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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Influence of Carbon Source, Nitrogen and PEG on Caffeic Acid Derivatives Production in Callus Cultures of *Echinacea purpurea* L.

Echinacea purpurea L. Kallus Kültürlerinde Karbon Kaynağı, Nitrojen ve PEG'in Kafeik Asit Türevlerinin Üretimine Etkisi

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Abstract

This study aimed to determine the effects of polyethylene glycol (PEG) as an abiotic elicitor and nutritional factors (different ammonium/nitrate ratios, carbon source and amount) in the culture medium on the production of Caffeic Acid Derivatives (CADs) in callus cultures of *Echinacea purpurea* L. Petiole and root explants were cultured on MS medium modified in terms of different types (sucrose and maltose) and amounts (sucrose 15, 45, 60 g l^{-1}, and maltose 15, 30, 45, 60 g l^{-1}) of carbon source, different concentrations (5, 10, 15 g l^{-1}) of PEG and ammonium nitrate ratios (0:35, 5:25, 15:15, 35:0 mM). The amounts of CADs in the callus obtained at the end of the 10-week culture period were analysed. In both explant types, the highest amount of CADs were obtained from the medium containing 15 g l^{-1} sucrose and 15 or 30 g l^{-1} maltose applications, while the highest amount of CADs was obtained in the medium containing 0:35 mM ammonium/nitrate in nitrogen applications. While the highest amount of CADs in root explant was obtained from the medium containing 10 g l^{-1} PEG applications, CADs content could not be obtained in petiole explant. As a result, the highest amounts of caftaric, chlorogenic, caffeic, and chicoric acids (respectively, 9.38, 0.71, 0.29, and 34.77 mg g^{-1}) were determined at callus obtained from root explant cultured on MS medium containing 30 g l^{-1} sucrose and 0:35 mM ammonium/nitrate. In conclusion, optimization of culture conditions and different elicitor applications were made to increase secondary metabolite content in *E. purpurea* L. under *in vitro* conditions and the results obtained were presented comparatively.

Keywords: Sucrose, Maltose, Nitrogen, PEG, Caffeic acid derivatives, *Echinacea purpurea* L., HPLC

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Öz

Bu çalışmada, abiyotik bir elisitör olarak polietilen glikolün (PEG) ve besin ortamındaki farklı beslenme faktörlerinin (farklı amonyum/nitrat oranları, karbon kaynağı ve miktarı) *Echinacea purpurea* L. kallus kültürlerinde kafeik asit türevlerinin miktarlarına etkisinin belirlenmesi amaçlanmıştır. Petiyol ve kök eksplantları karbon kaynağının farklı türleri (sakkaroz ve maltoz) ve miktarları (sakkaroz 15, 45, 60 g l^{-1} ve maltoz 15, 30, 45, 60 g l^{-1}), farklı amonyum nitrat oranları (0:35, 5:25, 15:15, 35:0 mM) ve farklı konsantrasyonlarda (5, 10, 15 g l^{-1}) PEG içeren MS besin ortamlarında 10 hafta boyunca kültür alınmış ve kültür süresi sonunda elde edilen kalluslarda kaftarik, klorojenik, kafeik ve kikorik asit miktarları tespit edilmiştir. Her iki eksplant türünde de en yüksek kafeik asit türevlerinin miktarları, karbon kaynağı uygulamalarında, 15 g l^{-1} sakkaroz ile 15 ve 30 g l^{-1} maltoz; nitrojen uygulamalarında ise 0:35 mM amonyum/nitrat içeren besin ortamlarında uyarılan kalluslarda tespit edilmiştir. PEG uygulamalarında ise, en yüksek miktarlarda kafeik asit türevleri, 10 g l^{-1} PEG içeren besin ortamından kök eksplantından uyarılan kalluslarda elde edilirken, petiyol eksplantından elde edilen kallus dokularında kafeik asit türevlerinden hiçbiri tespit edilememiştir. *E. purpurea* L kallus kültürlerinde, kafeik asit türevlerinin miktarını artırmak için, *in vitro* şartlar altında, abiyotik elisitör olarak PEG ve farklı beslenme faktörlerinin optimizasyonu araştırıldığı bu çalışmada, elde edilen sonuçlar karşılaştırmalı olarak sunulmuştur. Tüm uygulamalar değerlendirildiğinde; sonuç olarak, en yüksek kaftarik, klorojenik, kafeik ve kikorik asit miktarları (sırasıyla 9.38, 0.71, 0.29 ve 34.77 mg g^{-1}) 0:35 mM amonyum/nitrat ve 30 g l^{-1} sakkaroz içeren MS ortamında kültüre alınan kök eksplantından elde edilen kallus dokularında tespit edilmiştir.

Anahtar Kelimeler: Sakkaroz, Maltoz, Azot, PEG, Kafeik asit türevleri, *Echinacea purpurea* L., HPLC

1. Introduction

Echinacea purpurea L. is a medicinal herb mostly used to reduce the symptoms and duration of colds, influenza, and upper respiratory tract infections (Rajasekaran et al., 2013; Shahrajabian et al., 2020). It is rich in medicinally important secondary metabolites, such as alkamides (alkylamides), phenolic compounds (caffeic acid derivatives-CADs), glycoproteins and polysaccharides (Bruni et al., 2018). In particular, CADs namely cichoric acid, chlorogenic acid, caftaric acid, caffeic acid and echinacoside are characteristic phenolic compounds of *Echinacea* spp. and has important pharmacological properties (Murthy et al., 2014; Rady et al., 2018). Cichoric acid, caftaric acid and chlorogenic acid are the most studied CADs due to their anticancer, antiviral, anti-inflammatory, and antioxidant effects (Lin et al., 1999; Thygesen et al., 2007; Pleschka et al., 2009; Aarland et al., 2017; Chiou et al., 2017; Sharif et al., 2021)

Plant cell/tissue culture is a promising alternative technique to produce plant secondary metabolites. With cell/tissue culture techniques, secondary metabolites are produced in a short time independent of environmental factors (i.e. climate, geographical difficulties, seasonal restrictions, diseases and pests). At the same time, it is possible to increase the production of secondary metabolites with plant cell/tissue culture techniques, by optimizing the culture conditions (medium content, carbon source, growth regulators etc.) and elicitor sources (Rao and Ravishankar, 2002; Sökmen and Gürel, 2002; Tanur Erkoyuncu and Yorgancılar, 2015).

Secondary metabolites can be produced by using different biotechnological approaches, such as cell/callus culture techniques (Ram et al., 2013; Royandazagh and Pehlivan, 2016). Callus cultures are usually effected by various *in vitro* conditions including explant type, plant growth regulators, nutrient supply, carbohydrate source and other environmental conditions (Pehlivanet al., 2017; Khan et al., 2018). Optimization of callus cultures and different elicitor sources should be investigated in order to both increase biomass and increase the production of secondary metabolites. There are various studies on the optimization of *in vitro* culture conditions (different plant growth regulators types and concentrations) and different elicitor applications (methyl jasmonate, silver nanoparticle, yeast extract and fungal) to increase secondary metabolite production in *E. purpurea* L. (Ramezannezhad et al., 2019; Erkoyuncu and Yorgancilar, 2021; Demirci, 2022; Elshahawy et al., 2022), However, in different *in vitro* cultures (callus, shoot, cell suspension cultures) of *E. purpurea* L. research could be extended to increase both biomass production and the amount of CADs.

This study aims to investigate the effects of polyethylene glycol (PEG) as an abiotic elicitor and nutritional factors (different ammonium/nitrate ratios, carbon source and amount) in the culture medium on the production of CADs in callus cultures of *E. purpurea* L.

2. Materials and Methods

2.1. Sterilization and culture of seeds

Echinacea purpurea seeds obtained from plants cultivated in Selçuk University, Konya, Türkiye were used as starting material and sterilization procedure were applied as specified in Erkoyuncu and Yorgancilar (2021). Sterile seeds were cultured on MS (Murashige and Skoog, 1962) medium without plant growth regulators and 8-week-old sterile seedlings were used as explant sources throughout the study. All cultures were incubated in a growth cabinet (Sanyo: MLR-351H) at 24±2 ºC, 65 % humidity, 5 LS light intensity, and a photoperiod of 16/8 hours.

2.2. Preparation of different carbon sources and amount, ammonium/nitrate ratio and PEG containing medium

As a result of a previous study by Erkoyuncu and Yorgancılar, 2021, the highest CADs amounts in callus cultures of *E. purpurea* were obtained from callus induction from petiole and root explants. In this study, for optimum callus induction from petiole and root explant, the best combination of growth regulators (1.0 mg l^{-1} naphthalene acetic acid (NAA)+0.5 mg l^{-1} thidiazuron (TDZ); 0.5 mg l^{-1} NAA+0.5 mg l^{-1} benzylaminopurine (BAP), respectively), culture time (10 weeks), solid MS medium containing 30 g l^{-1} sucrose and 8 g l^{-1} agar were determined.

Both types of explants (root and petiole) were cultured on MS media containing the determined growth regulators combinations, modified in terms of carbon source and amounts, ammonium/nitrate ratios and PEG applications at different concentrations. Experiments were set up with 20 replications, 10 explants per petri dish, in order to obtain

sufficient material for CADs analysis. Cultures were maintained for a 10-week culture period under controlled conditions at 24±2 ºC, 65 % humidity, 5 LS light intensity, 16/8 photoperiod *(Figure 1).*

Different carbon sources and amount: Petiole and root explants were cultured on MS media containing suitable growth regulators combinations (1.0 mg l^{-1} NAA+0.5 mg l^{-1}TDZ; 0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP, respectively) and different types and amount of carbon sources (sucrose 15, 45, 60 g l^{-1}, and maltose 15, 30, 45, 60 g l^{-1}). Since 30 g l^{-1} sucrose was used as a standard in previous studies, it was excluded in this study.

Different ammonium/nitrate ratios: Petiole and root explants were cultured on MS medium was modified in terms of ammonium/nitrate ratio (0:35, 5:25, 15:15, 35:0 mM) containing suitable growth regulators combinations (1.0 mg l^{-1} NAA+0.5 mg l^{-1} TDZ; 0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP, respectively).

Polyethylene glycol (PEG): Petiole and root explants were cultured in MS media containing suitable growth regulators combinations (1.0 mg l^{-1} NAA+0.5 mg l^{-1} TDZ; 0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP, respectively) and different concentrations (5, 10, 15 g l^{-1}) PEG.

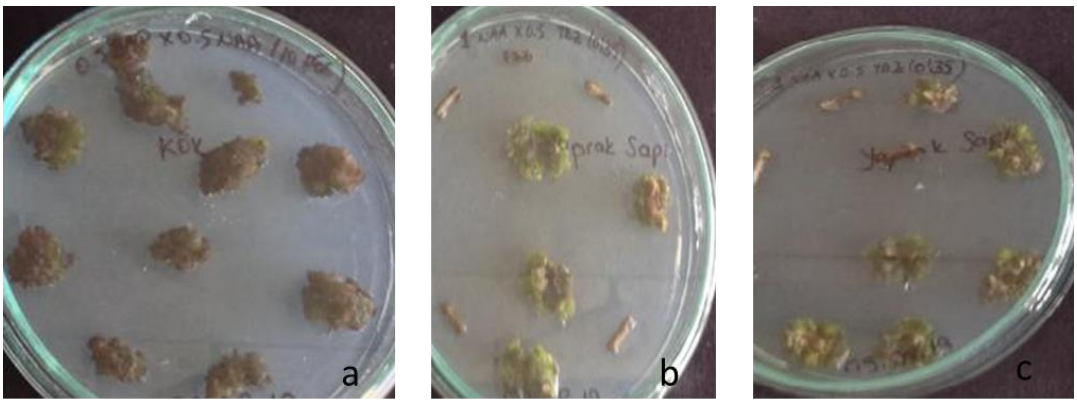

Figure 1. Callus tissues of different applications a. callus from root explant b. callus from petiole explant c. callus from petiole explant

2.3. Quantification of caffeic acid derivates

Cichoric, chlorogenic, caftaric and caffeic acid contents of 10 weeks old calli from all treatments were analysed. Following oven-drying for 1 d at 37 ºC, plant materials ground into fine powders (0.2 g) were extracted in an ultrasonic bath with 8 ml of methanol 70 % (v/v) for 15 min. The extracts were diluted with methanol 70 % (v/v) to make up the volume to 10 ml, filtered through a 0.45µm membrane filter and transferred to vials (Taha et al., 2009).

Standard stock solutions related CADs were prepared at 1, 5, 10, 25, 50 and 100 ppm, and were run at HPLC, the retention times of the standards were identified, and the calibration graphic was created with the absorption values read against the concentration values. According to these graphics, R2 ~ 0.99, and the results were evaluated according to the formulas in the graphics (*Figure 2*).

The HPLC–DAD method was employed for the analyses of caffeic acid derivates in the extracts. All the specifics of HPLC–DAD analyses, equipment's, and conditions were according to Erkoyuncu and Yorgancilar (2021).

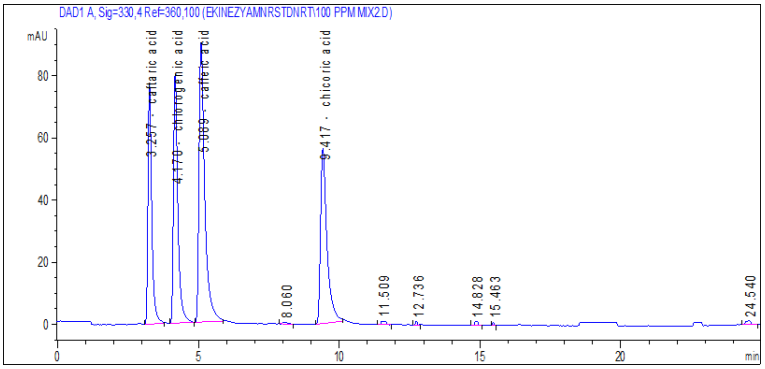

Figure 2. Chromatogram showing the separation times of the mixture of caffeic acid derivatives

3. Results and Discussion

3.1. The effect of carbon sources and amounts on the accumulation of CADs.

In order to determine the effect of the different carbon source on the amount of secondary metabolites, MS medium was modified in terms of sucrose (15, 45, 60 g l^{-1}) and maltose (15, 30, 45, 60 g l^{-1}) containing the growth regulator with the best callus formation (1.0 mg l^{-1} NAA+0.5 mg l^{-1} TDZ for petiole, and 0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP for root explants) was reported (Erkoyuncu and Yorgancilar, 2021). The amounts of caffeic acid derivatives were analysed at the callus tissues obtained at the end of the 10-week culture period in both explant types. Dry weights (mg/calli) and content of CADs of calli developed in the applications are given in *Table 1* and *2*.

Table 1. Quantities of CADs in calli obtained from different sucrose levels (mg g^{-1})

Explant sources	Sucrose (g l^{-1})	Callus dry weight (mg/calli)	Caffeic acid derivatives (mg g^{-1})			
			Caftaric	Chlorogenic	Caffeic	Chicoric
Root derived	**15**	6.9	**0.83±0.02**	**0.16±0.05**	-	**2.57±0.24**
	45	7.1	0.14±0.03	-	-	0.40±0.01
	60	8.6	-	-	-	-
Petiole derived	**15**	4.6	**0.54±0.02**	**0.21±0.01**	-	**1.27±0.04**
	45	6.9	-	-	-	-
	60	5.4	-	-	-	0.09

When *Table 1* is examined, the highest amounts of caftaric (0.83 mg g^{-1}), chlorogenic (0.16 mg g^{-1}), and chicoric (2.57 mg g^{-1}) acids were detected in callus tissues obtained on MS medium containing 15 g l^{-1} sucrose from root explant. Similarly, the highest amounts of caftaric (0.54 mg g^{-1}), chlorogenic (0.21 mg g^{-1}), and chicoric (1.27 mg g^{-1}) acids were detected 15 g l^{-1} sucrose from petiole explants.

According to *Table 2*, the highest amounts of caftaric (1.28 mg g^{-1}), chlorogenic (0.37 mg g^{-1}), caffeic (0.10 mg g^{-1}), and chicoric (3.76 mg g^{-1}) acids were detected in callus tissues obtained after 10 weeks of culture MS medium containing 1.0 mg l^{-1} NAA+0.5 mg l^{-1} TDZ and 15 or 30 g l^{-1} maltose from root explants. Similarly, the highest amounts of caftaric (0.96 mg g^{-1}), chlorogenic (0.59 mg g^{-1}), caffeic (0.12 mg g^{-1}), and chicoric (3.75 mg g^{-1}) acids were detected 15 g l^{-1} maltose from petiole explants.

Table 2. Quantities of CADs in calli obtained from different maltose levels (mg g^{-1})

Explant sources	Maltose (g l^{-1})	Callus dry weight (mg/calli)	Caffeic acid derivatives (mg g^{-1})			
			Caftaric	Chlorogenic	Caffeic	Chicoric
Root derived	**15**	16.9	1.24±0.01	0.37±0.25	0.11±0.08	2.69±0.05
	30	12.3	**1.28±0.05**	**0.20±0.08**	**0.10±0.10**	**3.76±0.02**
	45	9.4	0.30±0.07	0.06±0.01	0.06±0.14	0.33±0.03
	60	29.2	0.30±0.01	-	-	0.33±0.04
Petiole derived	**15**	20.4	**0.96±0.05**	**0.59±0.20**	**0.12±0.01**	**3.75±0.05**
	30	31.5	0.57±0.08	0.18±0.01	0.06±0.01	1.16±0.04
	45	27.4	0.85±0.09	0.15±0.08	0.06±0.02	2.30±0.10
	60	20.0	0.90±0.10	0.55±0.06	0.09±0.03	1.73±0.05

Caffeic acid derivatives could not be detected in callus tissues developed from 45 and 60 mg l^{-1} sucrose applications in almost both explant types. For both explant types, the efficiency of using maltose was lower than sucrose in terms of amounts of caffeic acid derivatives. As can be understood from here, 15 or 30 g l^{-1} sucrose or maltose can be recommended for further studies for the production of CADs in callus culture of *E. purpurea* L. Erkoyuncu and Yorgancilar (2021) reported that they used 30 g l^{-1} sucrose when determining the best culture medium for callus development and production of caffeic acid derivatives.

Different carbon sources such as sucrose, maltose, fructose and glucose are used separately or in combination to support the growth of cell and tissue cultures (Kretzschmar et al., 2007). In addition to their effects on growth, carbon

sources play an active role in the biosynthetic pathways of many compounds by regulating the expression of a significant number of genes. (Ali et al., 2016). Moreover, in *in vitro* cultures, carbon sources are also commonly used to generate osmotic stress factors, so it is very difficult to separate the osmotic stress effect and nutritional role of carbon sources (Liu and Cheng, 2008). Although the osmotic stress applied by different carbon sources varies depending on the plant species and culture system, it has been reported in various studies that it can lead to the production of biomass and secondary metabolites (Suan et al., 2011). In our study, it was determined that high concentrations of sucrose and maltose applications in the medium did not have a positive effect on the amounts of caffeic acid derivatives in both explant types. Among the carbon sources, the highest amounts of caftaric, chlorogenic, caffeic and chicoric acids were obtained in both explant types in medium containing 30 g l^{-1} sucrose.

Similar to our results, Liu et al. (2006) investigated the capacity of *E. purpurea* L. to produce caffeic acid derivatives in hairy root cultures, the highest biomass and amounts of chicoric acid (19.21 mg g^{-1}), caftaric acid (3.56 mg g^{-1}) and chlorogenic acid (0.93 mg g^{-1}) were obtained in MS medium containing 30 g l^{-1} sucrose.

Romero et al. (2009), in their study investigating alkamide production from *in vitro* cultures of three different *Echinacea* species, were reported ½ B5 medium containing 3% (30 g l^{-1}) sucrose was twice as effective as medium containing 1%, 2%, 4%, 5% (respectively, 10, 20, 40, and 50 g l^{-1}). The effectiveness of carbon source type and concentration in the medium varies depending on the plant species, but is also affected by other chemical compounds of the medium.

Wu et al. (2006), in a study investigating the effects of different auxin types (IAA, IBA, NAA) and concentrations (1.0, 2.0, 4.0, 6.0 mg l^{-1}) and different sucrose (1%, 3%, 5%, 7%, 9%), ammonium/nitrate ratio (0:40, 0:35, 0:30, 5:25, 10:20, 15:15, 20:10, 25:5, 30:0), medium strength (1/4, 1/2, 3/4, 1/1, 3/2, 2/2) and pH (4, 5, 6, 7, 8, 9) levelson biomass increase, total phenol and flavonoid accumulation in adventitious root culture of *E. angustifolia*, the highest biomass increase and total phenol and flavonoid content, obtained from 1/2 MS medium containing 5% sucrose, 5:25 (mM) ammonium/nitrate, pH:6.0., 2 mg l^{-1} IBA.

Cui et al. (2013) in a similar study investigating the effects of different sucrose concentrations (0 %, 1 %, 3 %, 5 %, 7 %, 9 %) on the biomass increase and accumulation of caffeic acid derivatives in adventive root cultures of *E. angustifolia*, the highest biomass and amounts of chlorogenic acid (2.26 mg g^{-1}), echinacoside (4.66 mg g^{-1}), cynarine (1.57 mg g^{-1}) and chicoric acid (1.57 mg g^{-1}) were determined in ¼ MS medium containing 5% sucrose.

These findings reveal that many factors such as the chemical composition of the medium, the type and concentrations of growth regulators used, culture type, plant species and explant type should be evaluated together in order to obtain and increase secondary metabolite production *in vitro*. Therefore, optimization of *in vitro* culture conditions is very important. In addition, studies on different medicinal plant species carried out for similar purposes also support our findings. On the other hand, Khan et al. (2018) investigated the effects of carbon sources of different types (sucrose, maltose, glucose and fructose) and concentrations (1 %, 3 % and 5 %) on biomass and secondary metabolite production in Fagonia indica callus cultures, with the highest biomass increase. Total phenol and flavonoid contents were determined in calli obtained from medium containing 3 % sucrose and 3 % maltose, respectively.

3.2. The effect of different nitrogen (ammonium/nitrate ratios) applications on the accumulation of CADs

In order to determine the effect of the different nitrogen source on the amount of caffeic acid derivatives, root and petiole explants of *E. purpurea* were cultured on MS medium with modified 0:35, 5:25, 15:15, 35:0 mM ammonium/nitrate ratio. In both explant types, callus stimulation did not occur in medium containing 35:0 mM ammonium/nitrate ratio, while healthy calli were obtained in other applications. The amounts of caffeic acid derivatives in the callus tissues obtained at the end of the 10-week culture period were analysed and the values obtained are given in the *Table 3*.

The highest amounts of caftaric, chlorogenic, caffeic and chicoric acids (respectively, 9.39, 0.71, 0.29, and 34.77 mg g^{-1}) were determined in callus tissues obtained from root explant cultured in MS medium containing 0:35 mM ammonium/nitrate. The highest amounts of caffeic acid derivatives in the petiole explant were also obtained from the 0:35 mM ammonium/nitrate (*Figure 3*).

As a result, the highest amounts of caftaric, chlorogenic, caffeic and chicoric acids were determined in callus tissues obtained from root explant after 10 weeks of culture in MS medium containing 0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP and 0:35 mM ammonium/nitrate ratio.

Table 3. Quantities of CADs in calli obtained from different ammonium/nitrate ratios (mg g^{-1})

Explant sources	Ammonium/Nitrate rate (mM)	Callus dry weight (mg/calli)	Caffeic acid derivatives (mg g^{-1})			
			Caftaric	Chlorogenic	Caffeic	Chicoric
Root derived	00:35	10.4	**9.38±0.10**	**0.71±0.04**	**0.29±0.30**	**34.77±0.09**
	15:15	4.6	0.09±0.01	0.09±0.09	0.08±0.25	0.22±0.01
	05:25	9.5	1.04±0.02	-	0.08±0.12	1.61±0.03
Petiole derived	00:35	61.4	**4.51±0.05**	**0.67±0.01**	**0.25±0.04**	**7.93±0.78**
	15:15	8.1	-	0.08±0.08	-	-
	05:25	11.5	3.37±0.09	0.39±0.09	0.07±0.15	6.78±0.05

Nitrogen sources are very important in the medium for the synthesis of secondary metabolites *in vitro*. In particular, the ratio of NH4+ to NO3- in the medium affects not only the growth of plant cell cultures (Veliky and Rose, 1973), but also the production of secondary metabolites (Smetanska, 2008). In our study, the highest amounts of caffeic acid derivatives were obtained in the two different explant types of *E. purpurea*, growth regulator combinations with the best callus development and 0:35 mM ammonium/nitrate ratio.

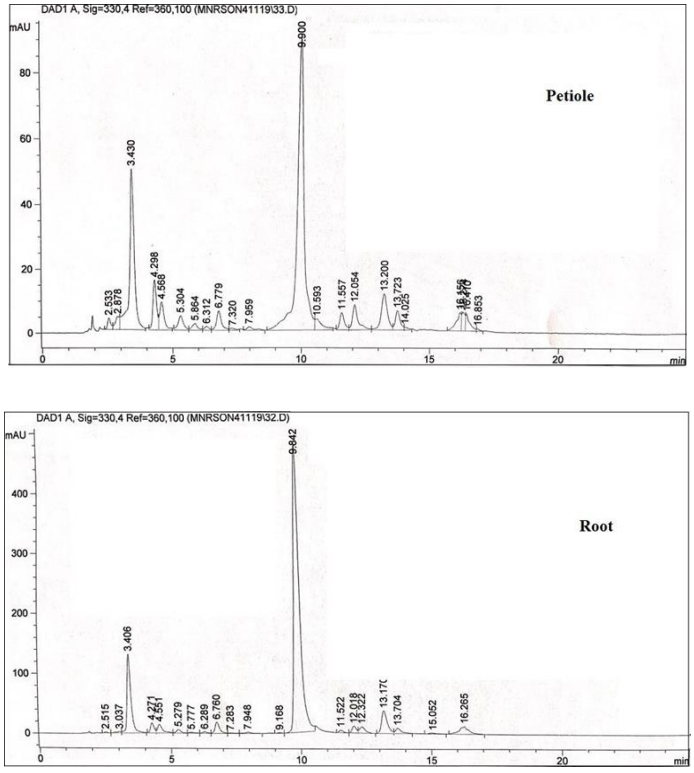

Figure 3. HPLC chromatogram of callus tissues obtained in 0:35 amonium/nitrate

Wu et al. (2006) obtained the highest total phenol and flavonoid content in adventive root culture of *E. angustifolia* in ½ MS medium containing 5:25 mM ammonium/nitrate, 2.0 mg l^{-1} IBA. For a similar purpose, Lee et al. (2011) examined the amount of secondary metabolites in MS medium containing different ammonium/nitrate ratios and in mulberry callus cultures, they found the highest amount of rutin in the medium containing 34/66 ammonium/nitrate and 5.0 mg l^{-1} IAA. Cui et al. (2010), obtained the highest hypersin content of 0:30 mM ammonium/nitrate, and total phenol and flavonoid content in ½ MS medium containing 10:20 mM

ammonium/nitrate in St. John's Wort adventitious root cultures. In our results, the highest amounts of caftaric, chlorogenic, caffeic, and chicoric acids were found in callus tissues obtained from root explant after 10 weeks of culture period on MS medium containing 0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP and 0:35 mM ammonium/nitrate ratio. The difference between these studies is due to the genotype, which clearly shows that each genotype has its own specific response. The findings revealed that medium optimization is very important in secondary metabolite production *in vitro* and optimization conditions specific to each genotype should be determined.

3.3. The effect of different Polyethylene glycol (PEG) applications on the accumulation of CADs

To determine the effect of drought stress on the amount of caffeic acid derivatives, root and petiole explants of *E. purpurea* L. were cultured on MS medium containing the growth regulator with the best callus formation and different polyethylene glycol (PEG) concentrations (5, 10, 15 g l^{-1}). The amounts of caffeic acid derivatives were analysed at the callus tissues obtained at the end of the 10-week culture period in both explant types, but none of the caffeic acid derivatives could be detected in any of the callus tissues obtained from the petiole explant. For this reason, caffeic acid derivatives determined only at calli obtained from root explants are given in *Table 4*.

Table 4. Quantities of CADs in calli obtained from different PEG concentrations (mg g^{-1})

PEG (g l^{-1})	Callus dry weight (mg/calli)	Caffeic acid derivatives (mg g^{-1})			
		Caftaric	Chlorogenic	Caffeic	Chicoric
5	14.4	0.34±0.01	0.05±0.01	-	0.60±0.17
10	**27.3**	**1.88±0.12**	**0.58±0.05**	**0.07±0.01**	**6.07±0.08**
15	26.3	0.10±0.01	-	-	0.13±0.05

The highest amounts of caftaric (1.88 mg g^{-1}), chlorogenic (0.58 mg g^{-1}), caffeic (0.07 mg g^{-1}) and chicoric (6.07 mg g^{-1}) acids were detected at the callus tissues obtained from the root explant after 10 weeks of culture on MS medium containing 0.5 mg l^{-1} NAA+0.5 mg l^{-1} BAP, 30 g l^{-1} sucrose and 10 g l^{-1} PEG.

Water stress is one of the most important environmental stresses that can regulate the growth and development of plants, limit plant production, and change the physiological and biochemical properties of plants. It is known that water stress increases the production of secondary metabolites as an abiotic elicitor in plants (Zobayed et al., 2007). PEG is an osmotic agent that is used in many plants to induce water stress and cannot be taken up by the plant (Lemcoff et al., 2006). According to study was conducted on *Hypericum perforatum* by Pavlik et al. (2007) researches were studied the effect of PEG (1.25, 2.5, 5, 10, 15 g l^{-1}) and sucrose (10, 20, 30 g l^{-1}) on secondary metabolite amount. At the mentioned study it was found that mostly low concentrations of PEG (1.25 and 5 g l^{-1}) have increased the production of hypericin and hyperforin. In our study, unlike this, 10 g l^{-1} PEG application was more effective. Similarly, Yamaner and Erdag (2013) were reported that *Hypericum adenotrichum in vitro* shoot cultures obtained on modified MS medium containing different concentrations of PEG (2.5, 10, 15 g l^{-1}) during different culture periods, in 10 g l^{-1} PEG application for 15 days. They reported that the amounts of hypericin (2.1 times) and pseudohypericin (2.3 times) increased at the end of the culture period. Osmotic stress caused by PEG application increased secondary metabolite production in many plants; Ex. iridoid glycosides (catalpol, harpagoside, aucubin and harpagide) in the roots of *Scrophularia ningpoensis* Wang et al. (2010), production of paclitaxel in *Taxus chinensis* cell suspensions Kim et al. (2001) and phenethanoid glycosylates in *Cistanche deserticola* cell cultures Liu and Cheng (2008) were increased by PEG applications. However, in order to increase secondary metabolite production by PEG application *in vitro*, it is necessary to determine the optimum PEG concentration for each plant species and explant type.

4. Conclusion

Optimization of culture conditions and different elicitor applications were made to increase secondary metabolite content in *E. purpurea* L. under *in vitro* conditions and the results obtained were presented comparatively.

The effect of the changes in the nutritional factors (nitrogen amount, type and amount of carbon source) in the medium and the abiotic elicitor (drought) formed in the medium on the amounts of caffeic acid derivatives were determined.

Among these applications, the highest amounts of caftaric acid (9.38 mg g^{-1}), chlorogenic acid (0.71 mg g^{-1}), caffeic acid (0.29 mg g^{-1}) and chicoric acid (34.77 mg g^{-1}), it was reached in callus tissues obtained from root explant at the end of 10-week culture period in medium containing 0:35 mM ammonium/nitrate and 30 g l^{-1} sucrose.

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Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Tanur Erkoyuncu M., Yorgancılar M.; Design Tanur Erkoyuncu M., Yorgancılar M.; Data Collection or Processing: Tanur Erkoyuncu M.; Statistical Analyses: Tanur Erkoyuncu M., Yorgancılar M.; Literature Search: Tanur Erkoyuncu M.; Writing, Review and Editing: Tanur Erkoyuncu M., Yorgancılar M.

References

Aarland, R. C., Bañuelos-Hernández, A. E., Fragoso-Serrano, M., Sierra-Palacios, E. d. C., Díaz de León-Sánchez, F., Pérez-Flores, L. J. and Mendoza-Espinoza, J. A. (2017). Studies on phytochemical, antioxidant, anti-inflammatory, hypoglycaemic and antiproliferative activities of *Echinacea purpurea* and *Echinacea angustifolia* extracts. *Pharmaceutical Biology, 55*(1): 649-656.

Ali, M., Abbasi, B. H., Ahmad, N., Ali, S. S., Ali, S. and Ali, G. S. (2016). Sucrose-enhanced biosynthesis of medicinally important antioxidant secondary metabolites in cell suspension cultures of *Artemisia absinthium* L. *Bioprocess and Biosystems Engineering,* 39(12): 1945-1954.

Bruni, R., Brighenti, V., Caesar, L. K., Bertelli, D., Cech, N. B. and Pellati, F. (2018). Analytical methods for the study of bioactive compounds from medicinally used *Echinacea* species. *Journal of Pharmaceutical and Biomedical Analysis,* 160: 443-477.

Chiou, S. Y., Sung, J.-M., Huang, P. W. and Lin, S. D. (2017). Antioxidant, antidiabetic, and antihypertensive properties of E*chinacea purpurea* flower extract and caffeic acid derivatives using *in vitro* models. *Journal of Medicinal Food,* 20(2): 171-179.

Cui, H.-Y., Baque, M. A., Lee, E. J. and Paek, K. Y. (2013). Scale-up of adventitious root cultures of *Echinacea angustifolia* in a pilot-scale bioreactor for the production of biomass and caffeic acid derivatives. *Plant Biotechnology Reports,* 7(3): 297-308.

Cui, X. H., Murthy, H. N., Wu, C. H. and Paek, K. Y. (2010). Adventitious root suspension cultures of *Hypericum perforatum*: effect of nitrogen source on production of biomass and secondary metabolites. *In Vitro Cellular and Developmental Biology-Plant,* 46(5): 437-444.

Demirci, T. (2022). Determination of secondary metabolite production efficiency in *Echinacea purpurea* callus, shoot, and root *in vitro* cultures with methyl jasmonate applications. *Acta Physiologiae Plantarum,* 44(12): 1-12.

Elshahawy, O. A., Zeawail, M. E. F., Hamza, M. A., Elateeq, A. A. and Omar, M. A. (2022). Improving the production of total phenolics and flavonoids and the antioxidant capacity of *Echinacea purpurea* callus through biotic elicitation. *Egyptian Journal of Chemistry,* 65(12): 137-149.

Erkoyuncu, M. T. and Yorgancilar, M. (2021). Optimization of callus cultures at *Echinacea purpurea* L. for the amount of caffeic acid derivatives. *Electronic Journal of Biotechnology,* 51: 17-27. https://doi.org/10.1016/j.ejbt.2021.02.003

Khan, T., Abbasi, B. H., Zeb, A. and Ali, G. S. (2018). Carbohydrate-induced biomass accumulation and elicitation of secondary metabolites in callus cultures of Fagonia indica. *Industrial Crops and Products,* 126: 168-176.

Kim, S. I., Choi, H. K., Kim, J. H., Lee, H. S. and Hong, S. S. (2001). Effect of osmotic pressure on paclitaxel production in suspension cell cultures of Taxus chinensis. *Enzyme and Microbial Technology,* 28(2-3): 202-209.

Kretzschmar, F. S., Oliveira, C. J. and Braga, M. R. (2007). Differential sugar uptake by cell suspension cultures of Rudgea jasminoides, a tropical woody *Rubiaceae*. *In Vitro Cellular and Developmental Biology-Plant,* 43(1), 71-78.

Lee, Y., Lee, D. E., Lee, H. S., Kim, S. K., Lee, W. S., Kim, S. H. and Kim, M. W. (2011). Influence of auxins, cytokinins, and nitrogen on production of rutin from callus and adventitious roots of the white mulberry tree (*Morus alba* L.). *Plant Cell, Tissue and Organ Culture,* 105(1): 9-19.

Lemcoff, J. H., Ling, F. and Neumann, P. M. (2006). Short episodes of water stress increase barley root resistance to radial shrinkage in a dehydrating environment. *Physiologia Plantarum,* 127(4): 603-611.

Lin, Z., Neamati, N., Zhao, H., Kiryu, Y., Turpin, J. A., Aberham, C. and Pannecouque, C. (1999). Chicoric acid analogues as HIV-1 integrase inhibitors. *Journal of Medicinal Chemistry,* 42(8): 1401-1414.

Liu, C. Z., Abbasi, B. H., Gao, M., Murch, S. J. and Saxena, P. K. (2006). Caffeic acid derivatives production by hairy root cultures of *Echinacea purpurea*. *Journal of Agricultural and Food Chemistry,* 54(22): 8456-8460.

Liu, C. Z. and Cheng, X. Y. (2008). Enhancement of phenylethanoid glycosides biosynthesis in cell cultures of *Cistanche deserticola* by osmotic stress. *Plant Cell Reports,* 27(2): 357-362.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum,* 15(3): 473-497.

Murthy, H. N., Kim, Y. S., Park, S. Y. and Paek, K. Y. (2014). Biotechnological production of caffeic acid derivatives from cell and organ cultures of *Echinacea* species. *Applied Microbiology and Biotechnology,* 98(18): 7707-7717.

Pavlik, M., Vacek, J., Klejdus, B. and Kubáň, V. (2007). Hypericin and hyperforin production in St. John's wort *in vitro* culture: influence of saccharose, polyethylene glycol, methyl jasmonate, and *Agrobacterium tumefaciens*. *Journal of Agricultural and Food Chemistry,* 55(15): 6147-6153.

Pehlivan, E. C., Kunter, B. and Royandazagh, S. D. (2017). Choise of explant material and media for *in vitro* callus regeneration in Sultana grape cultivar (*Vitis vinifera* L.). *Journal of Tekirdag Agricultural Faculty,* The Special Issue of 2nd International Balkan Agriculture Congress, 30-34.

Pleschka, S., Stein, M., Schoop, R. and Hudson, J. B. (2009). Anti-viral properties and mode of action of standardized *Echinacea purpurea* extract against highly pathogenic avian influenza virus (H5N1, H7N7) and swine-origin H1N1 (S-OIV). *Virology Journal,* 6(1): 1-9.

Rady, M. R., Aboul-Enein, A. M. and Ibrahim, M. M. (2018). Active compounds and biological activity of *in vitro* cultures of some *Echinacea purpurea* varieties. *Bulletin of the National Research Centre,* 42(1): 1-12.

Rajasekaran, D., Palombo, E. A., Chia Yeo, T., Lim Siok Ley, D., Lee Tu, C., Malherbe, F. and Grollo, L. (2013). Identification of traditional medicinal plant extracts with novel anti-influenza activity. *Plos One*, 8(11): e79293.

Ram, M., Prasad, K., Singh, S., Hada, B. and Kumar, S. (2013). Influence of salicylic acid and methyl jasmonate elicitation on anthocyanin production in callus cultures of *Rosa hybrida* L. *Plant Cell, Tissue and Organ Culture*, 113(3): 459-467.

Ramezannezhad, R., Aghdasi, M., and Fatemi, M. (2019). Enhanced production of cichoric acid in cell suspension culture of *Echinacea purpurea* by silver nanoparticle elicitation. *Plant Cell, Tissue and Organ Culture*, 139(2): 261-273.

Rao, S. R. and Ravishankar, G. (2002). Plant cell cultures: chemical factories of secondary metabolites. *Biotechnology Advances*, 20(2): 101-153.

Romero, F. R., Delate, K., Kraus, G. A., Solco, A. K., Murphy, P. A. and Hannapel, D. J. (2009). Alkamide production from hairy root cultures of *Echinacea*. *In Vitro Cellular and Developmental Biology-Plant*, 45(5): 599.

Royandazagh, S. D. and Pehlivan, E. C. (2016). *In vitro* micropropagation of *Lilium candidum* L. and alkaloids. *Journal of Tekirdag Agricultural Faculty*, 13(3): 100-110.

Shahrajabian, M. H., Sun, W. and Cheng, Q. (2020). Traditional herbal medicine for the prevention and treatment of cold and flu in the autumn of 2020, overlapped with COVID-19. *Natural Product Communications*, 15(8): 1431.

Sharif, K. O. M., Tufekci, E. F., Ustaoglu, B., Altunoglu, Y. C., Zengin, G., Llorent-Martínez, E. and Baloglu, M. C. (2021). Anticancer and biological properties of leaf and flower extracts of *Echinacea purpurea* (L.) Moench. *Food Bioscience*, 41: 101005.

Smetanska, I. (2008). Production of Secondary Metabolites Using Plant Cell Cultures. In Food Biotechnology (pp. 187-228): Springer.

Sökmen, A. and Gürel, E. (2002). Secondary Metabolite Production (TR, Trans.). In M. Babaoğlu, E. Gurel, and S. Ozcan (Eds.), Plant Biotechnology, Tissue Culture and Application (2. ed., Vol. 1, pp. 211-261). Konya: Selçuk University Press, Turkey.

Suan See, K., Bhatt, A. and Lai Keng, C. (2011). Effect of sucrose and methyl jasmonate on biomass and anthocyanin production in cell suspension culture of *Melastoma malabathricum* (*Melastomaceae*). *Revista de Biologia Tropical*, 59(2): 597-606.

Taha, H., Abd, E. R. ve Maharik, N. (2009). *In vitro* production of caffic acid derivatives in calli and regenerate cultures of *Echinacea angustifolia* Dc and *Echinacea pallida* Nutt, *Journal of Applied Sciences Research*, 5(1): 13-20.

Tanur Erkoyuncu, M. and Yorgancılar, M. (2015). Plant Tissue Culture forthe Production of Secondary Metabolites. *Selcuk Journal of Agriculture and Food Sciences*, 2(1): 66-76.

Thygesen, L., Thulin, J., Mortensen, A., Skibsted, L. H. and Molgaard, P. (2007). Antioxidant activity of cichoric acid and alkamides from *Echinacea purpurea*, alone and in combination. *Food Chemistry*, 101(1): 74-81.

Veliky, I. and Rose, D. (1973). Nitrate and ammonium as nitrogen nutrients for plant cell cultures. *Canadian Journal of Botany*, 51(10): 1837-1844.

Wang, D., Du, F., Liu, H. and Liang, Z. (2010). Drought stress increases iridoid glycosides biosynthesis in the roots of *Scrophularia ningpoensis* seedlings. *Journal of Medicinal Plants Research*, 4(24): 2691-2699.

Wu, C. H., Dewir, Y. H., Hahn, E. J. and Paek, K. Y. (2006). Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of *Echinacea angustifolia*. *Journal of Plant Biology*, 49(3): 193.

Yamaner, O. and Erdag, B. (2013). Effects of sucrose and polyethylene glycol on hypericins content in *Hypericum adenotrichum*. *Eurasian Journal of Biosciences*, 7: 101-110.

Zobayed, S., Afreen, F. and Kozai, T. (2007). Phytochemical and physiological changes in the leaves of St. John's wort plants under a water stress condition. *Environmental and Experimental Botany*, 59(2): 109-116.