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Influence of Selected Heavy Metals on Cell Growth and Camphor Secretion in *Achillea gypsicola* Hub. Mor. *In vitro* Cell Cultures

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Abstract: The use of abiotic and biotic elicitors for increasing the accumulation of pharmaceutical active ingredients in plant tissues has gained an increasing interest worldwide. This study was intented to provide promoting accumulation of camphor and phenolic compound using cadmium chloride ($CdCl_2$) and silver nitrate ($AgNO_3$) in cell culture of *Achillea gypsicola*. Growing cells from 8-day-old cultures were treated with three concentrations (5, 25 and 50 µM) of $CdCl_2$ and $AgNO_3$, along with the control. The quantification of camphor and phenolic compound were performed using Headspace-GC-MS and spectrophotometer, respectively. The content of camphor and phenolic compound, cell number and cell dry weight were significantly affected by increasing doses of $AgNO_3$ and $CdCl_2$. The highest significant change in camphor content was observed in cell treated with 25 µM $CdCl_2$ and $AgNO_3$ with a 6.88 and 6.32 fold increase, respectively. The application of 50 µM $AgNO_3$ and $CdCl_2$, however, resulted in a rapid decine in all attributes studied, implying that culture of *A. gypsicola* is susceptible to elicitation by high concentrations of these elicitors. In conclusion, using $AgNO_3$ and $CdCl_2$ elicitors in cultured tissues of *A. gypsicola* would be of great importance to enhanced production of desired bioactive compounds of medicinal importance.

Keywords: Compositae, Elicitor, Natural products, Terpenoid

Achillea gypsicola Hub. Mor. *In vitro* Hücre Kültürlerinde Seçilmiş Ağır Metallerin Hücre Büyümesi ve Kamfor Birikimi Üzerindeki Etkisi

Öz: Farmasötik aktif bileşenlerin bitki dokularında birikimini arttırmak için abiyotik ve biyotik elisitörlerin kullanımı dünya çapında artan bir ilgi kazanmıştır. Bu çalışma, *Achillea gypsicola* hücre kültüründe kadmiyum klorür ($CdCl_2$) ve gümüş nitrat ($AgNO_3$) kullanılarak kamfor ve fenolik bileşik birikimini teşvik etmeyi amaçlamıştır. Sekiz günlük kültürlerden büyüyen hücreler, kontrol ile birlikte üç konsantrasyonda (5, 25 ve 50 uM) $CdCl_2$ ve $AgNO_3$ ile muamele edildi. Kamfor ve fenolik bileşik miktar tayini sırasıyla Headspace-GC-MS ve spektrofotometre kullanılarak yapıldı. Kamfor ve fenolik bileşik miktarı, hücre sayısı ve hücre kuru ağırlığı, artan $AgNO_3$ ve $CdCl_2$ dozlarından önemli ölçüde etkilenmiştir. Kamfor miktarındaki en önemli değişiklik, sırasıyla 6.88 ve 6.32 kat artışla 25 uM $CdCl_2$ ve $AgNO_3$ ile muamele edilen hücrede gözlendi. Bununla birlikte, 50 uM $AgNO_3$ ve $CdCl_2$'nin uygulanması, çalışılan tüm özelliklerde hızlı bir düşüşle sonuçlandı ve bu, *A. gypsicola*'nın süspansiyon kültürünün bu elisitörlerin yüksek konsantrasyonları tarafından ortaya çıkmaya duyarlı olduğunu ima etti. Sonuç olarak, *A. gypsicola*'nın kültürlenmiş dokularında $AgNO_3$ ve $CdCl_2$ elisitörlerinin kullanılması, tıbbi öneme sahip arzu edilen biyoaktif bileşiklerin üretiminin arttırılması için büyük önem taşıyacaktır.

Anahtar Kelimeler: Compositae, Elisitör, Doğal bileşikler, Terpenoid

1.Introduction

Plant secondary metabolites (SMs) have been used for centuries in traditional folk medicine around the world to meet many different needs due to their many biological activities. In accordance with, the qualitative and quantitative evaluations of medicinal plants mostly focusing on the enhancement of SM synthesis and accumulation have increased over the past decades (Açıkgöz, 2020a; Dağlioğlu et al., 2022). It has been well documented that, SMs accumulate in small amounts in specialized tissues of various plant organs in most plants presumably in a certain stage of growth and development (Bourgaud et al., 2001; Murthy et al., 2014). SMs from medicinal and aromatic plants were obtained from wild or cultivated plants, with the majority of commercial supply is obtained from collected wild plants throughout the world (Nosov, 2012). In concequence of this, their consistent production directly from wild and/or field grown plants to meet the commercial demand becomes a challenging task (Verma & Shukla, 2015; Ramirez-Estrada et al. 2016). The need, therefore, is evident to develop some

reliable and feasible approaches enabling the production of valuable bioactive compounds with sufficient amount and good quality (Matkowski, 2008; Jamwal et al., 2018). Cell and tissue cultures can offer several potential advantages over the extraction of compounds from wild and field grown plants, the principal ones being a rapid, continuous, sustainable and economical production of valuable bioctive compounds with high concentration and purity (Davies & Deroles, 2014). The potential benefits of plant cell and tissue culture techniques in producing these valuable phytochemicals for commercial use have long been recognized (Rao & Ravishankar, 2002; Smetanska, 2008; Srivastava et al., 2019). In spite of its common usage, however, plant cell and tissue cultures have a dilemma of low yield of valuable plant bioactive compounds of commercial importance (Zhao et al. 2005; Isah et al. 2018). Therefore, various strategies have been developed for stimulating the synthesis of SMs using plant cell and tissue culture (Açıkgöz et al., 2018a; Açıkgöz et al., 2018b; Halder et al., 2019).

The synthesis of biologically active chemicals frequently occurs in plants exposed to environmental stresses comprising diverse elicitors or signal molecules (Ramakrishna & Ravishankar, 2011; Yue et al., 2016). SM synthesis and accumulation can be stimulated by the treatment of elicitors, activating plant defense system and triggering stress response, to overcome the low yield in cell and tissue cultures (Georgiev et al., 2009; Thakur et al., 2019). A number of studies have shown promising results in that employing biotic and abiotic elicitors to plant tissues to initiate, stimulate or enhance the biosynthetic pathways leading to enhanced production of important bioactive compounds (Verma & Shukla, 2015; Isah et al., 2018).

Various elicitors, with biological and non-biological origin (heavy metal salts, yeast, silver nitrate, salicylic acid, cadmium chloride, and chitosan etc.) have been widely used to induce the synthesis and increase the accumulation of bioactive compounds *in vitro* culture (Açıkgöz et al., 2022). The use of abiotic elicitors with chemical and physical stimuli *in vitro* cultures has atracted less interest as compared to the biotic elicitors (Radman et al., 2003). It has been proven that heavy metal induced stress activates signaling pathways which influence the synthesis of certain plant metabolites (Srivastava et al., 2019; Batı Ay et al., 2022). Silver nitrate and cadmium chloride have been proven to be associated with the accumulation of SMs *in vitro* cultures (Cetin et al., 20014; Park et al., 2016).

As in many parts of the world, bioactive substances of medicinal and aromatic plants were predominantly obtained form the plants grown wild in Turkey, with well over 11 000 flowering plant species, about one third of them is endemic (Baser, 2002). Intensive collection of economically important medicinal plants from their natural habitats has recently threatened their existence in the wild throughout the country. The genus *Achillea*, a member of *Asteraceae* family, commonly known as yarrow is represented by 59 species in Anatolia, 31 of which are endemic to Turkey, with a long history of use in traditional folk medicine (Mohammadhosseini et al., 2017; Demirci et al., 2018). *A. gypsicola*, an endemic species to Turkey, is of considerable importance as a good source of camphor with well-known medicinal properties (Açıkgöz, 2019; Açıkgöz, 2020b).

To our knowledge, there is no published work related with effect of $CdCl_2$ and $AgNO_3$ elicitors on production of SMs in *A. gypsicola.* In view of this, the current study intented to study the effect of $AgNO_3$ and $CdCl_2$ elicitors on cell growth and accumulation of phenolic compounds and camphor in *A. gypsicola* cell suspension culture.

2. Materials and Methods

2.1. Preparation of cell suspension cultures

The seeds of *A. gypsicola* were collected from the wild plants present in its natural habitat in Central Anatolia of Turkey (40°73′ N, 34°47′E) and properly identified at Ordu University, Turkey. For surface sterilization, the seeds were subjected to 70% (v/v) ethanol for 2 min and 25% (v/v) NaOCl for 45 min. Afterwards, the seeds were rinsed 3-4 times using distilled water for removing ethanol traces. The seeds soaked in 200 µM KNO_3 solution for 48 h were cultured in MS medium containing 2 mg/l GA_3 in a growth chamber at 25 ºC and for 16/8 (L/D) photoperiod and sterile plantlets were grown (Açıkgöz et al. 2019). The stem segments of sterile plantlets were used to establish cell suspension cultures. The stem explants were transferred to B_5 medium enriched with 0.5 mg/l BAP + 0.5 mg/l NAA. The obtained calluses were agitated at 105 rpm on a rotary shaker at 25 ºC for 16/8 (L/D) photoperiod.

2.2. Elicitation process and extraction

Approximately, 2,5 g cell suspension cultures were placed in 250 mL Erlenmeyer flasks having liquid medium of 50 mL and four concentrations (0, 5, 25 and 50 µM) of $AgNO_3$ and $CdCl_2$ were added. The cell suspension cultures were collected at 8, 48 and 72 h after

elicitation. After aseptically filtered and washed using deionized water, harvested suspensions were placed in a deep-freezer (-20 ^{o}C) till to extraction. Before subjecting to chemical analysis, the suspensions were powdered using a mortar and pestle. The procedure explained by Açıkgöz, (2021) was employed to prepare ethanol extracts. Briefly, 10 ml 96% ethanol was added to 2 g suspensions and homogenized for 2 min. The resulting mixture was placed in water bath at 45 ^{o}C for 12 hours before centrifuging at 4000 rpm on a rotary shaker for 5 min. The supernatants were evaporated for complete dryness in a rotary evaporator at 75 ^{o}C. The dried extracts were dissolved in 1 ml methanol and used for further analysis.

2.3. Determination of phenolic compounds, camphor and cell growth

To determine the total phenolic contents, Folin-Ciocalteu reagent based spectrophotometric assay was used as explained by Slinkard and Singleton (1977). The standard curve of gallic acid solution was used and the absorbance was recorded at 765 nm. The total phenolic content was estimated as milligram gallic acid equivalent per gram of fresh cell weight (mg GAE/g fresh weight). Dimethylamino cinnamaldehyde (DMAC) chromogenic reagent was employed to determine total flavanol according to (Prior et al. (2010). The absorbance at 640 nm was monitored and total flavanol values were presented as milligram cathecin equivalent per gram of fresh cell wight (m CTE/g fresh weight). For determination of total flavonol contents, Neu solution was used as described by Dai et al., (1995). Briefly, 1% 2-aminoethyl diphenylborinate and methanol was added to the extracts. The absorbance of resulting mixture was displayed at 410 nm and presented as milligram rutin equivalent per gram of fresh cell wight (m RE/g fresh weight). The protocol of Qu et al. (2006) was performed to determine total anthocyanin using McIIvaine's buffer and absorbance was recorded at 570 nm. As an indicator of anthocyanin content, color value (CV) of the extract was estimated using the equation of CV = 0.1 × Absorbance × Dilution factor (CV g^{-1} FCW).

The content of camphor was quantified through a Headspace GC-MS integrated with Shimadzu QP2010 ultra and Shimadzu AOC-5000 plus auto sampler was used. The capillary separation was performed on an RTX-5M 30 m column. Before the analysis, the device was uploaded with camphor standard and the retention times and mass fragments of the solution were determined. The calibration curve was plotted and the content of camphor was given in µg g^{-1}. The working layouts of GC-MS were given as below; carrier gas of helium, 250 ^{o}C injection temperature, 0.5 ml injection volume, 70 eV ionization voltage, 100 ^{o}C temperature and 10 min heating period.

Growth parameters such as cell number, cell viability (%), and cell dry weight (g l^{-1}) were recorded in cell suspension cultures. The Nageotte Counting Chamber as given by Moroff et al. (1994) was used to determine the number cells in suspensions. In measuring cell viability, the trypan blue staining technique described by Laloue et al. (1980) was employed. The filtered cells stored in an oven at 55 ^{o}C for 48 h were used to estimate cell dry weight.

2.4. Design of experiment and data analysis

All treatments were performed in triplicates. Variance analysis of the data was carried out based on completely randomized design using Minitab 17 statistical software. The significance test among the treatment means was performed by Tukey test at the 5% level.

3. Results

$AgNO_3$ and $CdCl_2$ elicitors significantly affected the accumulation of phenolic compounds and camphor along with cell growth in general. Exposing *A. gypsicola* cell suspension cultures to different concentrations of $AgNO_3$ caused significant alterations in all parameters studied, except total phenolic content (Tables 1 and 2). The influence of culture time was, however, non-significant for any of the attributes evaluated. The interaction between $AgNO_3$ doses and culture periods was significant for total phenolic content, indicating that the effect of $AgNO_3$ doses differed depending on culture period.

Significant and regular increases corresponding to 5 and 25 µM $AgNO_3$ doses were observed in all parameters, but the additional dose of 50 µM $AgNO_3$ resulted in significant decreases. The maximum production of camphor (1.6711 µg g^{-1}) was recorded with the use of 25 µM $AgNO_3$, which is 6.32 times higher compared to the control culture. Similarly, treatment of 25 µM $AgNO_3$ produced 31.5%, 42.8%, 30.25%, and 3.05% increases in total flavanol, total anthocyanin, cell number and cell dry weight in comparison to the control culture, respectively.

Subjecting *A. gypsicola* cell suspension cultures to several concentrations of $CdCl_2$ created a significant influence on total phenolic, total flavanol, total flavonol, camphor content, total anthocyanin, cell dry weight and

cell number (Tables 3 and 4). Increasing concentrations of $CdCl_2$, on the contrary, created no significant changes in cell viability. Culture period did not show any significant effects on the attributes evaluated, whereas interaction effect between $CdCl_2$ doses and culture periods was significant for cell viability.

Table 1. Contents of phenolic compounds and camphor in cell suspension cultures of *A. gypsicola* treated with silver nitrate

Çizelge 1. *Gümüş nitrat ile muamele edilmiş A. gypsicola hücre süspansiyon kültürlerinde fenolik bileşikler ve kamfor içeriği.*

Silver nitrate doses (µM)	Culture periods (hours)			Mean
	8	48	72	
Total phenolic (mg g^{-1})				
0 (control)	0.6369 abc*	0.6382 ab	0.6418 ab	0.6389
5	0.6396 abc	0.6452 ab	0.6548 ab	0.6465
25	0.6774 a	0.6851 a	0.6925 a	0.6850
50	0.6659 a	0.6558 ab	0.6536 ab	0.6584
Mean	0.6549	0.6560	0.6606	
Total flavanol (mg g^{-1})				
0 (control)	0.0099	0.0111	0.0115	0.0108 b*
5	0.0117	0.0123	0.0123	0.0121 b
25	0.0136	0.0141	0.0150	0.0142 a
50	0.0111	0.0106	0.0111	0.0109 b
Mean	0.0441	0.0434	0.0448	
Total flavonol (mg g^{-1})				
0 (control)	0.0426	0.0436	0.0429	0.0430 b*
5	0.0448	0.0459	0.0476	0.0461 a
25	0.0437	0.0419	0.0458	0.0438 ab
50	0.0453	0.0422	0.0428	0.0434 b
Mean	0.0441	0.0434	0.0448	
Total anthocyanin (CV g^{-1})				
0 (control)	0.0053	0.0058	0.0055	0.0056 b*
5	0.0059	0.0062	0.0059	0.0060 a
25	0.0075	0.0080	0.0086	0.0080 a
50	0.0041	0.0039	0.0052	0.0044 c
Mean	0.0057	0.0059	0.0063	
Camphor (µg g^{-1})				
0 (control)	0.2561	0.2624	0.2740	0.2642 d*
5	0.3707	0.3768	0.3676	0.3717 c
25	1.6661	1.6710	1.6760	1.6711 a
50	0.8497	0.8349	0.8273	0.8373 b
Mean	0.7857	0.7863	0.7862	

* The lowercase letters represent significance at 0.05 level ($P<0.05$).

Table 2. Cell number, cell dry weight and cell viability in *A. gypsicola* cell suspension cultures treated with silver nitrate for various hours.

Çizelge 2. *Çeşitli saatler boyunca gümüş nitrat ile muamele edilmiş A. gypsicola hücre süspansiyon kültürlerinde hücre sayısı, hücre kuru ağırlığı ve hücre canlılığı.*

Silver nitrate doses (µM)	Culture periods (hours)			Mean
	8	48	72	
Cell number				
0 (control)	82.840	82.760	83.120	82.906 d*
5	92.920	93.240	94.200	93.453 b
25	106.300	108.640	109.000	107.980 a
50	84.640	84.500	85.640	84.927 c
Mean	91.675	92.285	92.990	
Cell dry weight (g l^{-1})				
0 (control)	9.2462	9.2452	9.2578	9.2497 d*
5	9.4612	9.4405	9.4427	9.4481 b
25	9.5192	9.5294	9.5461	9.5316 a
50	9.3378	9.3390	9.3443	9.3403 c
Mean	9.3911	9.3885	9.3977	
Cell viability (%)				
0 (control)	97.9733	97.7467	97.3467	97.6889 a*
5	97.5400	98.0667	98.1400	97.9156 a
25	98.2067	97.3467	98.2167	97.9233 a
50	95.6800	94.7000	95.3000	95.2267 b
Mean	97.3515	96.9650	97.2508	

* The lowercase letters represent significance at 0.05 level ($P<0.05$).

Table 3. Contents of phenolic compounds and camphor in cadmium chloride tretaed *A. gypsicola* cell suspension cultures.

Çizelge 3. *Kadmiyum klorür ile muamele edilmiş A. gypsicola hücre süspansiyon kültürlerinde fenolik bileşikler ve kamfor içeriği*

Cadmium chloride doses (µM)	Culture periods (hours) 8	48	72	Mean
Total phenolic (mg g^{-1})				
0	0.6369	0.6382	0.6418	0.6390 c*
5	0.6371	0.6388	0.6410	0.6390 c
25	0.6807	0.6870	0.6921	0.6866 a
50	0.6562	0.6503	0.6455	0.6507 b
Mean	0.6549	0.6410	0.6551	
Total flavanol (mg g^{-1})				
0	0.0099	0.0111	0.0115	0.0108 b*
5	0.0111	0.0113	0.0121	0.0115 b
25	0.0136	0.0140	0.0156	0.0144 a
50	0.0120	0.0118	0.0114	0.0117 b
Mean	0.0117	0.0121	0.0126	
Total flavonol (mg g^{-1})				
0	0.0426	0.0436	0.0429	0.0430 b*
5	0.0427	0.0429	0.0443	0.0433 b
25	0.0448	0.0459	0.0476	0.0461 a
50	0.0415	0.0431	0.0416	0.0421 b
Mean	0.0429	0.0438	0.0441	
Total anthocyanin (CV g^{-1})				
0	0.0053	0.0058	0.0055	0.0056 b*
5	0.0062	0.0045	0.0055	0.0054 b
25	0.0081	0.0078	0.0074	0.0078 a
50	0.0053	0.0043	0.0038	0.0045 b
Mean	0.0062	0.0056	0.0056	
Camphor (µg g^{-1})				
0	0.2561	0.2624	0.2740	0.2642 d*
5	0.4708	0.4757	0.4666	0.4710 c
25	1.7890	1.8154	1.8515	1.8186 a
50	0.9535	0.9488	0.9446	0.9490 b
Mean	0.8673	0.8755	0.8841	

* The lowercase letters represent significance at 0.05 level (P<0.05).

Table 4. Cell number, cell dry weight and cell viability in *A. gypsicola* cell suspension cultures treated with cadmium chloride for various hours.

Çizelge 4. *Çeşitli saatler boyunca kadmiyum klorür ile muamele edilmiş A. gypsicola hücre süspansiyon kültürlerinde hücre sayısı, hücre kuru ağırlığı ve hücre canlılığı.*

Cadmium chloride doses (µM)	Culture periods (hours) 8	48	72	Mean
Cell number				
0	82.800	82.700	83.100	82.867 c*
5	84.000	83.800	85.000	84.267 c
25	108.000	108.700	109.000	108.567 a
50	96.600	97.500	98.700	97.600 b
Mean	92.850	93.175	93.950	
Cell dry weight (g l^{-1})				
0	9.2462	9.2452	9.2578	9.2497 c*
5	9.3378	9.3390	9.3443	9.3403 c
25	9.5253	9.5294	9.5461	9.5336 a
50	9.4612	9.4405	9.4427	9.4481 b
Mean	9.3926	9.3896	9.3977	
Cell viability (%)				
0	97.9733 a*	97.7467 a	97.3467 a	97.6889
5	98.4200 a	98.3433 a	97.8267 a	98.1966
25	98.0500 a	98.2333 a	97.6600 a	97.9811
50	96.7567 a	94.5567 b	93.6767 b	94.9967
Mean	97.8000	97.2200	96.6275	

* The lowercase letters represent significance at 0.05 level (P<0.05).

The content of camphor reached its maximum of 1.8186 μg g^{-1} at with 25 μM $CdCl_2$, indicating a 6.88-fold increase compared to the starting culture. Treatment of 25 μM $CdCl_2$ produced 33.3%, 39.2%, and 31.0 increases in total flavanol, total anthocyanin and cell number compared with untreated culture as control, respectively. Similarly, suspension cultures treated with 25 μM $CdCl_2$ ended up with a 7.4 and 7.2-fold increases in total phenolic and total flavonol as compared to control. Similar to $AgNO_3$, a significant decrease in all parameters was noticed in suspension cultures treated with 50 μM $CdCl_2$.

4. Discussion

It has been well documented that heavy metals stimulate the biosynthesis of numerous bioactive compounds of economic importance in plant tissue culture (Kim et al. 2004; Zhao et al., 2010; Park et al., 2016; Srivastava et al., 2019; Zafar et al., 2020). For example, Ag+ has been proven to be associated with ethylene pathways which regulates the synthesis of SMs as a defense response of plant tissues (Pitta-Alvarez et al., 2000; Li et al., 2016). However, little information is available in scientific literature regarding heavy metal induced accumulation of SMs in Achillea species (Ghanati et al., 2014). There is no published study on the effects of $AgNO_3$ and $CdCl_2$ in *A. gypsicola* cell suspension culture.

In an attempt to increase the content of camphor, phenolic compounds and cell growth, we exposed cell suspension cultures to various levels of $AgNO_3$ and $CdCl_2$ for different exposure times. Our results indicated that both $AgNO_3$ and $CdCl_2$ could elicit cell growth and the production of phenolic compounds and camphor in *A. gypsicola* suspension culture. It is interesting to note that the effect of $AgNO_3$ and $CdCl_2$ on the content of camphor, phenolic compounds and cell growth were almost the same. The treatments of $AgNO_3$ and $CdCl_2$ up to 25 μM significantly enhanced the content of almost all phenolic compounds and camphor along with cell growth.

The effect of $AgNO_3$ and $CdCl_2$ was quite more obvious in camphor content in which 25 μM treatments of both elicitors resulted in more than 6-fold increase as compared to the initial culture. Regarding well-known medicinal properties of camphor, therefore, it sounds reasonable to conclude that both $AgNO_3$ and $CdCl_2$ would be effectively used as potent elicitors in suspension culture of *A. gypsicola*. The application of 50 μM $AgNO_3$ and $CdCl_2$, however, produced a rapid decline in all attributes studied, implying that cell suspension culture of *A. gypsicola* is susceptible to elicitation by high concentrations of $AgNO_3$ and $CdCl_2$. These data suggest that the stimulatory effects of $AgNO_3$ and $CdCl_2$ elicitors appear to be concenration dependent (Li et al., 2016). Among others, the concentration of elicitors is a key factor affecting the magnitude of the response and it varies depending on plant species, culture conditions and inoculation period (Isah et al., 2018;). Studying with cell suspension cultures of *Silybum marianum*, Ashtiani et al., (2010) indicated that Ag+ in low concentrations positively elicited silymarin production and cell growth, whereas a high dose of Ag+ showed inhibition. Similarly, Cetin et al., (2014) reported that treatment with $CdCl_2$ in 1.5 mM gave the lowest amounts of total phenolics, while application of 1.0 mM $CdCl_2$ produced the highest values in *Vitis vinifera* cell suspension culture.

In the present study, an increase in all phenolic compounds including anthocyanin was observed in cell suspension cultures treated with 5 and 25 μM $AgNO_3$ and $CdCl_2$. In an earlier study, however, we found that salicylic acid significantly enhanced synthesis of anthocyanin in *A. gypsicola* cell suspension culture, while methyl jasmonate resulted in a significant decrease (Açıkgöz et al., 2019). Furthermore, Ghanati et al., (2014) reported that treatment of *Achillea millefolium* with silver nanoparticles significantly increased camphor content, whereas the level of anthocyanin decreased. Based on these findings, it appears reasonable to conclude that different elicitors somehow produce metabolite-dependent responses. Moreover, elicitors do not function equally in all species and the response to an elicitor might be species-specific (Cai et al., 2012; Pitta-Alvarez et al., 2000).

Elicitor specificity and concentration, type of culture medium and time duration of elicitor exposure are among major regulating factors responsible for enhanced synthesis of SMs. In the current study, however, no significant variation was observed in the effect of culture period in any of the parameters in $AgNO_3$ and $CdCl_2$ treated cell suspensions. In a previous study, we found that culture period significantly affected total flavanol, camphor content and cell number in cell suspension culture of *A. gypsicola* treated with various doses of MeJA and SA (Açıkgöz, 2017; Açıkgöz et al., 2019). In a study, Namdeo et al. (2002) reported that suspension cultures of *Catharanthus roseus* elicited using *T. viride* for 48 h produced a 3-fold increase in ajmalicine content. A longer period of elicitor exposure, however, adversely affected ajmalicine production.

5. Conclusion

To our knowledge, this is the first instance of using $AgNO_3$ and $CdCl_2$ elicitors for enhancing camphor, phenolic compounds and cell growth in cell suspension cultures of *A. gypsicola*. The addition of $AgNO_3$ and $CdCl_2$ significantly increased the accumulation of camphor in cell suspension cultures. As compared to the starting culture, the addition of 25 µM $CdCl_2$ and $AgNO_3$ to cell suspension cultures brought about 6.88 and 6.32-fold increases in camphor content, respectively. The production of phenolic compounds and cell growth were also stimulated by $AgNO_3$ and $CdCl_2$ elicitors. Furthermore, culture period caused no significant variation in any of the attributes studied. The results of the current study revealed that $AgNO_3$ and $CdCl_2$ as elicitors could have a good potential in increasing the synthesis of phenolic compounds and camphor in *A. gypsicola* cell suspension culture.

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References

Açıkgöz, M. A. 2017. *Achillea gypsicola* türünde kallus kültürü ile sekonder metabolit üretim potansiyelinin belirlenmesi. Ordu University (Doctoral dissertation, PhD Thesis (Printed), Ordu).

Açıkgöz, M. A., Yarılgaç, T., & Kara, Ş. M. 2018a. Enhancement of phytochemical compounds using biotic and abiotic elicitors in purple coneflower (*Echinacea purpurea* L.). Indian J Pharm Educ Res, 52, 140-145. https://doi.org/10.5530/ijper.52.4s.90

Açıkgöz, M. A., Kara, Ş. M., Ebru, B. A., & Odabaş, S. 2018b. Effect of light on biosynthesis of alkamide, caffeic acid derivatives and echinacoside in *Echinacea purpurea* L. callus cultures. Akademik Ziraat Dergisi, 7(2), 179-184. https://doi.org/10.29278/azd.476349

Açıkgöz, M. A. 2019. Evaluation of Phytochemical Compositions and Biological Properties of *Achillea gypsicola* at Different Phenological Stages. Chemistry & Biodiversity, 16(12), e1900373. https://doi.org/10.1002/cbdv.201900373

Açıkgöz, M.A., Kara, S.M., Aygun, A., Ozcan, M.M. & Ay, E.B. 2019. Effects of methyl jasmonate and salicylic acid on the production of camphor and phenolic compounds in cell suspension culture of endemic Turkish yarrow (*Achillea gypsicola*) species. *Turkish Journal of Agriculture and Forestry* 43:19. https://doi.org/10.3906/tar-1809-54

Açıkgöz, M. A. 2020a. Establishment of cell suspension cultures of *Ocimum basilicum* L. and enhanced production of pharmaceutical active ingredients. Industrial crops and products, 148, 112278. https://doi.org/10.1016/j.indcrop.2020.112278

Açıkgöz, M. A. 2020b. Determination of essential oil compositions and antimicrobial activity of *Achillea gypsicola* Hub.-Mor. at different plant parts and phenological stages. Journal of Essential Oil Research, 32(4), 331-347. https://doi.org/10.1080/10412905.2020.1750496

Açıkgöz, M. A. 2021. Effects of sorbitol on the production of phenolic compounds and terpenoids in the cell suspension cultures of *Ocimum basilicum* L. Biologia, 76(1), 395-409. https://doi.org/10.2478/s11756-020-00581-0

Açıkgöz, M. A., Ebru, B. A., Aygün, A., & Kara, Ş. M. 2022. Light-Mediated Biosynthesis of Phenylpropanoid Metabolites in Cell Suspension Cultures of Turkish Yarrow (*Achillea gypsicola* Hub. Mor.). Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi, 25(6), 1234-1242. https://doi.org/10.18016/ksutarimdoga.vi.926160

Ashtiani, S.R., Hasanloo, T. & Bihamta, M.R. 2010. Enhanced production of silymarin by Ag+ elicitor in cell suspension cultures of *Silybum marianum*. *Pharmaceutical Biology* 48 (6): 708-715. https://doi.org/10.3109/13880200903264426

Baser, K.H.C. 2002. Aromatic biodiversity among the flowering plant taxa of Turkey. *Pure Applied Chemistry* 74: 527-545. https://doi.org/10.1351/pac200274040527

Bourgaud, F., Gravot, A., Milesi, S. & Gontier, E. 2001. Production of plant secondary metabolites, a historical perspective. *Plant Science* 5: 839-851. https://doi.org/10.1016/S0168-9452(01)00490-3

Cai, Z., Kastell, A., Knorr, D. & Smetanska, I. 2012. Exudation: an expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. *Plant Cell Reports* 31: 461-477. https://doi.org/10.1007/s00299-011-1165-0

Cetin, E.S., Babalik, Z., Hallac-Turk, F. & Gokturk-Baydar, N. 2014. The effects of cadmium chloride on secondary metabolite production in *Vitis vinifera* cv. cell suspension cultures. *Biological Research* 47:47. https://doi.org/10.1186/0717-6287-47-47

Dağlioğlu, Y., Açıkgöz, M. A., Özcan, M. M., & Kara, Ş. M. 2022. Impact of application of alumina oxide nanoparticles on callus induction, pigment content, cell damage and antioxidant enzyme activities in *Ocimum basilicum*. Journal of International Environmental Application and Science, 17(1), 22-33.

Dai, G.H., Andary, C., Mondolot, L. & Boubals, D. 1995. Involment of phenolic compounds in the resistance of grapevine callus to downy mildew (*Plasmopara viticola*). *European Journal of Plant Pathology* 101: 541-547. https://doi.org/10.1007/BF01874479

Davies, K.M. & Deroles, S.C. 2014. Prospects for the use of plant cell cultures in food biotechnology. *Current Opinion in Biotechnology* 26:133-140. https://doi.org/10.1016/j.copbio.2013.12.010

Demirci, B., Baser, K.H.C., Aytac, Z., Khan, S.I., Jacob, M.R. & Tabanca, N. 2018. Comparative study of three *Achillea* essential oils from eastern part of Turkey and their biological activities. *Records of Natural Products* 12: 195-200. https://dx.doi.org/10.25135/rnp.09.17.03.019

Ebru, B. A., Kocaman, B., Öner, E. K., & Açıkgöz, M. A. (2022). Effects of elicitors on secondary metabolite (SM) production and antioxidant activity in sweet basil (*Ocimum basilicum* L.) cell suspension cultures. *Notulae Scientia Biologicae*, 14(2), 11246-11246. https://doi.org/10.55779/nsb14211246

Georgiev, M.I., Weber, J. & Maciuk, A. 2009. Bioprocessing of plant cell cultures for mass production of targeted compounds. *Applied Microbiology and Biotechnology* 83: 809-823. https://doi.org/10.1007/s00253-009-2049-x

Ghanati, F., Bakhtiarian, S., Parast, B.M. & Behrooz, M.K. 2014. Production of new active phytocompounds by *Achillea millefolium* L. after elicitation with silver nanoparticles and methyl jasmonate. *Biosciences Biotechnology Research Asia* 11(2): 391-399. http://dx.doi.org/10.13005/bbra/1287

Halder, M., Sarkar, S. & Jha, S. 2019. Elicitation: A biotechnological tool for enhanced production of secondary

metabolites in hairy root cultures. *Engineering in Life Sciences* 19: 880-895. https://doi.org/10.1002/elsc.201900058

Isah, T., Umar, S., Mujib, A., Sharma, M.P., Rajasekharan, P.E., Zafar, N. & Frukh, A. 2018. Secondary metabolism of pharmaceuticals in the plant *in vitro* cultures: strategies, approaches, and limitations to achieving higher yield. *Plant Cell, Tissue and Organ Culture* 132: 239-265. https://doi.org/10.1007/s11240-017-1332-2

Jamwal, K., Bhattacharya, S. & Puri, S. 2018. Plant growth regulator mediated consequences of secondary metabolites in medicinal plants. *Journal of Applied Research on Medicinal and Aromatic Plants* 9: 26-38. https://doi.org/10.1016/j.jarmap.2017.12.003

Kim, O.T., Kim, M.Y., Hong, M.H., Ahn, J.C. & Hwang, B. 2004. Stimulation of asiaticoside accumulation in the whole plant cultures of *Centella asiatica* (L.) Urban by elicitors. *Plant Cell Reportes* 23: 339-344. https://doi.org/10.1007/s00299-004-0826-7

Laloue, M., Courtois, D. & Manigault, P. 1980. Convenient and rapid fluorescent staining of plant cell nuclei with "33258" Hoechst. *Plant Science Letter* 17: 175-179. https://doi.org/10.1016/0304-4211(80)90145-5

Matkowski, A. 2008. Plant *in vitro* culture for the production of antioxidants – A review. *Biotechnology Advances* 26: 548-560. https://doi.org/10.1016/j.biotechadv.2008.07.001

Mohammadhosseini, M., Sarker, S.D. & Akbarzadeh, A. 2017. Chemical composition of the essential oils and extract of Achillea species and their biological activities: a review. *Journal of Ethnopharmacology* 199: 257-315. https://doi.org/10.1016/j.jep.2017.02.010

Moroff, G., Eich, J. & Dabay, M. 1994. Validation of use of the Nageotte hemocytometer to count low levels of white cells in white cell reduced platelet components. *Transfusion* 34: 35-38. https://doi.org/10.1046/j.1537-2995.1994.34194098600.x

Murthy, H.N., Lee, E.J. & Paek, K.Y. 2014. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell, Tissue and Organ Culture* 118:1-16. https://doi.org/10.1007/s11240-014-0467-7

Namdeo, AG., Patil, S. & Fulzele, D.P. 2002. Influence of fungal elicitors on production of ajmalicine by cell cultures of *Catharanthus roseus*. *Biotechnology Progress* 18: 159-162. https://doi.org/10.1021/bp0101280

Nosov, A.M. 2012. Application of cell technologies for production of plant derived bioactive substances of plant origin. *Applied Biochemistry and Microbiology* 48(7): 609-624. https://doi.org/10.1134/S000368381107009X

Park, W.T., Arasu, M.V., Al-Dhabi, N.A., Yeo, S.K., Jeon, J., Park, J.S. & Park, S.U. 2016. Yeast extract and silver nitrate induce the expression of phenylpropanoid biosynthetic genes and induce the accumulation of rosmarinic acid in *Agastache rugosa* cell culture. *Molecules*, 21: 426. https://doi.org/10.3390/molecules21040426

Pitta–Alvarez, S.I., Spollansky, T.C. & Giulietti, A.M. 2000. The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. *Enzyme and Microbial Technology* 26: 252-258. https://doi.org/10.1016/S0141-0229(99)00137-4

Prior, R.L., Fan, E., Ji, H., Howell, A., Nio, C., Payne, M.J. & Reed, J. 2010. Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *Journal of the Science of Food and Agriculture* 90(9): 1473-1478. https://doi.org/10.1002/jsfa.3966

Radman, R., Saez, T., Bucke, C. & Keshavarz, T. 2003. Elicitation of plants and microbial cell systems. *Biotechnology and Applied Biochemistry* 37: 91-102. https://doi.org/10.1042/BA20020118

Ramakrishna, A. & Ravishankar, G.A. 2011. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal Behavior* 6(11): 1720-1731. https://doi.org/10.4161/psb.6.11.17613

Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioswki, M., Cusidó, R.M. & Palazon, J. 2016. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules* 21: 182. https://doi.org/10.3390/molecules21020182

Rao, S.R. & Ravishankar, G.A. 2002. Plant cell cultures: chemical factories of secondary metabolites. *Biotechnology Advances* 20: 101-153. https://doi.org/10.1016/S0734-9750(02)00007-1

Qu, J.G., Zhang, W., Jin, M.F. & Yu, X.J. 2006. Effect of homogeneity on cell growth and anthocyanin biosynthesis in suspension cultures of *Vitis vinifera*. *Chinese Journal of Biotechnology* 22: 805-510. https://doi.org/10.1016/S1872-2075(06)60057-5

Smetanska, I. 2008. Production of secondary metabolites using plant cell cultures. *Advances in Biochemical Engineering/Biotechnology* 111: 187-228. https://doi.org/10.1007/10_2008_103

Srivastava, M., Singh, G., Sharma, S., Shukla, S. & Misra, P. 2019. Elicitation enhanced the yield of glycyrrhizin and antioxidant activities in hairy root cultures of *Glycyrrhiza glabra* L. *Journal of Plant Growth Regulation* 38: 373-384. https://doi.org/10.1007/s00344-018-9847-2

Thakur, M., Bhattacharya, S., Khosla, P.K. & Puri, S. 2019. Improving production of plant secondary metabolites through biotic and abiotic elicitation. *Journal of Applied Research on Medicinal and Aromatic Plants* 12: 1-12. https://doi.org/10.1016/j.jarmap.2018.11.004

Verma, N. & Shukla, S. 2015. Impact of various factors responsible for fluctuation in plant secondary metabolites. *Journal of Applied Research on Medicinal and Aromatic Plants* 2: 105-113. https://doi.org/10.1016/j.jarmap.2015.09.002

Yue, W., Ming, Q.L., Lin, B., Rahman, K., Zheng C.J., Ting, H. & Qin, L.P. 2016. Medicinal plant cell suspension cultures: pharmaceutical applications and high-yielding strategies for the desired secondary metabolites. *Critical Reviews in Biotechnology* 36 (2): 215-232. https://doi.org/10.3109/07388551.2014.923986

Zhao, J., Davis, L.C. & Werpoorte, R. 2005. Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances* 23: 283-333. https://doi.org/10.1016/j.biotechadv.2005.01.003

Zhao, J.L., Zhou, L.G. & Wu, J.Y. 2010. Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures. *Applied Microbiology and Biotechnology* 87: 137-144. https://doi.org/10.1007/s00253-010-2443-4

Zafar, N., Mujib, A., Ali, M., Tonk, D., Gulzar, B.., Malik, M.O., Mamgain, J. & Sayeed, R. 2020. Cadmium chloride (CdCl2) elicitation improves reserpine and ajmalicine yield in *Rauvolfia serpentina* as revealed by high-performance thin-layer chromatography (HPTLC). *3 Biotech* 10: 344. https://doi.org/10.1007/s13205-020-02339-6