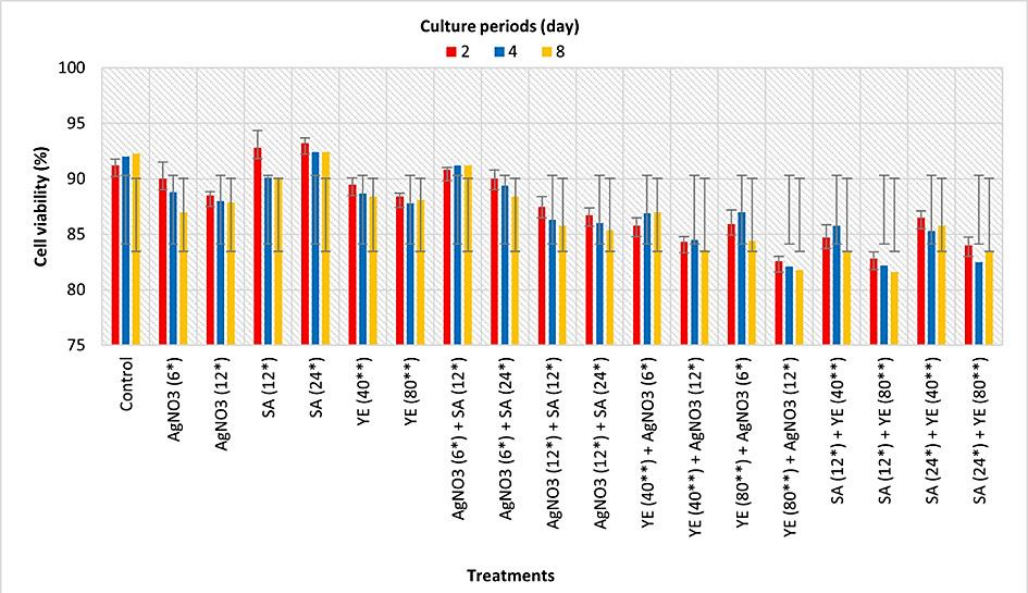

Figure 4. The effect of abiotic and biotic elicitor treatments on cell viability (%)
The result are expressed as means ± standard deviation (n=3). Error bars show standard deviation values. $AgNO_3$ = silver nitrate, SA = salicylic acid, YE = yeast extract, * = μM, ** = mg/l

Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis

The rosmarinic acid, chicoric acid, isoquercetin, and rutin contents were evaluated by RP-HPLC analysis and the values were demonstrated as mg/g DW and RP-HPLC chromatograms of the standards and extracts were shown in Figure 5 and Figure 6. The effects of elicitor treatments applied to *O. basilicum* cell suspension cultures on rosmarinic acid, chicoric acid, isoquercetin, and rutin accumulation are presented in Table 1 and Table 2. Accordingly, the rosmarinic and chicoric acid contents were found to vary from 8.15 mg/g DW to 20.19 mg/g DW and from 2.32 mg/g DW to 7.55 mg/g DW, respectively, through elicitor treatments. While elicitor treatments is generally seen to increase rosmarinic acid and chicoric acid biosynthesis, the treatment of $AgNO_3$ (12 μM) + SA (12 μM) reduced the biosynthesis of rosmarinic acid, and YE (40 mg/l) + $AgNO_3$ (12 μM) and YE (80 mg/l) + $AgNO_3$ (12 μM) treatments reduced the biosynthesis of chicoric acid compared with the control culture (Table 1). On the other hand, the highest increase in the rosmarinic and chicoric acid content in elicitor treatments compared with the control culture was 118% and 123% [(SA (24 μM) + YE (80 mg/l)], respectively, obtained from the cells harvested at the end of the 4th day.

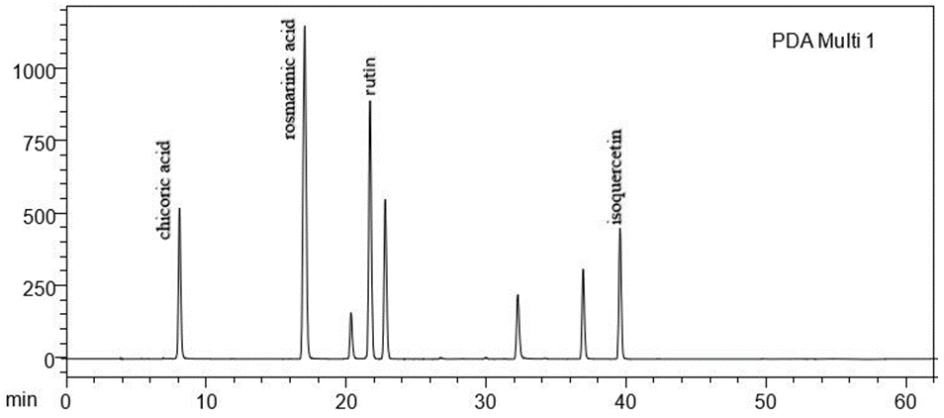

Figure 5. RP-HPLC chromatogram of mix standards

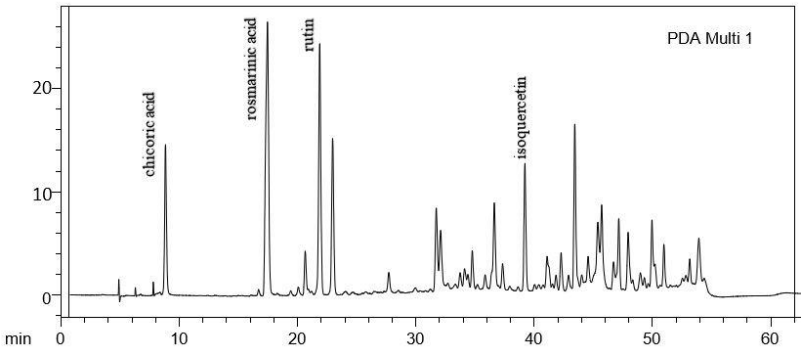

Figure 6. Representative RP-HPLC chromatograms for quantification of chicoric acid, rosmarinic acid, rutin and isoquercetin extract of *O. basilicum* suspension culture

Table 1. Rosmarinic acid (mg/g DW) and chicoric acid (mg/g DW) recorded in *Ocimum basilicum* L. cell suspension cultures subjected to different elicitor treatments for various days

Elicitors treatments	Rosmarinic acid (mg/g DW)			Chicoric acid (mg/g DW)		
	Culture periods (day)			Culture periods (day)		
	2	4	8	2	4	8
Control	9.120 ± 1.20^{l}	9.250 ± 0.94^{k}	9.220 ± 0.70^{k}	3.25 ± 0.52^{h}	3.38 ± 0.75^{g}	3.45 ± 0.27^{g}
$AgNO_3$ (6*)	9.900 ± 3.30^{k}	10.44 ± 1.75^{i}	10.75 ± 0.92^{g}	5.40 ± 0.15^{d}	5.83 ± 0.64^{d}	6.08 ± 0.45^{c}
$AgNO_3$ (12*)	10.02 ± 2.72^{k}	10.50 ± 1.40^{i}	10.84 ± 0.70^{g}	6.20 ± 0.18^{b}	6.57 ± 1.02^{c}	6.70 ± 1.12^{b}
SA (12*)	11.25 ± 0.64^{h}	11.78 ± 1.13^{h}	12.10 ± 0.41^{f}	5.74 ± 1.74^{c}	6.20 ± 0.80^{d}	6.16 ± 0.32^{b}
SA (24*)	13.02 ± 1.47^{f}	13.65 ± 3.00^{f}	13.94 ± 0.56^{d}	6.85 ± 1.24^{a}	7.10 ± 0.95^{b}	7.26 ± 2.43^{a}
YE (40**)	18.19 ± 1.70^{c}	18.53 ± 1.75^{c}	18.20 ± 0.92^{b}	6.02 ± 1.40^{b}	6.45 ± 0.80^{c}	5.85 ± 0.35^{d}
YE (80**)	19.16 ± 2.00^{b}	19.40 ± 1.15^{b}	19.52 ± 3.10^{a}	5.80 ± 0.71^{c}	6.32 ± 2.40^{c}	6.64 ± 0.84^{a}
$AgNO_3$ (6*) + SA (12*)	9.320 ± 1.08^{l}	9.980 ± 0.44^{j}	9.570 ± 0.65^{g}	4.38 ± 2.20^{f}	4.87 ± 0.50^{f}	4.10 ± 0.18^{f}
$AgNO_3$ (6*) + SA (24*)	10.47 ± 0.80^{j}	10.78 ± 1.00^{i}	10.90 ± 3.32^{g}	4.63 ± 0.85^{e}	5.22 ± 1.60^{e}	5.55 ± 1.41^{d}
$AgNO_3$ (12*) + SA (12*)	8.200 ± 0.65^{m}	8.470 ± 0.70^{l}	8.152 ± 0.58^{h}	3.00 ± 1.05^{h}	3.25 ± 0.27^{h}	3.56 ± 0.90^{g}
$AgNO_3$ (12*) + SA (24*)	9.170 ± 0.30^{l}	9.510 ± 2.38^{k}	9.654 ± 3.00^{i}	3.38 ± 0.20^{g}	3.62 ± 0.42^{g}	3.29 ± 1.35^{h}
YE (40**) + $AgNO_3$ (6*)	11.35 ± 2.36^{h}	10.80 ± 0.85^{i}	10.47 ± 0.45^{h}	3.10 ± 0.76^{h}	3.48 ± 0.30^{g}	3.60 ± 0.71^{g}
YE (40**) + $AgNO_3$ (12*)	10.81 ± 1.00^{j}	10.50 ± 0.70^{i}	10.14 ± 0.50^{h}	2.74 ± 0.80^{i}	2.89 ± 0.22^{i}	3.00 ± 0.30^{h}
YE (80**) + $AgNO_3$ (6*)	12.28 ± 1.44^{g}	12.60 ± 1.58^{g}	12.58 ± 1.78^{e}	3.57 ± 0.43^{g}	3.20 ± 1.65^{h}	2.95 ± 1.56^{i}
YE (80**) + $AgNO_3$ (12*)	10.43 ± 0.96^{j}	10.00 ± 2.00^{i}	9.750 ± 1.10^{i}	2.80 ± 0.19^{i}	2.32 ± 0.64^{i}	2.48 ± 2.04^{j}
SA (12*) + YE (40**)	15.44 ± 1.70^{e}	15.79 ± 1.75^{e}	18.00 ± 2.38^{b}	4.72 ± 0.35^{e}	5.10 ± 0.58^{e}	5.20 ± 0.50^{e}
SA (12*) + YE (80**)	17.43 ± 2.00^{d}	17.98 ± 1.15^{d}	17.52 ± 2.77^{c}	5.15 ± 0.66^{d}	5.08 ± 2.00^{e}	4.90 ± 0.54^{e}
SA (24*) + YE (40**)	17.60 ± 0.64^{d}	18.00 ± 1.13^{d}	18.17 ± 4.10^{b}	5.65 ± 0.51^{c}	5.98 ± 0.27^{d}	6.25 ± 0.30^{c}
SA (24*) + YE (80**)	19.53 ± 1.47^{a}	20.19 ± 3.00^{a}	19.60 ± 0.92^{a}	6.96 ± 1.47^{a}	7.55 ± 0.73^{a}	6.70 ± 1.05^{b}

The result are expressed as means ± standard deviation (n=3). Mean followed by the same letter in the same column do not differ statistically at $p < 0.05$ according to Tukey test. $AgNO_3$ = silver nitrate, SA = salicylic acid, YE = yeast extract, * = μM, ** = mg/l, DW = dry weight

Table 2. Isoquercetin (mg/g DW) and rutin (mg/g DW) recorded in *Ocimum basilicum* L. cell suspension cultures subjected to different elicitor treatments for various days

Elicitors treatments	Isoquercetin (mg/g DW)			Rutin (mg/g DW)		
	Culture periods (day)			Culture periods (day)		
	2	4	8	2	4	8
Control	1.35 ± 0.39^{g}	1.52 ± 0.70^{g}	1.64 ± 0.12^{f}	2.30 ± 0.13^{e}	2.66 ± 0.40^{f}	2.80 ± 0.58^{f}
$AgNO_3$ (6*)	2.88 ± 0.48^{b}	3.24 ± 0.22^{b}	3.91 ± 0.41^{a}	3.46 ± 0.62^{c}	3.70 ± 0.27^{d}	3.43 ± 0.10^{e}
$AgNO_3$ (12*)	2.92 ± 0.60^{b}	3.16 ± 0.19^{b}	3.00 ± 0.52^{c}	4.15 ± 0.75^{b}	4.54 ± 0.72^{c}	4.67 ± 1.30^{c}
SA (12*)	2.02 ± 0.44^{e}	2.25 ± 0.18^{e}	2.46 ± 0.82^{d}	3.00 ± 0.27^{d}	3.53 ± 0.76^{d}	3.35 ± 1.00^{e}
SA (24*)	2.38 ± 0.60^{d}	2.61 ± 1.13^{d}	2.77 ± 0.76^{c}	3.60 ± 0.94^{c}	3.32 ± 0.55^{e}	3.40 ± 0.50^{e}
YE (40**)	2.66 ± 0.50^{c}	2.97 ± 0.95^{c}	3.30 ± 0.73^{b}	6.10 ± 0.40^{a}	6.24 ± 0.30^{b}	6.48 ± 0.72^{a}
YE (80**)	3.31 ± 0.54^{a}	3.88 ± 0.98^{a}	3.73 ± 0.60^{a}	4.15 ± 1.75^{b}	4.54 ± 0.81^{c}	4.67 ± 0.62^{c}
$AgNO_3$ (6*) + SA (12*)	1.24 ± 0.25^{g}	1.00 ± 0.50^{h}	1.05 ± 0.42^{h}	1.94 ± 0.90^{f}	2.15 ± 0.46^{g}	2.00 ± 0.70^{h}
$AgNO_3$ (6*) + SA (24*)	1.35 ± 0.77^{g}	1.56 ± 0.35^{g}	1.50 ± 0.40^{f}	2.49 ± 1.40^{e}	2.20 ± 0.63^{g}	1.85 ± 0.25^{h}
$AgNO_3$ (12*) + SA (12*)	1.60 ± 0.09^{f}	1.87 ± 0.62^{f}	1.39 ± 0.93^{g}	2.10 ± 0.21^{f}	2.53 ± 0.30^{f}	2.66 ± 0.64^{f}
$AgNO_3$ (12*) + SA (24*)	1.65 ± 0.20^{f}	1.48 ± 0.13^{g}	1.56 ± 0.58^{f}	2.78 ± 1.36^{d}	2.40 ± 0.75^{g}	2.03 ± 0.29^{h}
YE (40**) + $AgNO_3$ (6*)	2.15 ± 0.67^{d}	1.90 ± 1.30^{f}	1.91 ± 0.66^{e}	2.02 ± 0.19^{g}	1.70 ± 0.25^{i}	1.85 ± 0.37^{h}
YE (40**) + $AgNO_3$ (12*)	2.20 ± 0.18^{d}	2.51 ± 0.57^{d}	2.60 ± 0.91^{d}	1.90 ± 0.42^{f}	2.13 ± 0.64^{g}	1.96 ± 1.00^{h}
YE (80**) + $AgNO_3$ (6*)	3.12 ± 0.18^{a}	3.27 ± 0.72^{b}	3.15 ± 0.15^{b}	6.35 ± 1.42^{a}	6.57 ± 0.57^{a}	6.20 ± 1.55^{b}
YE (80**) + $AgNO_3$ (12*)	2.03 ± 1.00^{e}	1.58 ± 0.39^{g}	1.51 ± 0.73^{f}	1.96 ± 0.45^{f}	2.10 ± 0.50^{h}	1.91 ± 0.33^{h}
SA (12*) + YE (40**)	2.35 ± 0.09^{d}	2.53 ± 0.72^{d}	2.51 ± 0.60^{d}	2.88 ± 0.39^{d}	3.10 ± 0.19^{e}	3.24 ± 1.27^{e}
SA (12*) + YE (80**)	2.90 ± 0.51^{b}	2.79 ± 0.39^{c}	2.78 ± 0.46^{c}	2.74 ± 0.58^{d}	2.55 ± 0.34^{f}	2.40 ± 0.78^{g}
SA (24*) + YE (40**)	3.01 ± 0.44^{b}	2.80 ± 0.53^{c}	2.81 ± 0.93^{c}	3.65 ± 2.34^{c}	3.81 ± 3.00^{d}	3.77 ± 0.82^{d}
SA (24*) + YE (80**)	2.57 ± 0.20^{c}	2.55 ± 0.27^{d}	2.63 ± 0.70^{d}	2.76 ± 1.92^{d}	2.71 ± 1.00^{f}	2.75 ± 1.29^{f}

The result are expressed as means ± standard deviation (n=3). Mean followed by the same letter in the same column do not differ statistically at $p < 0.05$ according to Tukey test. $AgNO_3$ = silver nitrate, SA = salicylic acid, YE = yeast extract, * = μM, ** = mg/l, DW = dry weight.

Rosmarinic and chicoric acid, two of the most important phenolic compounds found in *Ocimum* genus, can be produced quickly and easily in plant cell cultures, and in some cases, their production levels in plant tissue culture systems may be even higher than the wild plant (Petersen and Simmonds, 2003). To date, many studies have been conducted in plant tissue culture systems to increase these bioactive compounds with elicitor treatments in *Ocimum* species, but these studies have been limited to PGRs and spectral light treatments (Bais *et al.*, 2002; Duran *et al.*, 2019; Nadeem *et al.*, 2019; Nazir *et al.*, 2019). As far as we know, the effects of the applications of $AgNO_3$, an ethylene inhibitor (Hegde *et al.*, 2017; Yang *et al.*, 2017), SA, which plays a key role in the growth-development and defense systems of plants (Ptak *et al.*, 2017; Yang *et al.*, 2017), and YE elicitor, one of the endophytic fungi (Dowom *et al.*, 2017; Sing *et al.*, 2017), individually or in combination on the biosynthesis of these bioactive compounds in *O. basilicum* cell suspension cultures have not yet been investigated. However, the studies using other cultivars have reported that the combined use of YE + Ag^+ (silver ion), YE + Ag^+ + MJ (methyl jasmonate) and YE + SA elicitors was more effective in increasing certain bioactive compounds compared with the use alone (Zhao *et al.*, 2001; Li *et al.*, 2003; Cheng *et al.*, 2013; Pourianezhad *et al.*, 2019).

Studies on rosmarinic and chicoric acid biosynthesis have been focused mainly on hairy root cultures of two cultivars. It has been reported by many researchers that the treatments of $AgNO_3$ and YE in *Salvia miltiorrhiza* cause to increase in the biosynthesis of rosmarinic acid (Yan *et al.*, 2006; Xiao *et al.*, 2010), and that the incubation temperature, photoperiod, and ultrasound treatments increased the chicoric acid biosynthesis in *Echinacea purpurea* (Wu *et al.*, 2007; Liu *et al.*, 2012). In addition, the studies in other cultivars have reported that $AgNO_3$, SA and YE treatments are effective in rosmarinic acid biosynthesis (Park *et al.*, 2016; Dowom *et al.*, 2017). Like previous studies, the results of the present study have shown that rosmarinic acid and chicoric acid biosynthesis can be increased by $AgNO_3$, SA and YE treatments. Furthermore, it has been demonstrated in this study that the combined use of SA and YE elicitors in *O. basilicum* cell suspension cultures [(SA (24 μM) + YE (80 mg/l)] may be more effective in rosmarinic acid and chicoric acid biosynthesis.

Compared with the control culture, maximum biosynthesis of isoquercetin and rutin was 3.88 mg/g DW [YE (80 mg/l)] at the end of the 4th day and 6.35 mg/g DW [YE (80 mg/l) + $AgNO_3$ (6 μM)] at the end of the 2nd day. In isoquercetin and rutin biosynthesis, these values were followed by 3.12 mg/g (cells harvested at the end of 2nd day) and YE (80 mg/l) + $AgNO_3$ (6 μM), 6.10 mg/g (cells harvested at the end of 2nd day) and YE (40 mg/l), respectively (Table 2).

Isoquercetin, also known as quercetin-3-O-glucoside, and rutin polyphenols, also known as vitamin P, are bioactive compounds dominant in *O. basilicum* (Vlase *et al.*, 2014; Mousavi *et al.*, 2019). The obtained results showed that $AgNO_3$, SA and YE treatments were effective in isoquercetin and rutin biosynthesis in accordance with previous studies (Azeez and Ibrahim, 2013; Zhao *et al.*, 2014). On the other hand, as in this study, researchers have also reported that isoquercetin synthesized in plant tissue culture systems can be produced at higher levels than organs growing in natural conditions (Kuo *et al.*, 2015). Some of these studies used hairy root culture, thus, increased the rutin content by 9.5 times compared with the wild plant roots (Kim *et al.*, 2009). Bálintová *et al.* (2019) studied 8 different *Hypericum* species and reported that the biotic elicitors added to the culture increased rutin biosynthesis by 1.2-3.3 times and isoquercetin biosynthesis by 1.5 to 13 times. Contrary to these studies, another study of *Hypericum perforatum* callus culture reported that rutin biosynthesis was lower than in wild-growing plants, while isoquercetin biosynthesis could not be detected in callus cultures (Tusevski *et al.*, 2016).

Headspace solid-phase microextraction-gas-chromatography-mass-spectrometry (HS-SPME–GC/MS) analysis

The estragole and linalool contents were evaluated by HS-SPME–GC/MS analysis and the values were demonstrated as μg/g DW. Additionally, HS-SPME–GC/MS chromatograms of the standards and extracts

were shown in Figure 7 and Figure 8. The effects of elicitor treatments on estragole and linalool biosynthesis are given in Table 3. Accordingly, the highest values of estragole compared with the control culture was 4.50 µg/g DW [$AgNO_3$ (6 µM) + SA (24 µM)]. On the other hand, the highest values of linalool compared with the control culture was 3.02 µg/g DW [SA (24 µM)] (Table 3). These values were followed by SA (24 µM) and $AgNO_3$ (12 µM) treatments with an increase of 0.54-fold (from 2.55 µg/g to 3.92 µg/g DW) and 1.62-fold (from 1.10 µg/g to 2.88 µg/g DW), respectively. Among elicitor applications, YE (80 mg/l) + $AgNO_3$ (6 µM) and YE (40 mg/l) treatments resulted in the minimal estragole and linalool biosynthesis (1.70 µg/g and 1.00 µg/g, respectively).

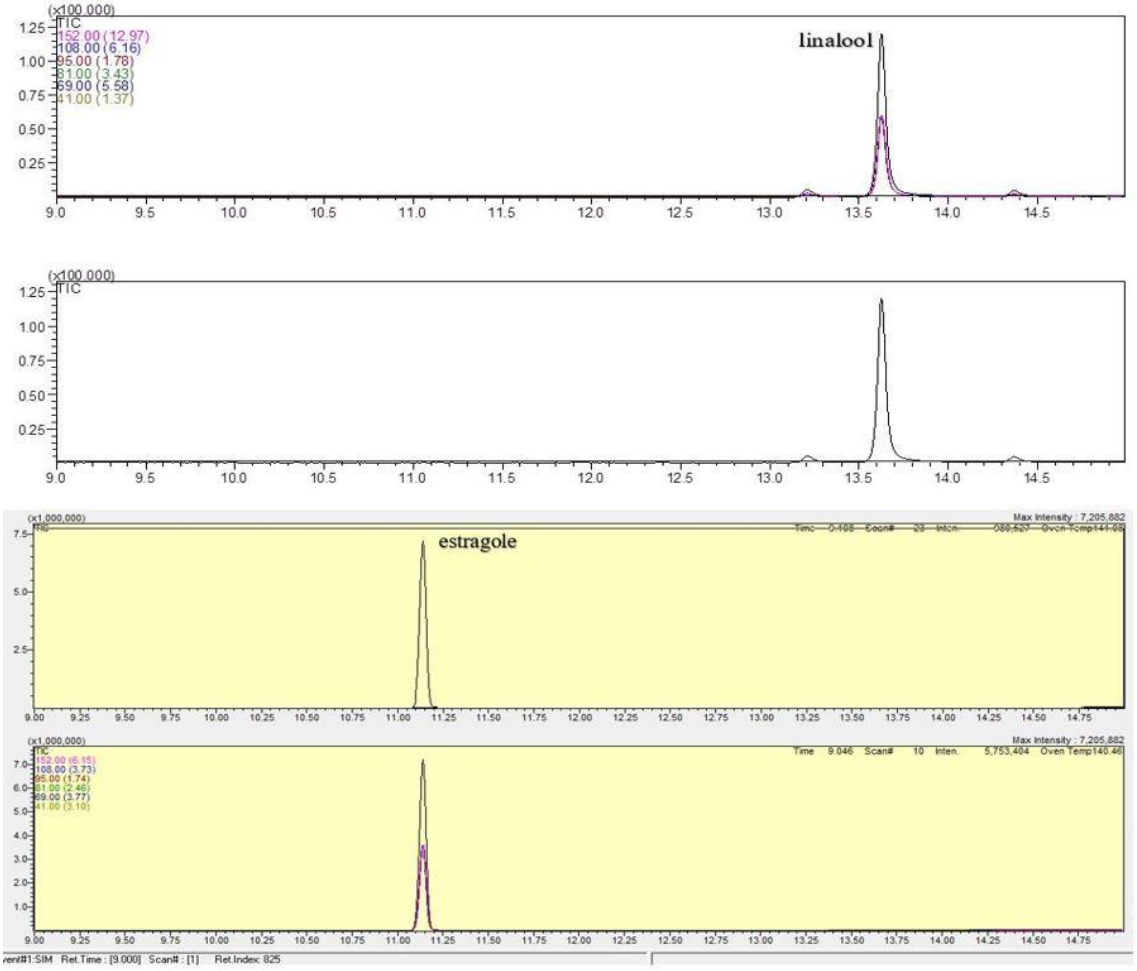

Figure 7. HS-SPME–GC/MS chromatogram of linalool and estragole standards

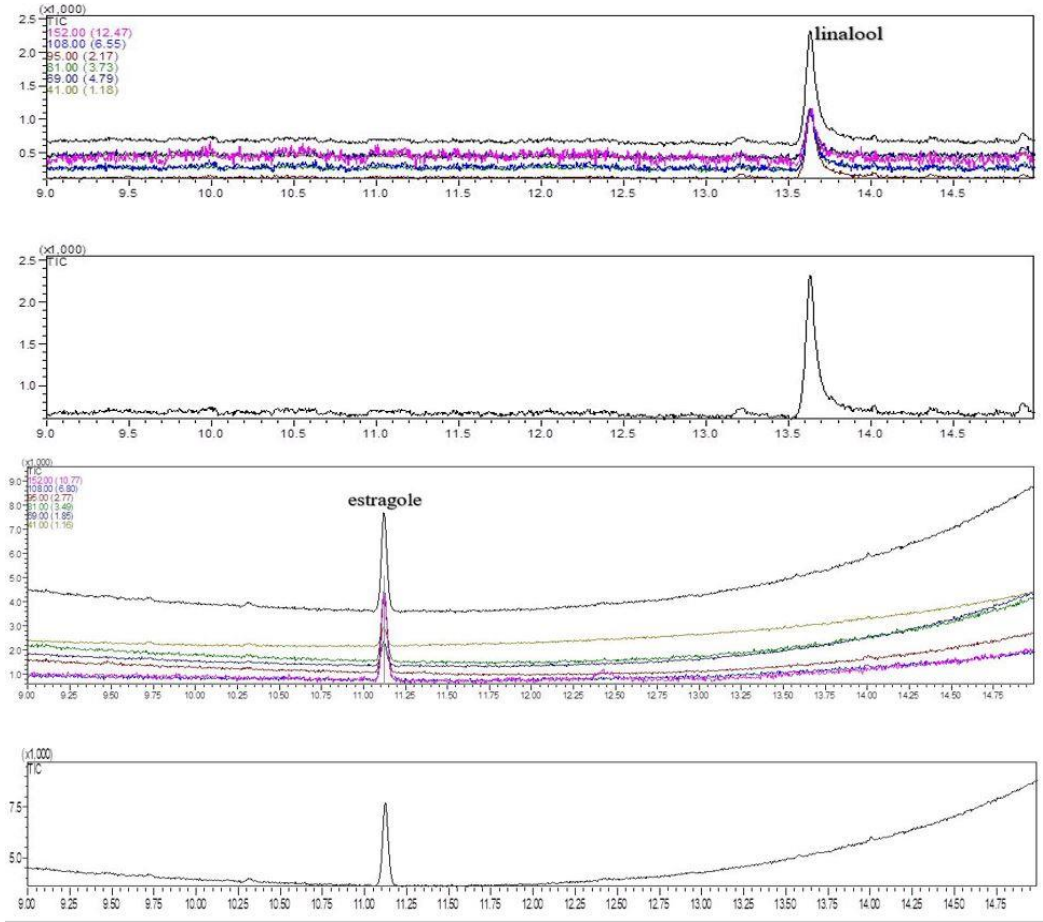

Figure 8. Representative HS-SPME–GC/MS chromatograms for quantification of linalool and estragole extract of *O. basilicum* suspension culture

Table 3. Estragole (µg/g DW) and linalool (µg/g DW) recorded in *Ocimum basilicum* L. cell suspension cultures subjected to different elicitor treatments for various days

Elicitors treatments	Estragole (µg/g DW)			Linalool (µg/g DW)		
	Culture periods (day)			Culture periods (day)		
	2	4	8	2	4	8
Control	2.33 ± 0.42^{e}	2.55 ± 1.00^{f}	2.82 ± 0.40^{fg}	1.10 ± 0.30^{i}	1.43 ± 0.30^{hi}	1.68 ± 0.18^{gh}
$AgNO_3$ (6*)	3.55 ± 0.31^{b}	3.74 ± 0.92^{c}	3.28 ± 0.19^{bc}	2.26 ± 0.48^{c}	2.70 ± 0.25^{b}	3.08 ± 0.56^{b}
$AgNO_3$ (12*)	3.19 ± 0.75^{c}	3.66 ± 0.64^{c}	3.35 ± 1.20^{b}	2.88 ± 0.20^{b}	3.27 ± 0.34^{a}	3.00 ± 0.73^{b}
SA (12*)	2.73 ± 0.94^{d}	2.98 ± 0.88^{d}	3.00 ± 1.10^{c}	2.35 ± 0.61^{c}	2.47 ± 0.72^{c}	2.28 ± 0.40^{f}
SA (24*)	3.54 ± 0.40^{b}	3.92 ± 0.63^{b}	4.22 ± 2.25^{a}	3.02 ± 1.23^{a}	3.34 ± 0.25^{a}	3.35 ± 2.29^{a}
YE (40**)	2.13 ± 0.56^{f}	2.28 ± 0.17^{g}	2.17 ± 0.72^{gh}	1.00 ± 0.15^{ij}	0.83 ± 0.09^{kl}	0.65 ± 0.78^{kl}
YE (80**)	2.42 ± 0.48^{e}	2.70 ± 1.00^{e}	2.88 ± 0.80^{fg}	1.15 ± 0.54^{ij}	1.27 ± 0.36^{ij}	0.92 ± 0.42^{jk}
$AgNO_3$ (6*) + SA (12*)	2.51 ± 0.56^{e}	2.30 ± 0.95^{fg}	2.25 ± 0.75^{gh}	1.52 ± 0.11^{gh}	1.77 ± 0.65^{ef}	1.86 ± 0.56^{fg}
$AgNO_3$ (6*) + SA (24*)	3.82 ± 1.00^{a}	4.50 ± 0.47^{a}	4.19 ± 0.09^{a}	2.00 ± 0.58^{de}	2.50 ± 0.50^{bc}	2.64 ± 0.19^{cd}
$AgNO_3$ (12*) + SA (12*)	2.26 ± 0.39^{e}	2.25 ± 0.20^{gh}	2.10 ± 0.41^{gh}	1.30 ± 0.74^{hi}	1.19 ± 0.30^{ij}	1.28 ± 0.32^{ij}
$AgNO_3$ (12*) + SA (24*)	2.15 ± 1.12^{f}	2.17 ± 0.42^{gh}	2.12 ± 0.27^{gh}	1.24 ± 0.13^{hi}	1.49 ± 1.00^{gh}	1.56 ± 0.78^{gh}
YE (40**) + $AgNO_3$ (6*)	2.40 ± 0.72^{e}	2.71 ± 0.55^{e}	2.70 ± 0.94^{ef}	1.63 ± 0.20^{fg}	1.91 ± 0.72^{de}	1.88 ± 0.40^{fg}
YE (40**) + $AgNO_3$ (12*)	2.48 ± 0.18^{e}	2.60 ± 1.20^{ef}	2.35 ± 2.10^{gh}	1.51 ± 0.58^{gh}	1.63 ± 0.50^{fg}	1.45 ± 0.19^{hi}
YE (80**) + $AgNO_3$ (6*)	1.70 ± 0.53^{h}	1.94 ± 0.46^{i}	2.15 ± 0.98^{gh}	1.00 ± 0.50^{ij}	1.34 ± 0.85^{hi}	1.59 ± 0.23^{gh}
YE (80**) + $AgNO_3$ (12*)	2.00 ± 0.10^{gh}	2.40 ± 1.51^{gh}	2.80 ± 1.00^{fg}	1.82 ± 0.67^{ef}	1.80 ± 0.17^{ef}	2.12 ± 0.22^{ef}
SA (12*) + YE (40**)	2.21 ± 0.72^{ef}	2.10 ± 0.55^{gh}	2.19 ± 0.20^{gh}	1.06 ± 0.17^{ij}	1.25 ± 0.22^{ij}	1.20 ± 0.37^{ij}
SA (12*) + YE (80**)	2.35 ± 0.15^{e}	2.52 ± 0.29^{ef}	2.50 ± 0.35^{fg}	1.37 ± 0.30^{hi}	1.59 ± 0.10^{fg}	1.45 ± 0.15^{hi}
SA (24*) + YE (40**)	2.46 ± 1.56^{e}	2.00 ± 0.75^{hi}	2.17 ± 1.44^{gh}	1.26 ± 0.36^{hi}	1.53 ± 0.40^{gh}	1.59 ± 0.49^{gh}
SA (24*) + YE (80**)	2.68 ± 2.13^{d}	2.80 ± 0.96^{e}	2.77 ± 2.00^{fg}	1.76 ± 0.67^{ef}	1.63 ± 1.24^{fg}	1.64 ± 1.00^{gh}

The result are expressed as means ± standard deviation (n=3). Mean followed by the same letter in the same column do not differ statistically at $p < 0.05$ according to Tukey test. $AgNO_3$ = silver nitrate, SA = salicylic acid, YE = yeast extract, * = µM, ** = mg/l, DW = dry weight.

In previous studies, it has been reported that various treatments such as PGRs, copper sulfate, MJA, melatonin can be used effectively in estragole and linalool biosynthesis in plant tissue culture systems, moreover,

estragole and linalool, which are not in the parent plant organs, can be synthesized with elicitor treatments in plant tissue culture systems (Tavares *et al.*, 2004; Trettel *et al.*, 2018; Coskun *et al.*, 2019; El-Kader *et al.*, 2019). On the other hand, as far as we know, the effects of $AgNO_3$, SA and YE treatments individually or in combination on estragole and linalool biosynthesis in *O. basilicum* cell suspension cultures have not been investigated before. Studies to date have mostly been limited to plant PGRs, copper sulfate, chitosan, methyl jasmonate, and methyl salicylate elicitors. In some of these studies, estragole and linalool biosynthesis were increased by 1.3 times and 1.1 fold respectively compared with control culture (Deschamps and Simon, 2006; Gupta *et al.*, 2018; Monfort *et al.*, 2018), while in others (copper sulfate) linalool biosynthesis was decreased by 8.4 fold (Trettel et al., 2018). Cells that are in different growth stages in plant tissue culture systems have various levels of mRNA and protein synthesis (Chong *et al.*, 2005). This is why cells can react differently to elicitor treatments, causing different levels of bioactive compounds to accumulate (Kang *et al.*, 2009). Therefore, elicitor types, doses, combinations and application times should be adjusted very well in triggering the signal molecules in the cells. Some concentrations and combinations of $AgNO_3$, SA and YE treatments used in this study were found to be more successful in estragole and linalool biosynthesis compared with cell culture without elicitor treatment.

Total flavonoid (TFC) and phenolic (TPC) content

The total flavonoid and phenolic contents of the samples obtained from the elicitor-treated cell cultures are presented in Table 4. Accordingly, the total flavonoid content in elicitor treatments ranged from 1.42 to 3.12 (µgQE/g DW), while the total phenolic content ranged from 13.30 to 17.91 µgGAE/g DW. Among elicitor treatments, the highest total flavonoid content was obtained from SA (12 µM) + YE (80 mg/l) treatment as 3.12 µgQE/g DW harvested at the end of the 8th day, and the lowest total flavonoid content was obtained from the control culture harvested at the end of the 2nd day as 1.42 µgQE/g DW. The highest total phenolic content was obtained from SA (24 µM) + YE (80 mg/l) treatment harvested at the end of 4th day as 17.91 µgGAE/g DW, and the lowest total phenolic content was 13.30 µgGAE/g obtained from YE (40 mg/l) + $AgNO_3$ (6 µM) at the end of 4th day. The treatment, which mostly increased the total flavonoid and phenolic content compared with the control culture, was SA (24 µM) + YE (40 mg/l) and SA (24 µM) + YE (80 mg/l) treatments (104% and 28%, respectively).

Table 4. Total flavonoid content (TFC) (µgQE/g DW) and total phenolic content (TPC) (µgGAE/g DW) recorded in *Ocimum basilicum* L. cell suspension cultures subjected to different elicitor treatments for various days

Elicitors treatments	TFC (µgQE/g DW)			TPC (µgGAE/g DW)		
	Culture periods (day)			Culture periods (day)		
	2	4	8	2	4	8
Control	1.42 ± 0.76^{h}	1.47 ± 0.54^{h}	2.05 ± 0.83^{h}	13.87 ± 0.90^{i}	14.40 ± 0.52^{h}	15.31 ± 0.38^{f}
$AgNO_3$ (6*)	1.60 ± 0.19^{g}	1.86 ± 0.50^{g}	2.16 ± 0.27^{g}	15.37 ± 3.28^{g}	15.62 ± 1.35^{f}	16.20 ± 0.74^{d}
$AgNO_3$ (12*)	1.70 ± 0.40^{g}	1.87 ± 0.17^{g}	2.24 ± 0.81^{f}	14.28 ± 2.55^{h}	14.00 ± 3.76^{j}	14.18 ± 1.79^{j}
SA (12*)	1.43 ± 0.94^{h}	1.66 ± 0.88^{h}	1.84 ± 1.10^{i}	13.70 ± 0.61^{j}	13.95 ± 1.72^{j}	14.28 ± 2.40^{j}
SA (24*)	2.10 ± 0.40^{d}	2.31 ± 0.63^{e}	2.28 ± 2.25^{f}	14.40 ± 1.23^{g}	14.72 ± 0.80^{g}	15.20 ± 2.29^{g}
YE (40**)	2.00 ± 0.62^{e}	2.15 ± 0.55^{f}	2.47 ± 0.93^{d}	14.60 ± 3.15^{g}	14.64 ± 4.09^{g}	15.10 ± 0.85^{g}
YE (80**)	1.84 ± 0.39^{f}	2.08 ± 0.25^{f}	2.29 ± 0.35^{f}	15.42 ± 1.54^{e}	15.70 ± 2.36^{f}	15.75 ± 2.42^{e}
$AgNO_3$ (6*) + SA (12*)	1.72 ± 0.36^{f}	1.80 ± 0.21^{g}	1.77 ± 0.38^{i}	15.50 ± 3.30^{e}	15.63 ± 0.32^{f}	15.88 ± 0.88^{e}
$AgNO_3$ (6*) + SA (24*)	1.98 ± 0.41^{e}	2.15 ± 0.40^{f}	2.51 ± 0.76^{d}	16.40 ± 1.34^{c}	16.66 ± 1.30^{c}	16.42 ± 0.71^{c}
$AgNO_3$ (12*) + SA (12*)	1.26 ± 0.55^{i}	1.48 ± 0.61^{h}	1.35 ± 0.19^{i}	13.72 ± 0.96^{j}	14.00 ± 1.74^{j}	14.31 ± 2.02^{j}
$AgNO_3$ (12*) + SA (24*)	1.55 ± 0.27^{g}	1.53 ± 0.57^{h}	1.79 ± 1.05^{i}	14.18 ± 2.32^{h}	14.40 ± 1.40^{h}	14.53 ± 0.27^{i}
YE (40**) + $AgNO_3$ (6*)	2.40 ± 0.94^{c}	2.64 ± 0.91^{c}	3.00 ± 0.43^{b}	13.97 ± 1.63^{i}	13.80 ± 2.72^{k}	13.64 ± 3.40^{k}
YE (40**) + $AgNO_3$ (12*)	2.17 ± 1.00^{d}	2.15 ± 0.75^{f}	2.40 ± 0.56^{e}	12.95 ± 0.58^{k}	13.30 ± 1.77^{l}	13.50 ± 2.12^{k}
YE (80**) + $AgNO_3$ (6*)	2.63 ± 0.63^{b}	2.50 ± 0.66^{d}	2.70 ± 0.45^{c}	14.35 ± 1.20^{g}	14.30 ± 2.15^{h}	14.73 ± 1.95^{h}
YE (80**) + $AgNO_3$ (12*)	1.94 ± 0.45^{e}	2.10 ± 0.21^{f}	2.05 ± 0.30^{h}	14.10 ± 0.47^{h}	14.18 ± 1.54^{i}	14.50 ± 0.80^{i}
SA (12*) + YE (40**)	2.37 ± 0.58^{c}	2.55 ± 0.49^{c}	2.38 ± 0.70^{e}	15.62 ± 0.51^{e}	15.98 ± 0.97^{e}	16.10 ± 1.30^{d}
SA (12*) + YE (80**)	2.52 ± 0.40^{b}	2.77 ± 0.85^{b}	3.12 ± 0.74^{a}	16.01 ± 3.10^{d}	16.23 ± 3.38^{d}	16.30 ± 2.70^{c}
SA (24*) + YE (40**)	2.75 ± 0.19^{a}	3.00 ± 0.48^{a}	3.03 ± 1.62^{b}	16.75 ± 0.76^{b}	17.12 ± 1.28^{b}	17.10 ± 1.00^{b}
SA (24*) + YE (80**)	2.86 ± 0.71^{a}	2.82 ± 0.30^{b}	2.30 ± 0.27^{f}	17.70 ± 2.20^{a}	17.91 ± 0.75^{a}	17.76 ± 1.43^{a}

The result are expressed as means ± standard deviation (n=3). Mean followed by the same letter in the same column do not differ statistically at $p < 0.05$ according to Tukey test. $AgNO_3$ = silver nitrate, SA = salicylic acid, YE = yeast extract, * = µM, ** = mg/l, DW = dry weight.

As far as we know, no studies have been conducted on the effects of $AgNO_3$, SA and YE treatments on the total flavonoid and phenolic content in *O. basilicum* cell suspension cultures, either individually or in combination. However, there are studies on the effects of other elicitor sources on the total phenolic and flavonoid content. Similar to our study, it has been reported that the treatment of $AgNO_3$, SA and YE individually or in combination increases the total flavonoid and phenolic content (Yan *et al.*, 2006; Gonçalves *et al.*, 2019). Contrary to these studies, there are studies reporting that $AgNO_3$, SA and YE treatments reduce the total flavonoids and phenolics content (Reyes-Martínez *et al.*, 2019). Cells may react to some elicitor treatments in a short time and some elicitor treatments in a longer period of time (Ali *et al.*, 2007; Cai *et al.*, 2013). Therefore, proper selection of the elicitor types, culture system and application time of elicitors in plant tissue culture systems may increase the chances of success in the synthesis of these valuable bioactive compounds. In this study, many alternatives were tried in the establishment of *O. basilicum* cell suspension culture and in elicitor treatments, and some treatments were found to be successful in increasing the total flavonoid and phenolic content.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) activity

DPPH assays, which are widely used to evaluate antioxidant activity, were made and the results were also shown in Figure 9. While elicitor treatments generally showed increases in radical scavenging capacity, SA (24 µM) + YE (80 mg/l) was the most effective treatment with values ranging from 87.8% to 88.7%. This was followed by SA (12 µM) + YE (80 mg/l) treatment with 86.5%-87.4%. The strongest radical scavenging capacity among elicitor treatments was obtained as 88.7% from SA (24 µM) + YE (80 mg/l) at the end of 8th day, the weakest radical scavenging capacity was detected as 77.9% in cells without elicitor treatment at the end of 2nd day. The highest increase in radical scavenging capacity was observed in cells harvested at the end of the 8th day in the treatment of SA (24 µM) + YE (80 mg/l). In addition, the combined use of elicitor treatments was found to increase radical scavenging capacity more than individual uses (Figure 4). Because, elicitors ($AgNO_3$, YE, and SA) stimulated some genes of antioxidant enzymes such as superoxide dismutases (SODs), catalases (CATs) and ascorbate peroxidases (APXs) to preserve cells in response to reactive oxygen species (ROS) (El-Esawi *et al.*, 2017).

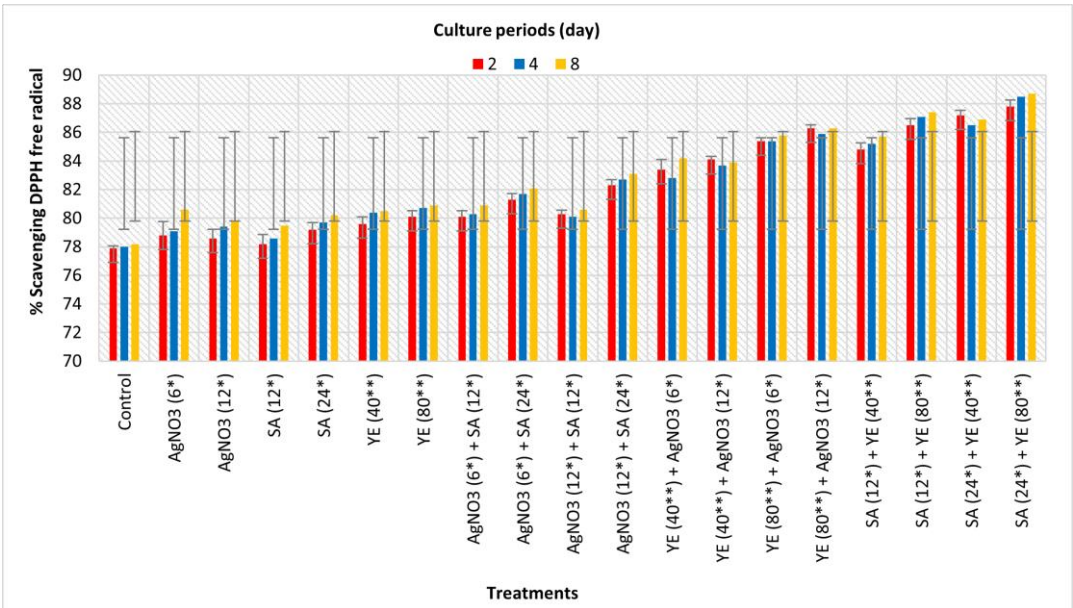

Figure 9. The effect of abiotic and biotic elicitor treatments on % scavenging DPPH free radical
The result are expressed as means ± standard deviation (n=3). Error bars show standard deviation values. $AgNO_3$ = silver nitrate, SA = salicylic acid, YE = yeast extract, * = µM, ** = mg/l, DPPH = 2, 2-diphenyl

Like previous studies, our results have shown that there is parallelity between TPC, TFC, and radical scavenging capacity (Nadeem *et al.*, 2018; Gonçalves *et al.*, 2019). This is because flavonoids and phenolics are mostly responsible for antioxidant activity in plants (Abraham *et al.*, 2011; Fazal *et al.*, 2016). Depending on elicitor treatments, the type and concentration of phenolic acids and flavonoids may be at different levels in cell suspension cultures (Sabir *et al.*, 2012; Xu *et al.*, 2016). Therefore, these differences between antioxidant activities can be explained by the differences in the phenolic and flavonoid content during elicitor treatment (Krishnan *et al.*, 2015; Sarkate *et al.*, 2017). In addition, previous research has reported that the concentrations, duration of application and plant cultivars used for $AgNO_3$, SA and YE treatments may be effective in radical scavenging capacity change. A study conducted on *Thymus lotocephalus* shoot cultures reported that 50 µM $AgNO_3$ and 500 mg/L YE treatments increased radical scavenging capacity significantly after 6 weeks compared with the cell culture without elicitor treatments (Gonçalves *et al.*, 2019), while in a study on *Vitis vinifera* cell suspension cultures, 5 and 50 µM Cd treatment (24 hours later) increased radical scavenging capacity compared with the cell culture without elicitor treatments, but 25 µM Ag treatment (24 hours later) reduced radical scavenging capacity (Cai *et al.*, 2013).

Conclusions

As far as we know, the effects of elicitor ($AgNO_3$, SA and YE) treatments applied individually or in combination in *O. basilicum* cell suspension cultures on cell growth (cell count and cell dry weight), cell viability (%), total phenolic content (TPC) and total flavonoid content (TFC), and phenolic compounds and terpenoids biosynthesis has been investigated for the first time. Among elicitor treatments, SA treatment was the only treatment that increased cell count, cell dry weight and cell viability. SA (24 µM) + YE (40 mg/l) and SA (24 µM) + YE (80 mg/l) treatments increased the total amount of flavonoids, total phenolic amount and antioxidant activity the most, compared with the control culture. The maximum production level of rosmarinic and chicoric acid compared with the control culture was obtained as 20.19 and 7.55 mg/g DW from SA (24 µM) + YE (80 mg/l) treatment, respectively. Compared with the control culture, maximum biosynthesis of isoquercetin and rutin was 3.88 mg/g DW [YE (80 mg/l)] and 6.35 mg/g DW [YE (80 mg/l) + $AgNO_3$ (6 µM)], respectively. Estragole and linalool's highest values were 4.50 µg/g DW [$AgNO_3$ (6 µM) + SA (24 µM)] and 3.02 µg/g DW [SA (24 µM)], respectively. Results clearly show that the elicitor treatment could enhance the biosynthesis of phenolic compounds and terpenoid (SMs) content in cell suspension cultures of *O. basilicum* and may be used for commercial supply in the future for therapeutic applications.

Authors' Contributions

EBA performed the experiments, BK performed the experiments, EKÖ collected data and analyzed data, MAA performed the experiments, collected data, analyzed data and wrote the manuscript.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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