Original Article

Enhancement of Phytochemical Compounds Using Biotic and Abiotic Elicitors in Purple Coneflower (*Echinacea purpurea* L.)

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ABSTRACT

Background: Phytochemicals also known secondary metabolites, naturally occurring in medicinal and aromatic plants, are of considerable importance for plant survival and human health. **Objective:** The objective of this study was to increase accumulation of caffeic acid and alkamide, using biotic and abiotic stresses conditions driving cell defense systems, in cell suspension cultures in purple coneflower (*Echinacea purpurea* L.). **Methods:** As biotic and abiotic elicitors, yeast extract (0, 25, 50 and 100 mg l^{-1}), chitosan (0, 25, 50 and 100 mg l^{-1}), sorbitol (0, 5, 25 and 50 g l^{-1}), cadmium chloride (0, 5, 25 and 50 μM) and silver nitrate (0, 5, 25 and 50 μM) solutions were used in an eight-day cell culture. The cells were daily harvested up to the third day of the culture in cadmium chloride and silver nitrate, whereas up to the seventh day of the culture in the other applications. Alkamide and caffeic acid contents in harvested cells were determined using GC-MS device. **Results:** The contents of alkamide and caffeic acid, as compared to the control, increased by 1.3 and 0.5 times with 50 mg l^{-1} yeast application. In the applications of 25 g l^{-1} sorbitol and 100 mg l^{-1} chitosan, alkamide content increased by 0.8 and 1.5 fold, but the amounts of caffeic acid increased by 2.5 and 3.1 fold, in comparison to the control culture. The highest amounts of alkamide in cadmium chloride and silver nitrate (105 and 127 μg g^{-1} dw, respectively) were obtained after 24 h from starting culture, while caffeic acid content reached its highest value (27 and 38 μg g^{-1} dw, respectively) after 72 h. **Conclusion:** This study indicated that biotic and abiotic stress factors, by driving cell defense systems, had a great potential for increasing caffeic acid and alkamide *in vitro* conditions in purple coneflower.

Key words: Alkamide, Caffeic acid, Callus culture, Secondary metabolites.

INTRODUCTION

Phytochemicals, naturally produced in some high-structured plants and important for human health, are produced in a great number and diversity in plants. The plants are affected positively or negatively from the ecological conditions of their natural habitat. In this case, the produced secondary metabolites are inhibited having a certain level of quality and standard. Furthermore, the production of plants with known classical methods and also the production of secondary metabolites from these plants is rather costly and time consuming compared to cell cultures. Therefore, tissue culture techniques in the production of secondary metabolites are frequently used. Cell suspension culture, one of these techniques, allow to produce secondary metabolites in specific qualities and standards and to obtain new compounds not being in the main plant as independently of geographical, seasonal and environmental factors. Most phytochemicals such as ginseng, slymarine, paclitaxel, plumbagin and puerarin are produced using this technique. *Echinacea purpurea* L., with the origin of North America, is a species having antibacterial, antiviral and antifungal properties. Naturally occurring

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certain important phytochemicals such as caffeic acid and alkamide have made this species as one of the most studied plant species in recent years.[1-3] The effect of various elicitors (lighting period, light intensity, U.V B and C rays, jasmonic acid, methyl jasmonate, salicylic acid and incubation temperature and duration) on caffeic acid and alkamide accumulation have carried out in Echinacea species until today, using adventitious root cultures in Echinacea species. At the end of these studies, some appropriate protocols for the production of these valuable phytochemicals with bioreactors have been developed.[4-9] In cell suspension cultures, however, the effects of elicitors on the accumulation of phytochemicals have not been sufficiently investigated. The objective of this study is to enhance biosynthesis of caffeic acid and alkamide in cell suspension cultures. For this reason, various elicitors (yeast extract, chitosan, silver nitrate, cadmium chloride and sorbitol) were applied to cell cultures and their effects on the accumulation of phytochemicals were studied.

MATERIAL AND METHOD

In the study, coneflower (*Echinacea purpurea* L.) seeds were used as plant material. The seeds were germinated in a medium containing 2.0 mg/l BAP + 0.01 mg/l IBA + 2.0 mg/l GA_3 and sterilized plantlets were obtained. The leaf and stem explants of these plantlets were cultured in MS and B5 media with different combinations of plant growth regulators (using 2, 4-D x BAP and NAA x KIN combinations) at 25°C (16 h light / 8 h darkness). Cell suspension cultures were generated from B5 medium supplemented with 1 mg/l BAP + 2 mg/l NAA hormone combination in which stem explant was used. Afterward, the concentrations of 0 (control) -25, 50 and 100 mg l-1 of yeast extract and chitosan were applied to cell cultures, as biotic elicitors. As abiotic elicitors, sorbitol with the doses of 0, 5, 25 and 50 g l^{-1}, cadmium chloride ($CdCI_2$) and silver nitrate ($AgNO_3$) with the doses of 0, 5, 25 and 50 µM were applied to cell cultures. In the first three applications, the samples were harvested with 24 h intervals from the first day (after 24 h) to the 8th day, but the samples were harvested with 24 h intervals from the first day (after 24 h) to the 4th day in the last two applications. Each of the applications was carried out with three replicates in 8-day cell cultures. All applications with completed sampling were filtered and washed in a sterile cabinet to be used in caffeic acid and alkamide analysis and stored in deep freeze (-20 °C) until extraction. Content of caffeic acid and alkamide was determined using GC-MS device. For headspace analysis, Shimadzu QP2010 ultra waC-MS device and capillary colon separation was done with RTX-5M 30 m. The device was first given a standard of compounds and then mass fragments and retention time were determined. Calibration curves of the compounds were then drawn and the amount of the samples was determined as µg/g. In statistical evaluation, a two-way analysis of variance was used. The differences among the means were determined by the Tukey test and the results were given as mean±standard error. A 5% significance level was used in calculations and interpretations as well.

RESULTS AND DISCUSSION

In all elicitor applications, the amount of caffeic acid and alkamide content increased according to the concentration used and the time spent. At the concentration of 50 mg/l of the yeast extract, the amount of caffeic acid increased from 7.1 µg/g to 10.8 µg/g at the end of the 5th day, increasing by 52% over the control group. At the same concentration, alkamide content raised from 24.2 µg/g to 55.7 µg/g, increasing by 1.33 fold (Table 1). On the other hand, as shown in Table 2, chitosan doses increased accumulation of caffeic acid continuously starting at the 1st day and the highest caffeic acid of 30.2 µg/g was attained with 100 mg/l chitosan dose at the end of the 7th day. At the same dose, alkamide content increased continuously from the 1st day and at the end of the 6th day it reached up to 60.2 µg/g, as compared to the control. In the application of 25 g/l of sorbitol solution, the amount of caffeic acid, compared to control culture, increased from 7.37 µg/g to 24.37 µg/g at the end of the 7th day. Alkamide content of 23.7 µg/g in the initial culture increased to the level of 42.66 µg/g at the end of the 3rd day, at the same concentration (Table 3). Cadmium chloride application of 25 µM produced the highest caffeic acid (27 µg/g) and alkamide content (105 µg/g) after 72 and 24 h from the application, respectively (Table 4). The highest caffeic acid (38 µg/g) was obtained after 72 h from the application of 50 µM silver nitrate. At the same concentration, alkamide content reached its highest value of 127 µg/g after 24 h from the application (Table 5). The caffeic acid and alkamide accumulation reached the highest value at 50 mg/l doses in yeast elicitor applications.

There are a number of studies indicating that the yeast elicitors promote the accumulation of phytochemicals, while the doses used rather vary according to plant species. For example, the accumulation of tanshinone terpene was promoted the most by 100 mg/l yeast application, while its accumulation in another species was promoted the most by 200 mg/l yeast application.[10-13] On the other hand, the present study revealed that the

Table 1: Descriptive statistics related to the effect of yeast extract (mg/l) and sampling time (day) on caffeic acid (µg/g) and alkamide (µg/g) accumulation in cell suspension cultures of *Echinacea purpurea* species.

s.t.	Yeast extract (mg/l)							
	Caffeic acid (µg/g)				Alkamide (µg/g)			
	0	25	50	100	0	25	50	100
1.d	6.8±0.3n**	7.8±0.2j-m	8.4±0.3b-j	9.7±0.1b-e	23±0.1m**	24± 0.3klm	24±0.2j-m	23±0.5m
2.d	7.0±0.4mn	7.9±0.2j-m	8.8±0.2e-ı	9.7±0.2b-e	23.4±0.1lm	32.4±0.3gh	37.47±0.7d	28.1±0.3ı
3.d	7.4±0.1lmn	8.4±0.2h-k	9.1±0.1d-h	9.5±0.2c-f	23.70±0.1klm	32.9±0.4fg	46.6±0.5d	34.4±0.3e
4.d	7.4±0.1l-n	8.9±0.2e-ı	9.8±0.3bcd	9.6±0.2b-e	24.2 ±0.4j-m	33.7±0.2ef	50.37±0.7b	38.2±0.3d
5.d	7.1±0.5mn	9.6±0.2b-e	10.8±0.5a	10.4±0.4ab	24.2 ±0.1j-m	33.1±0.1fg	55.7±0.2a	31.3±0.5h
6.d	7.6±0.2k-n	8.3±0.4h-k	9.9±0.3bc	9.2±0.3c-g	24.1±0.4klm	33.3±0.1efg	51.4±0.3b	21.50±0.8m
7.d	7.3±0.2lmn	8.3±0.6h-k	9.6±0.3b-f	9.6±0.3b-f	23.4±0.6lm	32.6±0.35fg	51.1±0.8b	17.4±0.8o

**: The differences among the means without common letter are significant (p<0.01); s. t: sampling time; d: day

Table 2: Descriptive statistics related to the effect of chitosan (mg/l) and sampling time (day) on caffeic acid (µg/g) and alkamide (µg/g) accumulation in cell suspension cultures of *Echinacea purpurea* species.

s.t.	Chitosan (mg/l)							
	Caffeic acid (µg/g)				Alkamide (µg/g)			
	0	25	50	100	0	25	50	100
1.d	6.8±0.3o**	10.3±0.7n	14.9±0.4ı	17.7±0.4gh	23.4±0.1k**	23.8±0.1k	24.3±0.3k	24.0±0.5k
2.d	7.0±0.4o	11.2±0.2mn	16.9±0.4h	21.8±0.4ef	23.4±0.1k	25.8±0.3j	27.0.±0.3ı	27.8±0.1hı
3.d	7.4±0.1o	11.9±0.3lm	18.5±0.4g	22.7±0.7e	23.7±0.2k	28.2±0.4h	31.2±0.5g	34.9±0.5f
4.d	7.4±0.1o	12.8±0.4kl	21.4±0.4f	25.1±0.2d	24.2±0.4k	30.9±0.4g	37.0±0.5e	48.5±0.6d
5.d	7.1±0.5o	13.4±0.5jk	24.5±0.5d	26.6±0.5bc	24.2±0.9k	49.1±0.2d	53.9±0.4c	54.6±0.4c
6.d	7.6±0.2o	14.6±0.6ıj	25.6±0.7cd	27.7±0.5b	24.1±0.4k	54.9±0.3c	58.2±0.4b	60.2±0.3a
7.d	7.4±0.2o	16.8±0.6h	27.7±0.6b	30.2±0.4a	23.4±0.6k	59.2±0.3ab	59.6±0.6a	59.4±0.4a

**: The differences among the means without common letter are significant (p<0.01); s. t: sampling time; d: day

Table 3: Descriptive statistics related to the effect of sorbitol (g/l) and sampling time (day) on caffeic acid (µg/g) and alkamide (µg/g) accumulation in cell suspension cultures of *Echinacea purpurea* species.

s.t.	Sorbitol (g/l)							
	Caffeic acid (µg/g)				Alkamide (µg/g)			
	0	5	25	50	0	5	25	50
1.d	6.80±0.3p**	7.56±0.1op	8.51±0.2n	10.8±0.3kl	23.4±0.1n**	25.4±0.2kl	26.1±0.1jk	26.8±0.1j
2.d	7.0±0.4p	8.1±0.3no	10.5±0.1kl	13.9±0.3hı	23.4±0.1n	35.7±0.9f	37.6±0.4e	30.7±0.4g
3.d	7.4±0.1op	9.5±0.2m	12.1±0.4j	17.7±0.2ef	23.7±0.1n	38.8±0.7d	42.7±0.2a	29.4±0.4h
4.d	7.4±0.1op	10.3±0.2lm	18.3±0.2ı	18.3±0.3e	24.2±0.4mn	39.0±0.6d	40.7±0.3c	28.1±0.1ı
5.d	7.1±0.5p	11.2±0.2k	15.9±0.1g	19.9±0.4d	24.2±0.1mn	40.9±0.5bc	41.0±0.2bc	25.4±0.1kl
6.d	7.6±0.2op	12.1±0.2j	17.2±0.1f	22.4±0.4c	24.1±0.4mn	40.8±0.3c	41.1±0.3bc	24.9±0.4lm
7.d	7.4±0.2op	14.4±0.6h	25.5±0.5a	24.4±0.2b	23.4±0.6n	41.5±0.3bc	41.9±0.3ab	25.0±0.5lm

**: The differences among the means without common letter are significant (p<0.01); s. t: sampling time; d: day

Table 4: Descriptive statistics related to the effect of cadmium chloride (µM) and sampling time (day) on caffeic acid (µg/g) and alkamide (µg/g) accumulation in cell suspension cultures of *Echinacea purpurea* species.

s.t	Cadmium chloride (µM)							
	Caffeic acid (µg/g)				Alkamide (µg/g)			
	0	5	25	50	0	5	25	50
1.d	6.8±0,26g**	11.3±0,32f	16.5±0,35e	17.9±0,26f	23.4±0,10h	86.5±0,46e	105±0,41a	79±0,13f
2.d	7.0±0,36g	17.4±0,20de	24.7±0,60b	26.0±0,21a	23.4±0,14h	94.4±1,14d	97±0,13c	77±0,44g
3.d	7.4±0,1g	18.9±0,13c	27.0±0,33a	26.6±0,46a	23.7±0,14h	94.3±0,36d	99±0,32b	77±0,44g

**: The differences among the means without common letter are significant (p<0.01); s.t: sampling time; d: day

Table 5: Descriptive statistics related to the effect of silver nitrate (µM) and sampling time (day) on caffeic acid (µg/g) and alkamide (µg/g) accumulation in cell suspension cultures of *Echinacea purpurea* species.

s.t.	Silver nitrate (µM)							
	Caffeic acid (µg/g)				Alkamide (µg/g)			
	0	5	25	50	0	5	25	50
1.d	6,8±0,26n**	11.8±0.150g	15.4±0.261f	15.7±0.640f	23.4±0,1h**	96.2±0,64g	105±1,0d	127±0,50a
2.d	7,0±0,36mn	17.2±0.480e	25.1±0.492c	25.6±0.484c	23.4±0,14h	97.6±0,42fg	109±0,82c	104±0,90d
3.d	7,4±0,10lmn	20.1±0.303d	28.9±0.308b	38.0±0.260a	23.7±0,14h	98.7±0,46f	115±1,31b	101±0,19e

**: The differences among the means without common letter are significant (p<0.01); s. t: sampling time; d: day

dose of 50 mg/l of yeast extract was much more effective. There have been several studies reporting that the elicitors of chitosan, sorbitol, cadmium chloride and silver nitrate in cell suspension cultures increased plant resistance and induced the activity of phenylalanine ammonium liyaz (PAL).[14-18] The findings of this present study also support the previous studies, revealing that biotic and abiotic elicitors increase the accumulation of caffeic acid and alkamide. In previous studies carried out in *Echinacea purpurea* species, root hair cultures were used and higher caffeic acid and alkamide accumulation were obtained, compared to our study. In the previous studies, root hair cultures were used whereas stem cells were used in our study. This differences in cell culture may cause different responses to elicitors and the synthesis of phytochemicals in different amounts.[19-22]

CONCLUSION

In general, several studies related to increasing the amount of caffeic acid and alkamide have been carried out on Echinacea species. These studies have indicated that root hair cultures are successful in increasing caffeic acid and alkamide accumulation. In this study using stem cell culture, it was also revealed that the accumulation of caffeic acid and alkamide could increase with certain elicitors applied to cell suspension cultures of *Echinacea purpurea* species.

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CONFLICT OF INTEREST

The authors have no conflict of interest

ABBREVIATION

dw: dry weight; **s. t:** sampling time; **d:** day; **µg:** microgram; **µM:** micro molar; **GC-MS:** Gases Chromatography-Mass Spectrometer; **U. V:** ultraviolet light; **MS:** Murashige and Skoog; **B5:** Gamborg; **BAP:** benzyladenine; **2,4-D:** 2,4-diklorofenoksi acetic acid; **IBA:** indol-3-butirik acid; **GA_3:** gibberellic acid; **NAA:** naftalinacetic acid; **KIN:** kinetin; **$CdCI_2$:** cadmium klorit; **$AgNO_3$:** silver nitrate.

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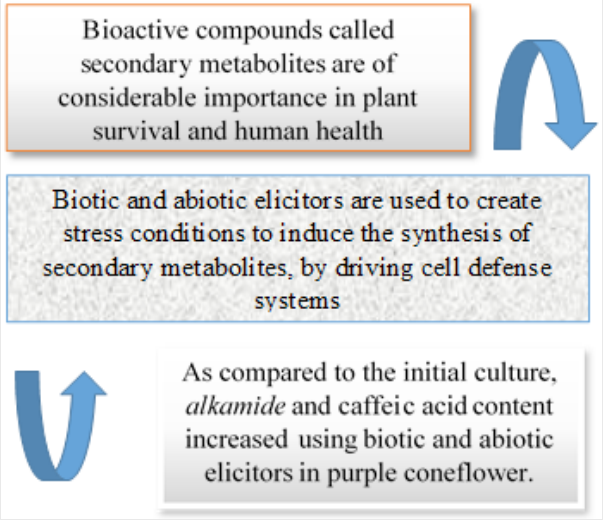
PICTORIAL ABSTRACT



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SUMMARY

- Producing secondary metabolites using biotic and abiotic elicitors *in vitro* has recently become important worldwide.
- The cell suspension cultures were subjected to biotic (chitosan and yeast extract) and abiotic (cadmium chloride, silver nitrate and sorbitol) elicitors to increase accumulation of caffeik acid and alkamide.
- All biotic and abiotic elicitors increased the content of *alkamide* and caffeik acid.
- Biotic and abiotic elicitors showed a great potential for increasing *alkamide* and caffeic acid in purple coneflower.

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