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Light-Mediated Biosynthesis of Phenylpropanoid Metabolites in Cell Suspension Cultures of Turkish Yarrow (*Achillea gypsicola* Hub. Mor.)

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ABSTRACT
Turkish yarrow (*Achillea gypsicola* Hub. Mor.) contains medicinally valuable bioactive metabolites. Light plays a key role in many physiological events in plants such as the accumulation of phenylpropanoid metabolites, particularly photosynthesis. The aim of this study was to determine the effects of light on the biosynthesis of phenylpropanoid metabolites and biomass growth in Turkish yarrow cell suspension cultures. *In vitro* plantlets obtained from seeds were used as explant sources and cell cultures of *A. gypsicola* were established using stem segment-derived callus tissues. Then, 8-day-old cell cultures kept in a B5 medium supplemented with 0.5 mg/L BA (benzylaminopurine)+0.5 mg/L NAA (naphthalene acetic acid) were incubated in light and dark environments. Cells were harvested five times (1st, 2nd, 3rd, 6th, and 12th day) in total. The amount of camphor was determined by gas chromatography-mass spectrometry (GC–MS). Other parameters were calculated using the spectrophotometric method. The total phenolic amount increased depending on the sampling time in dark and light conditions. Phenolic accumulation was increased by 29.25% compared to the initial culture. Also, light application increased the amount of camphor 1.12 times, the amount of flavanol 4.9 times, and the amount of flavonol 0.5 times. These results clearly demonstrate the effect of light on cell growth and phenylpropanoid metabolite accumulation in *A. gypsicola* cell cultures.

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Türk Civanperçemi (*Achillea gypsicola* Hub. Mor.) Hücre Süspansiyon Kültürlerinde Fenilpropanoid Metabolitlerin Işık Aracılığıyla Biosentezi

ÖZET
Türk civanperçemi (*Achillea gypsicola* Hub. Mor.) tıbbi açıdan değerli metabolitler içerir. Işık, bitkilerde başta fotosentez olmak üzere fenipropanoid metabolitlerin birikimi gibi birçok fizyolojik olayda kilit bir rol oynar. Bu çalışmanın amacı, ışığın Türk civanperçemi hücre süspansiyon kültüründe fenilpropanoid metabolitlerin biyosentezi ve biyokütle artışı üzerindeki etkilerini belirlemektir. Tohumlardan elde edilen *in vitro* bitkicikler eksplant kaynağı olarak kullanılmış ve gövde segmentinden türetilen kallus dokularından hücre kültürleri oluşturulmuştur. Daha sonra, 0.5 mg/L BA (benzilaminopurin)+0.5 mg/L NAA (naftalen asetik asit) ile desteklenmiş B5 ortamında muhafaza edien 8 günlük hücre kültürleri, aydınlık ve karanlık ortamlarda inkübe edilmiştir. Toplamda beş kez (1, 2, 3, 6 ve 12. gün) hücre hasadı yapılmıştır. Kamfor miktarı gas chromatography-mass spectrometry (GC-MS) ile belirlenmiştir. Diğer parametreler spektrofotometrik yöntem kullanılarak hesaplanmıştır. Toplam fenolik miktarı karanlık ve aydınlık koşullarda örnek alma zamanına bağlı olarak artış göstermiştir. Fenolik madde birikimi başlangıç kültürüne göre %29.25 artmıştır. Işık uygulaması kamfor miktarını 1.12 kat,

Tarımsal Biyoteknoloji

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 22.04.2021
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Anahtar Kelimeler
Abiyotik elisitör
Biyoaktif bileşikler
Kallus kültürü
Sekonder metabolit

flavanol miktarını 4.9 kat ve flavonol miktarını 0.5 kat artırmıştır. Bu sonuçlar bize *A. gypsicola* hücre kültürlerinde ışığın hücre büyümesi ve sekonder metabolit birikimindeki etkisini açıkça ortaya koymuştur.

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INTRODUCTION

Achillea gypsicola Hub. Mor. is an important herb with medicinal properties such as antimicrobial and anticarcinogenic activities (Saeidnia et al., 2011). *A. gypsicola* essential oil was composed of various volatile compounds, and the main compounds in *A. gypsicola* essential oil were borneol, camphor, and 1,8-cineole (Açıkgöz, 2019; Açıkgöz, 2020).Camphor terpene has potential benefits for health in antimicrobial (antibacterial, antifungal, antiviral), antitussive, antinociceptive, antimutagenic, anticarcinogenic, and cardiovascular terms (Edris, 2007; Lin et al., 2007; Cheng et al., 2009; Zuccarini, 2009; Abdel-Rahman et al., 2015). The contents and components of these compounds differ according to several factors such as diseases, used plant parts, growing stage, harvesting time, soil, and climate conditions (Açıkgöz, 2019; Açıkgöz, 2020). Therefore, there is a need to develop some methods to support the production of high-value bioactive metabolites in terms of quantity and quality. Among these methods, plant cell culture techniques to be used for secondary metabolite production is of great importance (Batı Ay et al., 2022). Further, plant cell cultures reduce the demand for expensive chemicals due to their simple extraction methods. Various strategies can be used to develop valuable secondary metabolite products in plant cell cultures. It is known that biotic and abiotic stress factors are used especially to initiate or increase the synthesis of phenylpropanoid metabolites (Açıkgöz, 2021; Dağlioğlu et al., 2022).

Among abiotic stress factors, light is one of the most significant environmental factors that regulate the growth, development, and metabolism of plants. Light is not only effective in photosynthesis, growth, and development, but also has a significant function in the biosynthesis of primary and secondary metabolites by increasing phenylalanine ammonia-lyase (PAL) in cell cultures (Khan et al., 2013; Murthy et al., 2014).

The reactions of plant cells to light occur through the interactions of the main photomorphogenic 1/suppressor of phytochrome A (COP1/SPA) proteins associated with pokeweed antiviral protein (PAP). In the dark, these two proteins are suppressed by ubiquitin ligase. In light environments, it causes instability of SPA1 and SPA2 proteins, which are among the SPA proteins, thus causing the COP1 protein to be more active (Liu et al., 2011; Zuo et al., 2011; Weidler et al., 2012). This causes various metabolic activities such as the biosynthesis of chlorophyll and anthocyanins as well as development and growth responses such as phototropism and flowering induction (Kami et al., 2010).

Light also plays an active role in the generation of important phytochemicals in most medicinal plant species. Many phenolic compounds, cinnamic acid, flavonoids, anthocyanins, and caffeic acid derivatives cichoric, caftaric, chlorogenic, and caffeic acid in plants are produced by PAL initiated phenylpropanoid pathway (Shohael et al., 2006; Sreelakshmi and Sharma, 2008). In previous studies (Zhao et al., 2010; Ali and Abbasi, 2014; Tariq et al., 2014; Georgieva et al., 2015; Ahmad et al., 2016; Açıkgöz et al., 2018), it has been shown that light conditions increase the dry weight of the cell, total phenolic, anthocyanin, flavanol, and flavonol compared to dark conditions. This study was conducted to determine the effect of light on phenylpropanoid metabolite production in cell cultures of *A. gypsicola*.

MATERIAL and METHOD

Plant material

As plant material, seeds of *A. gypsicola*, endemic to Turkey, collected from native flora are used. *A. gypsicola* plants were detected in the hills on the right side of "Çorum–İskilip" at the 26th and 47th kilometers and plant samples were taken for species identification. The plant samples were subjected to the herbarium and kept in room conditions until identification. Species diagnosis was made by Prof. Dr. Hayri Duman, Gazi University, Faculty of Science, Department of Biology.

Callus establishment

In vitro plantlets obtained from these seeds were used as explant sources and cell suspension cultures of *A. gypsicola* were established using stem segment-derived callus tissues (Açıkgöz, 2017). The cultures were maintained in a B5 medium supplemented with 0.5 mg/L benzylaminopurine+0.5 mg/L naphthalene

acetic acid and grown in three 250 mL Erlenmeyer flasks containing 50 mL of liquid medium and 2.5 g of green fragile calluses. Then, they were cultured on a rotary shaker at 105 rpm at 25 °C and a light/dark photoperiod 16/8.

Treatment of light

After the cell cultures were placed in a shaker at an average speed of 105 rpm, they were kept at 25 °C and under continuous light of an average of 8.000 lux for different periods. The control group was cultured at 25 °C in dark conditions. For the light application, sampling was done five times, starting from the 1st day to the 2nd, 3rd, 6th, and 12th days, and the 1st-day sampling was done 8 h after the application.

Sample extraction

The extraction was done based on the method of Abbasi et al. (2012). Briefly, 2 g of the crushed samples were taken and 10 ml of 96% ethanol was added. After 2 min of mixing in the homogenizer, they were kept in a water bath at 45°C for one night. At the end of this period, the samples were centrifuged at 4000 rpm for 5 min and the supernatant part containing phenolic compounds was removed and evaporated in a rotary evaporator until completely dry at 75°C.

Determination of cell number

Cell number was determined according to Moscatiello et al. (2013) with the following formula:

$acn = \Sigma c40i=1 \cdot 20 \cdot df$

acn: average cell number

c: cell number

df: dilution factor

Determination of dry cell weights

It was obtained by weighing the filtered cells after being kept in the drying oven at 55 °C for 48 h.

Determination of total phenolic content

The extracts evaporated in a rotary evaporator were dissolved in 1 ml of methanol and used in the phenolic compound analysis. Analysis was performed using the Folin Ciocalteu reagent based on the Chan et al. (2007) method. Spectrophotometer readings were carried out at a wavelength of 765 nm, and the total phenolic compound amounts were determined as gallic acid equivalent mg/g fresh cell weight using the curve prepared from the standard gallic acid solution.

Determination of total flavanol content

The dimethylamino cinnamaldehyde method used by Sun et al. (1998) was used to determine the total flavanols in cell suspension cultures. The readings were carried out in the spectrophotometer at 640 nm wavelength. The results were determined as mg/g fresh cell weight as the equivalent of catechin by using the curved prepared from the catechin standard.

Determination of total flavonol content

Total flavonol analysis was made according to the method used by Kumaran and Joel Karunakaran (2007), using the Neu solution. Accordingly, after adding a mixture of 1% 2-aminoethyl diphenylborinate solution and methanol to the extracts and mixing thoroughly, the absorbance values at 410 nm were determined. The amount of total flavonols was determined as mg/g fresh cell weight as a routine equivalent by using the curve prepared from the routine standard.

Determination of total anthocyanin content

Antochyanin analyzes were carried out using Mcllvain's buffer (pH=3) according to the method used by Giusti and Wrolstad (2000). The readings in the spectrophotometer were made at a wavelength of 570 nm and the anthocyanin amounts were calculated as color values and the results were given as CV/g fresh cell weight.

Determination of camphor content

Camphor compound was determined by GC-MS device in the study. For Headspace GC-MS analysis, Shimadzu QP2010 Ultra GC-MS was integrated into the device and Shimadzu AOC-5000 plus autosampler was used. Capillary column separation was determined by RTX-5M 30m. The camphor standard was given to the device first for analysis. The mass fragments and retention time of the compound were determined. the precision of the work and to increase the accuracy, 9 major ion peaks were selected. Later camphor the calibration curve of the compound was drawn and the amount of the sample was determined as µg/g.

Statistical analysis

One way factor analysis of variance was used to evaluate the data. Different averages were determined with the Tukey test and the results were expressed in the letter representation alongside the averages. Significance level (5%) was used in calculations and interpretations. All calculations were used with SPSS statistical package program.

RESULTS and DISCUSSION

Cell numbers, dry cell weight

In this study, the effect of light and dark situations on cell number and dry cell weight was investigated in the callus culture of *A. gypsicola* species. While the

number of cells was 85.300 in the initial culture in bright conditions, it was determined to reach 104.300 at the end of the 12th day. The lowest cell number was obtained from the initial cultures of dark and light treatments (84.100 and 85.300, respectively) (Figure 1). It has been determined that dry cell weight (g/l) increases in both light and dark conditions. While the highest dry cell weight (13.7219 g/l and 13.3981 g/l, respectively) was obtained from the cells that incubated in light for 6 and 12 days, in the cells incubated in the dark, this value was determined as 12. 128 g/l maximum on day 12. It has been reported by many researchers that stress factors are very efficient in the development of secondary metabolites and cell growth (Georgieva et al., 2015; Ahmad et al., 2016). The age of cell culture, the duration of exposure to the elicitor, and the type of elicitor are important in improving the effectiveness of these treatments. Light is one of the major elicitors that stimulates the production of secondary metabolites and developmental processes (Khan et al. 2013). Previous studies have revealed that light plays an important role in cell growth. Zhao et al. (2010), Ali and Abbasi (2014), Silva et al. (2017), and Anjum et al. (2017) reported in their studies that light promotes cell growth. Similarly, it has been shown that light has an important role in cell growth in A. gypsicola.

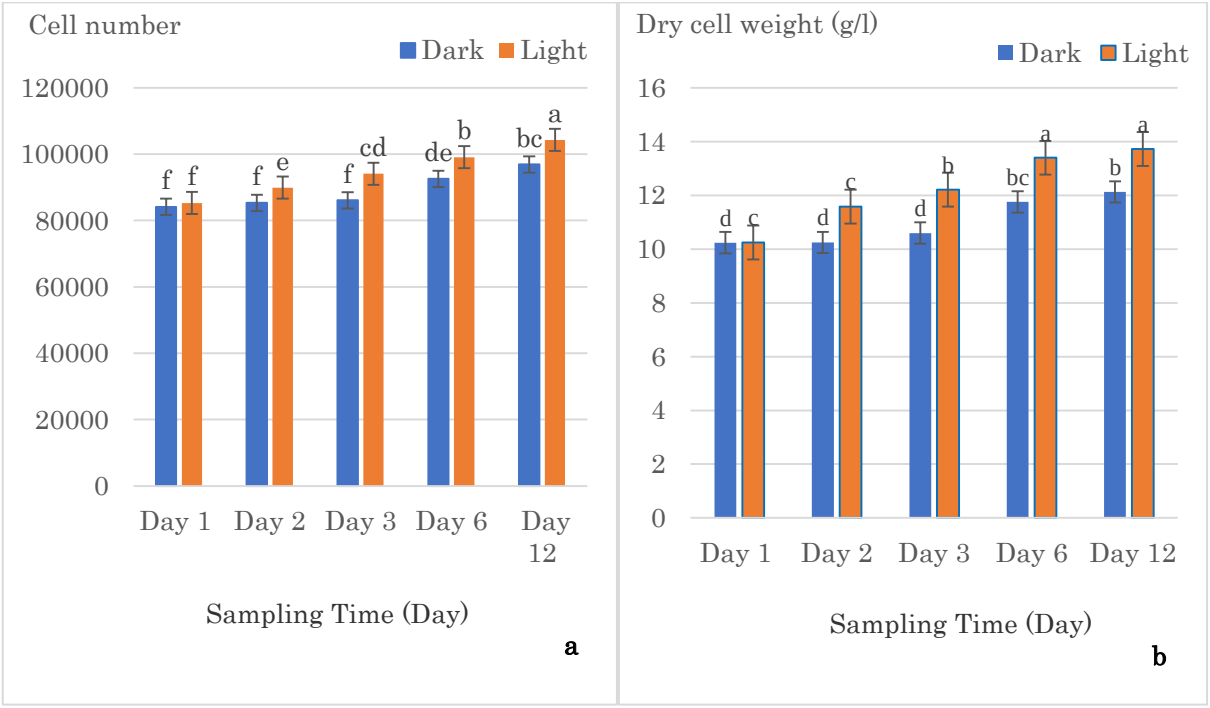

Figure 1. Effects of light treatments (dark and light) and sampling times (days) on cell number (number) (a), cell dry weight (g/l) (b) in cell suspension cultures of *A. gypsicola* species.

Şekil 1. A. gypsicola türünde hücre süspansiyon kültürlerinde ışık uygulamalarının (karanlık ve aydınlık) örnek alma zamanı (gün), hücre sayısına (adet) (a), hücre kuru ağırlığına (g/l) (b) etkileri

Error bars show standard deviation values. Means with a similar letter are not significant at the 5% level of the Tukey test.

Total phenolic, flavanol and flavonol content

Plant phenolics and flavonoids have strong biological activity. In this study, we researched the influence of light applied to *A. gypsicola* cultures for different periods on total phenolic, total flavanol, and total flavanol content. The total amount of phenolic substance (mg/g) increased regularly both in dark conditions and in the light environment depending on the application period (Figure 2). While the total amount of phenolic matter was an average of 0.7720 mg/g in the initial culture in the dark environment, it increased to 0.9543 mg/g at the end of the 12th day. In light application, the phenolic substance accumulation, which was 0.7744 mg/g in the initial culture, increased to 0.9978 mg/g at the end of the 12th day. It was determined that total flavanol accumulation (mg/g) was mostly in bright environments when light and dark environments were compared and flavanol accumulation increased significantly starting from the 1st day. The amount of flavanol, which was 0.0160 mg/g in the initial culture in bright conditions, increased to 0.0725 mg/g at the end of the 12th day. The amount of flavonol, which was 0.0123 mg/g in the initial culture under dark conditions, reached 0.0535 mg/g at the end of the 12th day. Total flavanol accumulation (mg/g) generally increased in dark and light environments. In the dark environment, while the total amount of flavanol in the initial culture was 0.0417 mg/g, it reached 0.0654 mg/g at the end of the 12th day. In light application,

the amount of flavanol, which was 0.0434 mg/g in the initial culture, increased to 0.0814 mg/g at the end of the 12th day. It was determined that light treatments were 53.24% more effective in flavanol accumulation when light and dark environments are compared. The remarkable point here is that the amounts of total phenolic, flavanol, and flavanol increase with light application. Light has an important function in the biosynthesis of primary and secondary metabolites by increasing PAL activity in cell cultures (Khan et al., 2013; Murthy et al., 2014). Many phenolic compounds, caffeic acid, cinnamic acid, flavonoids, anthocyanins, and caffeic acid derivates in plants are produced by the phenylpropanoid pathway initiated by PAL (Shohael et al., 2006; Sreelakshmi and Sharma, 2008). Therefore, it can be said that light increases the total amount of flavonoids by stimulating the biosynthetic pathway. In previous studies, Fazal et al. (2016), Nadeem et al. (2018), and Shohael et al. (2006) reported that light promotes secondary metabolite accumulation more than dark conditions. In this study, it was revealed that light conditions increase the amount of total phenolic, flavanol, and flovonol other active ingredients compared to dark conditions.

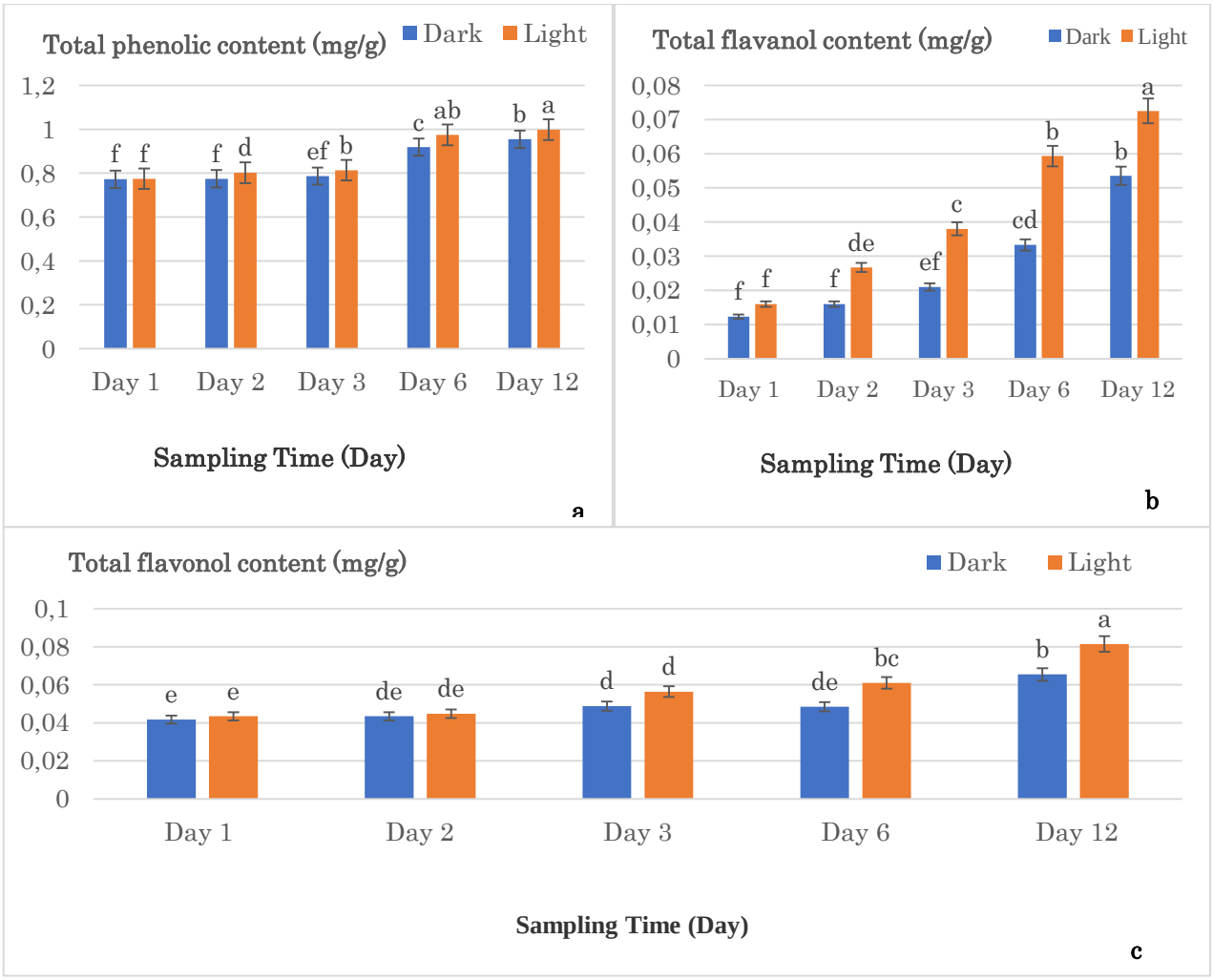

Figure 2. Effects of light treatments (dark and light) and sampling times (days) on total phenolic (mg/g) (a), flavanol (mg/g) (b), flavonol content (mg/g) (c) in cell suspension cultures of A. gypsicola species.

Şekil 2. A. gypsicola türünde hücre süspansiyon kültürlerinde ışık uygulamalarının (karanlık ve aydınlık) toplam fenolik madde (mg/g) (a), flavanol (mg/g) (b), flavonol içeriğine (mg/g) (c) etkileri

Error bars show standard deviation values. Means with a similar letter are not significant at the 5% level of the Tukey test.

Total anthocyanin content

Anthocyanins constitute an important part of flavonoids, a common group of secondary metabolites. Anthocyanins found in plant tissues are synthesized in different concentrations depending on the genetic structure of the plant, environmental factors, and the stress conditions that the plant is exposed to (Schwinn et al., 2004). In this study, when the total anthocyanin accumulation in dark and light environments was compared, it was determined that

the anthocyanin accumulation occurred highest in light with 0.0097 CV/g and the lowest value was in the cells incubated with 0.0058 CV/g in the dark (Figure 3). Therefore, we can say that the light increases anthocyanin accumulation. The role of anthocyanins against light stress has been widely documented (Merzlyak et al., 2000; Trojak et al., 2017).

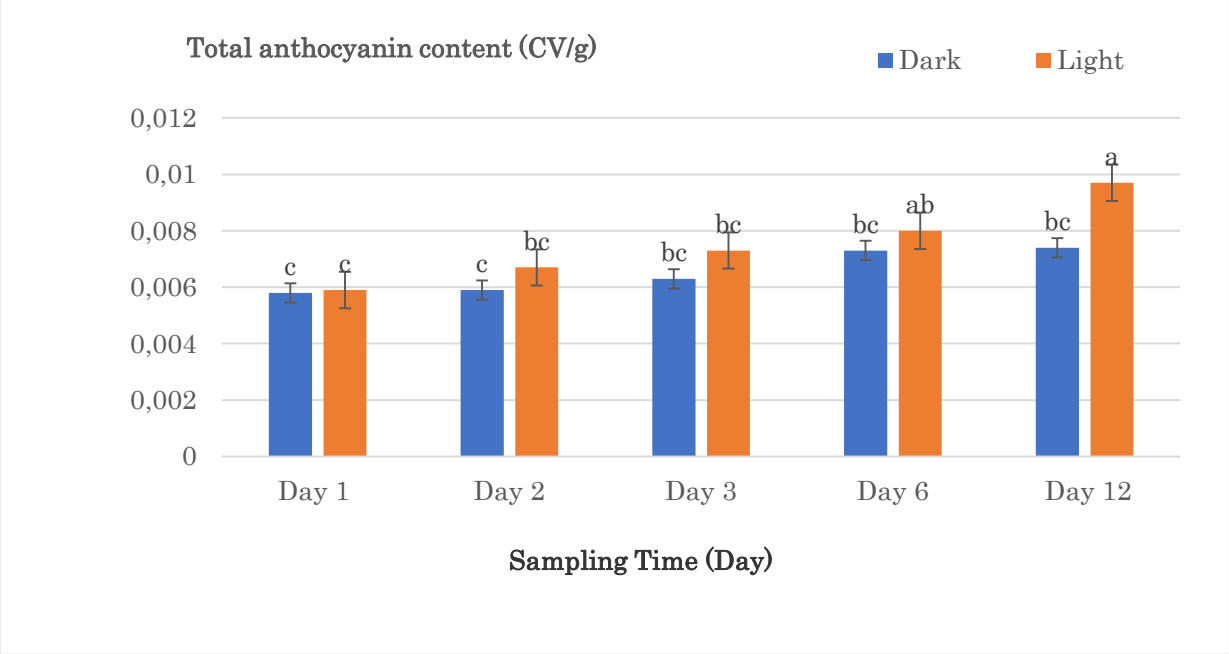

Figure 3. Effects of light treatments (dark and light) and sampling times (days) on the total anthocyanin content (CV/g) in cell suspension cultures of *A. gypsicola* species

Error bars show standard deviation values. Means with a similar letter are not significant at the 5% level of the Tukey test.

Şekil 3. A. gypsicola türünde hücre süspansiyon kültürlerinde ışık uygulamalarının (karanlık ve aydınlık) toplam antosiyanin miktarına (CV/g) etkileri

Camphor content

Camphor is an important ingredient found in the essential oil of plants that has medicinal effects such as antimicrobial and anticarcinogenic (Saeidnia et al., 2011). In the study, the amount of camphor, which was 0.7162 µg/g in the initial culture in the light environment, increased to 0.9693 µg/g 3 days after the application, to 1.1173 µ/g after 6 days, and after 12 days, it increased by 111.77% and reached 1.5167 µg/g (Figure 4). The highest camphor accumulation in cells incubated in the dark was 1.1153 µg/g at the end of the 12th day. The remarkable point here is that light treatments promote camphor accumulation more than dark conditions. Previously, Nieto-Trujillo et al. (2017) studies on *Tanacetum parthenium* and Razavizadeh et al. (2017) on *Carum copticum* species showed that stress factors raise the amount of phenylpropanoid metabolites. In addition, Silva et al. (2017) examined the effects of the light source applied to the culture environment at different intensities to increase the secondary metabolite accumulation in a study conducted on *Plectranthus amboinicus* species and they showed that carvacrol accumulation continuously increased depending on the intensity of light.

CONCLUSIONS

In this study, it was determined for the first time that light treatment in *A. gypsicola* cell suspension culture promoted cell number and dry cell weight, total flavanol, flavonol, phenolic, anthocyanin, and camphor content more than in dark conditions. Looking at the sampling times, it is seen that the highest value of cell growth and phenylpropanoid metabolite accumulation is reached on the 12th day, while the lowest value is reached in the initial cultures. The amount of camphor increased from 0.7162 µg/g in the initial culture to 1.5167 µg/g after 12 days with an increase of 111.77% in cells cultured in the light environment. It was determined that sampling times in dark environments do not have a significant effect on the amount of anthocyanin in total anthocyanin accumulation. The total amount of phenolic content increased regularly both in dark conditions and in the light environment and the accumulation of phenolic content in the initial culture in the light environment increased by 29.25% at the end of the 12th day. It has been determined that flavanol accumulation is mostly in bright environments. The amount of flavanol, which was 0.0160 mg/g on the 1st day, increased 4.9 times and reached 0.0725 mg/g at the end of the 12th day. When the light and dark environments are compared in

total flavonol accumulation, it was determined that light conditions were 53.24% more effective in flavanol accumulation. These results showed us that light treatments in *A. gypsicola* cell suspension cultures significantly promoted cell growth and phenylpropanoid metabolite accumulation.

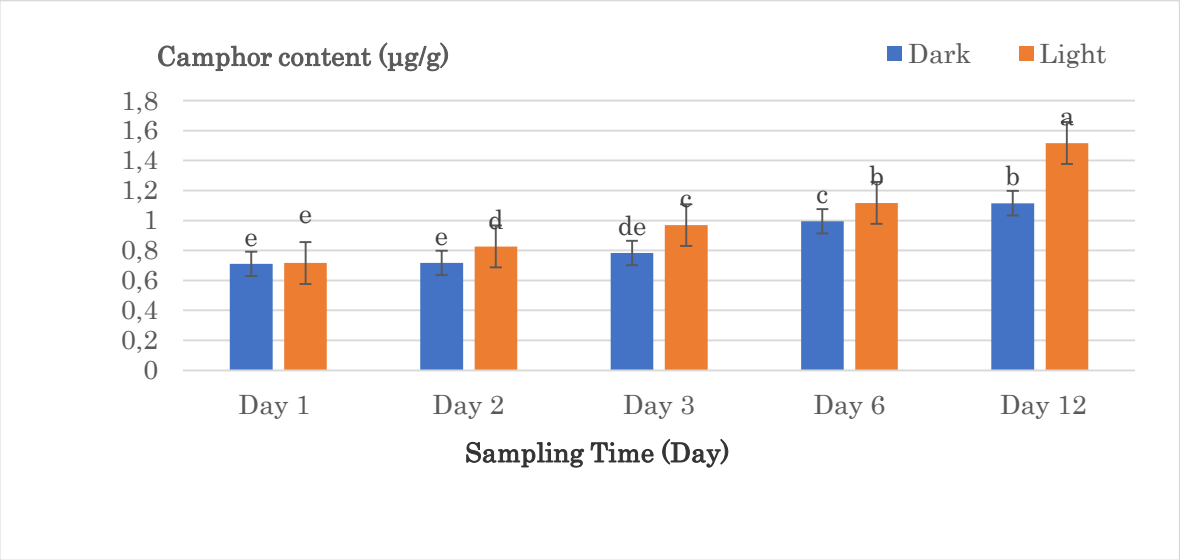

Figure 4. Effects of light treatments (dark and light) and sampling times (days) on camphor content (µg/g) in cell suspension cultures of *A. gypsicola* species

Error bars show standard deviation values Means with a similar letter are not significant at the 5% level of the Tukey test.

Şekil 4. *A. gypsicola* türünde hücre süspansiyon kültürlerinde ışık uygulamalarının (karanlık ve aydınlık) kamfor miktarına (µg/g) etkileri

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

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