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Optimization of callus cultures at *Echinacea purpurea* L. for the amount of caffeic acid derivatives

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ABSTRACT

Background: In order to produce an effective callus in *Echinacea purpurea* L.; determination of the explant type and growth regulators that best respond to callus induction and the optimization of the culture conditions to increase the amount of caffeic acid derivatives (CADs) in the obtained callus. CADs contents of callus cultures of *E. purpurea* were evaluated by establishing an effective callus induction system *in vitro*.

Results: Various medium containing different growth regulators were tested using leaf, petiole, cotyledon and root as the explants. The best callus development was achieved in MS medium with 1.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} BAP in leaf, 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} TDZ in petiole, 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} TDZ in cotyledon and 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP in roots. Upon optimisation of callus growth, each type of explant was cultured for 4, 6, 8 and 10 weeks in medium for the analyses of caftaric acid, chlorogenic acid, caffeic acid and chicoric acid contents. The highest amounts of caftaric acid (4.11 mg/g) and chicoric acid (57.89 mg/g) were found from petiole explants and chlorogenic acid (8.83 mg/g) from root explants at the end of the 10-week culture time.

Conclusions: As a result of the present study, the production of caffeic acid derivatives was performed by providing the optimization of *E. purpurea* L. callus cultures. Effective and repeatable protocols established in this study may offer help for further studies investigating the production of caffeic acid derivatives *in vitro*.

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1. Introduction

Echinacea purpurea L., which has been used in traditional medicine for a long time to treat various diseases, especially the common cold, sore throat, and other upper respiratory tract infections [1], is a very popular medicinal plant used widely as root extracts or tea all over the world today [2]. *E. purpurea* extracts have antioxidative, antibacterial, antiviral, and antifungal activities [3], and has a very important place in terms of medical use with positive effects in viral diseases [4,5], respiratory tract infections [6], skin diseases like atopic eczema [7]. In addition to these activities, it was also determined that it stops the growth of tumors [8] and cancer cells [9].

The most important components of *E. purpurea* are phenolic compounds, which include caffeic acid derivatives (CADs) [10]. The most common CADs are cichoric acid, echinacoside, chlorogenic acid, caftaric acid, caffeic acid and cynarin, the amounts of which vary according to the plant parts [11]. Cichoric acid is considered to be the most important CAD in terms of the medicinal value of *E. purpurea* [12], and the highest cichoric acid content was detected in *E. purpurea* [13]. It was determined that cichoric acid has immunostimulator and antiviral activities since it inhibits HIV-1 (Human Immunodeficiency Virus) integrase enzyme, and promotes phagocyte activity [14]. In addition, as an anti-hyaluronidase, it protects collagen, and has high free radical cleansing activities against cichoric acid and echinacoside reactive oxygen species; and therefore, has antioxidant and anti-inflammatory effects with its protective and therapeutic effects for the damage caused by UV rays on the skin [15]. It was reported that chlorogenic acid inhibits glucose absorption in the intestines, preventing diabetes, and creates anticarcinogenic effects by mitigating cancerogenic effects caused by *N*-nitroso compounds [9,16].

Since the medicinal activity of *E. purpurea* is not associated with one single chemical, plant production is required to obtain the full

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spectrum of all metabolites [16,17]. The plant production of *E. purpurea* remains inadequate for several reasons like environmental pollution, contamination of plant materials with microorganisms, the variability of active components, and lack of pure, standardized plant material for biochemical analysis [18]. In addition, it was also determined that these compounds are obtained from plants that grow in nature because of the difficult, costly, time-consuming, and insufficient herbal production in response to increased consumption [19]. In plants collected from nature, the desired standard and quality cannot be achieved, and there is always the danger of the extinction of the plant.

In particular, to produce medicinal plants through plant cell and tissue cultures by optimizing culture conditions; the environmental factors (i.e. climate, geographical difficulties, seasonal restrictions) encountered during the cultivation of the plant are eliminated, less land use is ensured, and the extinction of the plant is prevented by collecting it from nature. At the same time, plant cell and tissue cultures provide advantages such as being able to produce a sufficient amount of economically valuable metabolites in low amounts in plants and establishing homogeneity, standard quality and efficiency in production [20]. Production with plant cells and tissue cultures, which is considered as an alternative method in the production of secondary metabolites, can be carried out in a faster, simpler, reliable and predictable manner compared to conventional methods.

Studies in *E. purpurea* have been on establishing an effective regeneration system with callus and/or shoot cultures [21,22,23,24], providing mass production with the micropropagation system [25,26] and obtaining secondary metabolites of standard quality and efficiency from this production [27,28]. Although there are studies on different growth regulators and stress practices to increase secondary metabolite production in hairy and adventive root and cell suspension cultures were conducted [19,29,30,31], studies conducted especially on the optimization of callus cultures are limited in number. However, when optimization is achieved in callus cultures, biomass growth becomes easier, and in this way, an effective start is achieved to cell suspension cultures for mass production. Also, the production of secondary metabolites can be increased with this optimization.

In the present study, the purpose was to determine the explant type and the most appropriate growth regulators responding best to callus induction to produce an effective callus in *E. purpurea*, and to investigate the optimization of cultural conditions to increase the caffeic acid derivatives amounts in the resulting callus. Our study is important in this sense for obtaining high caffeic acid derivatives amounts in callus cultures.

2. Material and methods

2.1. Material and chemicals

E. purpurea seeds were used as the starting material, and were obtained from the plants cultured in Selcuk University, Turkey. Analytically sensitive chemicals of Sigma, Merck, Sigma-Aldrich and Duchefa brands were used throughout the study. All standards used in the analyses (i.e. cichoric acid, chlorogenic acid, caftaric acid, caffeic acid, purity 95% by HPLC) were from Sigma-Aldrich (St. Louis, MO, USA) and had Acetonitrile, methanol HPLC grade from Merck (Germany). Water was purified using a Milli-Q PLUS 185 system from Millipore (Milford, MA, USA).

2.2. Sterilization of plant material

The seeds were washed for 30 s in 70% ethanol (a/h) solution, 1–2 drops of Tween-20 was added, kept in 10% sodium hypochlorite (NaOCl) solution for 10 min, rinsed with sterile pure water 3 times. Sterile seeds were cultured in MS [32] medium without plant growth regulators, and the 8-week old sterile seedlings were used as explant sources throughout the study. All the cultures were kept in the growth cabinet (Sanyo: MLR-351H) at 24 ± 2 °C, 65% humidity, 5 LS light intensity, 16/8-h photoperiod throughout the study.

2.3. Callus cultures and optimization

The leaf, petiole, cotyledon and root explants taken from sterile seedlings were taken to culture in the combinations of NAA or 2.4-D (0.2, 0.5, 1.0, 2.0, 4.0 mg l^{-1}) and BAP, TDZ or KIN (0.2, 0.5, 1.0, 2.0 mg l^{-1}) in MS medium containing growth regulators as 10 explant/petri dish. Four weeks after the beginning of the culture, the percentage of the callus formation (%) was determined by rating the number of callus-forming explants to the total number of explants in 4 different explants. The callus that developed from each explant was weighed on a precision scale, and the fresh callus weight was recorded as mg [33]. The callus efficiency (mg/callus) was determined according to the equation of callus weight × callus formation rate/100.

2.4. Optimization of culture time in the production of caffeic acid derivatives

After determining the growth regulator and concentration, which yielded the best callus in 4 different explants (leaf, petiole, cotyledon and root), each explant was taken to the culture at different times (4-6-8-10 weeks) to determine how the amount of caffeic acid derivatives in the callus tissues of different explant types were affected by the culture time. At the end of these culture times, the caffeic acid derivatives amounts in the callus tissues of four different explants were determined.

2.5. Analysis of caffeic acid derivatives

2.5.1. Preparation of samples

The callus obtained as a result of different applications were subjected to drying for 2 d at −55 °C in a lyophilizer for use in the analyses. The dried samples were fragmented and made into powder with the help of a press.

2.5.2. Extraction

The extraction was carried out according to the protocol that was modified by Taha et al. [27]. The powdered 0.2 g sample was extracted in an ultrasonic bath with 70% of 8 ml methanol (v/v) for 15 min. The volume of the extract was made to 10 ml by diluting with methanol 70% (v/v). After the visible solid particles precipitated, the liquid part was filtered with a 0.45 μm membrane filter, and was then transferred to the vials with a syringe for HPLC analysis. The extraction procedure was repeated twice for each sample.

2.5.3. Preparation of standard solutions and creation of calibration graphic

Cichoric acid, chlorogenic acid, caftaric acid, caffeic acid standard stock solutions were prepared separately [16]. Each of the standards was weighed as 10 mg, and was dissolved in an ultrasonic bath with 5 ml 70% (v/v) methanol for 15 min, and the volume was made to 10 ml with 70% (v/v) methanol. Dilutions 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, $R^2 \sim 0.99$, and the results were evaluated according to the formulas in the graphics. Also, according to these graphics, the following were calculated; caftaric acid (y = 7.2575x − 0.4774,

R^2 = 0.9996), chlorogenic acid (y = 8.6587x − 5.2497, R^2 = 0.9998), caffeic acid (y = 12.805x − 7.7252, R^2 = 0.9996), and cichoric acid (y = 8.8418x − 7.9709, R^2 = 0.9967).

2.5.4. Chromatographic method and conditions

The HPLC analysis method described in the European Pharmacopoeia was selected to determine the quantity of caffeic acid derivatives. The Agilent 1200 Series HPLC System and Zorbax OD S4-type column were used. The movable phase solutions were set as 0.1% phosphoric acid (Movable Phase A), acetonitrile (Movable Phase B), and Flow-Type Gradient Solution. The injection volume was 10 µl. Diode-Array Detector (DAD) was run on a wavelength of 330 nm. Three injections were performed for each sample.

2.6. Statistical analysis

The averages of the callus cultures were subjected to variance analysis according to the factorial trial pattern in random parcels. They were subjected to Arcsine Transformation before Variance Analysis in the data calculated as percentages [34]. The data of the results of the callus cultures were compared in the JMP 13.0 Statistical Program with LSD multiple-comparison test at $P < 0.05$ significance level.

3. Result and discussion

3.1. Optimization of callus induction

3.1.1. Callus formation percentage

E. purpurea was subject to variance analysis with percentage values of callus formation in leaf, petiole, cotyledon, and root explants. According to the variance analysis results, the effects of the interaction of auxin applications, cytokinin applications, and auxin × cytokinin applications on the callus formation percentage were found to be statistically significant (P < 0.01).

The highest callus formation percentage (90%) was obtained in the following growth regulator combinations; 1.0 mg l^{-1} 2.4-D + 0.2/1.0/2.0 mg l^{-1} BAP and 4.0 mg l^{-1} 2.4-D in the leaf; 0.5 mg l^{-1} NAA + 0.2/0.5 mg l^{-1} BAP, 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} TDZ and 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} KIN in the petiole; 2.0 mg l^{-1} NAA + 0.5 mg l^{-1} TDZ in cotyledon; 0.2 mg l^{-1} NAA + 2.0 mg l^{-1} KIN, 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP, 1.0 mg l^{-1} 2.4-D + 1.0/2.0 mg l^{-1} BAP in the root (Table 1).

Callus induction varies depending on the genotype, explant type, and growth regulator concentration of the plant, and the balance between auxin and cytokinin, in particular, is an important factor for callus production [35]. Similar to our results, Taha et al. [27], and Butiuc-Keul et al. [28] identified optimum callus induction in different Echinacea types in MS with 2.4-D and BAP. However, Ramezannezhad et al. [31] obtained maximum callus induction from *E. purpurea* leaf explant, at different concentrations of NAA and 2.4-D in 1/2 MS that included combinations with KIN. This difference shows that the genotype, explant type, growth regulator concentration, and medium contents are so important in the callus induction [36]. In the petiole explant, Choffe et al. [21] found that callus induction was provided in MS that included NAA and BAP. In cotyledon explant, Zebarjadi et al. [24] achieved the highest callus induction with 93% in MS that included different NAA and BAP combinations. In our study; however, the use of TDZ together with NAA increased the induction of callus from the cotyledon explant to 100%. Murthy et al. [37] stated that TDZ is a synthetic growth regulator showing auxin and cytokinin effects, which can lead to strong regeneration depending on the concentration used, and caused the formation of callus. Similar to our results, Ramezannezhad et al. [31] achieved maximum callus induction in root explants in combinations of NAA or 2.4-D with KIN.

When callus are evaluated in general for the combination of growth regulators obtained, they differ in terms of morphology and organogenic potentials. It was observed that all NAA concentrations were green, granular and compact in the callus obtained with combinations with BAP, TDZ and KIN in different concentrations, and there was direct shoot induction, especially at low NAA concentrations, and there was organogenic potential. All 2.4-D concentrations, different BAP, TDZ and KIN concentrations obtained as a result of the combination of callus were yellowish, smooth and soft, and there were no organogenic features. Similarly, Coker and Camper [22] mostly achieved direct shoot and root induction in NAA-containing medium, and callus induction was mostly provided in medium with 2.4-D. Koroch et al. [23] achieved callus formation and shoot induction at low NAA concentrations at different concentrations with MS medium that included BAP and NAA; and callus induction increased at high NAA concentrations, and there was no shoot regeneration (Fig. 1).

Especially in NAA and BAP combinations at low concentrations (0.2, 0.5 mg l^{-1}), callus and direct shoot induction in leaf explant types were more than in other explant types. In NAA and KIN combinations; however, callus and root induction were detected especially in petiole and root explants. In NAA and TDZ combinations, on the other hand, green, granular and compact callus were formed in all explant types. Only callus induction occurred in all explant types in combinations of 2.4-D with BAP, TDZ and KIN (Fig. 2).

3.1.2. Callus weight and callus efficiency

One of the most important targets of cell and tissue cultures in medicinal plants is to obtain higher amounts of biomass accumulation to produce plant-derived products in greater amounts. For this reason, maximum biomass production is very important in *in vitro* cultures of medicinal plants [38]. In our study, the purpose was to determine the growth regulators that promoted the induction and weight of the callus at the maximum level to achieve maximum biomass production.

E. purpurea callus fresh weight values of the leaf, petiole, cotyledons and root explants were subjected to variance analysis separately. According to the variance analysis results, the effect of auxin × cytokinin applications on the weight of callus was found to be statistically significant in leaf and petiole explants ($P < 0.01$); however, the effect of cytokinin applications on the weight of the callus was found to be statistically insignificant. In cotyledon and root explants. On the other hand, the effect of auxin applications, cytokinin applications, and auxin × cytokinin applications on the callus weight (mg) was found to be statistically significant at $P < 0.01$.

When the effects of growth regulators at different types and concentrations were examined in four different explants on the fresh callus weight, the highest fresh callus weight values were obtained in the following medium; 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BAP and 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} TDZ in the leaf (507 mg and 502 mg); in the petiole (456 mg) 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} TDZ; in the cotyledon (2179 mg) 1.0 mg l^{-1} NAA + 0.2 mg l^{-1} TDZ; in the root (665 and 642 mg) 0.2 mg l^{-1} NAA + 1.0 mg l^{-1} TDZ and 2.0 mg l^{-1} NAA + 1 mg l^{-1} TDZ, respectively (Table 2).

In many plant species, it was reported that auxin and cytokinin were used in combination to achieve optimum callus development and avoid necrosis in the callus [39]. Also, the effect of growth regulators on plant biomass varies with the number and type of specific receptors in different parts of the plant [40]. For this reason, both the weight of the callus and the type of explant in which the callus is derived are very important. Similar to the results of our study, Jones et al. [25] reported that the size of the callus increased as the concentration of TDZ in the medium increased

Table 1
Callus formation percentages (%) in growth regulators of different explant types.

mg l^{-1}		NAA					2,4-D					
		0.2	0.5	1.0	2.0	4.0	0.2	0.5	1.0	2.0	4.0	Avg.
Leaf												
K	0	0u	22nu	45cr	36ht	70ah	24lu	25lu	73ae	51bq	90a	44ab
BAP	0.2	0u	28ku	71ag	47cr	54bo	26lu	37gt	90a	46cr	45cr	44ab
	0.5	0u	57am	34ıu	6tu	49cr	32ju	17qu	78ac	42ds	38ft	35ad
	1.0	37gt	28ku	50bq	30ku	0u	0u	45cr	90a	39et	9su	33bd
	2.0	6tu	48cr	0u	0u	21ou	0u	41ds	90a	39et	18qu	26ce
TDZ	0.2	23mu	45cr	39et	21ou	39et	17qu	0u	27lu	25lu	30ku	27ce
	0.5	18qu	41ds	45cr	0u	51bq	0u	18qu	41ds	25lu	18qu	26ce
	1.0	39et	47cr	15ru	40et	21ou	30ku	9su	0u	27lu	41ds	27ce
	2.0	21ou	56an	0u	45cr	0u	0u	17qu	34ıu	19pu	0u	19e
KIN	0.2	41ds	66aj	0u	45cr	72af	30ku	67aı	62ak	44cr	22nu	45a
	0.5	15ru	0u	40et	30ku	26lu	39et	53bp	79ac	50bq	41ds	37ac
	1.0	31ku	0u	58al	0u	69ah	30ku	26lu	75ad	47cr	57am	39ab
	2.0	32ju	0u	84ab	30ku	69ah	18qu	40et	51bq	45cr	75ad	45a
Avg.		20e	34bd	37bc	25de	42b	19e	30cd	61a	38bc	37bc	
$LSD_{0.05\ (cytokinin)}$: 10.95					$LSD_{0.05\ (auxin)}$: 9.62				$LSD_{0.05\ (cytokininxauxin)}$: 34.69			
Petiole												
K	0	43fy	56bq	90a	11y\	52bt	35k[	51bu	79ad	52bt	58ap	53ab
BAP	0.2	53bs	90a	0\	60ao	69aj	73ag	65al	58ap	57aq	43fy	57a
	0.5	37ı[	90a	0\	45ex	39h[	66ak	41gz	84ab	49cv	9z\	46bc
	1.0	28o\	55br	0\	66ak	0\	36j[	20s\	84ab	40g[	13x\	34ef
	2.0	18u\	81ac	0\	32l\	62an	36j[	15w\	47dw	32l\	6[\	33ef
TDZ	0.2	30m\	84ab	75af	67ak	45ex	22r\	19t\	6[\	24q\	34k[	41ce
	0.5	42fz	24q\	90a	26p\	63am	28o\	38ı[	15w\	25p\	0\	35df
	1.0	51bu	84ab	66ak	60ao	55br	39h[	30m\	12x\	29n\	25p\	45bd
	2.0	39h[	70aı	52bt	39h[	17v\	24q\	11y\	18u\	24q\	32l\	33ef
KIN	0.2	63am	62an	47dw	67ak	0\	50cv	69aj	30m\	45ex	30m\	46bc
	0.5	32l\	51bu	78ae	57aq	20s\	27o\	49cv	39h[	34k[	9z\	40ce
	1.0	49cv	60ao	72ah	90a	34k(	21s\	62an	28o\	39g[	41gz	50ac
	2.0	43fy	15w\	60ao	24q\	41gz	21s\	20s\	15w\	22r\	17v\	28f
Avg.		41bd	63a	48bc	50b	38d	37d	38d	40cd	36d	24e	
$LSD_{0.05\ (cytokinin)}$: 10.62					$LSD_{0.05\ (auxin)}$: 9.31				$LSD_{0.05\ (cytokininxauxin)}$: 33.59			
Cotyledon												
K	0	0n	13ln	0n	22jn	30gn	0n	22jn	22jn	22jn	22jn	15e
BAP	0.2	35fm	64af	43dl	51bj	22jn	35fm	18kn	60ag	39em	43dl	41c
	0.5	30gn	64af	63af	51bj	35fm	43dl	43dl	47ck	42dl	30gn	45bc
	1.0	26hn	26hn	51bj	81ab	0n	30gn	13ln	60ag	42dl	60ag	39c
	2.0	39em	26hn	31gm	68ae	13ln	31gm	43dl	64af	41el	26hn	38c
TDZ	0.2	56bh	60ag	47ck	68ae	64af	30gn	47ck	38em	45dk	55bh	51ab
	0.5	51bj	0n	72ad	90a	47ck	47ck	51bj	47ck	43dl	22jn	47ac
	1.0	30gn	68ae	51bj	77ac	64af	55bh	43dl	77ac	50cj	35fm	55a
	2.0	39em	59bg	52bj	60ag	51bj	56bh	68ae	51bj	53bı	47ck	54ab
KIN	0.2	31gm	13ln	31gm	13ln	47ck	0n	13ln	39em	23ın	13ln	22de
	0.5	0n	0n	55bh	9mn	39em	22jn	30gn	35fm	35fm	43dl	27d
	1.0	13ln	43dl	35fm	0n	22jn	9mn	13ln	39em	30gn	34fm	24de
	2.0	0n	0n	39em	0n	39em	0n	43dl	34fm	28hn	0n	18de
Avg.		27e	34de	44ac	45ab	36cd	28e	34de	47a	38bd	33de	
$LSD_{0.05\ (cytokinin)}$: 9.60					$LSD_{0.05\ (auxin)}$: 8.46				$LSD_{0.05\ (cytokininxauxin)}$: 31.28			
Root												
K	0	0[	28oz	69aı	0[	30nz	58ap	36ız	78ad	52br	34kz	39df
BAP	0.2	30nz	75af	75af	46dv	12wz	45dw	58ap	43fx	45dw	35jz	46bd
	0.5	62an	90a	75af	33lz	6z	49ct	17tz	56bp	44ex	49ct	48ad
	1.0	58ap	81ac	81ac	46dv	11xz	30nz	6z	90a	41gy	38hz	48ad
	2.0	35jz	54br	26pz	60ao	0[	48cu	26pz	90a	51bs	55bq	45ce
TDZ	0.2	81ac	62an	62an	64am	67ak	27oz	55bq	39gz	47du	81ac	59a
	0.5	48cu	71ah	69aı	84ab	66al	72ag	47du	45dw	44ex	0[	55ab
	1.0	56bp	72ag	54br	46dv	47du	62an	40gy	78ad	50cs	22qz	53ac
	2.0	36ız	68aj	69aı	58ap	54br	45dw	53br	78ad	54br	40gy	55ab

Table 1 (*continued*)

mg l^{-1}		NAA					2,4-D					
		0.2	0.5	1.0	2.0	4.0	0.2	0.5	1.0	2.0	4.0	Avg.
Leaf												
KIN	0.2	13vz	17tz	19sz	22qz	18sz	28oz	32mz	78ad	50cs	70ah	35ef
	0.5	9yz	19sz	41gy	11xz	0[	0[	39gz	54br	41gy	69aı	28f
	1.0	47du	41gy	15uz	45dw	51bs	30nz	41gy	46dv	44ex	53br	41de
	2.0	90a	28oz	39gz	12wz	38hz	21rz	77ae	66al	49ct	30nz	45ce
Avg.		43d	54b	53bc	41d	31e	40de	41d	65a	47bd	44cd	
$LSD_{0.05(cytokinin)}$: 10.38					$LSD_{0.05\ (auxin)}$: 9.11				$LSD_{0.05\ (cytokininxauxin)}$: 32.88			

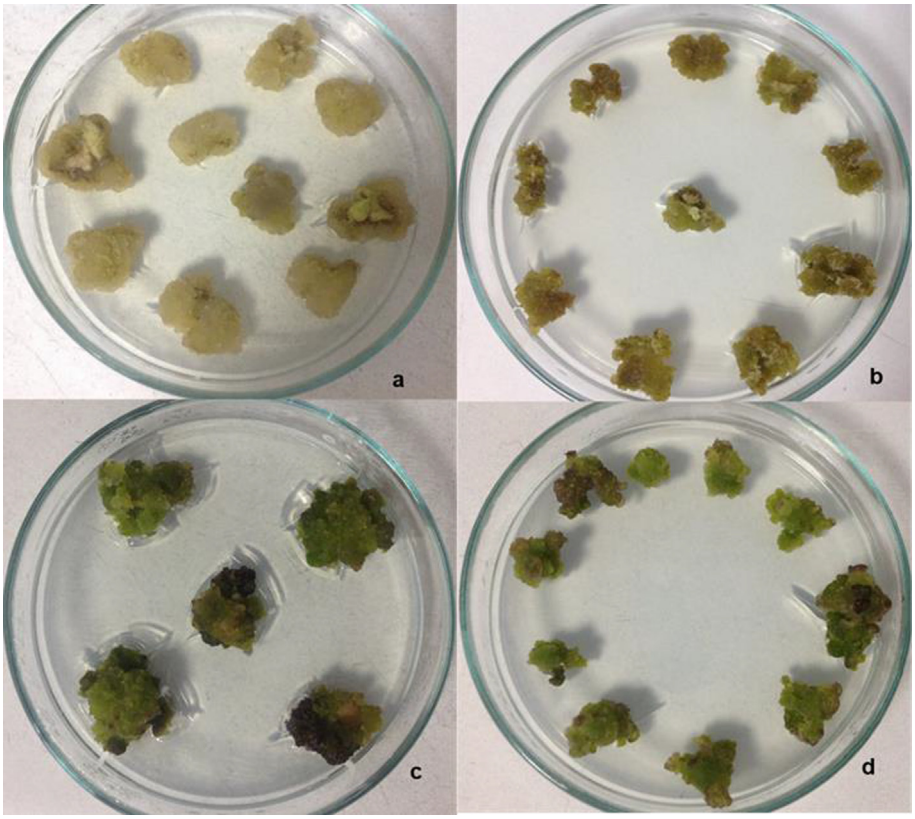

Fig. 1. Best callus development in different types of explants a) 1.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} BAP in the leaf, b) 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} TDZ in the petiole, c) 2.0 mg l^{-1} NAA + 0.5 mg l^{-1}TDZ in the cotyledon, d) 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP in the root.

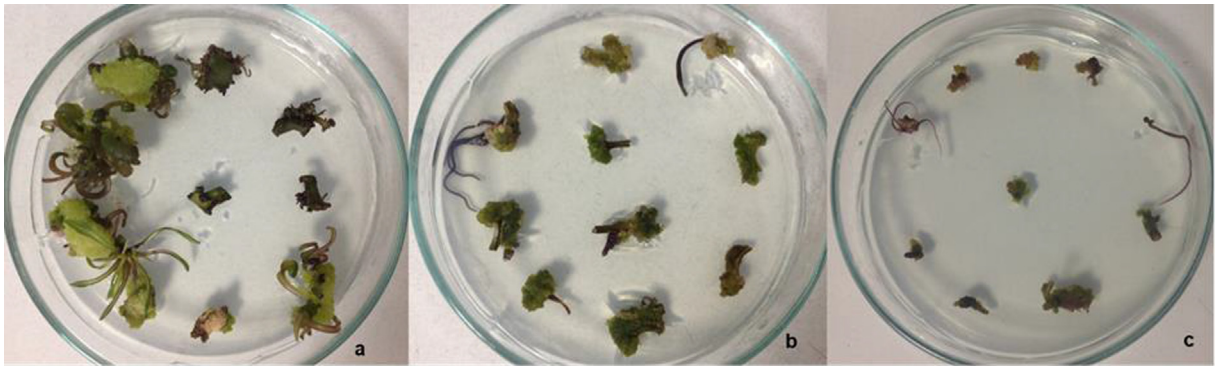

Fig. 2. Indirect shoot and root induction in different eksplants a) 0.5 mg l^{-1} NAA + 0.2 mg l^{-1} BAP in the leaf, b) 0.2 mg l^{-1} NAA + 0.2 mg l^{-1} KIN in the petiole c) 0.2 mg l^{-1} NAA + 0.2 mg l^{-1} KIN in the root.

Table 2
Callus weights (mg) in growth regulators of different explant types.

mg l^{-1}		NAA					2,4-D					
		0.2	0.5	1.0	2.0	4.0	0.2	0.5	1.0	2.0	4.0	Avg.
Leaf												
K	0	0m	54hm	91gm	37ım	50hm	24jm	13lm	157dm	58hm	37ım	52
BAP	0.2	0m	229bı	160dm	58hm	153em	51hm	29jm	361ac	122fm	46hm	121
	0.5	0m	237bh	230bı	17lm	109fm	67hm	23jm	301bf	109fm	46hm	114
	1.0	100gm	110fm	507a	19km	0m	0m	92gm	215ck	79gm	10lm	113
	2.0	13lm	113fm	0m	0m	1m	0m	347ae	420ab	204cl	50hm	115
TDZ	0.2	90gm	216cj	170cm	20jm	10lm	91gm	0m	3m	38ım	58hm	70
	0.5	73hm	83gm	88gm	0m	26jm	0m	25jm	13lm	26jm	67hm	40
	1.0	48hm	77hm	155dm	502a	32jm	51hm	38ım	0m	52hm	119fm	108
	2.0	72hm	181cm	0m	108fm	0m	0m	60hm	25jm	21jm	0m	47
KIN	0.2	147fm	89gm	0m	149fm	17lm	32jm	274bg	47hm	101gm	49hm	91
	0.5	28jm	0m	358ac	70hm	3m	58hm	200cl	18lm	81gm	49hm	87
	1.0	114fm	0m	171cm	0m	17lm	87gm	24jm	18lm	55hm	92gm	58
	2.0	68hm	0m	351ad	68hm	18lm	90gm	48hm	88gm	90gm	134fm	95
Avg.		58ce	107bc	175a	81be	34e	42de	90bd	128ab	80be	58ce	
$LSD_{0.05\ (cytokinin)}$: Ö.D.					$LSD_{0.05\ (auxin)}$: 53.9				$LSD_{0.05\ (cytokinin\ xauxin)}$: 194.96			
Petiole												
K	0	21no	168eo	272bı	8no	5o	20no	19no	44mo	21no	3o	58
BAP	0.2	141go	230cl	0o	159fo	69ko	76ko	60lo	51mo	49mo	10no	85
	0.5	137go	129go	0o	42mo	0o	40mo	69ko	142go	65lo	8no	63
	1.0	38mo	200dm	0o	141go	0o	13no	147go	91jo	66ko	13no	71
	2.0	65lo	125go	0o	31mo	19no	53lo	63lo	122ho	67ko	30mo	57
TDZ	0.2	74ko	350ad	65lo	153go	30mo	244ck	35mo	30	83jo	51mo	109
	0.5	258cj	124go	446ab	19no	26mo	63lo	40mo	12no	29mo	0o	102
	1.0	138go	126go	450ab	456a	9no	59lo	42mo	7o	32mo	23mo	134
	2.0	92jo	87jo	274bı	118ho	2o	98ıo	21no	23mo	42mo	23mo	78
KIN	0.2	71ko	295ah	338ae	125go	0o	81jo	103ıo	19no	52lo	5o	109
	0.5	83jo	127go	301ag	406ac	6o	14no	122ho	54lo	50mo	12no	117
	1.0	82jo	75ko	140go	139go	6o	7o	37mo	23mo	23mo	26mo	56
	2.0	54lo	10no	337af	186dn	48mo	53lo	100ıo	10no	52lo	43mo	89
Avg.		96b	157a	202a	153a	17c	63bc	66bc	46bc	49bc	19c	
$LSD_{0.05\ (cytokinin)}$: Ö.D.					$LSD_{0.05\ (auxin)}$: 49.19				$LSD_{0.05\ (cytokinin\ xauxin)}$: 177.57			
Cotyledon												
K	0	0ı	70ı	0ı	18ı	25ı	0ı	38ı	12ı	40ı	110hı	31f
BAP	0.2	275eı	261eı	1318b	292eı	67ı	78hı	17ı	58ı	49ı	43ı	246bd
	0.5	318eı	670cf	889bd	294eı	165gı	55ı	112hı	93hı	85hı	78hı	276bc
	1.0	238fı	106hı	914bd	377eı	0ı	14ı	50ı	145hı	58ı	21ı	192be
	2.0	61ı	98hı	325eı	270eı	7ı	135hı	141hı	55ı	101hı	73ı	127cf
TDZ	0.2	737ce	636cg	2179a	320eı	59ı	81hı	104hı	54ı	76ı	68ı	431a
	0.5	382eı	0ı	171gı	368eı	271eı	86hı	99hı	60ı	87hı	102hı	163bf
	1.0	225fı	565ch	246fı	1308b	80hı	147hı	72ı	72ı	95hı	88hı	290ab
	2.0	247fı	396eı	449dı	949bc	77ı	146hı	65ı	28ı	97hı	148hı	260bc
KIN	0.2	47ı	22ı	197fı	305eı	104hı	0ı	27ı	62ı	28ı	23ı	81ef
	0.5	0ı	0ı	380eı	93hı	66ı	37ı	52ı	162gı	85hı	91hı	97df
	1.0	8ı	58ı	178gı	0ı	7ı	47ı	72ı	28ı	58ı	84hı	54ef
	2.0	0ı	0ı	218fı	0ı	18ı	0ı	100hı	57ı	39ı	0ı	43ef
Avg.		195cd	222bc	574a	353b	73d	64d	73d	68d	69d	71d	
$LSD_{0.05\ (cytokinin)}$:153.27					$LSD_{0.05\ (auxin)}$:134.45				$LSD_{0.05\ (cytokinin\ xauxin)}$: 485.21			
Root												
K	0	0m	11lm	65km	0m	8lm	13lm	17lm	58km	26lm	16lm	21e
BAP	0.2	102hm	186em	311dı	35lm	60km	42lm	36lm	100hm	50km	23lm	95bd
	0.5	219dm	549ac	406bd	117hm	7lm	50km	17lm	75jm	41lm	21lm	150ab
	1.0	98ım	337cg	370be	158em	11lm	24lm	17lm	48km	32lm	38lm	113bc
	2.0	163em	264dk	76jm	588ab	0m	54km	12lm	59km	39lm	30lm	129ab
TDZ	0.2	288dj	153em	100hm	191dm	43lm	56km	18lm	26lm	40lm	62km	98bd
	0.5	318dh	62km	137gm	223dl	63km	108hm	35lm	34lm	44lm	0m	102bd
	1.0	665a	362cf	67km	642a	36lm	49km	8lm	23lm	25lm	20lm	190a
	2.0	170em	115hm	54km	370be	39lm	49km	22lm	23lm	34lm	41lm	92bd

Table 2 (*continued*)

mg l^{-1}		NAA					2,4-D					
		0.2	0.5	1.0	2.0	4.0	0.2	0.5	1.0	2.0	4.0	Avg.
Leaf												
KIN	0.2	13lm	33lm	150fm	59km	17lm	13lm	13lm	19lm	16lm	19lm	35de
	0.5	23lm	10lm	70jm	1m	0m	0m	13lm	20lm	15lm	26lm	18e
	1.0	76jm	166em	67km	30lm	17lm	19lm	14lm	16lm	19lm	26lm	45ce
	2.0	170em	61km	145fm	10lm	33lm	7lm	32lm	23lm	20lm	17lm	52ce
Avg.		177a	187a	155a	186a	26b	37b	20b	40b	31b	26b	
$LSD_{0.05\ (cytokinin)}$: 69.12					$LSD_{0.05\ (auxin)}$: 60.62				$LSD_{0.05\ (cytokinin\ xauxin)}$: 218.59			

in the induction of callus from the leaf explants *E. purpurea*. Lu [41] reported that TDZ promotes cell division and the growth of callus tissues. Similarly, Yorgancilar and Erisen [42] obtained the maximum callus weight in NAA and TDZ combinations in the callus tissues derived from the petiole explant. The highest value in terms of callus weight was determined in the callus tissues derived from the cotyledon among other explant types. Just as it was the case in callus induction, the fresh callus weight varies depending on the genotype, the type of growth regulator, and the type of explant of the plant. In the study, in which different explant types were compared in terms of callus weight, the highest callus weight value was determined in the callus derived from cotyledon among the leaf, hypocotyl and cotyledon explants [43]. Rabie et al. [44] obtained the maximum biomass in the cotyledon explant among different explant types of *E. purpurea.* No studies were detected on the effectiveness of TDZ on the stimulation of callus and biomass from the root explant in Echinacea species. Zeng et al. [45] and Khan et al. [46] reported that callus weight with the increase in TDZ concentration in callus induction and biomass from root explant was conducted for similar purposes in different plant species. The reason for the increase in growth parameters in callus cultures induction by TDZ was associated with the ability of TDZ to trigger the production of purine cytokines for advanced cellular growth [47].

In the starting of callus cultures in different explant types, the purpose was to establish an effective and repeatable protocol, to determine the growth regulator that would provide the best callus development in each explant, and to determine the effect of these growth regulators on caffeic acid derivative amounts in callus tissues. For this purpose, it was aimed to reach the maximum biomass by determining the growth regulator that provides optimum callus development in terms of both callus weight and callus formation percentage values for each explant type. Thus, the callus efficiency value was calculated for each explant with the equation of "callus weight × callus formation rate/100", and the type and concentration of the growth regulator with the highest efficiency value for the explant in question were determined according to this result.

When callus efficiency values were evaluated for each explant type, the growth regulator combinations that provided the best development of callus were determined as follows; 1.0 mg l^{-1} 2. 4-D + 2.0 mg l^{-1} BAP in the leaf; 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} TDZ in the petiole; 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} TDZ in the cotyledon; 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP in the root; and these growth regulator combinations continued for optimizing the culture times for the production of caffeic acid derivatives in callus (Fig. 3).

3.2. Analysis of caffeic acid derivatives

After the growth regulator type and concentration that responded the best to callus development in four different explants (leaf, petiole, cotyledon, root) were determined as a result of the callus cultures, the amounts of caftaric acid, chlorogenic acid, caffeic acid, and cichoric acid amounts in the callus tissues obtained from different explants of the plant in these growth regulators were determined at the end of different cultural times (Table 3).

When the Table 3 is examined, it is seen that caffeic acid derivatives were not detected in the callus obtained from other explants aside from the root after a 4-week culture time. With prolonged culture time, the caftaric, chlorogenic and cichoric acid amounts increased in the callus obtained from all explants; however, no caffeic acid was detected in any of them. The highest caftaric acid and cichoric acid amounts (4.11 and 57.89 mg/g, respectively) were obtained from the petiole explants at the end of the 10-week culture time; and the highest chlorogenic acid amount (8.83 mg/g) was obtained in the callus obtained from the root explants at the end of the 10-week culture time. When evaluated in general terms, the highest caffeic acid derivative amounts were detected in the callus obtained from petiole and root explants at the end of the 10-week culture time (Fig. 4).

In the present study, the highest cichoric acid amounts were detected in the callus obtained from roots and petiole explants (30.82 and 57.89 mg/g, respectively) at the end of the 10-week culture time.

Callus induction from the root explant was carried out in the medium that contained 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP, which gave the best callus efficiency, and callus induction from the petiole explant was carried out in the medium that contained 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} TDZ. Cichoric acid is the characteristic component of *E. purpurea,* and is often found in roots and vegetative parts [48]. Achieving the highest cichoric acid amounts in the root and petiole-induced callus show that the tissue part where the culture of callus is started is very important in the production of secondary metabolites. Ramezannezhad et al. [31] determined the cichoric acid content as 5.63 mg/g in the callus they obtained at the end of 8-week culture time of the root explants of *E. purpurea* in 2.0 mg/l 2.4-D + 1.0 mg/l in modified 1/2 MS medium that contained KIN. The type and concentration of the applied growth regulator and the content of the medium significantly affect secondary metabolite production under *in vitro* conditions [27,49]. In addition, culture times revealed serious changes in secondary metabolite production, especially in callus and cell suspension cultures [50,51]. Demirci et al. [52] investigated the effects of methyl jasmonate (MeJA) applications on the accumulation of caffeic acid derivatives in shoots and roots in *E. purpurea in vitro* conditions at different culture times, and found that increased culture time and MeJA applications increased caffeic acid derivatives in both shoot and roots compared to controls. Among the caffeic acid derivatives, cichoric acid, which is the characteristic component of *E. purpurea*, yielded the highest amount (54.87 mg/g) from the roots in 100 μM MeJA application after 45-day culture time. Both increased MeJA concentration and long culture time caused stress factors resulting in an increase in cichoric acid amount. No studies were found in the literature determining the quantities of caffeic acid derivatives in petiole-derived callus of *E. purpurea*. Taha et al. [27] obtained the highest cichoric acid amount (5.12 mg/g)

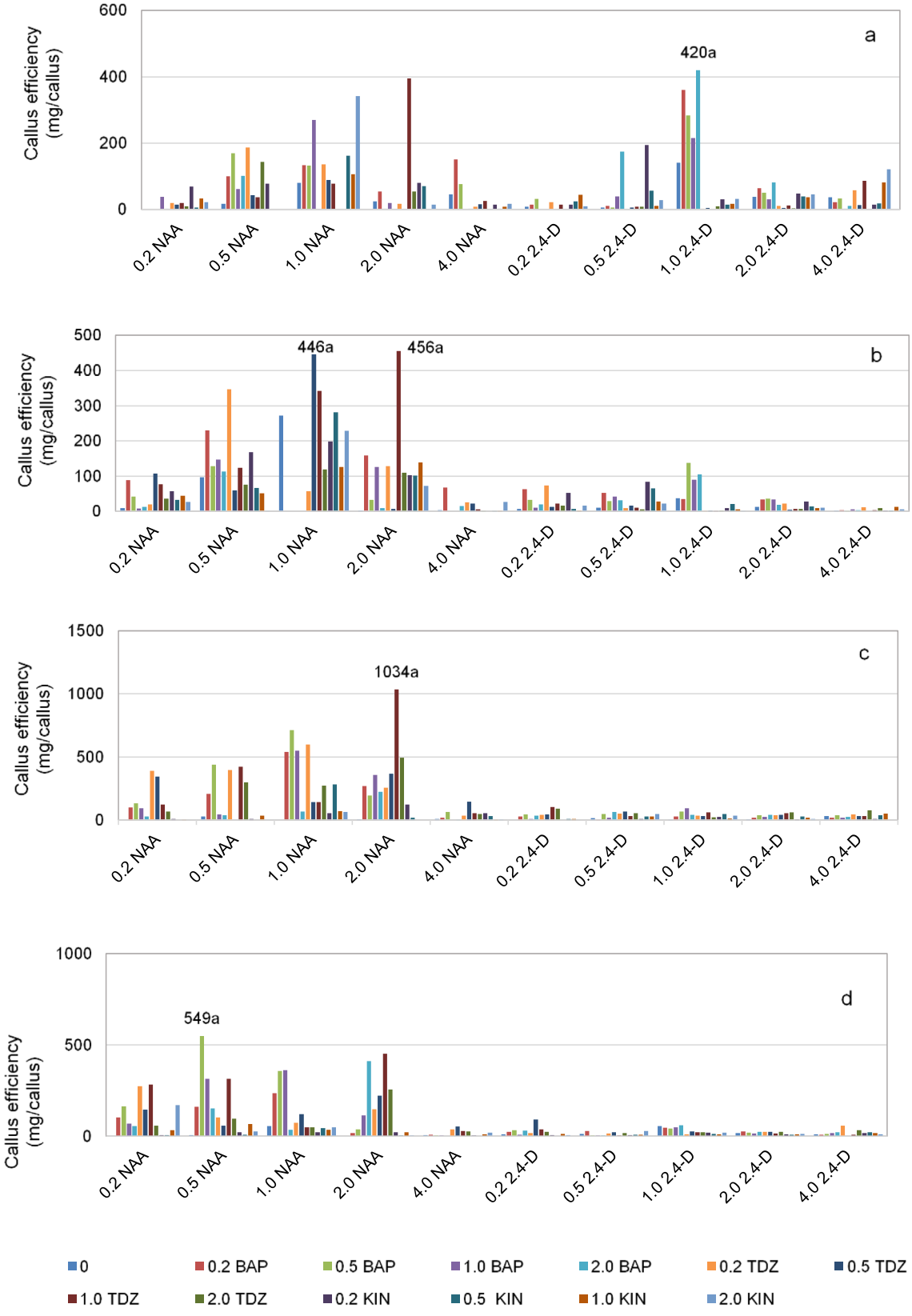

Fig. 3. a) Leaf b) petiole c) cotyledon d) root explant callus efficiency (mg/callus).

Table 3
Amounts of caffeic acid derivatives (mg/g) in callus obtained from different explant types at different culture times.

Culture times (Weeks)	Explant types	Caftaric acid	Chlorogenic acid	Caffeic acid	Cichoric acid
4	Root	–	0.07 ± 0.01	–	0.09 ± 0.00
	Petiole	–	–	–	–
	Leaf	–	–	–	–
	Cotyledon	–	–	–	–
6	Root	–	0.05 ± 0.00	–	0.06 ± 0.00
	Petiole	0.21 ± 0.00	0.08 ± 0.00	–	0.65 ± 0.22
	Leaf	–	–	–	–
	Cotyledon	–	–	–	–
8	Root	1.31 ± 0.02	0.63 ± 0.00	–	5.90 ± 0.24
	Petiole	0.98 ± 0.00	0.29 ± 0.00	–	2.56 ± 0.00
	Leaf	–	–	–	–
	Cotyledon	1.00 ± 0.01	0.70 ± 0.01	–	3.21 ± 0.01
10	Root	2.91 ± 0.01	8.83 ± 0.07	–	30.82 ± 1.14
	Petiole	4.11 ± 0.00	4.17 ± 0.02	–	57.89 ± 0.03
	Leaf	–	0.23 ± 0.03	–	–
	Cotyledon	1.78 ± 0.10	3.52 ± 0.18	–	9.87 ± 0.20

in petiole-derived callus of *E. angustifolia*, which is a different type of Echinacea, at different 2.4-D and KIN concentrations at the end of culture time of 4 weeks in 3.0 mg l^{-1} 2.4-D + 1.0 mg l^{-1} KIN. It is considered that the difference between the results of this study and the results of our study was due to the plant type, the type and concentration of the growth regulator, and the duration of the culture.

The highest amounts of chlorogenic acid were obtained in petiole and root-induced callus at the end of a 10-week culture time (4.17, 8.83 mg/g, respectively). Ramezannezhad et al. [31] obtained the highest chlorogenic acid amount (4 mg/g) in root-derived callus in the leaf and root-derived callus in modified 1/2 MS medium that contained 2.4-D and KIN at different concentrations in 8 weeks. Prolonged stays in culture created stress factor and caused the amounts of secondary metabolites to increase. Similar results were reported in other plant species by applying different culture periods under *in vitro* conditions in increasing the production of secondary metabolites [53,54].

The highest amounts of caftaric acid were obtained in petiole and root-induced callus at the end of the 10-week culture time (4.11 and 2.91 mg/g, respectively). No studies were detected in the literature review examining the amount of caftaric acid in the callus *of E. purpurea*. Wu et al. [30,55] determined the highest amount of caftaric acid (4.35 mg/g) in the adventive root cultures derived from the roots of *E. purpurea* in 1/2 MS medium that contained IBA 2.0 mg l^{-1} at the end of a 5-week culture time. These results show that the root explant is an accurate starting point to increase caftaric acid production under *in vitro* conditions.

Caffeic acid could not be detected in different explants of *E. purpurea* in any callus obtained at different cultural times. Caffeic acid, which is found in very small amounts in *E. purpurea,* was not affected by different explant growth regulators and culture times. However, Ramezannezhad et al. [31] detected 6.95 mg/g caffeic acid in root-derived callus obtained in 1/2 MS medium that contained 0.1/0.5 mg l^{-1} KIN + 2.0 mg l^{-1} 2.4-D. This result shows that caffeic acid production increases at a significant level in the combination of 2.4-D and KIN and in 1/2 MS. The growth regulator combinations applied in our study, the media, and the culture time were not found to be suitable for caffeic acid production.

When evaluated in general, maximum amounts of caftaric acid, chlorogenic acid, and cichoric acid were obtained at the end of the 10-week culture period in callus tissues obtained from the roots and petiole explants of *E. purpurea.* In previous studies conducted so far on *E. purpurea,* generally similar results were achieved in hairy and adventive root cultures as a result of different growth regulators and stress practices [19,29,56].

4. Conclusions

Maximum callus response was obtained by successfully induction callus with different growth regulator combinations in each of the leaf, petiole, cotyledon and root explants of *E. purpurea*. The type and concentration of growth regulator at which the highest callus efficiency value was obtained for each explant type was determined. In these growth regulators, the effects of different culture times on the amount of caffeic acid derivatives were determined in callus obtained from different parts of the plant. According to the results obtained here, the highest amount of caftaric acid (4.11 mg/g), chlorogenic acid (8.83 mg/g) and cichoric acid (57.89 mg/g) amounts were obtained in the callus of the petiole and root explants of *E. purpurea* at the end of a 10-week culture period. When evaluated in general terms, the amounts of caffeic acid derivatives increased in *E. purpurea* with increasing culture time.

As a result of the present study, the production of caffeic acid derivatives was performed by providing the optimization of *E. purpurea* callus cultures. In this way, infrastructure was created for the efforts to increase caffeic acid derivatives under *in vitro* conditions. Also, a standard was formed for mass production in an industrial sense.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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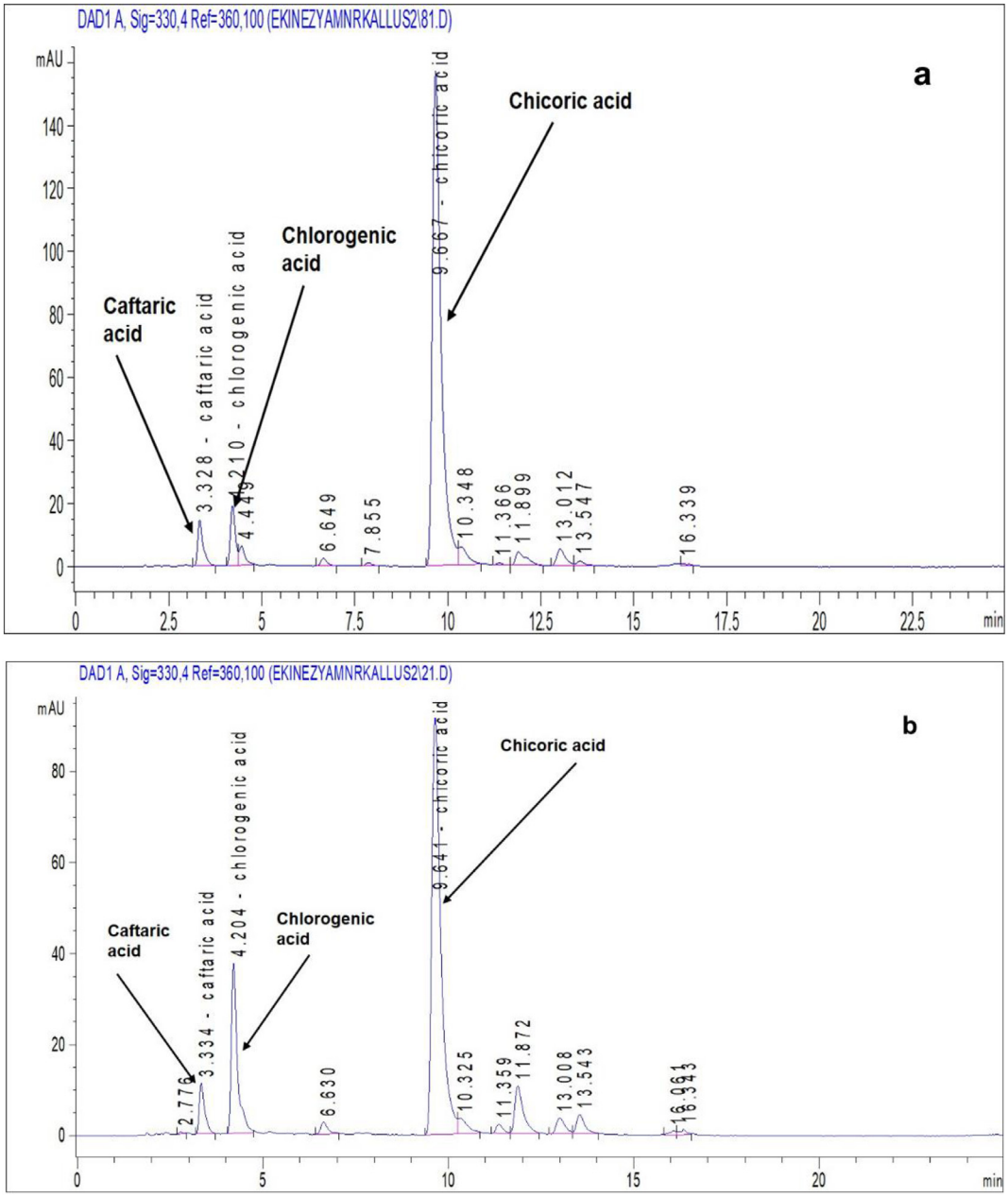

Fig. 4. a) HPLC chromatogram of callus tissues obtained from petiole explants at the end of the 10-week culture period b) HPLC chromatogram of callus tissues obtained from root explants at the end of the 10-week culture period.

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