

## **Effect of light on biosynthesis of alkamide, caffeic acid derivatives and echinacoside in *Echinacea purpurea* L. callus cultures\***

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### **Abstract**

Light is not only effective in photosynthesis, growth and development, but also play an important role in biosynthesis of primary and secondary metabolites. The present research was aimed to determine effect of light on biosynthesis of alkamide, caffeic acid derivatives and echinacoside in cell suspension cultures of *Echinacea purpurea* L. Stem explants derived from sterile plants were subjected to callus culture in a B5 media supplemented with 1.0 mg/L BAP and 2.0 mg/L NAA. Afterward, 8-day cell cultures were incubated in light and dark environments and cell harvesting, with a three-day interval, was carried out five times. The contents of alkamide, caffeic acid derivatives and echinacoside were determined using HPLC. Alkamide, caftaric acid and echinacoside content increased regularly with light application. Light application increased alkamide content by 57%, as an average, compared to darkness. The cell cultures exposed to 12 days of incubation in light produced the highest alkamide content, with a 2.11-fold increase, compared to dark. Subjecting the cell cultures to light for 12 days resulted in 70% and 63% increases in caftaric acid and echinacoside. This study showed that light application in *Echinacea purpurea* L. cell suspension culture had a great potential for increasing some important phytochemicals.

**Key words:** Caftaric acid, elicitor, purple coneflower, phytochemical compounds, secondary metabolites

### **ışığın *Echinacea purpurea* L. kallus kültüründe alkamide, kafeik asit türevleri ve echinacoside biyosentezi üzerine etkisi**

#### **Öz**

ışık sadece fotosentez, büyüme ve gelişmede etkili değildir, aynı zamanda birincil ve ikincil metabolizma ürünlerinin biyosentezinde de önemli bir rol oynamaktadır. Bu araştırmanın amacı ışığın *Echinacea purpurea* L. hücre süspansiyon kültüründe alkamide, kafeik asit türevleri ve echinacoside biyosentezine etkisini tespit etmektir. Steril bitkilerin gövde eksplantlarından kalluslar elde edilmiş ve 1.0 mg/L BAP ve 2.0 mg/L NAA ilave edilmiş B5 ortamında kültüre alınmışlardır. Daha sonra, 8 günlük hücre kültürleri, aydınlık ve karanlık ortamlarda inkübe edilmiştir ve hücreler, üç günde bir olmak üzere, toplamda beş kez hasat edilmiştir. Alkamide, kafeik asit türevleri ve echinacoside içerikleri HPLC ile belirlenmiştir. Alkamide, kaftarik asit ve echinacoside içeriği ışık uygulamalarıyla düzenli olarak artmıştır. ışık uygulaması ile alkamide içeriği karanlık ortama göre ortalama olarak %57 artmıştır. En yüksek alkamide birikimi ışıktaki 12 günlük inkübasyona maruz bırakılan hücre kültüründe tespit edilmiş ve karanlık ortama göre 2.11 katlık bir artış gerçekleşmiştir. Hücre kültürlerinin 12 gün süreyle ışığa maruz bırakılması, kaftarik asit ve echinacoside birikiminde %70'lik ve %63'lük bir artışa yol açmıştır. Bu araştırma, ışık uygulamasının *Echinacea purpurea* L. hücre süspansiyon kültüründe bazı önemli fitokim-

yasalların artırılmasında büyük bir potansiyele sahip olduğunu göstermiştir.

**Anahtar kelimeler:** Kaftarik asit, elisitör, koni çiçeği, fitokimyasal bileşikler, sekonder metabolitler

## Introduction

Multipurpose multi-use of bioactive substances, called secondary metabolites, found in the chemical structures of plants used for medicinal and aromatic purposes make these plants rather important in many different ways. Secondary metabolites obtained directly from plants grown in natural habitat are produced in a great number and variety in plants. These phytochemicals, varying according to plant species, variety, plant organ, growing stage, harvest time and postharvest processes and also prevailing climatic and soil factors, are greatly affected positively or negatively by the environmental conditions surrounding the plants. In this case, the produced compounds are hardly difficult to be in the same quality standards (Murthy et al., 2014; Gehlot et al., 2017). Cell suspension cultures, one of the most common tissue culture applications, may lead to obtain these compounds in specific quality standards, generate new compounds not found in original plant, and produce commercially these economically highly valuable phytochemicals independently of ecological conditions (Srivastava et al., 2011). *Echinacea purpurea* has recently gained an increasing interest by virtue of its important phytochemicals such as caffeic acid and alkamide (Xu et al., 2014; Manayi et al., 2015; El Aal et al., 2016). In *Echinacea* species, so far, the effect of several elicitors (U.V B and C rays, light intensity, jasmonic acid, methyl jasmonate, salicylic acid, and incubation temperature and duration) on alkamide, caffeic acid derivatives and echinacoside accumulation has been investigated using adventitious root cultures. At the end of these studies, appropriate protocols for the production of these valuable phytochemicals with bioreactors have been developed (Wu et al., 2007; Romero et al., 2009; Liu et al., 2012; Gualandi et al., 2014). However, the effects of light applications on the accumulation of phytochemicals in cell suspension cultures have not been adequately investigated (El-Aal et al., 2016). In view of this, the present research was carried out to determine the effects of light and dark conditions on accumulation of phytochemicals such as alkamide, caffeic acid derivatives and echinacoside along with cell growth and cell

viability of *Echinacea purpurea* using cell suspension cultures.

## Materials and Methods

*Echinacea purpurea* L. seeds were germinated in a medium supplemented with 2.0 mg/L BAP + 0.01 mg/L IBA + 2.0 mg/L GA<sub>3</sub> and sterile plantlets were grown. The leaf and stem explants of these sterile plantlets were cultured in MS and B5 media containing different combinations of plant growth regulators (BAP with NAA and NAA with KIN). Each combination was planted in 4 petri dishes including 10 explants and cultured at 25 °C and 16/8 h light/dark condition for 4 weeks. Thereafter, the obtained calluses were sub-cultured twice in hormone free conditions. The calluses formed after eight weeks following subculture were evaluated in terms of callus weight and callus forming explant ratio to determine the most effective growth medium, hormone combination, and explant source. As result, the combination of B5 medium supplemented with 1.0 mg/L BAP + 2.0 mg/L NAA and stem explant was used to produce cell suspension cultures.

### Preparation of cell suspension culture creation

The obtained calluses were weighted to 0.5 g in a sterile cabinet and placed in a 250 mL Erlenmeyer containing 125 mL of liquid nutrient medium. Afterwards, the cell cultures were matured for 8 days on a rotary shaker with an average speed of 105 rpm and then they were cultured at 25 °C with a 10 000 Lux luminous condition. The control cells were cultured at 25 °C in darkness. In both light and dark environments, the samples were taken 5 times (in the 1<sup>st</sup>, 3<sup>rd</sup>, 6<sup>th</sup>, 9<sup>th</sup> and 12<sup>th</sup> days) and the first day samples were obtained 8 hours after incubation. The contents of alkamide, caffeic acid derivatives (caftaric, chlorogenic, and cichoric acid) and echinacoside in harvested samples were determined using Prominence LC-20A Modular HPLC system with L-2400 UV detector and 100 RP-18e column (250 mm × 4.6 mm × 5 µm, Shimadzu). Flow rate was 1.5 mL/min and wavelength of the UV-detector was arranged to 330 nm. The device was first given a standard of compounds and mass fragments and retention times were determined. Calibration curves of the compounds were then drawn and the amount of samples was determined as µg/g dw.

### Determination of cell growth and viability

Cell growth was determined by the average number of cells (n) and cell dry weight (g/L). The number of

cells was determined with the Nageotte counting chamber using the below formula of Moroff et al. (1994).

$$n = \sum_{i=1}^{40} c \cdot 20 \cdot sf \quad (1)$$

Where,

n: the average number of cells

c: the number of cells counted in each rectangular

sf: dilution factor

The cell dry weight was obtained by weighting the filtered cells kept in an oven at 55 °C for 48 hours. Cell viability was determined using trypan blue staining technique developed by Laloue et al. (1980). According to this technique, 50 µl of cell suspension culture, 125 µl of trypan blue, and 75 µl of phosphate buffer were added to the Eppendorf tube in order to increase the staining efficiency while keeping the cells alive in during counting. The data were analyzed through a one-way ANOVA using Minitab 17 statistical software program. The means were compared with the use of Duncan's Multiple Range Test.

## Results and Discussion

The effect of light and dark condition along with incubation period on alkamide, caftaric acid, echina-

coside, cell number and cell dry weight was statistically significant, but not on cell viability (Table 1 and Table 2). The amount of alkamide ( $\mu\text{g g}^{-1}$  dw) increased steadily in both the dark and the light conditions, depending on the incubation period. In darkness, the amount of alkamide in the starting culture was  $166.09 \mu\text{g g}^{-1}$ , but increased to  $250.90 \mu\text{g g}^{-1}$  after 12 days of incubation. In light application, the amount of alkamide, which was  $184.44 \mu\text{g g}^{-1}$  in the starting culture, increased to  $530 \mu\text{g g}^{-1}$  at the end of 12<sup>th</sup> day of culture. The highest accumulation of alkamide was found at the end of the 12<sup>th</sup> day in both dark and light environments and the amount of alkamide deposited in light medium was found to be  $279.10 \mu\text{g g}^{-1}$  higher than those in dark. Compared with light and dark environments, higher caftaric acid accumulation was observed in light, with a significant increases starting from the first day of incubation. In light medium, the amount of caftaric acid, which was  $38.60 \mu\text{g g}^{-1}$  in the starting culture, reached to  $120.60 \mu\text{g g}^{-1}$ , increasing 3.12 fold at the end of a 12-day culture. On the other hand, the amount of caftaric acid went up over 71% from  $33.50 \mu\text{g g}^{-1}$  in the starting culture to  $57.40 \mu\text{g g}^{-1}$  after 12 days of incubation, in dark condition.

Table 1. Alkamide, caftaric acid and echinacoside contents of *Echinacea purpurea* L. cell suspension cultures stored at dark and light conditions for several days

Medium	Incubation period (day)	Alkamide ( $\mu\text{g g}^{-1}$ dw)	Caftaric acid ( $\mu\text{g g}^{-1}$ dw)	Echinacoside ( $\mu\text{g g}^{-1}$ dw)
Dark	1	166.09 f**	33.50 f**	67.20 g**
	3	195.20 f	35.20 f	72.34 f
	6	200.78 ef	44.12 ef	80.55 de
	9	225.87 d	51.33 de	82.36 d
	12	250.90 d	57.40 d	84.87 d
Mean		207.77 B**	44.30 B**	77.46 B**
Luminous	1	184.44 f	38.60 f	70.52 ef
	3	255.64 d	44.20 ef	80.40 d
	6	387.26 c	74.40 c	120.80 c
	9	458.43 b	100.92 b	164.40 b
	12	530.00 a	120.63 a	196.31 a
Mean		363.15 A	75.40 A	126.49 A

\*, \*\*: The means with the same lower and upper case letter in the same column are not significantly different from each other ( $P > 0.05$  and  $P > 0.01$ , respectively).

The content of echinacoside showed an overall increase in dark and light environments, depending on the period of incubation; the longer the period of culture, the higher the content of echinacoside. In the dark, the amount of echinacoside in the initial culture was  $67.20 \mu\text{g/g}$ , whereas it increased to  $80.51 \mu\text{g/g}$  at the 6<sup>th</sup> day and to  $84.87 \mu\text{g/g}$  at the 12<sup>th</sup> day. The echinacoside content of  $70.52 \mu\text{g/g}$  in the starting culture of the luminous medium reached to the levels of  $120.80 \mu\text{g/g}$  in the 6<sup>th</sup> day and  $196.32$

$\mu\text{g/g}$  in the 12<sup>th</sup> day. In general, the comparison of light and dark conditions indicated that light application was more effective on alkamide, caftaric acid echinacoside accumulation than dark (Table 1). The higher number of cells was counted in the illuminated environment and it showed significant increases starting from the 1<sup>st</sup> day of culture, in comparison to darkness. In light condition, the number of cells in the starting culture was 85.40 and increased to 95.40 and 102.76 at the 6<sup>th</sup> and the 12<sup>th</sup> day, respec-

tively. The lowest number of cells, on the other hand, was obtained from the initial culture of dark and light applications, as 84.50 and 85.40, respectively. Depending on the period of incubation, cell dry weights increased in the light and dark conditions.

While respective dry weights were 12.33 g/L and 12.55 g/L of the cells incubated at 9 and 12 days in light, these values were found to be as 10.70 g/L and 11.12 g/L after 9 and 12 days, respectively (Table 2).

Table 2. Cell number, cell dry weight and cell viability in *Echinacea purpurea* L. cell suspension cultures stored at dark and light conditions for several days

Medium	Incubation period (day)	Cell number	Cell dry weight (g/L)	Cell viability (%)
Dark	1	84.50 f**	9.22 d**	98.22
	3	84.21 f	9.30 d	97.55
	6	85.30 f	9.44 d	98.30
	9	88.40 de	10.70 bc	99.42
	12	91.82 bc	11.12 b	98.50
Mean		91.83 B*	9.96 B*	98.41
Luminous	1	85.40 f	9.24 d	98.54
	3	88.70 e	10.42 c	99.42
	6	95.40 cd	11.20 b	98.50
	9	98.20 b	12.33 a	97.56
	12	102.76 a	12.55 a	98.72
Mean		94.09 A	11.15 A	98.55

\*, \*\*: The means with the same lower and upper case letter in the same column are not significantly different from each other ( $P > 0.05$  and  $P > 0.01$ , respectively).

Light, perceived by plants through a variety of photoreceptors that detect red and infrared lights, is one of the most important environmental factors that regulate the growth, development and metabolism of plants (Chaves et al., 2011; Heijde and Ulm, 2012). The response of plant cells to light occurs by virtues of interaction between proteins of COP1/SPA connected with PAP proteins. In darkness, these two proteins are suppressed by ubiquitin ligase. In light conditions, however, SPA1 and SPA2 proteins become unstable and consequently COP1 proteins become more active (Shalitin et al., 2002; Balcerowicz et al., 2011; Weidler et al., 2012). This leads to various metabolic activities such as chlorophyll and anthocyanin biosynthesis as well as growth and development responses such as phototropism and flowering induction (Kami et al., 2010).

Light is not only effective in photosynthesis, growth and development, but also has an important role in biosynthesis of primary and secondary metabolites by increasing PAL activity in cell cultures (Halliday and Fankhauser, 2003; Khan et al., 2013). Many phenolic compounds, caffeic acid, cinnamic acid, flavonoids, anthocyanin and caffeic acid derivatives (cichoric acid, caftaric acid, chlorogenic acid and caffeic acid) are produced by the phenylpropanoid pathway initiated by PAL (Winkel-Shirley, 2001; Shohael et al., 2006; Sreelakshmi and Sharma, 2008). According to previous studies (Zhao et al., 2010; Tariq et al., 2014; Georgieva et al., 2015; Ahmad et

al., 2016) cell growth, the contents of anthocyanin, total phenolic substance, alcamide, caftaric acid and other active ingredients were stimulated much more by light as compared to dark. It is a well-known fact that light plays a key role in primary and secondary metabolism and various plant developmental processes. A positive correlation between increasing light intensity and levels of phenolics has been reported (Bennett and Wallsgrove, 1994). Ali and Abbasi (2014) reported that continuous light may turn the cell suspension cultures to stress condition and act as a triggering factor for enhanced accumulation of phenolic metabolites, which is in agreement with our results presented here. Similarly, positive effect of continuous light on anthocyanin production was reported by Chan et al. (2010) in cultures of *Melastoma malabathricum*. The cultures exposed to 10-d continuous darkness showed the lowest pigment content, while the cultures exposed to 10-d continuous irradiance showed the highest pigment content.

### Conclusion

The present study revealed that light application stimulated *Echinacea purpurea* cell suspension cultures to accumulate more alcamide, caftaric acid and echinacoside than darkness did. Similarly, the number and dry weight of the cells cultured in light environment was more fostered than in dark. Furthermore, caffeic acid derivatives of chlorogenic, and cichoric acids could not be detected in *Echinacea purpurea* cell suspension cultures. The effect of light

increased with increasing incubation time; the longer the period of culture, the higher the content of alkalamide, gallic acid and echinacoside. In conclusion, this study indicates that light application in cell suspension culture of *Echinacea purpurea* L. possess a great potential for increasing important secondary metabolites such as alkalamide, gallic acid and echinacoside.

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