L-Phenylalanine Applications and Culture Duration Affect Root Growth and Production of Tropane Alkaloids and Phenolics in Adventitious Root Cultures of *Hyoscyamus Niger* L.

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Abstract

This study was aimed to determine the effects of different concentrations of L-phenylalanine (0.25, 0.50 and 1.00 mM L-Phe) applied to adventitious root cultures for different culture durations on root growth and production of tropane alkaloids and phenolics in *Hyoscyamus niger*. For this purpose, adventitious roots obtained from petiole explants of *in vitro* seedlings were used as plant materials. After adventitious roots were cultured in liquid MS medium, L-Phe was applied to the adventitious roots. Then the roots were harvested on the 1st, 3rd, and 7th day of the applications, fresh and dry root weights, root growth indexes were analysed. It was also examined the contents of tropane alkaloids (hyoscyamine and scopolamine) and phenolics (gallic acid, catechin, epicatechin, vanillin, cinnamic acid, rosmarinic acid, p-coumaric, o-coumaric acid, rutin, caffeic acid, ferulic acid, chlorogenic acid, and quercetin) by HPLC in both adventitious roots and culture medium. As a result, the greatest root growth values were obtained from the roots harvested on the 3rd and 7th days after 0.50 and 1.00 mM L-Phe applications. The maximum scopolamine and hyoscyamine amounts were detected in the cultures with the application of 0.50 mM L-Phe for 3 days. In terms of phenolics, 0.50 mM and 1.00 mM L-Phe application with 1st and 3rd days were selected as the most appropriate L-Phe applications and culture duration. Consequently, it has been determined that *in vitro* secondary metabolite production may be increased in *H. niger* root cultures with the selection of appropriate L-Phe concentrations and culture durations.

Introduction

Hyoscyamus niger L. is a species belonging to the Solanaceae family, which contains very important tropane alkaloids such as hyoscyamine, apohyoscine, apohyoscine, scopolamine, skimmianine, apoatropine, a-belladonnine, b-belladonnine, and tropine. The most important and most abundant ones in *H. niger* roots are hyoscyamine and scopolamine (Al-Snafi 2018). Hyoscyamine and scopolamine use in the treatment of stomach pain, ulcer, kidney, and liver diseases due to their pain-relieving and spasm-resolving effects (John et al. 2010). The economic values of these metabolites are quite high since they have important biological activities, and are found in very low amount in plants. The long and complexity of the biosynthesis pathways make it difficult to the obtaining of these alkaloids synthetically, economically and practically, so the use of wild-collected plants for getting these alkaloids remains a common method today (Dehghan et al. 2012). However, the continuous collection of plants, especially those containing root-derived metabolites, brings with it the danger of extinction. Besides, the infestation of plants by microorganisms, plant production dependent on the seasons, lack of pure and standard plant material, geographical difficulties, and ecological differences prevent the secondary metabolite production of standard quality by collecting from nature (Dörnenburg and Knorr 1997).

In vitro secondary metabolite production techniques create an important potential day by day; to eliminate all the negativities in traditional secondary metabolite isolation by wild-collected plants (Jones et al. 2009). Besides, *in vitro* secondary metabolite production enables many applications that cannot be applied in nature to be carried out within *in vitro* conditions to increase the metabolite efficiency to much higher levels. Changes in culture medium, conditions, application of biotic and abiotic elicitors, as well as

the addition of precursors or intermediates in the biosynthesis pathways are among the approaches that can be applied to increase the *in vitro* production of commercial metabolites such as alkaloids and phenolics (Demirci et al. 2021; Pitta-Alvarez et al. 2000).

In *H. niger*, few studies have been conducted to increase the synthesis of tropane alkaloids with *in vitro* conditions, and all these studies focused on the callus, cell suspension cultures or *in vitro* plantlets (Aljibouri et al. 2012; Ghorbanpour et al. 2013a, 2013b). However, *in vitro* root cultures in many plant species containing root-derived metabolites such as *H. niger* are used effectively to produce many metabolites, including tropane alkaloids and phenolics (Moharrami et al. 2017).

L-Phe, is an aromatic amino acid, commence the biosynthesis of various compounds produced from the pathways of phenylpropanoid and flavonoid in cells. A small number of previous studies have stated that L-Phe can increase secondary metabolite production as an inexpensive and effective application for *in vitro* conditions (Demirci et al. 2020, 2021; Koca and Karaman 2015). It is known that the biosynthesis of hyoscyamine and scopolamine is initiated by the transformation of L-Phe to phenylpyruvate with aromatic amino acid aminotransferase (Bedewitz et al. 2014). Many studies have included applications that generally create stress signals in plants to increase the production of hyoscyamine and scopolamine (Ghorbanpour et al. 2013a; Hong et al. 2012). However, studies on precursors in the biosynthesis pathway of tropane alkaloids are almost non-existent. For this reason, L-Phe was used as a precursor in this research to increase tropane alkaloids and phenolics in adventitious root cultures. The culture duration is an important criterion that changes the amount of metabolite yield for *in vitro* secondary metabolite production (Shakeran et al. 2015). So determining the most appropriate culture duration is of great importance in terms of obtaining high metabolite yield.

This study, it was aimed to determine the effects of L-Phe application at different concentrations and culture duration on the root growth development, accumulation of tropane alkaloids and phenolics. In addition, phenylalanine ammonia lyase (PAL) activity was also determined to clarify how the applications made changed PAL activity and to reveal the relationship between PAL activity and secondary metabolite production. With this study, L-phenylalanine was used for the first time to increase the yield in the production of hyoscyamine and scopolamine from *in vitro H. niger* root cultures. It provides important information for future work.

Method

Plant material

In this study, adventitious roots derived from petiole explants of *in vitro* seedlings of *H. niger* were used as plant materials. The seeds were obtained from Zeytinburnu Municipality Medicinal and Aromatic Plants Garden Directorate, Istanbul, Turkey.

Obtaining adventitious root cultures

In order to overcome dormancy and initiate germination, seeds were kept in 250 mg l^{-1} gibberellic acid (GA_3, Sigma-Aldrich, Germany) solution for 48 h before being transferred to the culture medium (Ghorbanpour et al. 2013b). After GA_3 application, seeds were rinsed 4 times with double distilled water (ddH_2O) and mixed with 70% ethyl alcohol (%99, Tekkim, Turkey) for 10 s. Then seeds were sterilized with 0.1% mercury chloride ($HgCl_2$, Sigma-Aldrich, Germany) solution containing a few drops Tween 20 (Sigma-Aldrich, Germany) for 10 min. Finally, they were rinsed 4 times with sterile ddH_2O and transferred to a germination medium (Aljibouri et al. 2012).

Murashige and Skoog (MS) medium (Duchefa Biochemie, Netherland) containing 30 g L^{-1} sucrose (Sigma-Aldrich, Germany) and 6 g l^{-1} agar (Sigma-Aldrich, Germany) were used for the germination of seeds (Murashige and Skoog 1962). Ten seeds were transferred to each of the sterile Petri dishes containing 30 ml of MS medium and cultured for 15 days at 25°C in the dark (Fig. 1-A) (Aljibouri et al. 2012). After the 15-day germination period, the plantlets that emerged from the seed were transferred to 50 ml of the same medium in 250 ml flasks. Then they were grown for 15 more days in a photoperiod of 16 h light / 8 h dark, 50 µmol / m^2 fluorescent light intensity at 25°C. The petiole segments of 1 cm length cut with a sterile scalpel from the plantlets were transferred to MS medium containing 2 mg l^{-1} Indole-3-butyric acid (IBA, Sigma-Aldrich, Germany), 30 g l^{-1} sucrose, 6 g l^{-1} agar and were cultured for 6 weeks at 25°C for adventitious root formation (Fig. 1-B, 1-C) (Hong et al. 2012). To reproduce the adventitious roots formed at the end of six weeks, they were transferred to 30 ml liquid MS mediums containing 2 mg l^{-1} Indole-3-butyric acid and 30 g l^{-1} sucrose in 100 ml Erlenmeyer (Fig. 1-D). Liquid root cultures were grown at 25°C, dark conditions, on an orbital shaker at 90 rpm rotational speed for 3 week periods. Subculture was applied 3 times at 3-week intervals.

L-Phe applications

Roots propagated in liquid MS medium were weighed 1.5 grams in sterile conditions with the help of analytical scales and transferred to another liquid MS medium containing 0.5 mg l^{-1} IBA and 30 g l^{-1} sucrose. After culturing at 25°C for 3 weeks, the roots were used in the L-Phe (Sigma-Aldrich, Germany) applications. The stock solution of L-Phe was prepared with distilled water and filter-sterilized. Then L-Phe at 0.25, 0.50, and 1.00 mM concentrations were added to the medium with 3% Tween-20 which has been reported to have positive effects on the accumulation of tropane alkaloids (Boitel-Conti et al. 2000). To the control group, only tween 20 was added. A completely randomized design was used during the applications and analysis (4 application x 3 replication x 4 flasks). Adventitious roots were harvested on the 1st, 3rd and 7th days after the applications. At the harvest, culture mediums were transferred to amber bottles and stored at -20°C and adventitious roots were reserved for later analysis.

Determination of root growth parameters

Roots, harvested on the 1st, 3rd and 7th days, were washed with ddH_2O and excess water was removed with blotting paper. Then fresh weights of adventitious roots were weighted with analytical scales and presented as g 100 ml^{-1}. Growth indexes (GI) were calculated with the formula:

GI: (harvested FW (g) - inoculated FW (g)) / inoculated FW (g)

Then roots were dried at 40°C for 72 h and weighted with the help of an analytical balance. The dried roots weights were expressed as g 100 ml^{-1}.

Extraction of tropan alkaloids and phenolics from adventitious roots and culture medium

For the extraction from adventitious roots, completely dried roots were powdered with the help of mortar and pestle. Then, 200 mg of powdered roots were extracted with 20 ml of methanol (HPLC grade, Sigma Aldrich, Germany) - ddH_2O (60:40 (v/v)) solution in an ultrasonic water bath for 15 min. After the mixture was centrifuged at 10.000 rpm for 15 min, the liquid fraction was collected in a separate test tube. The same processes were repeated twice by adding 20 ml of methanol-ddH_2O mixture to the pelleted part again. The liquid fractions were combined and evaporated under pressure at 45°C with the aid of a rotary evaporator to obtain dry extracts. After evaporation, dried extracts were dissolved in 1.5 ml pure methanol and passed through 0.45 µm filters and stored at -20 ° C until used in the analysis.

The extraction of tropane alkaloids from the culture mediums was done according to the method of Boitel-Conti et al. (2000). Accordingly, after adding 3 ml of 28% ammonium hydroxide (NH_4OH, Sigma-Aldrich, Germany) to 40 ml of filtered culture medium, the alkaloids were extracted 3 times with 30 ml of chloroform. Then, chloroform was removed in a rotary evaporator and the residue was dissolved with 2 ml of methanol After filtration, extracts were used in HPLC analysis.

The phenolic contents in the culture medium were performed according to the method of Vuong et al. (2014). Shortly, 10 ml of 100% ethyl acetate was added to 10 ml of culture medium and mixed rapidly for 5 min. The mixture was kept immobile for 30 min at room temperature, allowing the ethyl acetate phase to be separated from the culture medium. Then, the phase was stored in a separate bottle. The same process was repeated two more times by adding ethyl acetate into the culture medium. After the extraction process was completed, the ethyl acetate was completely evaporated. The residue was then dissolved in 1.5 ml methanol and filtered by 0.45 µm pore size membrane filters (Millipore Co. Bedford, MA), and then used in HPLC analysis.

Determination of tropane alkaloids and phenolics by HPLC

In order to detect tropan alkaloids, an HPLC system manufactured by Shimadzu Corp. (Kyoto, Japan) was used. The separation of hyoscyamine and scopolamine was carried out by Agilent Eclipse XDB-C18 HPLC column (250 × 4.6 mm i.d. 5 µm, Wellborn, Germany). In the separation process performed with the gradient program at 0.8 ml min^{-1} flow rate, the injection volume was 20 µl, the column temperature was set at 40°C, 2% acetic acid (mobile phase A, HPLC grade, Sigma-Aldrich) and 100% acetonitrile (mobile phase B, HPLC, Sigma-Aldrich) were used according to modify the method by Boitel-Conti et al. (2000). The gradient program was as follows; 0–12 min, 0–12% B; 12–13 min, 12–20% B; 13–33 min, 20–28% B; 33–48 min, 28–100% B. Scopolamine and hyoscyamine analytical standards obtained from Sigma-

Aldrich were prepared in different dilutions and run by HPLC. The correlation coefficient (R^2) for scopolamine was found to be 0.9887 and for hyoscyamine 0.9981. The results of HPLC runs on roots and culture mediums were calculated as mg g^{-1} dry root weight, mg 100 ml^{-1} in culture mediums and mg per flask.

The amounts of gallic acid, catechin, chlorogenic acid, caffeic acid, epicatechin, vanillin, rosmarinic acid, *p*-coumaric acid, *o*-coumaric acid, ferulic acid, rutin, cinnamic acid and quercetin in root and culture medium were determined by the same HPLC system and conditions. The gradient elution; mobile phase A contained 2% Acedic acid (HPLC, Sigma-Aldrich), solvent B contained Methanol (HPLC, Sigma-Aldrich). The following gradient was used: 0–12 min, 0–12% B; 12–13 min, 12–20% B; 13–33 min, 20–28% B; 33–48 min, 28–30% B; 48–53 min, 30–38% B; 53–68 min, 38–40% B; 68–70 min, 40% B; 70–90 min, 40–50% B; 90–105 min, 50–60% B; 105–107 min, 60–100% B; 107–112 min, 100% B; 112–117 min, 0% B (Demirci et al. 2021). During the determination of the amounts of phenolics by HPLC, analytical standards obtained from Sigma-Aldrich were used. The standards were prepared in different dilutions and run by HPLC and the analysis results were presented as mg g^{-1} dry root weight, mg 100 ml^{-1} culture medium, and mg per flask according to analytical standards.

For HPLC calculation of tropane alkaloids and phenolics, scopolamine and hyoscyamine examined at 220 nm; gallic acid, catechin, epicatechin, vanillin, rosmarinic acid, cinnamic acid, and o-coumaric acid at 278 nm; p-coumaric acid at 309 nm; caffeic acid and ferulic acid at 320 nm; chlorogenic acid at 325 nm; routine and quercetin at 370 nm (Supplementary Material 1). HPLC was run on all flasks at least 2 times to examine the amounts of tropane alkaloids and phenolics in adventitious roots, culture medium and per flask (4 application x 3 replication x 4 flasks x 2 runs). HPLC data were calculated with Shimadzu Class-VP Chromatography Laboratory Automatic Software System.

Determination of total phenolic content (TPC)

TPCs of the adventitious roots and culture medium were determined using Folin–Ciocalteu method (Singleton and Rossi 1965). The absorbances of the extracts were measured at 765 nm by a T70 Plus Dual Beam spectrophotometer. Total phenolic contents in adventitious roots and culture medium were calculated by the calibration curve prepared according to the gallic acid analytical standard.

Determination of phenylalanine ammonia lyase (PAL) activity

For the analysis of PAL activity, 0.5 g of fresh adventitious roots were extracted in 50 mM K-phosphate buffer (pH = 7) containing 1.0 mM EDTA and 1% polyvinylpyrrolidone. The supernatant of the enzyme extract, which was centrifuged at 10,000 x g for 15 min at 4°C, was collected for analyses. The determination of PAL enzyme activity was carried out according to the method of D'Cunha et al. (1996). Absorbances at 290 nm were determined as a measure of trans-cinnamic acid produced.

Statistical analyses

Data were subjected to analysis of variance with mean separation by Duncan's multiple range tests. Differences were considered statistically significant at the $p \leq 0.05$ levels.

Results

Effects on the roots growth parameters

The effects of L-Phe applied to *H. niger* adventitious root cultures at different concentration and duration on fresh root weight, root growth index and dry root weight were examined, and the results are presented in Fig. 2. As a result of the variance analysis on the root growth parameters, it was found that the interactions of application*culture duration were not statistically significant. On the other hand, when the applications and culture duration were examined separately, there were significant differences ($p \leq 0.05$). It was determined that as L-Phe concentration and culture duration increased, root growth parameters parallelly increased. The greatest values of fresh root weights (11.33–11.64 g 100 ml^{-1}), growth indexes (1.27–1.33) and dry root weights (1.08–1.11 g 100 ml^{-1}) were obtained from the roots applied with 0.50 mM and 1.00 mM of L-Phe and harvested at 3rd and 7th days.

Effects on the production of tropane alkaloids

In order to understand the effects of L-Phe applications and culture durations on the production of tropane alkaloids, the amounts of hyoscyamine and scopolamine were determined. For this, the amount of alkaloids in adventitious roots and in the culture medium were determined separately, and then the amount of alkaloids obtained per flask was calculated. Applications and culture durations interactions were statistically significant ($p \leq 0,05$), according to scopolamine and hyoscyamine accumulations in adventitious roots, culture medium, and per flask (Supplementary Material 2). When the scopolamine amounts were examined, it was seen that the amount of scopolamine increased in parallel with the dose with 0.25 and 0.50 mM L-Phe applications both in the roots, in the culture medium and in the production amount per flask (Fig. 3). However, there was a decrease in the upward trend in scopolamine production with 1.00 mM L-Phe. Scopolamine was mostly obtained in adventitious roots with 0.50 mM L-Phe as 1.96 and 1.97 mg g^{-1} on the 1st and 3rd days, respectively, and 6.12 times more in the control group. Similarly, it was determined that the amount of scopolamine with 0.50 mM L-Phe in the culture medium was higher than the other groups, and there was approximately 1.5 times more scopolamine. When the quantities of scopolamine produced per flask were calculated, the highest amounts were again obtained with 0.50 mM L-Phe.

Hyoscyamine accumulation occurred similarly to scopolamine (Fig. 4). In adventitious roots, the highest hyoscyamine was obtained with 0.50 mM L-Phe as 0.59 mg g^{-1} on the 3rd day. Approximately 2 times more hyoscyamine was produced compared to control roots. This amount decreased statistically significantly when the culture duration was extended. The amount of hyoscyamine in the culture medium was statistically higher than the other groups at 0.25 mM L-Phe application. While culture duration did not affect the amount of hyoscyamine in the control group and 1.00 mM L-Phe application, 0.25 and 0.50

mM L-Phe applications increased the amount of hyoscyamine compared to the culture duration. The highest amount of hyoscyamine produced per flask was 0.23 and 0.20 mg flask^{-1} on the 3rd and 7th days, respectively, with 0.50 mM L-Phe application.

Effects on the production of phenolics

In this study, changes in phenolic contents in *H. niger* adventitious root cultures depending on L-Phe applications and culture durations were also investigated. For this purpose, TPC and gallic acid, catechin, epicatechin, vanillin, cinnamic acid, rosmarinic acid, p-coumaric, o-coumaric acid, rutin, caffeic acid, ferulic acid, chlorogenic acid, and quercetin amounts were determined in adventitious roots and culture medium. Rutin, *o*-coumaric acid and quercetin could not be detected in cultures while amounts of other phenolics were determined according to L-Phe applications and culture duration. In adventitious roots, the effects on the phenolics are presented in Table 1. In terms of TPC and all phenolics except chlorogenic acid, the application*culture duration interactions were statistically significant ($p \leq 0.05$). In this study, 0.5 and 1.00 mM L-Phe applications and 3-day culture duration had stimulating effects on the TPCs in adventitious roots. While all L-Phe applications increased TPC compared to the control, the lowest value was obtained from the control roots harvested on the 1st day. Accordingly, it was found that the most effective application*culture duration combination in terms of TPC was 0.50 mM L-Phe application for 3 days. According to HPLC results, the phenolic accumulations significantly changed depending on the L-Phe applications and culture duration.

Table 1
Effects of L-Phenylalanine application and culture duration on the phenolic contents of adventitious roots in *Hyoscyamus niger* (Rutin, o-coumaric acid and quercetin could not be detected)

Phenolics	Applications	Culture duration (days)			
		1st	3rd	7th	Mean
Total phenolic contents (mg g^{-1} DW)	Control	5.14 ± 0.06 Cc*	6.85 ± 0.08 Ac	5.75 ± 0.07 Bd	5.92
	0.25 mM L-Phe	7.37 ± 0.15 Ab	7.46 ± 0.12 Abc	6.34 ± 0.03 Bc	7.06
	0.50 mM L-Phe	7.86 ± 0.05 Ba	8.82 ± 0.23 Aa	8.10 ± 0.03 Ba	7.97
	1.00 mM L-Phe	8.01 ± 0.08 Aa	8.13 ± 0.17 Aab	7.77 ± 0.03 Ab	8.26
	Mean	7.10	7.81	6.99	
Gallic acid (µg g^{-1} DW)	Control	20.57 ± 0.01 Bd	24.38 ± 0.42 Ad	25.31 ± 0.73 Ac	23.42
	0.25 mM L-Phe	40.56 ± 0.47 Ac	38.11 ± 0.49 Bc	36.27 ± 0.65 Bb	38.32
	0.50 mM L-Phe	64.67 ± 0.19 Aa	65.08 ± 0.24 Aa	54.31 ± 0.60 Ba	61.36
	1.00 mM L-Phe	45.77 ± 0.49 Ab	40.87 ± 0.18 Bb	37.94 ± 0.80 Cb	41.53
	Mean	42.89	42.11	38.46	
Catechin (µg g^{-1} DW)	Control	162.69 ± 1.9 Bd	227.02 ± 3.3 Ad	220.60 ± 0.4 Ab	203.44
	0.25 mM L-Phe	773.28 ± 3.6 Ab	358.47 ± 30.5 Bc	244.61 ± 11.7 Cb	458.79
	0.50 mM L-Phe	626.96 ± 24.8 Cc	903.88 ± 34.8 Aa	788.52 ± 1.2 Ba	773.12
	1.00 mM L-Phe	863.45 ± 27.4 Aa	670.16 ± 7.3 Bb	176.99 ± 6.2 Cc	570.20
	Mean	606.59	539.88	357.68	
Epicatechin (µg g^{-1} DW)	Control	16.24 ± 0.54 Cd	28.18 ± 0.43 Bd	35.94 ± 1.95 Ab	26.79
	0.25 mM L-Phe	26.03 ± 1.12 Cc	39.74 ± 0.46 Ac	34.51 ± 1.03 Bb	33.43

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

Phenolics	Applications	Culture duration (days)			
		1st	3rd	7th	Mean
	0.50 mM L-Phe	67.35 ± 24.8 Ba	87.93 ± 0.56 Aa	87.41 ± 0.98 Aa	80.90
	1.00 mM L-Phe	40.68 ± 0.77 Ab	42.37 ± 0.28 Ab	30.52 ± 0.68 Bb	37.85
	Mean	37.57	49.55	47.09	
Vanillin	Control	9.72 ± 0.63 Ac	9.43 ± 0.76 Ac	9.67 ± 0.18 Ad	9.61
(μg g^{-1} DW)	0.25 mM L-Phe	12.02 ± 0.06 Bb	15.18 ± 0.95 Ab	16.43 ± 0.31 Ab	14.54
	0.50 mM L-Phe	22.38 ± 0.04 Ba	27.99 ± 1.35 Aa	24.63 ± 0.29 ABa	25.00
	1.00 mM L-Phe	11.96 ± 0.06 Cb	13.52 ± 0.10 Abc	12.82 ± 0.21 Bc	12.77
	Mean	14.02	16.53	15.89	
Cinnamic acid	Control	3.18 ± 0.58 Ad	4.22 ± 0.11 Ac	3.84 ± 0.01 Ab	3.74
(μg g^{-1} DW)	0.25 mM L-Phe	6.42 ± 0.43 Bc	11.32 ± 0.29 Ab	10.57 ± 0.67 Ba	9.43
	0.50 mM L-Phe	9.44 ± 0.25 Ab	10.49 ± 0.41 Ab	9.27 ± 0.23 Aa	9.73
	1.00 mM L-Phe	14.37 ± 0.07 Aa	14.37 ± 0.26 Aa	10.08 ± 0.21 Ba	12.94
	Mean	8.35	10.10	8.44	
Rosmarinic acid	Control	8.20 ± 0.06 Cc	12.80 ± 0.40 Aab	10.41 ± 0.53 Bb	10.47
(μg g^{-1} DW)	0.25 mM L-Phe	10.49 ± 0.70 Bbc	19.30 ± 2.83 Aa	12.92 ± 0.10 Aba	14.24
	0.50 mM L-Phe	20.43 ± 0.93 Aa	10.91 ± 0.47 Cb	13.88 ± 0.31 Ba	15.07
	1.00 mM L-Phe	12.98 ± 0.03 Ab	12.83 ± 0.07 Aab	12.76 ± 0.24 Aa	12.86
	Mean	13.02	13.96	12.49	
p-coumaric acid	Control	3.34 ± 0.08 Ac	3.86 ± 0.95 Ab	0.00 ± 0.00 Bb	2.40

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

Phenolics	Applications	Culture duration (days)			
		1st	3rd	7th	Mean
(μg g^{-1} DW)	0.25 mM L-Phe	16.16 ± 0.42Aab	4.33 ± 0.58 Bb	0.00 ± 0.00 Cb	6.83
	0.50 mM L-Phe	13.32 ± 0.73 Ab	10.27 ± 1.03 Ba	0.00 ± 0.00 Bb	7.86
	1.00 mM L-Phe	19.46 ± 1.38 Aa	9.51 ± 0.96 Ba	3.24 ± 0.21 Ca	10.74
	Mean	13.07	6.99	0.81	
Caffeic acid (μg g^{-1} DW)	Control	0.69 ± 0.01 Ab	0.00 ± 0.00 Ba	0.00 ± 0.00 Ba	0.23
	0.25 mM L-Phe	4.57 ± 0.07 Aa	0.00 ± 0.00 Ba	0.00 ± 0.00 Ba	1.52
	0.50 mM L-Phe	0.36 ± 0.01 Ac	0.17 ± 0.01 Ba	0.00 ± 0.00 Ca	0.17
	1.00 mM L-Phe	0.74 ± 0.04 Ab	0.70 ± 0.35 Aa	0.00 ± 0.00 Ba	0.47
	Mean	1.59	0.22	0.00	
Ferulic acid (μg g^{-1} DW)	Control	4.58 ± 0.37 Aa	0.15 ± 0.02 Bc	0.00 ± 0.00 Bb	1.58
	0.25 mM L-Phe	2.76 ± 0.30 Abc	0.00 ± 0.00 Bc	0.00 ± 0.00 Bb	0.92
	0.50 mM L-Phe	1.61 ± 0.16 Ac	1.00 ± 0.20 Ab	0.00 ± 0.00 Bb	0.87
	1.00 mM L-Phe	2.93 ± 0.17 Ab	2.35 ± 0.06 Aa	1.14 ± 0.17 Ba	2.14
	Mean	2.97	0.88	0.29	
Chlorogenic acid (μg g^{-1} DW)	Control	963.91 ± 26.58	1233.91 ± 33.72	1025.95 ± 4.81	1074.59 c
	0.25 mM L-Phe	1364.79 ± 17.95	1787.08 ± 58.46	1549.57 ± 5.92	1567.15 b
	0.50 mM L-Phe	1410.41 ± 30.59	1842.83 ± 69.59	1690.52 ± 9.26	1647.92 ab
	1.00 mM L-Phe	1595.09 ± 32.21	1887.17 ± 53.14	1672.15 ± 16.7	1718.14 a

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

Phenolics	Applications	Culture duration (days)			
		1st	3rd	7th	Mean
	Mean	1333.55 C	1687.75 A	1484.55 B	

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

The data concerning the effects of L-Phe and culture duration on leakage of phenolics from roots to culture medium were presented in Table 2. Interaction between applications and cultures durations was not found to be significant in terms of TPC. However, 0.5 mM L-Phe and 3rd day were the most affecting L-Phe application and culture duration for TPC in culture medium. When the amounts of other phenolics were examined, phenolics leaking into the culture medium varied depending on the L-Phe concentration.

Table 2
Effects of L-Phenylalanine (L-Phe) application and culture duration on the phenolics in culture medium (Rutin, *o*-coumaric acid and quercetin could not be detected)

Phenolics	Applications	Culture duration (days)			
		1st	3rd	7th	Mean
TPC (mg 100 ml^{-1})	Control	0.86 ± 0.03*	1.22 ± 0.01	0.95 ± 0.01	1.01 c
	0.25 mM L-Phe	1.08 ± 0.01	1.41 ± 0.12	1.27 ± 0.07	1.25 b
	0.50 mM L-Phe	1.27 ± 0.04	1.53 ± 0.01	1.36 ± 0.06	1.38 a
	1.00 mM L-Phe	1.15 ± 0.03	1.32 ± 0.03	1.20 ± 0.07	1.22 b
	Mean	1.09 C	1.38 A	1.19 B	
Gallic acid (µg 100 ml^{-1})	Control	0.00 ± 0.00 Ac	0.00 ± 0.00 Ac	0.00 ± 0.00 Ad	0.00
	0.25 mM L-Phe	0.00 ± 0.00 Bc	0.00 ± 0.00 Bc	11.56 ± 1.19 Ab	3.85
	0.50 mM L-Phe	24.23 ± 0.42 Aa	23.01 ± 0.47 Aa	20.47 ± 0.67 Ba	22.57
	1.00 mM L-Phe	4.38 ± 0.32 Ab	3.80 ± 0.14 ABb	3.39 ± 0.09 Bc	3.86
	Mean	7.15	6.70	8.85	
Catechin (µg 100 ml^{-1})	Control	75.16 ± 5.20 Bc	132.04 ± 6.50 Ac	86.01 ± 0.34 Bd	98.40
	0.25 mM L-Phe	170.86 ± 0.74 Ba	289.59 ± 1.99 Aa	123.92 ± 0.32 Cb	194.79
	0.50 mM L-Phe	108.58 ± 0.34 Cb	224.33 ± 4.26 Ab	199.11 ± 1.01 Ba	177.34
	1.00 mM L-Phe	119.44 ± 3.46 Ab	118.23 ± 1.91 Ac	97.81 ± 1.10 Bc	111.83
	Mean	119.01	191.05	126.72	
Epicatechin (µg 100 ml^{-1})[c]	Control	10.15 ± 0.28	14.59 ± 0.71	13.18 ± 0.21	12.64 b
	0.25 mM L-Phe	11.20 ± 0.13	11.84 ± 0.86	11.43 ± 0.54	11.49 b

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

Phenolics	Applications	Culture duration (days)			
		1st	3rd	7th	Mean
	0.50 mM L-Phe	31.50 ± 0.53	42.45 ± 1.06	33.37 ± 0.50	35.77 a
	1.00 mM L-Phe	40.19 ± 1.10	36.43 ± 0.53	27.02 ± 22.67	35.54 a
	Mean	23.26	26.33	21.25	
Vanillin (µg 100 ml^{-1})	Control	4.02 ± 0.41 Bb	5.56 ± 0.22 Ab	5.98 ± 0.33 Ab	5.18
	0.25 mM L-Phe	10.60 ± 0.73 Aa	12.27 ± 0.27 Aab	11.51 ± 0.37 Aa	11.46
	0.50 mM L-Phe	12.45 ± 0.63 Ba	18.30 ± 0.31 Aa	12.07 ± 0.09 Ba	14.27
	1.00 mM L-Phe	9.80 ± 1.54 Aa	6.74 ± 3.42 Bb	4.39 ± 2.19 Cb	6.98
	Mean	9.21	10.72	6.98	
Cinnamic acid (µg 100 ml^{-1})	Control	0.54 ± 0.01 Ac	0.47 ± 0.04 Ab	0.28 ± 0.01 Bc	0.43
	0.25 mM L-Phe	0.20 ± 0.01 Cc	0.53 ± 0.02 Bb	0.95 ± 0.04 Ac	0.57
	0.50 mM L-Phe	4.34 ± 0.14 Ab	6.05 ± 0.15 Aa	5.56 ± 0.77 Aa	5.35
	1.00 mM L-Phe	19.87 ± 0.80 Aa	7.25 ± 0.52 Ba	2.84 ± 0.25 Cb	9.99
	Mean	6.27	3.58	2.41	
Rosmarinic acid (µg 100 ml^{-1})	Control	8.34 ± 0.09 Ad	7.42 ± 0.30 Ad	2.96 ± 0.36 Bc	6.24
	0.25 mM L-Phe	14.22 ± 0.06 Ab	10.50 ± 0.08 Bc	14.30 ± 0.39 Ab	13.00
	0.50 mM L-Phe	12.55 ± 0.55 Bc	16.58 ± 0.92 Ab	17.78 ± 0.97 Aa	15.63
	1.00 mM L-Phe	20.14 ± 0.08 Aa	21.74 ± 0.52 Aa	17.84 ± 0.62 Ba	19.91
	Mean	13.81	14.06	13.22	

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

Phenolics	Applications	Culture duration (days)			
		1st	3rd	7th	Mean
p-coumaric acid (mg 100 ml^{-1})	Control	3.94 ± 0.19 Ab	2.11 ± 0.29 Bc	0.23 ± 0.01 Ca	2.09
	0.25 mM L-Phe	0.18 ± 0.00 Bc	1.06 ± 0.17 Ac	0.00 ± 0.00 Cb	0.41
	0.50 mM L-Phe	2.19 ± 0.12 Bb	4.44 ± 0.20 Ab	0.00 ± 0.00 Cb	2.21
	1.00 mM L-Phe	15.83 ± 0.76Aa	10.07 ± 0.75 Ba	1.92 ± 1.92 Ca	9.28
	Mean	5.53	4.42	0.54	
Caffeic acid (µg 100 ml^{-1})	Control	0.01 ± 0.00 Ac	0.00 ± 0.00 Bb	0.00 ± 0.00 Ba	0.005
	0.25 mM L-Phe	2.25 ± 0.11 Aa	0.00 ± 0.00 Bb	0.00 ± 0.00 Ba	0.75
	0.50 mM L-Phe	0.88 ± 0.03 Ab	0.00 ± 0.00 Bb	0.00 ± 0.00 Ba	0.29
	1.00 mM L-Phe	0.71 ± 0.18 Ab	0.87 ± 0.02 Aa	0.00 ± 0.00 Ba	0.53
	Mean	0.96	0.22	0.00	
Ferulic acid (µg 100 ml^{-1})	Control	0.00 ± 0.00 Ac	0.00 ± 0.00 Ab	0.00 ± 0.00 Aa	0.00
	0.25 mM L-Phe	0.00 ± 0.00 Bc	0.29 ± 0.05 Ab	0.00 ± 0.00 Ba	0.10
	0.50 mM L-Phe	0.43 ± 0.01 Ab	0.00 ± 0.00 Bb	0.00 ± 0.00 Ba	0.14
	1.00 mM L-Phe	3.58 ± 0.04 Aa	2.76 ± 0.45 Aa	0.00 ± 0.00 Ba	2.11
	Mean	1.00	0.76	0.00	
Chlorogenic acid (µg 100 ml^{-1})	Control	17.76 ± 1.16 Cd	107.77 ± 3.05 Ac	37.85 ± 1.34 Bd	54.46
	0.25 mM L-Phe	40.71 ± 0.88 Cb	158.85 ± 3.23 Ab	94.77 ± 1.32 Bb	98.11

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

Phenolics	Applications	Culture duration (days)			
		1st	3rd	7th	Mean
	0.50 mM L-Phe	34.24 ± 1.36 Cc	189.36 ± 3.63 Aa	73.22 ± 5.37 Bc	98.94
	1.00 mM L-Phe	80.46 ± 1.20 Ca	200.41 ± 5.10 Aa	173.76 ± 3.08 Ba	151.55
	Mean	43.29	164.10	94.90	

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

The amounts of phenolic contents per flask obtained by collecting the phenolics accumulated in roots and leaked into the culture medium were presented in Table 3. When TPC per flask was examined, the greatest amount (3.39 mg flask^{-1}) was obtained from the flask applied with 0.5 mM L-Phe and harvested on the 3rd day of culture. Of the phenolics, the amounts of gallic acid, catechin, epicatechin and vanillin obtained per flask reached the greatest levels with 0.5 mM L-Phe application for 3 days. The greatest cinnamic acid (10.42 µg flask^{-1}), p-coumaric acid (10.80 mg flask^{-1}) and ferulic acid (1.63–1.98 µg flask^{-1}) were found in the cultures with 1.00 mM L-Phe application for 1 day while maximum rosmarinic acid (10.90 µg flask^{-1}), and chlorogenic acid (704.94 µg flask^{-1}) obtained from the flasks applied with 1.00 mM L-Phe for 3 days. Caffeic acid was found to reach the greatest value with 0.25 mM L-Phe application for 1 day.

Table 3
Effects of L-Phenylalanine (L-Phe) application and culture duration on the phenolics per flask (Rutin, o-coumaric acid and quercetin could not be detected)

Phenolics	Applications	Culture duration (days)			
		1st	**3rd**	**7th**	**Mean**
Total phenolic contents (mg flask^{-1})	Control	1.73 ± 0.07 Cc*	2.42 ± 0.08 Ac	2.11 ± 0.04 Bc	2.09
	0.25 mM L-Phe	2.49 ± 0.08 ABb	2.82 ± 0.09 Ab	2.43 ± 0.05 Bb	2.58
	0.50 mM L-Phe	2.73 ± 0.06 Bab	3.39 ± 0.08 Aa	3.15 ± 0.05 Aa	3.09
	1.00 mM L-Phe	2.83 ± 0.08 Ba	3.18 ± 0.08 Aab	3.04 ± 0.05 Aba	3.02
	Mean	2.45	2.95	2.68	
Gallic acid (µg flask^{-1})	Control	5.89 ± 0.25 Bd	7.32 ± 0.06 Ad	8.04 ± 0.34 Ad	7.08
	0.25 mM L-Phe	11.92 ± 0.39 Bc	12.22 ± 0.58 Bc	15.21 ± 0.27 Ab	13.12
	0.50 mM L-Phe	26.64 ± 0.70 ABa	28.58 ± 0.55 Aa	24.53 ± 0.15 Ba	26.59
	1.00 mM L-Phe	15.52 ± 0.41 Ab	15.10 ± 0.18 ABb	14.12 ± 0.13 Bc	14.91
	Mean	15.00	15.81	15.48	
Catechin (µg flask^{-1})	Control	69.72 ± 2.87 Bc	107.85 ± 4.24 Ad	95.83 ± 1.02 Ac	91.13
	0.25 mM L-Phe	278.40 ± 4.34 Aa	202.55 ± 15.3 Bc	116.38 ± 4.16 Cb	199.11
	0.50 mM L-Phe	220.64 ± 11.5 Bb	368.70 ± 18.1 Aa	326.79 ± 3.39 Aa	305.37
	1.00 mM L-Phe	303.55 ± 6.96 Aa	264.39 ± 4.35 Bb	90.48 ± 2.18 Cc	219.47
	Mean	218.08	235.87	157.37	
Epicatechin (µg flask^{-1})	Control	7.70 ± 0.27 Cd	12.84 ± 0.07 Bd	15.36 ± 0.69 Ab	11.97
	0.25 mM L-Phe	11.00 ± 0.28 Bc	16.29 ± 0.79 Ac	14.60 ± 0.31 Ab	13.96
	0.50 mM L-Phe	29.62 ± 0.65 Ba	42.03 ± 0.77 Aa	39.61 ± 0.10 Aa	37.09

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

	1.00 mM L-Phe	24.70 ± 0.58 Ab	25.40 ± 0.13 Ab	18.65 ± 6.60 Ab	22.91
	Mean	18.25	24.14	22.05	
Vanillin (μg flask^{-1})	Control	3.98 ± 0.04 Bc	4.50 ± 0.31 ABc	4.86 ± 0.07 Ac	4.45
	0.25 mM L-Phe	6.71 ± 0.28 Bb	8.54 ± 0.28 Ab	8.77 ± 0.09 Ab	8.01
	0.50 mM L-Phe	10.44 ± 0.02 Ca	14.80 ± 0.23 Aa	11.97 ± 0.15 Ba	12.40
	1.00 mM L-Phe	6.65 ± 0.53 Ab	6.64 ± 1.06 Abc	5.75 ± 0.64 Ac	6.35
	Mean	6.94	8.62	7.84	
Cinnamic acid (μg flask^{-1})	Control	1.08 ± 0.19 Ac	1.41 ± 0.06 Ad	1.30 ± 0.02 Ac	1.27
	0.25 mM L-Phe	1.95 ± 0.14 Bc	3.79 ± 0.16 Ac	3.70 ± 0.18 Ab	3.15
	0.50 mM L-Phe	4.16 ± 0.18 Bb	5.30 ± 0.10 Ab	4.81 ± 0.25 ABa	4.76
	1.00 mM L-Phe	10.42 ± 0.32 Aa	7.08 ± 0.26 Ba	4.33 ± 0.23 Cab	7.28
	Mean	4.40	4.40	3.54	
Rosmarinic acid (μg flask^{-1})	Control	4.85 ± 0.11 Bc	6.07 ± 0.12 Ab	4.19 ± 0.04 Cc	5.03
	0.25 mM L-Phe	7.35 ± 0.27 Ab	9.33 ± 0.88 Aa	8.47 ± 0.11 Ab	8.38
	0.50 mM L-Phe	9.88 ± 0.32 Aa	8.61 ± 0.49 Aa	10.03 ± 0.38 Aa	9.51
	1.00 mM L-Phe	10.07 ± 0.09 Ba	10.90 ± 0.13 Aa	9.76 ± 0.16 Ba	10.24
	Mean	8.04	8.73	8.11	
p-coumaric acid (mg flask^{-1})	Control	2.14 ± 0.08 Ac	1.81 ± 0.36 Ac	0.07 ± 0.00 Bb	1.34
	0.25 mM L-Phe	4.80 ± 0.07 Ab	1.72 ± 0.20 Bc	0.00 ± 0.00 Cb	2.14

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

	0.50 mM L-Phe	4.64 ± 0.23 Ab	4.75 ± 0.29 Ab	0.00 ± 0.00 Bb	3.13
	1.00 mM L-Phe	10.80 ± 0.66 Aa	6.27 ± 0.13 Ba	1.70 ± 0.50 Ca	6.25
	Mean	5.59	3.64	0.44	
Caffeic acid	Control	0.20 ± 0.01 Ac	0.00 ± 0.00 Bb	0.00 ± 0.00 Ba	0.07
(μg flask^{-1})	0.25 mM L-Phe	2.02 ± 0.01 Aa	0.00 ± 0.00 Bb	0.00 ± 0.00 Ba	0.67
	0.50 mM L-Phe	0.37 ± 0.01 Ab	0.06 ± 0.01 Bb	0.00 ± 0.00 Ca	0.14
	1.00 mM L-Phe	0.44 ± 0.06 Ab	0.50 ± 0.12 Aa	0.00 ± 0.00 Ba	0.31
	Mean	0.76	0.14	0.00	
Ferulic acid	Control	1.31 ± 0.07 Ab	0.05 ± 0.01 Bb	0.05 ± 0.00 Bb	0.45
(μg flask^{-1})	0.25 mM L-Phe	0.81 ± 0.08 Ac	0.09 ± 0.02 Bb	0.00 ± 0.00 Bb	0.30
	0.50 mM L-Phe	0.62 ± 0.06 Ac	0.33 ± 0.07 Bb	0.00 ± 0.00 Cb	0.32
	1.00 mM L-Phe	1.98 ± 0.05 Aa	1.63 ± 0.13 Aa	0.40 ± 0.06 Ba	1.34
	Mean	1.18	0.53	0.01	
Chlorogenic acid	Control	280.77 ± 4.73 Cc	403.16 ± 16.9 Ac	337.03 ± 4.70 Bc	340.32
(μg flask^{-1})	0.25 mM L-Phe	413.07 ± 8.28 Cb	619.08 ± 9.99 Ab	530.03 ± 4.67 Bb	520.73
	0.50 mM L-Phe	433.18 ± 19.4 Cb	670.00 ± 17.9 Aab	594.53 ± 7.89 Ba	565.00
	1.00 mM L-Phe	518.94 ± 9.80 Ca	704.94 ± 21.5 Aa	630.17 ± 17.9 Ba	618.02
	Mean	411.49	599.30	522.94	

* Different capital letters within the same row show significant differences between culture durations by Duncan test ($p \leq 0.05$). Different small letters within the same column show significant differences between L-Phe applications by Duncan test ($p \leq 0.05$).

Effects of L-Phe applications and culture duration on the PAL activity

PAL enzyme activity was another parameter studied in this study since it is an enzyme that takes part in the phenylpropanoid pathway in plants and the synthesis of many secondary metabolites by converting L-Phe to trans-cinnamic acid (Syklowska-Baranek et al. 2012). As a result of the analysis of variance in terms of PAL activity, the interaction of application*culture duration was found to be statistically significant ($p \leq 0.05$). The highest PAL enzyme activity was found in roots harvested on the 1st day in the control group with 3.45 µmol trans-cinnamic acid g-1 fresh root h-1 (Fig. 5). But PAL activity showed a sharp decrease in control roots harvested on day 3 and 7 as 0.91 and 0.49 µmol trans-cinnamic acid g-1 fresh root h-1, respectively. A similar pattern was observed with 0.5 mM L-Phe application. One of the most striking results was obtained with 0.25 mM L-Phe application. PAL activity, which was high on the 3rd day in this application, fell rapidly on the 7th day and decreased about 10 times. According to these results, it was determined that there was no relationship between PAL activity and the amount of tropane alkaloids.

Discussion

In this study, the effects of 0.25, 0.50, and 1.00 mM L-Phe applied to adventitious root cultures of *H. niger* on root growth and production of scopolamine, hyoscyamine and phenolics in roots and culture medium were investigated at different culture durations. Biotic and abiotic elicitors applied to cultures under *in vitro* conditions can increase the production of metabolites by stimulating the biosynthesis steps of secondary metabolites (Shakeran et al. 2015). Another approach to increase the *in vitro* secondary metabolite production is precursor addition to the culture medium. Increasing *in vitro* secondary metabolite production with precursor applications is a promising approach and is used in the production of many pharmaceutically effective secondary metabolites (Namdeo et al. 2007). However, success in precursor applications can vary significantly depending on the precursor, its concentrations, culture condition, application time, the targeted biosynthetic pathway and metabolite (Jackson and Attalla 2010). The syntheses of the metabolites reach the greatest levels in a certain period of the culture, and metabolite accumulation in cultures harvested before or after this period could significantly reduce (Lee et al. 2001; Moussous et al. 2018; Ono et al. 1998). Therefore, in this study, L-Phe applications as a precursor and different culture durations were used to determine their effects on root growth, alkaloid and phenolic production in *H. niger* adventitious root cultures and to create a protocol that provides high metabolite yield.

When the effects of L-Phe and culture durations on root fresh weight, growth index and root dry weight were assessed, important differences were found. Root growth parameters increased in parallel with increasing L-Phe concentration and culture duration. Stimulating effects of L-Phe on cell and root growth have been shown in *Arnebia euchroma* cell cultures (Syklowska-Baranek et al. 2012) and *Echinacea purpurea* adventitious root cultures (Mobin et al. 2015). The findings that L-Phe applications increase

biomass in different plants such as *Datura stramonium* (Moursy et al. 1988), *Hydrocotyle bonariensis* (Masoumian et al. 2011), *Origanum majorana* (Korkor et al. 2017) and *Ocimum basilicum* (Koca and Karaman 2015) support our study. The growth-promoting effect of L-Phe in plant cells is thought to be due to its positive effects on the gibberellin biosynthesis mechanism and the accumulation of amino acids (Musbah and Ibrahim 2019). On the other hand, high L-Phe concentrations inhibited cell growth (Edahiro et al. 2005) and even reduced fresh and dry cell weight (Palacio et al. 2011). Coruzzi and Last (2000) found that increasing L-Phe concentration increases the dissolved nitrogen in cells and increases the osmotic pressure, as a result, the cell wall that draws water from neighbouring cells becomes softer and thinner and can easily be broken down. Thus, it causes a decrease in the amount of biomass depending on the increase in L-Phe concentration. Demirci et al. (2020) found that L-Phe applications in *Echinacea purpurea* hairy roots did not have a significant effect on root growth, but fresh and dry root weights and growth index increased in parallel to the culture duration prolonged. As a result, the effect of L-Phe applications on biomass yield varies significantly depending on the L-Phe concentration and culture duration (Demirci et al. 2020; Masoumian et al. 2011; Palacio et al. 2011).

The changes in amounts of hyoscyamine and scopolamine were also examined according to the L-Phe applications and culture duration. The use of L-Phe in appropriate concentrations increased the alkaloid accumulation. Similarly, in the previous studies (Hashimoto and Yamada 1987; Hassan and Jassim 2018) showed that L-Phe application as a precursor significantly increased the accumulation of *in vitro* alkaloid production. Khanna et al. (2005) stated that the effect of L-Phe promoting alkaloid production is due to the role of L-Phe as a signal molecule in the expression of enzymes that catalyse the synthesis of alkaloids. It is known that L-Phe applications at the optimum concentrations can be used as an effective method for secondary metabolite production. However, high doses of L-Phe administration decreased metabolite production (Demirci et al. 2020, 2021). The precursor used in high doses can reduce the amount of metabolites by causing feedback inhibition in the secondary metabolite biosynthesis pathway (Ouyang et al. 2005). Therefore, determining the most appropriate concentration is of great importance in precursor applications. In this study, the amounts of scopolamine and hyoscyamine in roots, in the culture medium changed depending on the L-Phe concentration and the culture duration. Previous studies on different plants have also obtained results that support our findings (Boitel-Conti et al. 2000; Hassan and Jassim 2018; Masoumian et al. 2011).

Another important factor affecting *in vitro* secondary metabolite production is culture duration. In this study, the greatest alkaloid production was obtained from the cultures harvested on the 3rd day, and the amount of alkaloid decreased on the 7th day. Similarly, Lee et al. (2001) reported that secondary metabolite yield decreased with the increasing duration of in vitro cultures. Figuero et al. (2010) reported that hypericin decreased similarly in *Hypericum perforatum*, and the reason for this decrease was that the plant's energy was used for growth rather than producing secondary metabolites with the prolongation of the culture duration. The selection of the most appropriate culture duration may vary significantly depending on the genotype, applications, and the targeted secondary metabolites. The accumulation of different alkaloids following biotic and abiotic elicitor applications in different plants vary significantly according to the culture duration (Moharrami et al. 2017). Therefore, it is possible to increase metabolite

productivity by determining the most appropriate culture duration when synthesis is at the highest level for *in vitro* secondary metabolite production (Moussous et al. 2018). Secondary metabolite yield will be low if *in vitro* plants are not harvested during the period when secondary metabolite production is high. It is thought that the decrease in secondary metabolite yield with prolonged culture duration is caused by the biotransformation of secondary metabolites or the decomposition of applied elicitor and precursor materials over time and losing their effect (Ono et al. 1998).

In addition to tropane alkaloids, *H. niger* has many valuable phenolics (Jassbi et al. 2014). However, as a result of the literature reviews, there is no research on the production of phenolics in *H. niger* cultures. As a result of this study, phenolic contents were significantly changed according to the L-Phe concentrations and culture durations. Phenolics are synthesized from L-Phe and L-tyrosine via shikimic acid biosynthesis. Especially, L-Phe is the most important amino acid in this biosynthesis pathway and is the common precursor of most phenolics (Heleno et al. 2015). It is known that the amount of phenolics increases significantly with L-Phe application in different plants *in vitro* cultures (Mobin et al. 2015, Demirci et al. 2021, Koca and Karaman et al. 2015, Palacio et al. 2011). However, the L-Phe concentration is important. Reductions in the amount of phenolic substance have been reported with high concentration L-Phe (Demirci et al. 2020, Mobin et al. 2015).

According to the results, it may be possible to produce high amounts of phenolics by appropriate L-Phe application and culture duration. After L-Phe application, the greatest amounts of phenolics were detected in the roots harvested on the 1st and 3rd days of the culture, while it was observed that the amount of phenolics in the cultures decreased with the extension of the culture duration to 7 days. These results show that the culture duration is one of the most important criteria for secondary metabolite production and is in agreement with the results of previous studies performed on different plants (Demirci et al. 2020; 2021). It is stated that this stimulating effect of L-Phe in the production of phenolics is achieved by stimulating the enzymatic pathways in plants (Koca and Karaman 2015). Another reason for the decrease in secondary metabolite accumulation during the culture is that the elicitors or precursors used to increase the synthesis are broken down or depleted over time. Edahiro et al. (2005) stated that the decrease in L-Phe added to culture medium over time affects metabolite yield negatively.

The use of L-Phe, which acts as a precursor in the production of phenolics, is known to stimulate the expression of genes responsible for the PAL (Kikowska et al. 2012). PAL activity and phenolics amounts parallelly increased with methyl jasmonate, epibrassinolide, chitosan and L-Phe applications in previous studies (Koca and Karaman 2015, Kim et al. 2005). In this study, while PAL activity reached its highest level in cultures harvested on the 1st day except for 0.25 mM L-Phe application, it decreased on the 3rd and 7th days. The greatest amounts of tropane alkaloids were obtained from the cultures applied with 0.5 mM L-Phe for 3 days in this study. Similarly, Kim et al. (2006a and b) reported that the induced PAL activity reached a maximum in 24 hours and that its maximum effect could continue for up to 48 hours. So it was seen that the effects of L-Phe applications on PAL activity were inconsistent. Kim et al. (2006a and b) also reported that there is no consistent relationship between amounts of PAL and TPC and an increase in phenolics could be due to other enzymes involved in the phenylpropanoid pathway. Similarly,

Li et al. (2007) showed in their study that the cinnamate 4-hydroxylase (C_4H) significantly affects the formation of trans-cinnamic acid. On the other hand, contents of phenolic compounds were high in the 1st and 3rd days of cultures compared to the 7th day parallel to the PAL enzyme activity. In previous studies conducted with the idea that L-Phe, which is the precursor of trans-cinnamic acid, would increase PAL activity, it was reported that there was no statistically significant difference in PAL activity in plants treated with L-Phe (Koca 2013; Koca and Karaman 2015).

Conclusion

In this study, L-Phe applications and culture duration were examined on increasing the production of tropane alkaloids and phenolic compounds in adventitious root cultures of *H. niger*, and it was found that L-Phe and culture duration significantly effective in increasing them when the appropriate L-Phe concentration and culture duration were selected. Based on the results, the greatest values in terms of root development were obtained in the 1st and 3rd days after 0.50 and 1.00 mM L-Phe applications. 0.50 mM L-Phe and 3-day culture duration were determined as the most effective combination for scopolamine and hyoscyamine alkaloids, as well as TPC, gallic acid, catechin, epicatechin, and vanillin. 1.00 mM L-Phe for 1 day attracted attention as another combination that can be used in the production of phenolic compounds. The effects of L-Phe applications on PAL enzyme activity were inconsistent while PAL activity and phenolic compounds were similarly high on the 1st and 3rd days of culture. Briefly, when appropriate L-Phe concentrations and culture duration are used, it is possible to increase the production of tropane alkaloids and phenolic compounds in *H. niger* root cultures. To our best knowledge, this study is the first to examine the effects of L-Phe and culture duration on alkaloid and phenolic production from *H. niger* adventitious root cultures.

Declarations

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Compliance with ethical standards

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Figures

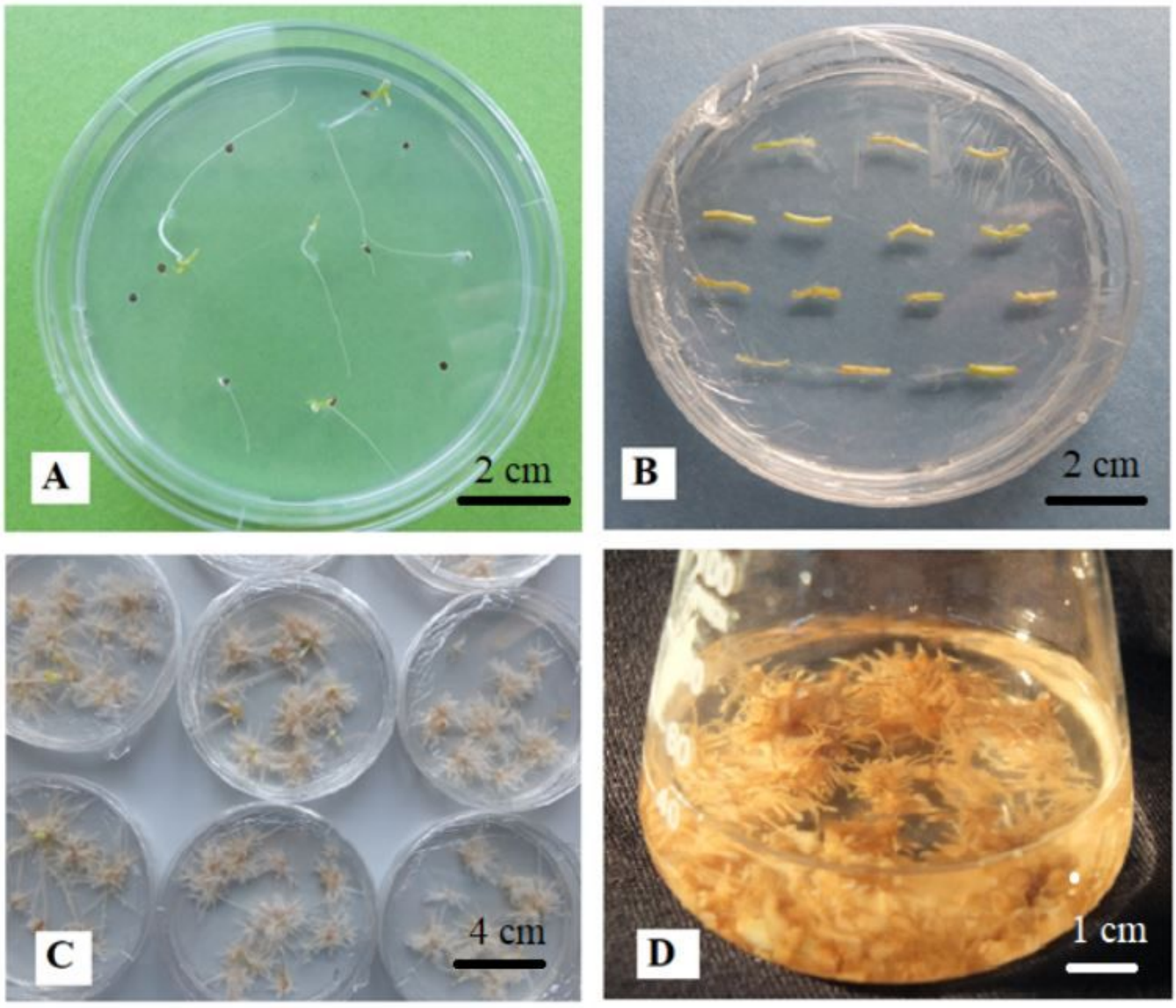

Figure 1

Obtaining adventitious root cultures in Hyoscyamus niger (A: germination of seeds, B: transferring explants to rooting medium, C: obtaining adventitious roots from explants, D: propagation of adventitious

roots in liquid medium)

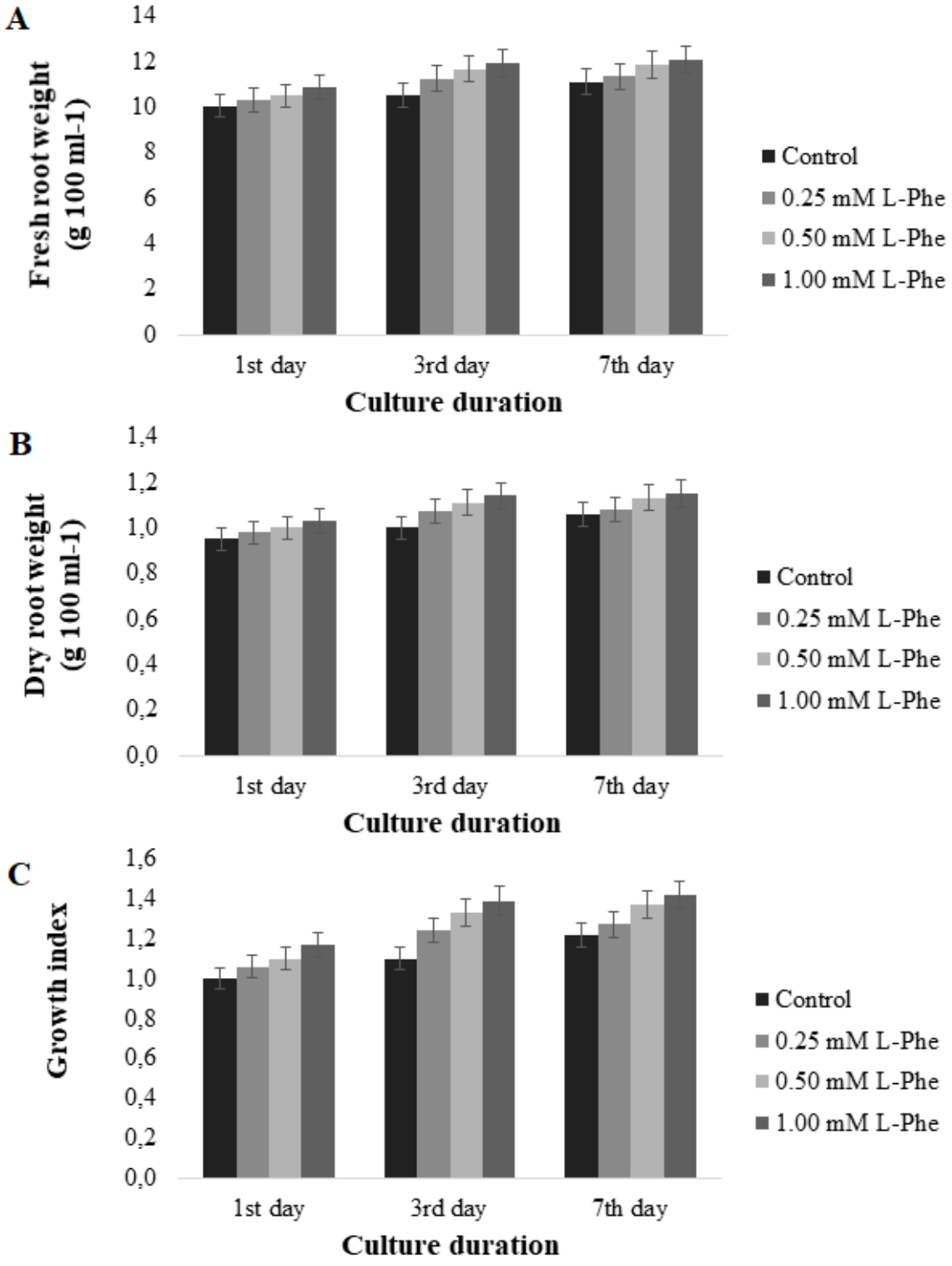

Figure 2

Effects of L-Phenylalanine (L-Phe) application and culture duration on growth parameter in adventitious roots of Hyoscyamus niger A: Fresh root weights (g 100 ml-1), B: Dry root weights (g 100 ml-1), C: Root growth indexes (Interaction among the L-Phe application and culture duration is not statistically significant)

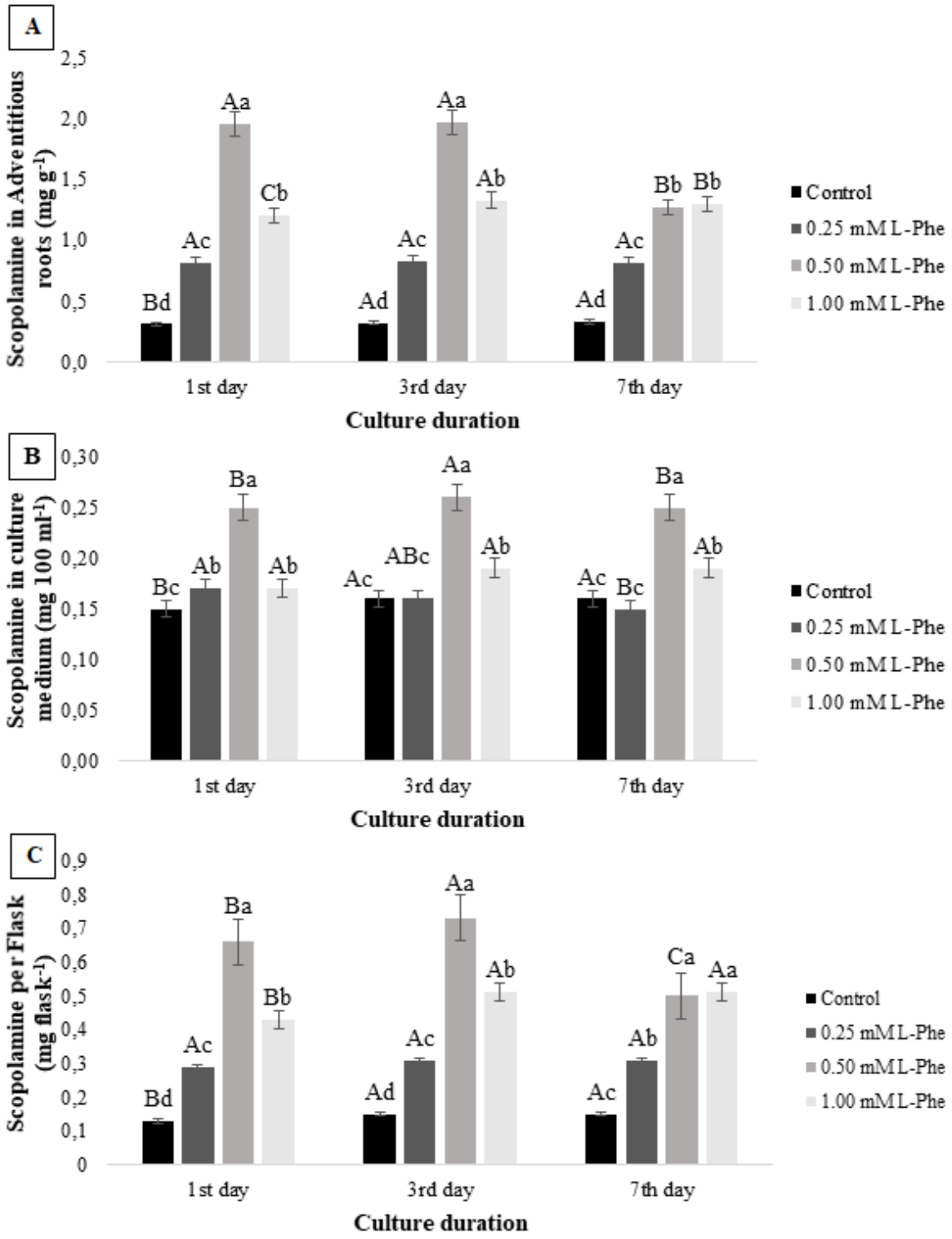

Figure 3

Effect of L-Phe and culture duration on scopolamine in adventitious root cultures of Hyoscyamus niger. A: scopolamine in adventitious roots (mg g-1), B: scopolamine in culture medium (mg 100 ml-1), C: production of scopolamine per flask (mg flask-1) (Differences between culture duration indicated by capital letters; differences between applications indicated by small letters)

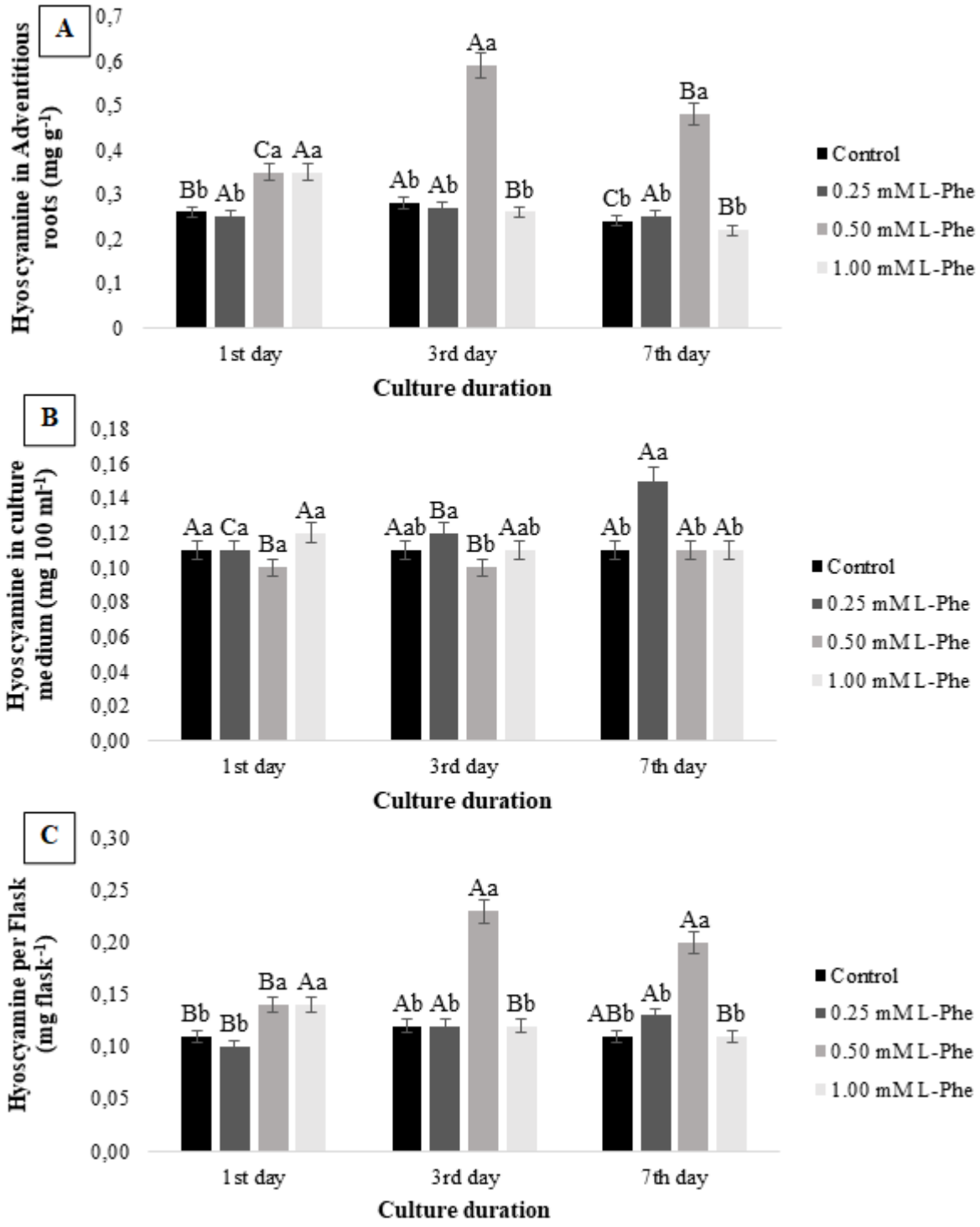

Figure 4

Effect of L-Phe and culture duration on hyoscyamine in adventitious root cultures of Hyoscyamus niger. A: hyoscyamine in adventitious roots (mg g-1), B: hyoscyamine in culture medium (mg 100 ml-1), C: production of hyoscyamine per flask (mg flask-1) (Differences between culture duration indicated by capital letters; differences between applications indicated by small letters)

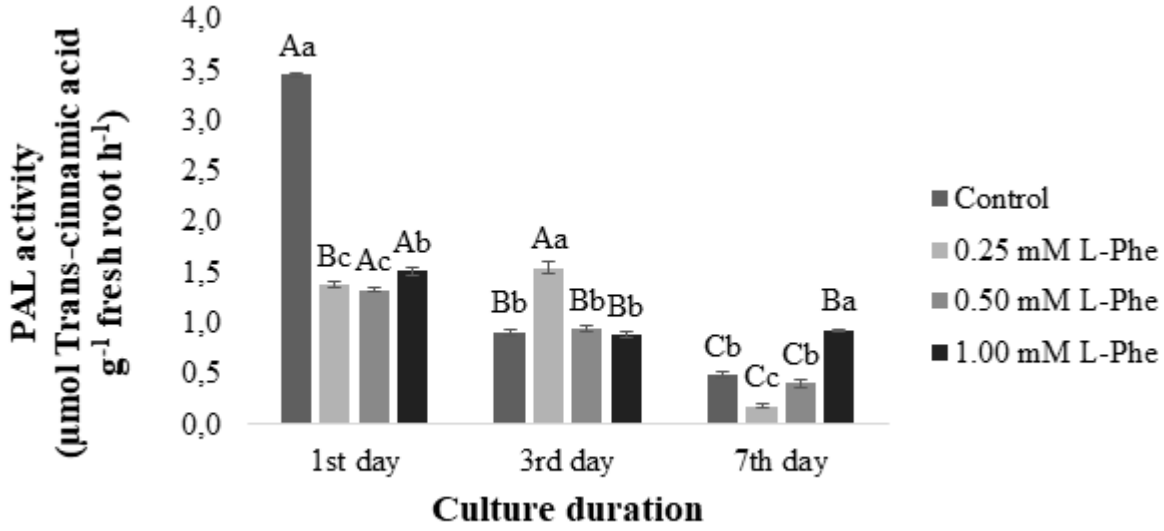

Figure 5

Effect of L-Phenylalanine and culture duration on PAL enzyme activity in adventitious roots of Hyoscyamus niger (Differences between culture duration indicated by capital letters; differences between applications indicated by small letters)

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