IJSM International Journal of Secondary Metabolite

International Journal of Secondary Metabolite
2022, Vol. 9, No. 2, 178–193

https://doi.org/10.21448/ijsm.1041324

Published at https://dergipark.org.tr/en/pub/ijsm Research Article

Effect of culture time on secondary metabolite production from MeJA-stimulated *in vitro* roots of *Hyoscyamus niger* L.

Hikmet Deveci[1,*], Tunhan Demirci[2], Nilgun Gokturk Baydar[1]

[1]Isparta University of Applied Sciences, Faculty of Agriculture, Agricultural Biotechnology Department, 32270, Isparta, Türkiye
[2]Suleyman Demirel University, Faculty of Pharmacy, Pharmaceutical Biotechnology Department, 32260, Isparta, Türkiye

Abstract: This study was conducted to determine the effects of different culture times on root growth and accumulation of secondary metabolites in adventitious roots from *Hyoscyamus niger* petioles. For this purpose, adventitious roots transferred to culture media containing 1 mM methyl jasmonate (MeJA) were harvested at 11 different culture times, starting from day 1 to day 30. After harvest, the fresh and dry weights of the roots and the root growth index as parameters of root growth, as well as the amounts of tropane alkaloids and phenolic compounds, were examined. It was found that the fresh and dry weights and the growth index of the roots harvested between the 12th and 30th day were the highest. The highest amounts of scopolamine were obtained from roots harvested on days 15, 18, and 21. The highest hyoscyamine accumulation was found on days 12 and 15. The highest total phenolic content was 19.33 mg g^{-1} in root cultures harvested on day 15. HPLC analyses revealed that ferulic acid, caffeic acid, *o*-coumaric acid, *p*-coumaric acid, rutin, and quercetin were not detected in the roots. On the other hand, the amounts of gallic acid, catechin, epicatechin, vanillin, cinnamic acid, rosmarinic acid, and chlorogenic acid showed significant changes depending on the culture times. Considering all the results, it was found that the optimum culture time for the production of secondary metabolites in *H. niger* adventitious root cultures stimulated with 1mM MeJA was 15 days.

ARTICLE HISTORY

Received: Dec. 24, 2021
Revised: Apr. 28, 2022
Accepted: May 10, 2022

KEYWORDS

Hyoscyamus niger,
Methyl jasmonate,
Adventitious roots,
Tropane alkaloids,
Culture time

1. INTRODUCTION

Tropane alkaloids (TAs), important secondary metabolites (SMs) with various pharmaceutical effects (Kohnen-Johannsen & Kayser 2019), occur in several families such as Proteaceae, Convolvulaceae, Brassicaceae, Euphorbiaceae, Rhizophoraceae, Solanaceae, and Erythroxylaceae (Jirschitzka *et al.,* 2012). In particular, plants of the Solanaceae family have been used as medicine for centuries due to their richness in TAs. *Hyoscyamus niger*, an annual or biennial herbaceous plant belonging to Solanaceae, is one of the most important species for the pharmaceutical industry due to its high content of TAs such as hyoscyamine and scopolamine, which are among the oldest drugs used in medicine (Dehghan *et al.,* 2012;

*CONTACT: Tunhan DEMIRCI tunhandemirci@sdu.edu.tr Suleyman Demirel University, Faculty of Pharmacy, Department of pharmaceutical Biotechnology, Isparta, Türkiye

ISSN-e: 2148-6905 / © IJSM 2022

Pudersell *et al.,* 2012). Hyoscyamine and scopolamine are generally used to treat stomach aches, earaches, sinusitis, ulcers, kidney, and liver ailments, as well as to relieve pain and as antispasmodics (John *et al.,* 2010; Sargın *et al.,* 2013). *H. niger* acts on the parasympathetic nervous system due to its TAs and is used to treat diseases of the ocular, anaesthetic, cardiac, and digestive systems (John *et al.,* 2010). Scopolamine has been found to have beneficial effects in patients with acute vertigo and to reduce gastric acid secretion during sleep in patients with nausea and ulcers caused by chemotherapy drugs in cancer patients (Clissold & Heel 1985). Scopolamine is a valuable pharmaceutical raw material because it has higher pharmacological activity and fewer side effects than hyoscyamine. Therefore, it is now known that the demand for scopolamine is about 10 times higher than that for hyoscyamine (Qin *et al.,* 2014; Shakeran *et al.,* 2017). Apart from these important medicinal effects, hyoscyamine and scopolamine can be highly toxic to the human body if consumed unknowingly. When the roots and leaves of *H. niger* are consumed as food, scopolamine and hyoscyamine can cause intoxication by paralysing the nerve endings of the parasympathetic system (Orbak *et al.,* 1998). *H. niger* poisoning has many clinical symptoms such as mydriasis, tachycardia, arrhythmia, agitation, convulsive thirst, dry mouth, nausea, vomiting, headache, blurred vision, auditory, visual, or tactile hallucinations, disorientation, and aggression (Alizadeh *et al.,* 2014).

H. niger is native to Europe and North Africa (Pokorny *et al.,* 2010). However, it is widely distributed in regions with temperate climates (Alizadeh *et al.,* 2014). It grows naturally in rocky areas, on undeveloped land, and on roadsides (Li *et al.,* 2011; Yücel & Yılmaz, 2014). An erect, viscid-haired, greasy, annual or biennial herb, 30-150 cm tall. Leaves are radical and cauline, neatly toothed or pinnately lobed. Flowers are bisexual, regular, pale yellowish-green or yellowish-white with a dark purple (reticulate) center, stalkless or subsessile, in axillary or terminal scorpion-like umbels. Capsules are surrounded by the globose base of the enlarged calyx. Seeds are oval, kidney- or knee-shaped, about 1.5 mm in diameter, brown, with fine but conspicuous reticulations. Flowering and fruiting occur from April to September.

The use of plants as medicines in traditional Chinese medicine dates back to 2700 BC, and the medicinal effects of many plants were discovered through trial and error (Faydaoğlu & Sürücüoğlu, 2013). Nowadays, medicinal plants, which occupy an important place in drug discovery studies, continue to be a natural source of pharmaceutical raw materials due to their content of SMs (D'yakova *et al.,* 2020; Zvezdina *et al.,* 2020). As the consumption of herbal medicines continues to increase, the reliance on medicinal plants to obtain active pharmaceutical ingredients has become even greater (Hong *et al.,* 2012). Synthetic production of pharmaceutical compounds, especially alkaloids found in plants, is more expensive and difficult than their isolation from plants due to the complexity of their chemical structures and the length of biosynthetic pathways (Dehghan *et al.,* 2012). For this reason, plant tissue cultures are now commonly used to achieve higher alkaloid production (Shah *et al.,* 2020). The production of SMs with medicinally important effects by *in vitro* methods should be provided in stable and desired quantities, regardless of seasonal variations compared to wild-collected or field-grown plants. At the same time, the use of these methods can prevent the uncontrolled collection of plants from their natural habitats (Ajungla *et al.,* 2009; Shah *et al.,* 2020; Roy 2021). *İn vitro* root cultures are one of the most successful methods for obtaining large amounts of valuable SMs from the roots of many plants (Kareem *et al.,* 2019; Roy 2021). However, low productivity often proves to be a major problem in the *in vitro* production of secondary metabolites (SMPs). To overcome this problem, some applications are made, such as changes in nutrient content, culture conditions, or the addition of new chemicals (Lu *et al.,* 2020). The use of biotic and abiotic elicitors *in vitro* root cultures is considered one of the most acceptable strategies to increase the production of many SMs such as phytoalexins and alkaloids in roots (Shah *et al.,* 2020). Various biotic and abiotic elicitors added to the culture medium can increase the production of SMs by activating the appropriate genes for synthesis or stimulating

physiological processes that lead to further accumulation of SMs (Shah *et al.,* 2020). Jasmonic acid and its methyl ester methyl jasmonate (MeJA) play a role in various physiological developmental processes such as seed germination, root growth, fertility, fruit ripening, and activation of the plant defense system against biotic and abiotic stresses (Zhang *et al.,* 2020; Zuniga *et al.,* 2020; Wang *et al.,* 2021). MeJA, first identified as a component of the essential oils of *Jasminum grandiforum* L. and *Rosmarinus officinalis* L., assist in the biosynthesis of various SMs such as terpenoids, indole alkaloids, nicotine, flavonoids, ginsenosides, benzophenanthridine alkaloids, and glucosinolates (Giri & Zaheer 2016; Tang *et al.,* 2020; Yousefian *et al.,* 2020). MeJA increases the amount of important TAs such as hyoscyamine and scopolamine by directly or indirectly affecting the synthesis of key enzymes such as Putrescine-N-methyl transferase (PMT) and hyoscyamine-6 beta-hydroxylase (H6H), which are responsible for the biosynthesis of these alkaloids (Kang *et al.,* 2004).

Culture time is another important factor determining yield in the *in vitro* production of plant SMs (Demirci *et al.,* 2020; Demirci *et al.,* 2021; Demirci *et al.,* 2022; Narayani and Srivastava 2017). In nature, each SM reaches its highest level at different time periods (Figueiredo *et al.,* 2008; Soni *et al.,* 2015). In *in vitro* cultures, the SM levels also change at different culture times, and it is important to determine the optimal culture time for each metabolite.

To our knowledge, there are no studies investigating the effects of different culture times on root growth, synthesis of TAs, and accumulation of phenolic compounds in MeJA-stimulated *H. niger* adventitious root cultures. The objective of this study was to find the optimal culture time in terms of root quantity, TAs, and phenolic compounds to achieve maximum yield from *H. niger* adventitious root cultures. This study provides important insights for future scale-up research with bioreactors.

2. MATERIAL and METHODS

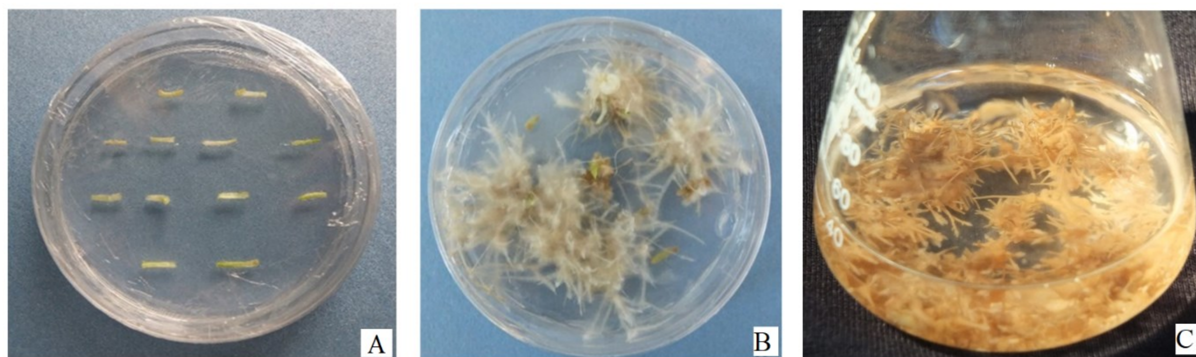
2.1. Plant Material

H. niger seeds used in this study were obtained from the Garden Directorate of Medicinal and Aromatic Plants of Zeytinburnu Municipality, Turkey. After the seeds germinated under *in vitro* conditions, petiole explants taken from the plantlets were used to form adventitious roots. These adventitious roots were then used as plant material in this study.

2.2. Obtaining Adventitious Roots

Seed germination and adventitious root collection followed the methods described in detail in our previous study (Demirci *et al.,* 2022). Briefly, seeds treated with gibberellic acid for 48 hours were rinsed with double distilled water (ddH_2O) and then sterilized by shaking with 70% ethanol (%99, Tekkim, Turkey) for 10 seconds and with 0.1% mercury chloride ($HgCl_2$, Sigma-Aldrich, Germany) for 10 min. The sterile seeds rinsed with sterile ddH_2O were transferred to Murashige and Skoog (1962) media (Duchefa Biochemie, Netherland) containing 3% sucrose (Sigma-Aldrich, Germany) and 0.6% agar (Sigma-Aldrich, Germany) and cultured in the dark at 25°C for 15 days. Germinated seeds were transferred to the media described above in tissue culture boxes with a volume of 250 ml and cultured for 15 days at 25°C (16 hours light/8 hours dark). Petiole explants of 1 cm length obtained from plantlets in growth media were cultured in MS media containing 2 mg l^{-1} indole-3-butyric acid (IBA, Sigma-Aldrich, Germany), 3% sucrose, and 0.6% agar for 6 weeks at 25°C in the dark to form adventitious roots (Figure 1-A). The adventitious roots formed (Figure 1-B) were transferred to 30 ml of liquid MS (2 mg l^{-1} IBA and 3% sucrose) medium in 100 ml culture boxes and cultured at 25°C (Figure 1-C). Roots growing on liquid media were subcultured onto the same liquid media at 3-week intervals.

Figure 1. Obtaining of *in vitro* adventitious roots in *Hyoscyamus niger* (A: inoculation of petiole explant, B: formation of adventitious roots, C: propagation of adventitious roots in liquid medium).



2.3. Stimulation of Adventitious Roots with MeJA

Healthy growing adventitious roots in liquid MS medium were weighed 1.25 g under aseptic conditions using an analytical balance and transferred to liquid MS medium supplemented with 2 mg l^{-1} IBA and 30 g l^{-1} sucrose. After the roots were cultured in these culture media at 25 °C for 7 days, 1 mM MeJA (Sigma-Aldrich, Germany) was added to the culture media. The stock solution of MeJA prepared with pure ethanol was added to each flask at 100 µl after filter sterilization. Roots were harvested on days 1, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 to study the effects of culture times after the application of 1mM MeJA. MeJA applications were made in 3 replicates for each harvest time and 4 flasks per replicate (11 harvest times X 3 replicates X 4 flasks). All applications and analyzes were planned as a completely randomized design.

2.4. Determination of Root Growth Parameters

Adventitious roots harvested at 11 different culture times were washed and the culture medium on the roots was completely removed. The fresh weights of the roots dehydrated with blotting paper were first determined on the analytical balance and calculated as g 100 ml^{-1}. The following formula was used to calculate the growth index of the roots whose fresh weights were determined: Growth index: (harvested FW (g) - inoculated FW (g)) / inoculated FW (g).

After the roots were completely dried in a drying oven at 40°C for 72 hours, they were weighed and the dry weights of the roots were calculated in g 100 ml^{-1}.

2.5. Extraction of Tropan Alkaloids and Phenolic Compounds from Adventitious Roots

To analyses the TAs and phenolics by chromatographic methods, the dried roots were ground with a pestle until they were powdered. Two hundred mg of the powdered roots were weighed and mixed with 20 ml of methanol solution (HPLC grade, Sigma Aldrich, Germany) (methanol:ddH2O, 60:40 (v/v)) in 50 ml test tubes. The mixture was shaken in an ultrasonic water bath for 15 min and centrifuged at 9000 rpm for 15 min. The same procedure was repeated two more times for the pellet fraction, while the supernatant fractions were transferred to flasks. Then, the collected supernatants were evaporated with a rotary evaporator at 45 °C under a vacuum to obtain dry extracts. To the dry extract, 1.5 ml of methanol was added and completely dissolved by vortexing. The extracts were stored at -20 °C after being filtered at 0.45 µm for analysis by HPLC and spectrophotometer (Jakabova *et al.,* 2012).

2.6. Determination of Tropane Alkaloids by HPLC

Chromatographic analyses of TAs were performed using a Shimadzu HPLC system (Kyoto, Japan). The HPLC system consisted of a LC -20AD pump, a DGU-20A3R degasser, a CTO - 10AS VP column heater, an SPD-M20A diode array detector, and a 250 × 4.6 mm i.d. 5 µm HPLC column (Agilent Eclipse XDB-C18, Wellborn, Germany). The modified method of

Boitel-Conti *et al.,* (2000) was used to determine the amount of hyoscyamine and scopolamine by HPLC. Mobile phase A was 2% acedic acid (Sigma-Aldrich, Germany) ultrapure water, and mobile phase B was 100% acetonitrile (HPLC grade, Sigma-Aldrich). Separation was performed according to the gradient programme; 0-12% B, 0-12 min; 12-20% B, 12-13 min; 20-28% B, 13-33 min; 28-100% B, 33-48 min. Flow rate and column temperature were 0.8 ml min^{-1} and 40 °C, respectively. The injection volume was 20 µl. Calculations were made at 220 nm in the instrument software (Shimadzu Class- VP Chromatography Laboratory Automated Software system) according to the calibration prepared with the analytical standards scopolamine (Sigma-Aldrich, Germany) and hyoscyamine (Sigma-Aldrich, Germany) and were expressed as mg g^{-1} dry weight. The data are the average of three measurements.

2.7. Determination of Phenolic Compounds by HPLC

The amounts of phenolic compounds including 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 adventitious roots were determined by HPLC. The HPLC system and column were the same as those used for the determination of TAs. For gradient elution, mobile phase A contained ultrapure water with 2% acetic acid, and mobile phase B contained 100% methanol (HPLC grade, Sigma-Aldrich). The HPLC gradient, 0-12% B, 0-12 min; 12-20% B, 12-13 min; 20-28% B, 13-33 min; 28-30% B, 33-48 min; 30-38% B, 48-53 min; 38-40% B, 53-68 min; 40% B ,68-70 min; 40-50% B ,70-90 min; 50-60% B ,90-105 min; 60-100% B ,105-107 min; 100% B ,107-112 min; 0% B ,112-117 min. Flow rate and column temperature were set at 0.8 ml min-1 and 40 °C. The injection volume was 20 µl. Calculations were performed in the instrument software (Shimadzu Class- VP Chromatography Laboratory Automated Software system) according to the calibration established with analytical standards and expressed as µg g^{-1} dry weight. The data are an average of three measurements.

2.8. Determination of Total Phenolic Content (TPC)

Total phenolics of adventitious roots were determined by the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). Accordingly, 40 µl of the liquid extracts obtained in the extraction phase were transferred to test tubes. Then, 2.4 ml of distilled water and 200 µl of Folin Ciocalteu were added to each. After waiting for 5 min, 600 µl of a saturated sodium carbonate solution at room temperature and 760 µl of distilled water were added to the mixtures. After vortexing, the mixture was incubated for 2 h at room temperature in the dark. Absorbances were measured at 765 nm using a spectrophotometer (T70 Plus Dual Beam/Arlington, USA). The TPC in the roots was calculated using the calibration curve of gallic acid standards and the results were expressed as mg gallic acid equivalents (GAE) g^{-1} DW).

2.9. Statistical Analyses

The root cultures were randomly selected during MeJA applications and roots in each culture medium in which the application occurred were examined in the experiments. Data were analyzed at the $p \leq 0.05$ level using Duncan' Multiple Range Test with the program IBM SPSS 22 (ANOVA).

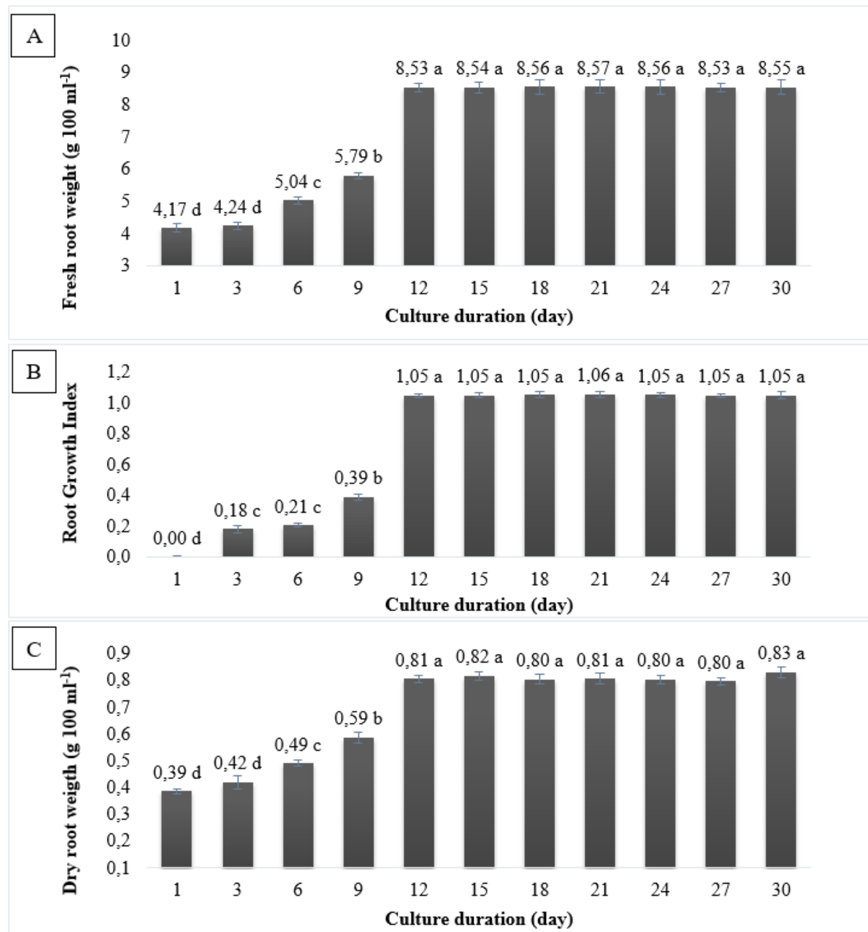
3. RESULTS

3.1. Effects of Culture Times on The Parameters of Root Growth

Root fresh weights, root growth indexes, and root dry weights were studied as root growth parameters. There were statistically significant differences in all growth parameters depending on the culture times ($p \leq 0.05$). The lowest fresh weights were found in roots harvested on the 1st and 3rd day after the application of 1 mM MeJA (4.17-4.24 g 100 ml^{-1}). Fresh weights increased significantly on days 6, 9, and 12 of culture with values of 5.04, 5.79, and 8.53 g 100 ml^{-1}, respectively (Figure 2A). However, no significant differences were observed between

harvests grown at 3-day intervals from day 12 to day 30. The root growth index increased slowly from day 3 and peaked (1.05) on day 12 (Figure 2B). Thereafter, the growth index remained constant until day 30 and showed no significant differences. Root dry weights were lowest on days 1 and 3 and increased significantly on days 6, 9, and 12, similar to fresh weight and growth index. While root dry weights ranged from 0.80 to 0.82 at all culture times, there were no significant differences between the 12th and 30th days. The lowest root dry weights ranged from 0.39 to 0.42 g 100 ml^{-1} and the highest root dry weights ranged from 0.80 to 0.83 g 100 ml^{-1}. Based on these results, it was determined that the optimum values in terms of root growth parameters were obtained for roots cultured during 12, 15, 18, 21, 24, 27, and 30 days.

Figure 2. Effects of culture times on the root growth parameters in *Hyoscyamus niger* adventitious roots stimulated with 1 mM MeJA (A: fresh root weight (FW, g 100 ml^{-1}), B: root growth index (GI), C: dry root weight (DW, g 100 ml^{-1})) (Duncan's multiple range test, $p \leq 0.05$).

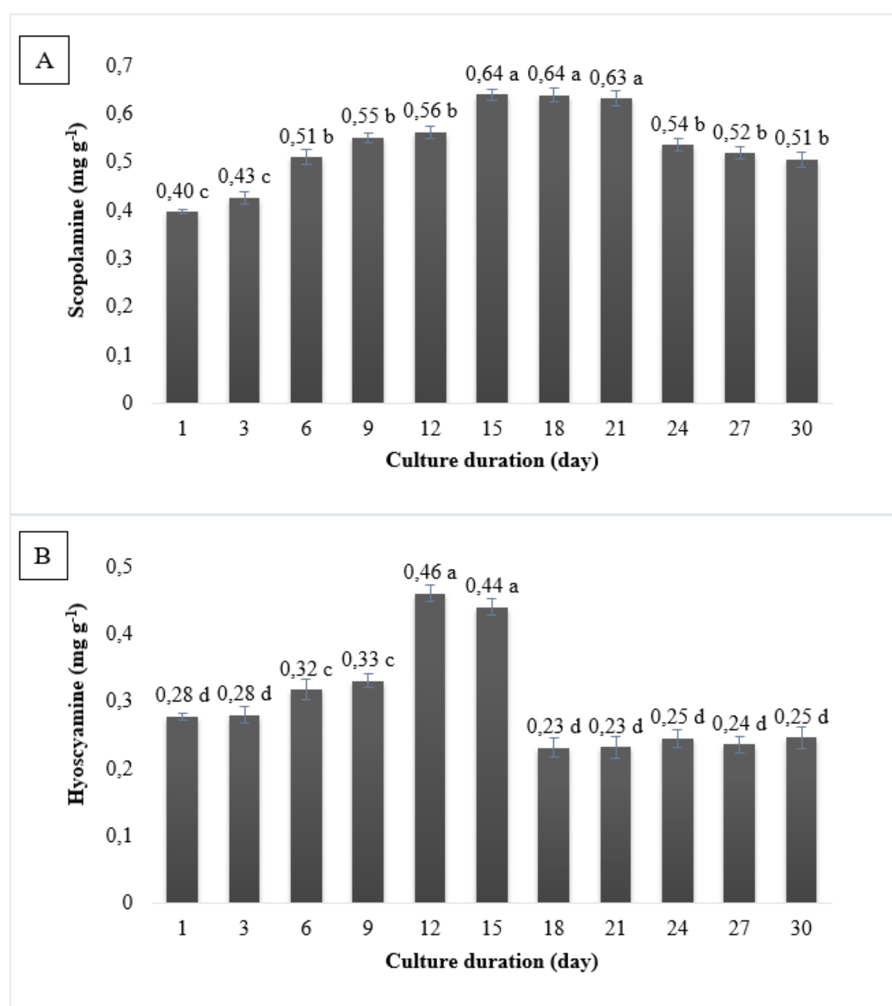


3.2. Effects of The Culture Times on The Production of Tropane Alkaloids

To understand the effects of culture times on the accumulation of tropane alkaloids in adventitious roots treated with 1 mM MeJA, the amount of hyoscyamine and scopolamine was determined by HPLC (Figure 3). The lowest amounts of scopolamine in the adventitious roots were detected at 0.40 and 0.43 mg g^{-1} on day 1 and day 3, respectively. The amount of scopolamine increased from day 3 to day 15, but there were no significant differences on days 6, 9, and 12 (0.51, 0.55, and 0.56 mg g^{-1}, respectively). The scopolamine level, which reached its maximum on day 15, remained constant between 0.63-0.64 mg g^{-1} on days 18 and 21, after which it decreased significantly on day 24 and remained constant until day 30 (Figure 3A). When the amount of hyoscyamine was analysed, it was 0.28 mg g^{-1} on days 1 and 3 and

increased to 0.32 and 0.33 mg g^{-1} on days 6 and 9, respectively. Then, it reached the highest values of 0.46 and 0.44 mg g^{-1} on days 12 and 15, respectively. However, the amount of hyoscyamine decreased from day 18 and remained between 0.23 mg g^{-1} and 0.25 mg g^{-1} until day 30 without significant change (Figure 3B). The analyses revealed that the 15-day culture period after the application of 1 mM MeJA was the optimum for the accumulation of hyoscyamine and scopolamine in adventitious root cultures of *H. niger*.

Figure 3. Effects of culture times on the scopolamine (A) and hyoscyamine (B) amounts in *H. niger* adventitious roots stimulated with 1 mM MeJA (Duncan's multiple range test, $p \leq 0.05$).



3.3. Effect of Culture Times on The Production of Phenolics

Total phenolic content (TPC) was determined by the spectrophotometric method to understand the effects of the culture times on the total phenolic accumulation in *H. niger* adventitious roots treated with 1 mM MeJA (Table 1). The TPC analyses revealed that the lowest TPCs were found in the roots harvested on day 1 at 5.59 mg g^{-1} and day 3 at 6.37 mg g^{-1}. The TPC increased from the 3rd day of culture and reached a maximum value of 19.33 mg g^{-1} on the 15th day. However, there was a significant decrease in the amount of TPC on the 18th day compared to the 15th day. It was found that there was no significant difference in TPC amount between the 18th and 21st day, while the decrease in TPC amount on the 24th day was not significant.

To determine the effects of culture times on the accumulation of some phenolic compounds, the amounts of gallic acid, catechin, epicatechin, vanillin, cinnamic acid, rosmarinic acid,

ferulic acid, caffeic acid, o-coumaric acid, p-coumaric acid, rutin, quercetin, and chlorogenic acid were also analysed by HPLC in this study. According to the results of HPLC analyses, caffeic acid, ferulic acid, rutin, quercetin, o-coumaric acid, and p-coumaric acid could not be detected in the roots. The amounts of gallic acid, catechin, epicatechin, vanillin, cinnamic acid, rosmarinic acid, and chlorogenic acid differed significantly according to culture time after the application of 1 mM MeJA ($p \leq 0.05$). Gallic acid levels reached their highest values on the 9th, 12th, and 15th days, with values ranging from 62.21 to 61.92 µg g^{-1}. From the 18th day, they began to decrease, and the lowest values were obtained in roots cultured for 27 and 30 days. Catechin showed no significant difference in the first 6 days of culture, but the increase that started on the 9th day reached the highest value on the 15th day. The lowest amount of catechin was found in the roots harvested on the 1st day with 97.49 µg g^{-1}, while the highest amounts of catechin were found in the roots collected on the 15th and 18th days with values of 598.77 and 596.10 µg g^{-1}, respectively. The accumulation of epicatechin in the roots reached its highest value of 28.02 µg g^{-1} on the 15th day. On day 18, the amount of epicatechin decreased drastically to 4.86 µg g^{-1}. While this drastic decrease in the amount of epicatechin continued until day 21, no epicatechin was detected on day 24 and the following days. HPLC analysis showed that the amount of vanillin increased rapidly from the first harvest period, reaching its highest value on day 6 and maintaining this value until day 15. However, on day 18 it began to decrease (14.15 µg g^{-1}) and reached its lowest value on day 21 (4.13 µg g^{-1}), decreasing even more. No major differences were observed between day 24 and day 30 concerning vanillin. The highest amounts of cinnamic acid were detected in the roots harvested on the 12th, 15th, 18th, and 21st days (26.98 - 28.87 µg g^{-1}), while the lowest values were found in the roots harvested on the 1st, 3rd, 27th and 30th days (4.02 - 5.97 µg g^{-1}). The highest levels of rosmarinic acid were detected between days 15 and 24, with an approximately 5-fold increase compared to day 1. It decreased dramatically from 56.79 µg g^{-1} on day 24 to 17.37 µg g^{-1} on day 27 and to 7.89 µg g^{-1} on day 30. Chlorogenic acid reached its highest levels on day 12 (2771.31 µg g^{-1}) and day 15 (2739.13 µg g^{-1}). The decrease in the amount of chlorogenic acid from day 18 continued gradually until day 30 and reached its lowest value on day 30 (1619.15 µg g^{-1}).

As a result of the study, it was concluded that the 15-day culture period after the application of 1 mM MeJA was the optimal culture period for phenol production in the adventitious roots of *H. niger*. Root growth parameters studied were root fresh weights, root growth indexes, and root dry weights. There were statistically significant differences in all growth parameters depending on the culture times ($p \leq 0.05$). The lowest fresh weights were found in roots harvested on the 1st and 3rd days after the application of 1 mM MeJA (4.17-4.24 g 100 ml^{-1}). Fresh weights increased significantly on days 6, 9, and 12 of culture with values of 5.04, 5.79, and 8.53 g 100 ml^{-1}, respectively (Figure 2A). However, no significant differences were observed between harvests grown at 3-day intervals from day 12 to day 30. The root growth index increased slowly from day 3 and peaked (1.05) on day 12 (Figure 2B). Thereafter, the growth index remained constant until day 30, with no significant differences. Root dry weights were lowest on days 1 and 3 and increased significantly on days 6, 9, and 12, similar to the trends in fresh weight and growth index. While root dry weights varied between 0.80 and 0.82 at all culture times, there were no significant differences between days 12 and 30 (Figure 2C). The lowest root dry weights ranged from 0.39 to 0.42 g 100 ml^{-1} and the highest root dry weights ranged from 0.80 to 0.83 g 100 ml^{-1}. Based on these results, it was found that the optimal values in terms of root growth parameters were obtained for roots cultured during 12, 15, 18, 21, 24, 27, and 30 days.

Table 1. Effects of culture times on the phenolic compounds in *H. niger* adventitious roots stimulated with 1 mM MeJA.

Culture times (day)	TPC (mg g^{-1})	Gallic acid (µg g^{-1})	Catechin (µg g^{-1})	Epicatechin (µg g^{-1})	Vanillin (µg g^{-1})	Cinnamic acid (µg g^{-1})	Rosmarinic acid (µg g^{-1})	Chlorogenic acid (µg g^{-1})
1	5.59 g*	24.75 ef	97.43 d	6.49 cd	15.48 c	4.02 d	10.08 ef	1690.54 ef
3	6.37 g	39.53 cd	126.90 d	6.54 cd	31.84 b	5.26 d	12.97 e	1889.99 de
6	10.97 de	54.05 b	133.12 d	9.67 c	37.84 a	12.97 c	23.63 c	2135.17 cd
9	11.42 d	62.21 a	307.81 c	9.38 c	40.01 a	20.98 b	26.08 c	2240.32 bc
12	15.68 c	62.58 a	485.62 b	15.08 b	39.12 a	26.98 a	35.02 b	2771.31 a
15	19.33 a	61.92 a	598.77 a	28.02 a	38.49 a	27.63 a	56.98 a	2739.13 a
18	17.16 bc	52.91 a	596.10 a	4.86 de	14.15 c	28.49 a	54.58 a	2494.44 b
21	17.45 b	49.90 bc	598.83 a	2.88 ef	4.13 d	28.87 a	55.78 a	2269.01 bc
24	11.71 d	34.34 de	500.66 b	0.00 f	4.24 d	12.60 c	56.79 a	2116.99 cd
27	9.68 ef	21.16 f	474.39 b	0.00 f	4.25 d	5.97 d	17.37 d	1919.85 de
30	8.66 f	18.05 f	459.43 b	0.00 f	4.26 d	5.56 d	7.89 f	1619.15 f

* Differences between means indicated by the same letters are not statistically significant (Duncan's multiple range test, $p \leq 0.05$)

4. DISCUSSION and CONCLUSION

In this study, the effects of different culture times on *H. niger* adventitious root cultures treated with 1 mM MeJA were evaluated in terms of root growth parameters and secondary metabolite accumulation. Root growth parameters, including root fresh weight, root growth index, and root dry weight, showed a similar pattern. Application of 1 mM MeJA resulted in no change in root growth on days 1, 3, and 6. However, on the 9th day, roots started to grow rapidly and reached their maximum growth on the 12th day. On the 12th day, root growth stopped and there was no statistically significant difference on the 12th, 15th, 18th, 21st, 24th, 27th, and 30th days. Similar to our study, it was reported that when 0.1 mM MeJA was applied to *Salvia miltiorrhiza* root cultures, root growth increased steadily until the 12th day and reached its maximum on the 12th day (Xiao *et al.,* 2009). It is known that there are differences in root growth as a function of MeJA concentration and culture time, as indicated by previous studies on different plants. Treatment of root cultures of *Glycyrrhiza glabra* (Shabani *et al.,* 2009) and *Setaria parviflora* (Kang *et al.,* 2004) with high concentrations of MeJA negatively affected root growth, and root growth decreased to the lowest level 24 and 72 h after application of 2 mM MeJA. In root cultures of *Datura metel*, root growth after application of various elicitors remained in the lag phase for the first 6 days, increased linearly for the next 21 days, and stopped after day 27 (Ajungla *et al.,* 2009). Application of chitosan in *Hypericum perforatum* root cultures (Brasili *et al.,* 2016) and application of pectinase in *Catharanthus roseus* root cultures (Rijhwani and Shanks 1998) suppressed root growth compared to the control group. Considering the results of the previous studies and this study, it is concluded that the effects of harvest periods on *in vitro* production of secondary metabolites vary considerably depending on the plant species, application of the elicitor, type of elicitor, and concentration of the elicitor. In particular, the application of elicitors that act as signaling molecules in the plant defense system, such as MeJA, inhibits the mitotic cycle in plant cells and reduces cell division and biomass increase by stopping cell division in G1 phase before entering S phase. For this reason, biomass production decreases or even stops after high concentrations of MeJA (Ho *et al.,* 2018; Kang *et al.,* 2004; Mendoza *et al.,* 2018).

This study also examined the effects of culture duration on the accumulation of hyoscyamine and scopolamine in adventitious roots of *H. niger*. Genotypes, metabolites, and elicitor applications have a great impact on determining the most appropriate culture time to achieve high efficiency *in vitro* production of secondary metabolites. MeJA is one of the most important elicitors used to enhance secondary metabolite production, and MeJA is known to significantly affect alkaloid production, especially as a function of culture time (Kang *et al.,* 2004; Zayed & Wink 2005). The 3-day application of MeJA to the roots of *Scopolia parviflora* significantly affected the amount of hyoscyamine and scopolamine as a function of MeJA concentration and culture time (Kang *et al.,* 2004). In the same study, MeJA was reported to regulate the synthesis of the enzymes putrescine N-methyl transferase (PMT) and hyoscyamine-6β-hydroxylase (H6H), thereby increasing alkaloid production. In *Peganum harmala*, the highest accumulation of alkaloids occurred in roots harvested on day 5 after MeJA application (Zayed & Wink 2005). Lee *et al.,* (2001) found that the alkaloid content in *Atropa belladonna* hair roots treated with 2 mM salicylic acid decreased by 35% on day 1, 80% on day 3, and 90% on day 7. The researchers explained the reason for this decrease as an increase in the amount of alkaloids entering the culture medium, explaining that salicylic acid accelerates the transfer of alkaloids from the roots to the culture medium (Lee *et al.,* 2001). In transformed A. belladonna hairy roots after phytosulfokine-α (PSK-α) application, the highest alkaloid production occurred in 4 weeks (Sasaki *et al.,* 2002). The optimal culture time for alkaloid production in root cultures varies considerably depending on elicitor application and genotypes, as previously reported (Harfi *et*

al., 2016; Moharrami *et al.,* 2017). The changes in phenolics in randomly selected roots after application of 1 mM MeJA were also investigated.

After the application of 1 mM MeJA, the changes in phenolics in adventitious roots grown at intervals of 3 days to 30 days were also studied. It was found that TPC varied significantly depending on the culture period. The highest TPC value was obtained in the roots harvested on day 15. The extensive literature search revealed that studies on alkaloid-rich plant species such as *H. niger* focused on alkaloid production under *in vitro* conditions, while phenolic compounds were not the main focus. However, *H. niger* is also rich in very valuable phenolics for the pharmaceutical and cosmetic industries. In studies investigating phenolic content as a function of culture time after biotic or abiotic elicitor applications, the highest total phenolic accumulation in hairy roots of *Harpagophytum procumbens* stimulated with *Agrobacterium rhizogenes* was found in roots collected on day 21 (Georgiev *et al.,* 2006). In cell cultures of *Thevetia peruviana* treated with MeJA, the highest total phenolic content was reached at the 96th hour (Mendoza *et al.,* 2018). The application of 100 µM cadmium to *Vaccinium corymbosum* caused the highest accumulation of total phenolic content on day 21 (Manquian-Cerda *et al.,* 2016). Culture time was shown to significantly alter phenolic content after elicitor applications on various plants such as *Pelargonium sidoides* (Yousefian *et al.,* 2020), *Polygonum hydropiper* (Ono *et al.,* 1998), *Capsicum frutescens* (Suresh & Ravishankar 2005), *Salvia miltiorrhiza* (Xiao *et al.,* 2009), *Agastache rugose* (Lee *et al.,* 2008), *Echinacea purpurea* (Demirci *et al.,* 2020). Ono *et al.,* (1998) reported that the changes in phenolic content during the culture period were due to the conversion of some phenols to other compounds. The researchers found that the amount of catechin, which increased until day 6, began to decrease later, which was due to the catechin being converted to procyanidin by bioconversion.

In the *in vitro* production of secondary metabolites, one of the most important reasons for the decrease in secondary metabolites after reaching the highest level in plant cells and tissues due to the increase in culture time is the increased release of metabolites from the explants to the culture medium (Lee *et al.,* 2001). Therefore, the accumulation of metabolites in plant materials decreases while it increases in culture media. In addition, conversion of metabolites to another compound by biodegradation or degradation of elicitor substances added to culture media to increase metabolite yield are other reasons for the decrease in secondary metabolite accumulation during culture (Ono *et al.,* 1998; Suresh and Ravishankar 2005). From the results, harvesting periods for *in vitro* production of secondary metabolites are the most important criterion for the production of large amounts of metabolites. However, many factors such as genotype, metabolite, application of elicitors, etc., play a role in determining the most appropriate culture time.

In this study, the effects of culture time on root growth, tropane alkaloids, and phenolics production in *H. niger* adventitious root cultures treated with 1 mM MeJA were investigated, and it was found that culture time significantly affected root growth and secondary metabolites production. Roots collected on day 15 after MeJA application was found to be the optimal culture time in terms of root growth and secondary metabolite content. However, further research is needed due to the lack of appropriate work to determine the optimal time of culture to increase yield in secondary metabolite production in plants rich in tropane alkaloids and *H. niger*. This work was the first to investigate the effects of culture time on highly efficient secondary metabolite production and optimal root growth in *H. niger* adventitious root cultures. Future experiments will use bioreactor systems in which pH, soluble oxygen, and culture media can be controlled to recover high levels of secondary metabolites from *H. niger* adventitious root cultures. The results of this study provide important guidance for future scale-up production using bioreactors to recover large amounts of alkaloids and phenols.

Acknowledgments

The authors thank Isparta University of Applied Sciences for supporting this research (2019-YL 1-0031).

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Hikmet Deveci: Formal analysis, Investigation. **Tunhan Demirci:** Conceptualization, Methodology, Formal analysis, Investigation, Writing- original draft, Writing-review & editing, Supervision. **Nilgun Gokturk Baydar:** Writing- review & editing, Supervision.

Orcid

Hikmet Deveci https://orcid.org/0000-0003-4393-8552

Tunhan Demirci https://orcid.org/0000-0001-8516-911X

Nilgün Gokturk Baydar https://orcid.org/0000-0002-5482-350X

REFERENCES

Ajungla, L., Patil, P.P., Barmukh, R.B., Nikam, T.D. (2009). Influence of biotic and abiotic elicitors on accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L. *Indian J Biotechnol. 8*, 317-322.

Alizadeh, A., Moshiri, M., Alizadeh, J., Balali-Mood, M. (2014). Black henbane and its toxicity - A descriptive review. *Avicenna J Phytomed. 4*, 297-311.

Boitel-Conti, M., Laberche, J.C., Lanoue, A., Ducrocq, C., Sangwan-Norreel, B.S. (2000). Influence of feeding precursors on tropane alkaloid production during an abiotic stress in *Datura innoxia* transformed roots. *Plant Cell, Tiss Org, 60*, 131-137. https://doi.org/10.1023/A:1006426314274

Brasili, E., Miccheli, A., Marini, F., Pratico, G., Sciubba, F., Di Cocco, M.E., Cechinel, V.F., Tocci, N., Valletta, A., Pasqua, G. (2016). Metabolic profile and root development of *Hypericum perforatum* L. *İn vitro* roots under stress conditions due to chitosan treatment and culture time. *Front Plant Sci, 7*, 507. https://doi.org/10.3389/fpls.2016.00507

Clissold, S.P., & Heel, R.C. (1985). Transdermal hyoscine (Scopolamine). A preliminary review of its pharmacodynamic properties and therapeutic efficacy. *Drugs, 29*, 189-207. https://doi.org/10.2165/00003495-198529030-00001

D'yakova, N.A., Slivkin, A.I., Gaponov, S.P. (2020). Assessment of Radionuclide Contents in Medicinal Plant Raw Material of the Central Black-Earth Belt and their Influence on Accumulation of Biologically Active Compounds. *Pharm Chem J, 54*, 626-630. https://doi.org/10.1007/s11094-020-02248-1

Dehghan, E., Hakkinen, S.T., Oksman-Caldentey, K.M., Ahmadi, F.S., 2012. Production of tropane alkaloids in diploid and tetraploid plants and *in vitro* hairy root cultures of Egyptian henbane (*Hyoscyamus muticus* L.). *Plant Cell Tiss Org, 110*, 35-44. https://doi.org/10.1007/s11240-012-0127-8

Demirci, T., Akcay, U.C., Baydar, N.G. (2020). Effects of 24-epibrassinolide and L-phenylalanine on growth and caffeic acid derivative production in hairy root culture of *Echinacea purpurea* L. Moench. *Acta Physiol Plant, 42*. https://doi.org/10.1007/s11738-020-03055-7

Demirci, T., Asci, O.A., Baydar, N.G. (2021). Influence of salicylic acid and L-phenylalanine on the accumulation of anthraquinone and phenolic compounds in adventitious root cultures

of madder (*Rubia tinctorum* L.). *Plant Cell Tiss Org, 144*, 313-324. https://doi.org/10.1007/s11240-020-01952-w

Demirci, T., Albayrak, İ. Göktürk Baydar, N. (2022). L-phenylalanine applications and culture duration affect root growth and production of tropane alkaloids and phenolics in adventitious root cultures of *Hyoscyamus niger* L. *Plant Cell Tiss Org, 149,* 295-311. https://doi.org/10.1007/s11240-021-02173-5

Faydaoğlu, E., & Sürücüoğlu, M. (2013). Tıbbi ve aromatik bitkilerin antimikrobiyal, antioksidan aktiviteleri ve kullanım olanakları. *Erzincan Üniversitesi Fen Bilimleri Enstitüsü Dergisi. 6*, 233-265. https://dergipark.org.tr/en/pub/erzifbed/issue/6027/80707

Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Scheffer, J.J.C. (2008). Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour Fragr. J, 23*, 213-226. https://doi.org/10.1002/ffj.1875

Georgiev, M., Heinrich, M., Kerns, G., Bley, T., Pavlov, A. (2006). Production of iridoids and phenolics by transformed *Harpagophytum procumbens* root cultures. *Eng. Life Sci, 6*, 593-596. https://doi.org/10.1002/elsc.200620160

Giri, C.C., & Zaheer, M. (2016). Chemical elicitors versus secondary metabolite production *in vitro* using plant cell, tissue and organ cultures: recent trends and a sky eye view appraisal. *Plant Cell Tiss Org, 126*, 1-18. https://doi.org/10.1007/s11240-016-0985-6

Harfi, B., Khelifi-Slaoui, M., Bekhouche, M., Benyammi, R., Hefferon, K., Makhzoum, A., Khelifi, L. (2016). Hyoscyamine production in hairy roots of three *Datura* species exposed to high-salt medium. *In vitro Cell Dev-Pl, 52*, 92-98. https://doi.org/10.1007/s11627-015-9725-6

Ho, T.T., Lee, J.D., Jeong, C.S., Paek, K.Y., Park, S.Y. (2018). Improvement of biosynthesis and accumulation of bioactive compounds by elicitation in adventitious root cultures of *Polygonum multiflorum*. *Appl Microbiol Biotechnol, 102*, 199-209. https://doi.org/10.1007/s00253-017-8629-2

Hong, M.L.K., Bhatt, A., Ping, N.S., Keng, C.L. (2012). Detection of elicitation effect on *Hyoscyamus niger* L. root cultures for the root growth and production of tropane alkaloids. *Rom Biotech Lett, 17*, 7340-7351.

Jakabova, S., Vincze, L., Farkas, A., Kilar, F., Boros, B., Felinger, A. (2012). Determination of tropane alkaloids atropine and scopolamine by liquid chromatography-mass spectrometry in plant organs of *Datura* species. *J Chromatogr A, 1232*, 295-301. https://doi.org/10.1016/j.chroma.2012.02.036

Jirschitzka, J., Schmidt, G.W., Reichelt, M., Schneider, B., Gershenzon, J., D'Auria, J.C. (2012). Plant tropane alkaloid biosynthesis evolved independently in the Solanaceae and Erythroxylaceae. *P Natl Acad Sci USA, 109*, 10304-9. https://doi.org/10.1073/pnas.1200473109

John, H., Binder, T., Hochstetter, H., Thiermann, H. (2010). LC-ESI MS/MS quantification of atropine and six other antimuscarinic tropane alkaloids in plasma. *Anal Bioanal Chem, 396*, 751-63. https://doi.org/10.1007/s00216-009-3209-7

Kang, S.M., Jung, H.Y., Kang, Y.M., Yun, D.J., Bahk, J.D., Yang, J.K., Choi, M.S. (2004). Effects of methyl jasmonate and salicylic acid on the production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia parviflora*. *Plant Sci, 166*, 745-751. https://doi.org/10.1016/j.plantsci.2003.11.022

Kareem, Z.J., Su, L., Rathgeb, A., Sirrenberg, A., Hadacek, F., Rashid, A.H.A.H., Karlovsky, P. (2019). Small-scale bioreactor for sterile hydroponics and hairy roots: Metabolic diversity and salicylic acid exudation by hairy roots of *Hyoscyamus niger*. *Appl Sci-Basel, 9.* https://doi.org/10.3390/app9153044

Kohnen-Johannsen, K.L., & Kayser, O. (2019). Tropane alkaloids: chemistry, pharmacology, biosynthesis and production. *Molecules, 24*, 796. https://doi.org/10.3390/molecules24040796

Lee, K.T., Hirano, H., Yamakawa, T., Kodama, T., Igarashi, Y., Shimomura, K. (2001). Responses of transformed root culture of *Atropa belladonna* to salicylic acid stress. *J Biosci Bioeng, 91*, 586-9. https://doi.org/10.1016/S1389-1723(01)80178-X

Lee, S.Y., Xu, H., Kim, Y.K., Park, S.U. (2008). Rosmarinic acid production in hairy root cultures of *Agastache rugosa* Kuntze. *World J. Microbiol. Biotechnol, 24*, 969-972. https://doi.org/10.1007/s11274-007-9560-y

Li, J., Shi, J., Yu, X., Sun, J., Men, Q., & Kang, T. (2011). Chemical and pharmacological researches on *Hyoscyamus niger*. *Chin. Herb. Med*, 117-126.

Lu, C.W., Ding, J.Z., Park, H.K., Feng, H. (2020). High intensity ultrasound as a physical elicitor affects secondary metabolites and antioxidant capacity of tomato fruits. *Food Control, 113*, 107176. https://doi.org/10.1016/j.foodcont.2020.107176

Manquian-Cerda, K., Escudey, M., Zuniga, G., Arancibia-Miranda, N., Molina, M., Cruces, E. (2016). Effect of cadmium on phenolic compounds, antioxidant enzyme activity and oxidative stress in blueberry (*Vaccinium corymbosum* L.) plantlets grown *in vitro*. *Ecotoxicol Environ Saf, 133*, 316-26. https://doi.org/10.1016/j.ecoenv.2016.07.029

Mendoza, D., Cuaspud, O., Arias, J.P., Ruiz, O., Arias, M. (2018). Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana*. *Biotechnol Rep (Amst), 19*, e00273. https://doi.org/10.1016/j.btre.2018.e00273

Moharrami, F., Hosseini, B., Sharafi, A., Farjaminezhad, M. (2017). Enhanced production of hyoscyamine and scopolamine from genetically transformed root culture of *Hyoscyamus reticulatus* L. elicited by iron oxide nanoparticles. *İn vitro Cell Dev Biol Plant, 53*, 104-111. https://doi.org/10.1007/s11627-017-9802-0

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant, 15*, 473-497.

Narayani, M., & Srivastava, S. (2017). Elicitation: A stimulation of stress in *in vitro* plant cell/tissue cultures for enhancement of secondary metabolite production. *Phytochem Rev, 16*, 1227-1252. https://doi.org/10.1007/s11101-017-9534-0

Ono, K., Nakao, M., Toyota, M., Terashi, Y., Yamada, M., Kohno, T., Asakawa, Y. (1998). Catechin production in cultured *Polygonum hydropiper* cells. *Phytochemistry, 49*, 1935-1939. https://doi.org/10.1016/S0031-9422(98)00426-9

Orbak, Z., Tan, H., Karakelleoğlu, C., Alp, H., Akdağ, R. (1998). *Hyoscyamus niger* (Henbane) poisonings in the rural area of east Turkey. *Eurasian J. Med, 30*, 145-148.

Pokorny, M.L., Mangold, J.M., Hafer, J., Denny, M.K. (2010). Managing spotted knapweed (Centaurea stoebe)–infested rangeland after wildfire. *Invasive Plant Sci Manag*, *3*, 182-189. https://doi.org/10.1614/IPSM-09-023.1

Pudersell, K., Vardja, T., Vardja, R., Matto, V., Arak, E. Raal, A. (2012). Inorganic ions in the medium modify tropane alkaloids and riboflavin output in *Hyoscyamus niger* root cultures. *Pharmacogn Mag, 8*, 73-77. https://dx.doi.org/10.4103%2F0973-1296.93330

Qin, B.F., Ma, L.L., Wang, Y.X., Chen, M., Lan, X.Z., Wu, N.B., Liao, Z.H. (2014). Effects of acetylsalicylic acid and UV-B on gene expression and tropane alkaloid biosynthesis in hairy root cultures of *Anisodus luridus*. *Plant Cell Tiss Org, 117*, 483-490. https://doi.org/10.1007/s11240-014-0454-z

Rijhwani, S.K., & Shanks, J.V. (1998). Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. *Biotechnol Prog, 14*, 442-449. https://doi.org/10.1021/bp980029v

Roy. A. (2021). Hairy root culture an alternative for bioactive compound production from medicinal plants. *Curr Pharm Biotechnol, 22*, 136-149. https://doi.org/10.2174/1389201021666201229110625

Sargın, S.A., Akçicek, E., & Selvi, S. (2013). An ethnobotanical study of medicinal plants used by the local people of Alaşehir (Manisa) in Turkey. *J. Ethnopharmacol*, *150*(3), 860-874. https://doi.org/10.1016/j.jep.2013.09.040

Sasaki, K., Ishise, T., Shimomura, K., Kobayashi, T., Matsubayashi, Y., Sakagami, Y., Umetsu, H., Kamada, H. (2002). Effects of phytosulfokine-α on growth and tropane alkaloid production in transformed roots of *Atropa belladonna*. *Plant Growth Regul, 36*, 87-90. https://doi.org/10.1023/A:1014786705829

Shabani, L., Ehsanpour, A.A., Asghari, G., Emami, J. (2009). Glycyrrhizin production by *in vitro* cultured *Glycyrrhiza glabra* elicited by methyl jasmonate and salicylic acid. *Russ J Plant Physl, 56*, 621-626. https://doi.org/10.1134/S1021443709050069

Shah, D., Kamili, A.N., Wani, A.A., Majeed, U., Wani, Z.A., Sajjad, N., Ahmad, P. (2020). Promoting the accumulation of scopolamine and hyoscyamine in *Hyoscyamus niger* L. through EMS based mutagenesis. *Plos One, 15*, e0231355. https://doi.org/10.1371/journal.pone.0231355

Shakeran, Z., Keyhanfar, M., Ghanadian, M. (2017). Biotic elicitation for scopolamine production by hairy root cultures of *Datura metel*. *Mol Biol Res Commun, 6*, 169-179. https://dx.doi.org/10.22099%2Fmbrc.2017.25776.1275

Singleton, V.L., & Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *AJEV, 16*, 144-158.

Soni, U., Brar, S., Gauttam, V.K. (2015). Effect of seasonal variation on secondary metabolites of medicinal plants. *Int. J. Pharm. Sci, 6*, 3654-3662. https://doi.org/10.13040/Ijpsr.0975-8232.6(9).3654-62

Suresh, B., & Ravishankar, G.A. (2005). Methyl jasmonate modulated biotransformation of phenylpropanoids to vanillin related metabolites using *Capsicum frutescens* root cultures. *Plant Physiol Biochem, 43*, 125-131. https://doi.org/10.1016/j.plaphy.2005.01.006

Tang, G.L., Ma, J.N., Hause, B., Nick, P., Riemann, M. (2020). Jasmonate is required for the response to osmotic stress in rice. *Environ Exp Bot, 175*. https://doi.org/10.1016/j.envexpbot.2020.104047

Wang, H.B., Cheng, X., Wu, C.E., Fan, G.J., Li, T.T., Dong, C. (2021). Retardation of postharvest softening of blueberry fruit by methyl jasmonate is correlated with altered cell wall modification and energy metabolism. *Sci Hortic-Amsterdam, 276*. https://doi.org/10.1016/j.scienta.2020.109752

Xiao, Y., Gao, S., Di, P., Chen, J., Chen, W., Zhang, L. (2009). Methyl jasmonate dramatically enhances the accumulation of phenolic acids in *Salvia miltiorrhiza* hairy root cultures. *Physiol. Plant*, *137*, 1-9. https://doi.org/10.1111/j.1399-3054.2009.01257.x

Yousefian, S., Lohrasebi, T., Farhadpour, M., Haghbeen, K. (2020). Effect of methyl jasmonate on phenolic acids accumulation and the expression profile of their biosynthesis-related genes in *Mentha spicata* hairy root cultures. *Plant Cell Tiss Org, 142*, 285-297. https://doi.org/10.1007/s11240-020-01856-9

Yücel, U.M., & Yılmaz, O. (2014). Total alkaloid amounts of two Hyoscyamus species (Henbane) grown in Van Region. *Van Vet J*, *25*, 77-80.

Zayed, R., & Wink, M. (2005). Beta-carboline and quinoline alkaloids in root cultures and intact plants of *Peganum harmala*. *Z Naturforsch C J Biosci, 60*, 451-458. https://doi.org/10.1515/znc-2005-5-614

Zhang, J.L., Liu, P., Liu, Y.B., Zhen, Z.X., Li, L., Gao, M.X. (2020). Effect of salicylic acid and methyl jasmonate on growth and pigment production in *Monascus purpureus*. *J Sci Ind Res India, 79*, 681-685. http://nopr.niscair.res.in/handle/123456789/55222

Zuniga, P.E., Castaneda, Y., Arrey-Salas, O., Fuentes, L., Aburto, F., Figueroa, C.R. (2020). Methyl jasmonate applications from flowering to ripe fruit stages of strawberry (Fragaria x ananassa 'Camarosa') reinforce the fruit antioxidant response at post-harvest. *Front Plant Sci, 11*, 538. https://doi.org/10.3389/fpls.2020.00538

Zvezdina, E.V., Dayronas, J.V., Bochkareva, I.I., Zilfikarov, I.N., Babaeva, E.Y., Ferubko, E. V., Guseynova, Z.A., Serebryanaya, F.K., Kaibova, S.R., Ibragimov, T.A. (20209. Members of the family Lamiaceae lindl. as sources of medicinal plant raw materials to obtain neurotropic drugs. *Pharm Pharmacol-Russ, 8,* 4-28. https://doi.org/10.19163/2307-9266-2020-8-1-4-28