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The effect of polyethylene glycol and chitosan on the production of hyoscyamine and scopolamine in *Hyoscyamus niger* L. cell suspension culture

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ABSTRACT

Elicitors can stimulate any defense-related pathways, leading to the synthesis of secondary metabolites in plants. The main aim of this study was to investigate the effects of polyethylene glycol (PEG) and chitosan elicitors on cell growth and the production of hyoscyamine and scopolamine in *Hyoscyamus niger* L. cell suspension cultures. Leaves from 6-week-old *in vitro* plantlets were used as explants for callus induction on Murashige and Skoog (MS) medium containing 0.25 mg/L 2,4-D. After establishing the cell suspension culture, different concentrations of chitosan (0, 25, 50, and 75 mg/L) and PEG (0, 2.5, 5, and 10%) were applied, and cells were harvested after 1, 2, and 3 days. The results showed that elicitor concentration and sampling time did not significantly affect cell growth, but accumulation and production of scopolamine and hyoscyamine varied with elicitor concentration, sampling time, and their interactions. Using chitosan and PEG as elicitors, the maximum scopolamine accumulation and production were 258.93 µg/g DW and 2475.18 µg/L, obtained with 50 mg/L chitosan after 2 days, and 25 mg/L chitosan for 2 days, respectively. The highest hyoscyamine accumulation and production were 146.81 µg/g DW and 2008.09 µg/L obtained 3 days after applying 2.5% PEG, respectively. These results indicate that chitosan is more effective for stimulating scopolamine production, while PEG is more suitable for hyoscyamine production in *H. niger* L cell suspension culture.

Key words: Elicitor, Henbane, High-performance liquid chromatography, Secondary metabolites, Viability.

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INTRODUCTION

Since ancient times, communities have widely used herbal medicines for their therapeutic properties (Akhbarizadeh *et al.*, 2023). Medicinal use extends to treating infectious and noninfectious diseases, often through traditinal preparation methods (Ibrahim and Kebede, 2020). *Hyoscyamus niger* L. is an important medicinal plant that contains tropane alkaloid compounds such as hyoscyamine and scopolamine (Ahmadpour *et al.*, 2024).

The genus *Hyoscyamus* contains various tropane alkaloids and used as is valued as a medical plant (Ebrahimzadeh and Ebrahimzadeh maboud, 2013). Plant secondary metabolites (phytochemicals) are a key source of active compounds used in pharmaceuticals, food, cosmetics, and agrochemicals. In addition, secondary metabolites play major roles in plant adaptation and interactions with their environment, helping plants cope with biotic and abiotic stresses (Abdulhafiz *et al.*, 2022). Cell suspension culture is easier and more cost-effective than traditional cultivation and wild-harvest collection, and can be used to preserve endangered species. (Wu *et al.*, 2021).

Different methods can be used to enhance secondary metabolite production, including biological and non-biological elicitors. An elicitor is a compound that not only induces phytoalexins accumulation but also stimulates defense-related pathways, leading to the synthesis of secondary metabolites (Ahmadian Chashmi *et al.*, 2010). Polyethylene glycol (PEG) is a non-plasmolyzing, non-toxic agent that induces osmotic stress and reduces photosynthetic rate. Stress in plants can affect photosystems, pigments, and electron transport chains, ultimately reducing photosynthesis. High molecular weight PEG does not pass through the cell wall. Evidence suggests that PEG addition to the culture medium reduces O_2 availability, mimiking drought stress (Ahmadi-Sakha *et al.*, 2022).

Chitosan, a natural polysaccharide known for biocompatibility, non-toxicity, and cost-effectiveness, is mainly obtained from crustacean exoskeleton. Chitosan and its derivatives play important roles in modern agriculture and biotechnology by enhancing plant defense mechanisms, promoting plant growth, increasing resistance to diseases and pests, and modulating signals processes, including cellular responses leading to programmed cell death (Yu *et al.*, 2025). A review of available resources indicate that the cell suspension culture of *H. niger* L. has not yet been prepared, and no prior studies have addressed this. Therefore, this study aims to establish *H. niger* L. cell suspension culture and to investigate the effects of PEG and chitosan on cell growth and, cell density, and the production and accumulation of the two secondary metabolites hyoscyamine and scopolamine.

MATERIALS AND METHODS

Disinfection, seed culture and explant preparing

H. niger L. seeds were obtained from the Imam Khomeini International University (Qazvin, Iran). To break seed dormancy, seeds were treated with 200 mg/L gibberellic acid for 2 days at 4 °C. Seeds were then washed with tap water and liquid soap for 30 minutes. Surface sterilization was performed with 70% ethanol for 45 s followed by 2.5% sodium hypochlorite for 20 min (Ahmadpour *et al*., 2023). Seeds were cultured on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) and germinated in a growth chamber under an 8/16 h dark/light photoperiod at 25±2 °C for 14 days. After 14 days, seedlings were transferred to culture jars containing MS basal medium (Figure 1A). leaves from these plants were used as an explant after 28 days (Figure 1B).

Callus induction and establishment of cell suspension culture

Leaves were cut into 1×1 cm segments and transferred to MS basal medium supplement with 0.25 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction, incubated in the dark at 25±2 °C, and sub-cultured every 21 days (Figure 1C). The culture medium contained 30 g/L sucrose, adjusted to pH 5.8, and sterilized by autoclaving at 121 °C and 1.2 bar pressure. For the cell suspension culture establishment, 120 mg of friable calli were transferred to a 100 mL Erlenmeyer containing 25 mL of liquid MS basal medium with 0.25 mg/L 2,4-D. Cultures were kept in the dark at 27±2 °C in a shaker at 130 rpm until the end of the experiment. After establishing the cell suspension culture, cells were maintained in 250 mL Erlenmeyer flasks containing 50 mL liquid MS medium with 0.25 mg/L 2,4-D and sub-cultured every 7 days.

Preparation of cell growth and viability curves

The cell growth and viability curves were developed using an initial cellular density of 4.9×10^5 cells/mL. Cells and viability were assessed at 2-days intervals. For cell counting, 1 mL of CrO_3 was added to 0.5 mL of cell suspension and incubated at 70 °C for 15 min, then, vortexed for 3 min. Cells were counted with a hemocytometer under an optical microscope (Farjaminezhad and Garoosi, 2019) (Figure 1D-G). For viability, 1 mL of cell suspension was transferred to a 2 mL tube, centrifuged at 1000 rpm for 5 min, the

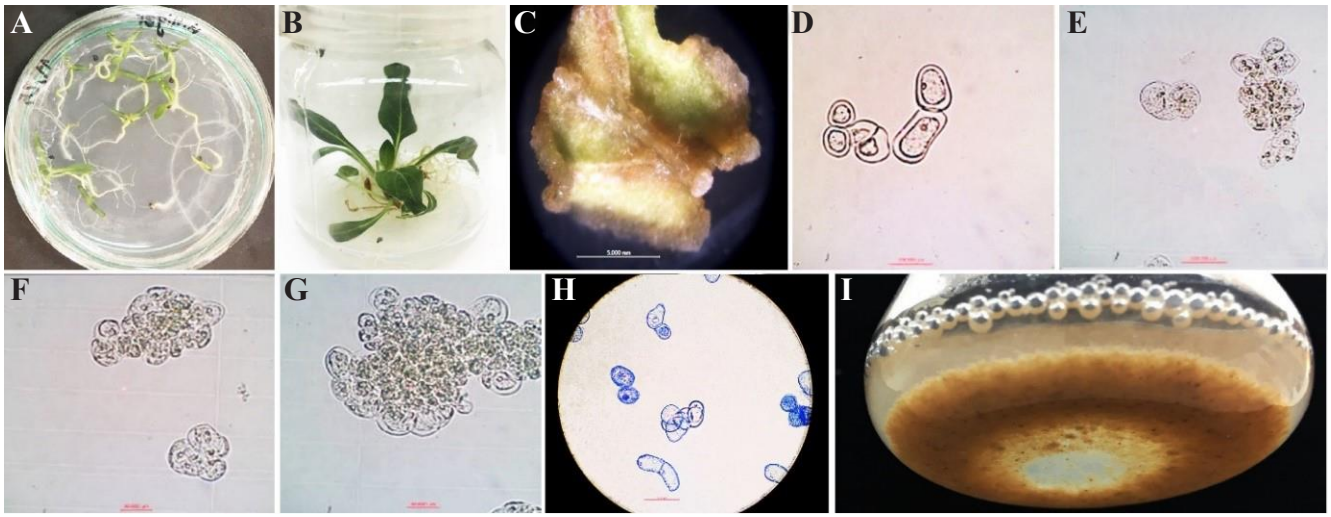

Figure 1. A: Seed germination, **B:** seedling used as explant, **C:** callus induction, **D-G:** over time gradual cell increasing in cell suspension culture, **H:** cell viability, where blue cytoplasmic staining indicates cell death, and **I:** cell suspension culture of *Hyoscyamus niger* L.

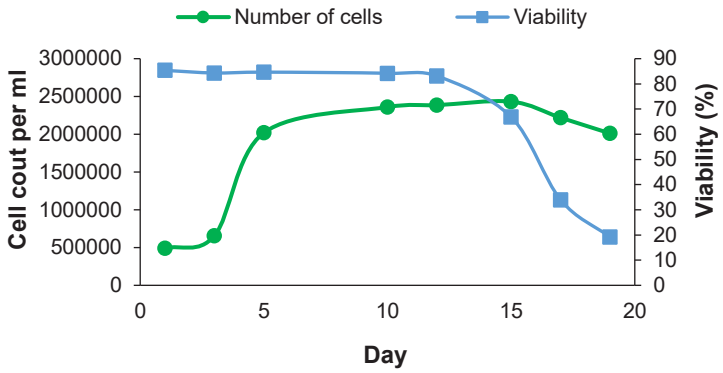

Figure 2. Growth and viability curves of *Hyoscyamus niger* L. cell suspension cultures.

supernatant discarded, and 50 µL of 1% Evans blue dye added and incubated for 10 min at room temperature. The mixture was re-centrifuged, the dye removed, 1 mL of distilled added, and cells were observed under an optical microscope by a hemocytometer to determine viability (Silva and Menéndez-Yuffá, 2006) (Figure 1H).

Applying elicitors to cell suspension culture

For elicitation, cell suspensions were cultured in 100 ml Erlenmeyer flasks containing 25 ml MS medium with 0.25 mg/L of 2,4-D and an initial cellular density of 4.9×10^5 cells/mL. According to the growth curve (Figure 2), on the 4th of cultivation, different concentrations of chitosan (0, 25, 50, and 75 mg/L) and PEG 4000 (0, 2.5%, 5%, and 10%) were added to the cell suspensions. Sampling was done on days 1, 2 and 3 post-elicitations, and fresh weight, dry weight, accumulation, and production of scopolamine and hyoscyamine were measured.

Measurement of fresh and dry weight of cells

Fresh weight was determined by collecting the cell mass on Whatman No. 1 filter paper using a Büchner funnel under vacuum, washing with 3 mL distilled water, applying vacuum for 30 s, and weighing immediately. Dry weight was estimated by drying the collected cells at 50 °C for 72 h.

Scopolamine and hyoscyamine extraction and HPLC-DAD analysis

To extract scopolamine and hyoscyamine, 100 mg of dry, powdered cells were placed in a 2 mL tube with 1 mL of 98.5% methanol and sonicated at 25 °C for 10 min. The mixtures were centrifuged at 12,000 rpm for 15 min, and the supernatant was collected and stored at -20 °C until analysis by HPLC-DAD (Ahmadpour *et al.*,

2024). Hyoscyamine and scopolamine contents were quantified using a Knauer HPLC-DAD system (DAD detector, Azura, Germany) with a modification to Vakili *et al.* (2012). The stationary phase was a C_{18} column (TSKgel-ODS C-18, 5 µm, 4.6×250 mm, Japan). The mobile phase consisted of 83% water, 15% methanol, 1.5% glacial acetic acid, and 0.5% triethylamine. Injection volume was 20 µL, column temperature was ambient, and flow rate was 0.5 mL/min. Absorbance for hyoscyamine and scopolamine was monitored at 258 nm. Quantification was performed using standards of hyoscyamine and scopolamine sulfate (Sigma, U.S.A), and the calibration curve. Production of scopolamine and hyoscyamine was calculated as (Srivastava and Srivastava, 2012):

(1) Production (µg/L) = biomass (g/L) × vinblastine and vincristine accumulation (µg/g DCW)

Statistical analysis

The study followed a factorial experiment in a completely randomized design with three replicate. The first factor was the different concentrations of chitosan and PEG 4000, and the second factor was sampling times. Variance analysis was performed using IBM SPSS statistics 27.0 (Armonk, NY, USA). Means were compared using Duncan's multiple range test at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

Seed and explant cultivation

Seeds were disinfected and immediately cultured. After 12 days, 72% of seeds germinated (Figure 1A). After three weeks, the seedlings were transferred to fresh, hormone-free MS culture medium in culture jars and maintained for approximately six weeks to promote robust growth (Figure 1B).

Callus induction and establishment of cell suspension culture

Callus induction is the initial step *in vitro*, culture , and it can result in irregular secondary metabolite levels due to variations in growth rate, genetic uniformity, or stability. As discussed by Chandran *et al.* (2020) optimizing this production is frequently associated with challenges cell suspension culture tends to be more uniform than callus culture and is favored in industry due to rapid growth, stable biomass production and high yields of secondary metabolites (Shah *et al.*, 2021). Callus obtained from the leaf explant on MS medium supplemented with 0.25 mg/L 2,4-D (Figure 1C) was sub-cultured to liquid MS medium containing 0.25 mg/L of 2,4-D and this hormone treatment was maintained until to end of the experiment (Figure 1I).

Cell growth and viability curves

Microscopic images of live and dead cells are shown in Figure 1D-H. Based on the cell growth curve (Figure 2), the initial stage comprises a two-day lag phase during which cells adapt to the new medium. Subsequently, cells enter the exponential growth phase and continue this phase until day 5. Thereafter, cells transition to the linear growth phase and maintain linear growth until day 15. The culture then enters the stationary phase, persisting until the day 19, followed by the death phase. According to the viability curve, cell survival reaches a maximum at day 12, with an 83.1% survival rate; thereafter, as culture medium compounds become depleted, survival declines to 19.5% on day 19. Cell suspension growth can be monitored estimating cell density through direct counting (Hazrati Jahan *et al.*, 2017; Abdulhafiz *et al.*, 2022). Our results showed a maximum viability of 85.35% (on day 1 post-initiation) and a maximum cell density of 2.43×10^6 per mL (on day 15). Studies on other species demonstrate similar approaches. Nagella and Murthy (2010) investigated various plant growth regulators (auxin, a combination of auxin, and cytokinin) to establish cell suspension culture in *Withania somnifera* (Solanaceae). Silva and Menéndez-Yuffá (2006) assessed cell viability in coffee cell suspension by staining dead cells with 1% Evans blue dye at different developmental stages.

The effect of chitosan on cell suspension culture growth, scopolamine, and hyoscyamine accumulation, and production

Using biotic and abiotic elicitors in plant cell suspension cultures stimulates molecular responses that, in some cases, lead to a simultaneous increase in metabolite production (Arya *et al.*, 2022). Based on variance analysis, the effects of different concentrations of chitosan, sampling time and their interactions on cell fresh and dry weight were not significant, whereas their effects on the accumulation and production of scopolamine and hyoscyamine were significant (Table 1A). Elicitation is one of the most effective strategies for increasing secondary metabolite production in cell suspension culture; however, while elicitors can enhance production, they often reduces biomass (Chandran *et al.*, 2020). The effect of chitosan on biomass production depends on its concentration; however, studies suggest that any negative impact on biomass can be offset by improved nutritional status (Vosoughi *et al.*, 2018; Ahmad *et al.*, 2019; Khan *et al.*, 2019). Chitosan can enhance biomass accumulation by improving nutrient uptake and modulating osmotic balance (Bautista-Baños *et al.*, 2003). Elicitation

Table 1. ANOVA results of the effects of chitosan, PEG and sampling time on cell suspension culture growth and the synthesis of scopolamine and hyoscyamine in *Hyoscyamus niger* L. cell suspension cultures.

Source of variation	df	Mean of square					
		FW	DW	Scopolamine accumulation	Scopolamine production	Hyoscyamine accumulation	Hyoscyamine production
A							
Chitosan (Ch)	3	290.88^{ns}	2.01^{ns}	22523.14**	2110831.11**	948.42**	92451.93**
Sampling time (ST)	2	77.83^{ns}	1.35^{ns}	17323.99**	1390070.37**	462.46**	32283.10**
Ch×ST	6	227.88^{ns}	1.84^{ns}	19630.13**	1504242.16**	758.20**	75526.46**
Error	24	139.92	1.26	46.05	25454.12	13.71	1973.61
Coefficient of variation (%)		16.08	12.17	6.62	17.02	10.16	13.14
B							
PEG	3	284.46^{ns}	3.32^{ns}	3826.29**	468486.01**	168.84**	24351.93*
Sampling time (ST)	2	67.21^{ns}	19.92^{ns}	7497.19**	1461494.86**	971.04**	226780.96**
PEG×ST	6	93.74^{ns}	5.92^{ns}	5859.49**	930099.86**	1037.12**	161373.20**
Error	24	115.19	3.62	85.12	11233.24	69.09	5567.37
Coefficient of variation (%)		15.31	19.18	16.03	17.69	24.90	21.58

^{ns}: non-significant, *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$. A: ANOVA analysis of the effect of chitosan and B: ANOVA analysis of the effect of PEG.

has been reported to stimulate biomass production in various systems, including neem cell suspensions (Farjaminezhad and Garoosi, 2021), several basil species (Mathew and Sankar, 2012), red sage (Zhao *et al.*, 2010), flax callus cultures (Ahmad *et al.*, 2019), and *Fagonia indica* (Khan *et al.*, 2019). Conversely, higher concentrations of chitosan can suppress biomass accumulation due to its elicitor effect (Shah *et al.*, 2021).

A major challenge of using cell suspension culture for secondary metabolite production is their low yield; elicitors can enhance production, addressing this limitation (Gorelick and Bernstein, 2014). Chitosan salts (lactate or acetate) are the most common derivatives, and their effectiveness depends on solubility (Kowalczyk *et al.*, 2015; Hawrylak-Nowak *et al.*, 2021). In this study, increasing chitosan concentration inhibited scopolamine accumulation; the highest scopolamine accumulation observed was 157.05 µg/g DW at 25 mg/L chitosan, which was 1.82-fold higher than the control. Sampling time also influenced scopolamine accumulation, with the highest accumulation on day 2 being 144.18 µg/g DW (Figure 3A and B). The maximum accumulation of scopolamine (258.93 µg/g DW) occurred at two days after elicitation with 50 mg/L chitosan, representing a 5.43-fold increase over the control on the day 2 (Table 2). Notably, while chitosan can stimulate scopolamine production, it can also inhibit production at higher concentrations; the maximum scopolamine production was 1529.03 µg/L with 25 mg/L chitosan, which was 1.87-fold higher than the control. Also, the peak production with 1315.22 µg/L occurred on day 2 (Figure 3A and B). Overall, the maximum scopolamine production (2475.18 µg/L) was achieved with 25 mg/L of chitosan for two days, 5.03-fold higher than the control on day 2 (Table 2).

Hyoscyamine accumulation increased with the increase of chitosan up to 25 mg/L and the declined with higher concentrations. With 25 mg/L chitosan, the accumulated hyoscyamine was 43.88 µg/g DW, 1.15-fold higher than the control. Sampling time was also influenced hyoscyamine accumulation; the highest accumulation on day 3 was 40.94 µg/g DW, with no significant difference from day 1 (Figure 3C and D). Interaction effects showed that, using chitosan, the greatest hyoscyamine accumulation (59.68 µg/g DW) occurred with of 25 mg/L for 1 day, which was 4.00-fold higher than the day-1 control (Table 2). Also, the highest hyoscyamine production was 419.74 µg/L with 25 mg/L chitosan to liquid MS medium, 1.13-fold higher than control, and among sampling times, the third day yielded the highest production (380.27 µg/L) on (Figure 3C and D). The maximum hyoscyamine production observed was 552.12 µg/L without chitosan after 3 days (Table 2).

Biological activity of chitosan underpins its use as an elicitor. Chitosan enhances plant resistance to stress

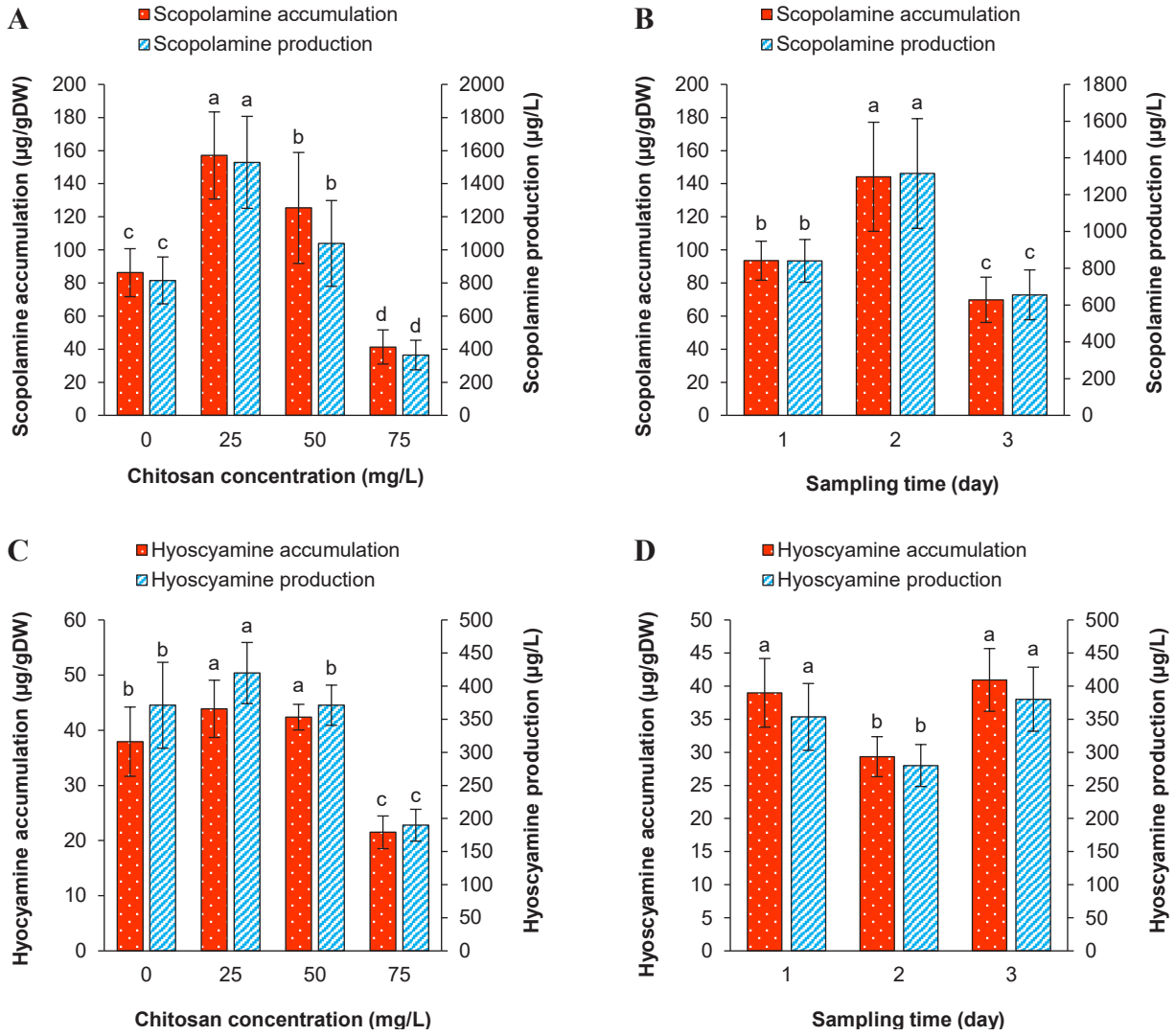

Figure 3. Effects of different **A and C:** chitosan concentrations and **B and D:** sampling times on the accumulation and production of hyoscyamine and scopolamine in *Hyoscyamus niger* L. cell suspension cultures.

Table 2. Effects of chitosan concentration and sampling time on the accumulation and production of scopolamine and hyoscyamine in *Hyoscyamus niger* L. cell suspension cultures.

Chitosan concentration (%)	Sampling time (day)	Scopolamine accumulation (µg/g DW)	Scopolamine production (µg/L)	Hyoscyamine accumulation (µg/g DW)	Hyoscyamine production (µg/L)
0	1	68.68±3.74^{f}	582.63±54.74^{d}	14.91±1.26^{f}	127.72±19.52^{d}
	2	47.68±4.92^{h}	491.92±32.40^{de}	41.62±3.42^{c}	433.43±44.17^{b}
	3	142.26±4.31^{d}	1371.73±49.36^{c}	57.26±1.16^{a}	552.12±15.76^{a}
25	1	159.23±5.26^{c}	1480.85±101.11^{c}	59.68±3.80^{a}	551.67±21.12^{a}
	2	246.83±3.23^{b}	2475.18±254.20^{a}	25.05±0.68^{e}	250.49±22.34^{c}
	3	65.10±2.53^{fg}	631.04±9.44^{d}	49.91±1.41^{bc}	457.06±31.08^{b}
50	1	63.52±6.03^{fg}	586.58±35.58^{d}	48.62±1.31^{b}	454.89±44.53^{b}
	2	258.93±6.49^{a}	2063.04±121.45^{b}	34.54±1.79^{d}	274.44±13.93^{c}
	3	53.61±1.19^{gh}	467.48±9.20^{de}	44.05±2.99^{bc}	384.05±25.13^{b}
75	1	82.39±0.99^{e}	710.64±63.75^{d}	32.81±2.6^{d}	279.79±16.32^{c}
	2	23.27±1.46^{i}	230.74±9.86^{ef}	16.15±0.59^{f}	161.98±16.16^{d}
	3	18.28±1.01^{i}	151.68±11.67^{f}	15.52±1.44^{f}	127.82±7.99^{d}

Means with different letters are significantly different ($p \leq 0.05$).

Table 3. Effects of PEG concentration and sampling time on cell suspension culture growth and the accumulation and production of scopolamine and hyoscyamine in *Hyoscyamus niger* L. cell suspension cultures.

PEG concentration (%)	Sampling time (day)	Scopolamine accumulation (µg/g DW)	Scopolamine production (µg/L)	Hyoscyamine accumulation (µg/g DW)	Hyoscyamine production (µg/L)
0	1	68.67±3.53^{b}	582.63±54.74^{cd}	14.91±1.26^{de}	131.67±35.26^{e}
	2	47.68±4.92^{cd}	491.92±32.40^{d}	41.62±3.42^{b}	433.43±44.17^{bc}
	3	135.59±8.50^{a}	1306.13±8.50^{b}	55.26±1.16^{a}	552.12±15.76^{b}
2.5	1	34.51±3.73^{de}	292.83±32.72^{e}	21.06±4.47^{cd}	184.45±53.32^{e}
	2	8.19±0.31^{g}	83.10±7.57^{f}	16.77±3.58^{de}	171.25±39.39^{d}
	3	146.81±11.00^{a}	2008.09±148.21^{a}	62.39±4.14^{a}	839.38±50.45^{a}
5	1	25.88±0.49^{ef}	233.18±9.79^{ef}	37.29±0.97^{b}	335.44±6.54^{cd}
	2	39.68±4.76^{de}	304.99±30.18^{e}	26.55±0.22^{c}	209.28±30.53^{de}
	3	47.35±3.10^{cd}	545.26±83.07^{cd}	40.02±1.86^{b}	454.83±83.22^{bc}
10	1	58.14±6.35^{bc}	498.04±55.42^{d}	27.94±0.51^{c}	240.68±17.65^{de}
	2	63.26±4.00^{bc}	692.06±25.55^{c}	42.07±3.80^{b}	464.89±58.02^{bc}
	3	14.68±2.69^{fg}	147.55±18.84^{ef}	12.62±0.88^{e}	130.86±17.44^{e}

Means with different letters are significantly different (p≤0.05).

by stimulating innate defense responses, including physiological and morphological changes such as oxidative stress, hydrogen peroxide accumulation, secondary metabolite synthesis, and growth regulation. It also modulates chromatin structure, inhibits H^{+}-ATPase activity at the plasma membrane, activates MAP kinases, and increases cytosolic Ca^{2+} concentration (Rabea *et al.*, 2003; El Hadrami *et al.*, 2010; Malerba and Cerana, 2016). Chitosan has been used as an elicitor in various studies; for example, its application in *stevia rebaudiana* increased production of rebaudioside A, biomass, and phenolic compounds (Stasińska-Jakubas and Hawrylak-Nowak, 2022). Other examples include enhanced growth and phenolic compounds in *Origanum vulgare* ssp. hirtum with different chitosan concentrations (Yin *et al.*, 2012). In *Stevia rebaudiana* chitosan elicitation improved tolerance salinity (Gerami *et al.,* 2020). Safikhan *et al.* (2018) reported chitosan stimulation of growth and physiological parameters in *Silybum marianum,* and chitosan mitigated drought stress in *Salvia officinalis*. Positive effects of chitosan on essential oil content, phenolic compounds and flavonoids content in *Salvia officinalis* have been reported (Vosoughi *et al.*, 2018). Chitosan also stimulated flavonoid content in *Ononis arvensis* (Tůmová and Bačkovská, 1999). In *Mentha piperita* suspension cultures, chitosan increased menthol accumulation (Chang *et al.*, 1998); greenhouse treatments with chitosan further enhanced phenolic compounds and flavonoids content, and antioxidant activity in *M. piperita* extracts (Salimgandomi and Shabrangi, 2016). In hairy root cultures of *Psammosilene tunicoides*, chitosan stimulated triterpenoid saponins production (Qiu *et al.*, 2021). Moreover, chitosan has been shown to stimulate biomass production in the cell cultures of three basil species: *Ocimum basilicum*, *O. sanctum*, and *O. gratissimum* (Mathew and Sankar, 2012).

The effect of PEG on cell suspension culture growth, scopolamine, and hyoscyamine accumulation and production

According to the ANOVA results, the effect of different PEG concentrations, sampling time and their interactions on cell fresh weight and dry weight were not significant; however, their effects on the accumulation and production of scopolamine and hyoscyamine were significant (Table 1B). In this study, increasing PEG concentration inhibited scopolamine accumulation, while the highest accumulation observed in the absence of PEG (83.98 µg/g DW). Sampling time also influenced scopolamine accumulation, with the maximum on day 3 being 86.11 µg/g DW (Figure 4A and B). The highest accumulated scopolamine (146.81 µg/g DW) was obtained three days after elicitation with 2.5% PEG, representing a 4.25-fold increase over the day-3 control (Table 3). It should be noted that PEG also inhibited scopolamine production; the maximum production (793.57 µg/L) was observed under control condition, and the peak production occurred on day 3 (1001.76 µg/L) (Figure 4A and B). Overall, the maximum production of scopolamine (2008.09 µg/L) was achieved with 2.5% of PEG for three days, which was 1.54-fold higher than the day-3 control (Table 3). The results showed that the hyoscyamine accumulation

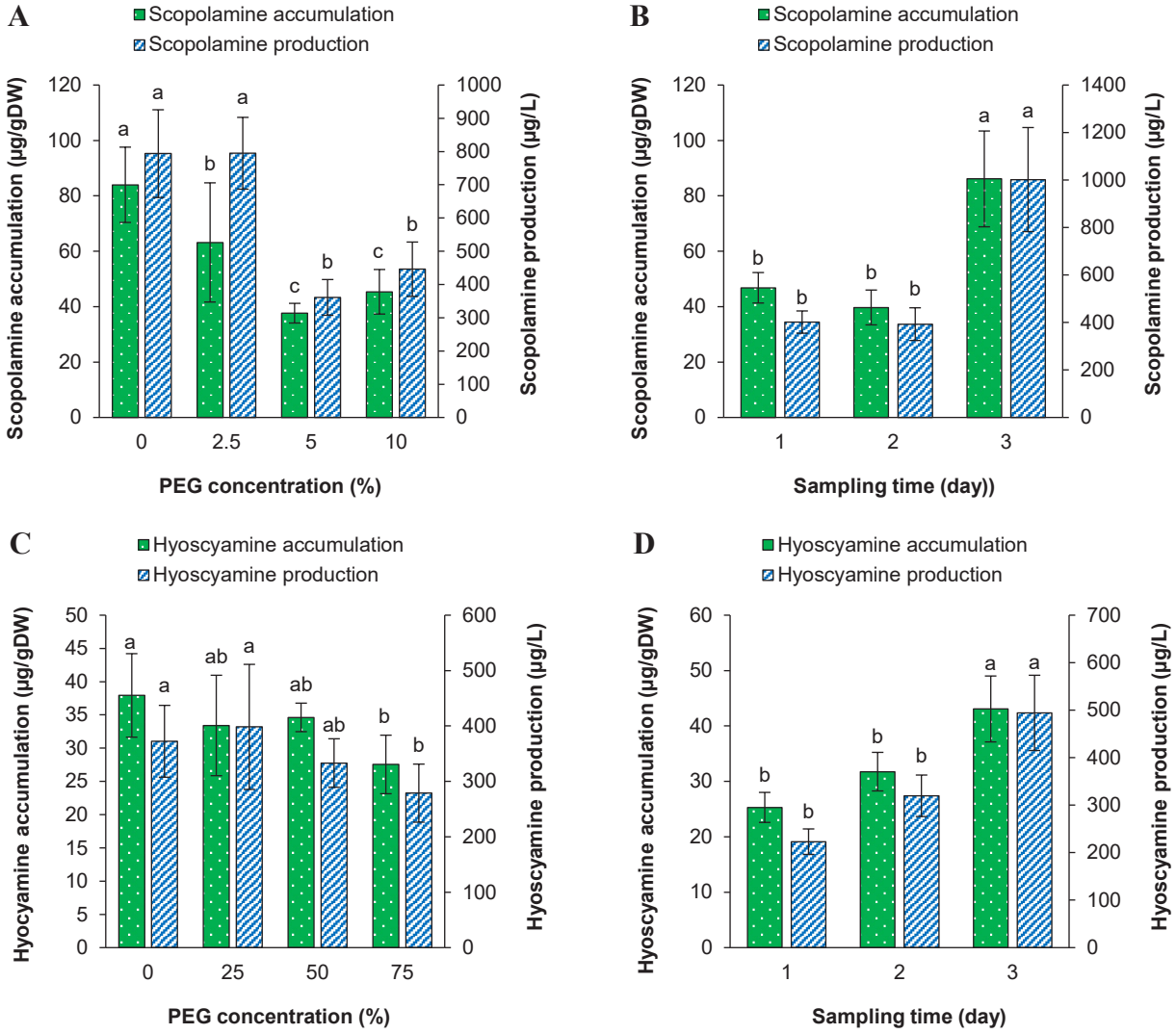

Figure 4. Effects of different **A and C:** PEG concentrations and **B and D:** sampling times on the accumulation and production of hyoscyamine and scopolamine in *Hyoscyamus niger* L.cell suspension cultures.

decreased with increasing of PEG. in the absence of PEG, the accumulated hyoscyamine was 37.93 µg/g DW. Sampling time also effected hyoscyamine accumulation; the highest accumulation on day 3 was 43.07 µg/g DW (Figure 4C and D).

PEG is a suitable non-toxic compound for inducing oxidative drought stress through osmotic potential in culture medium. The active production of reactive oxygen species (ROS) in plastids, peroxisomes, mitochondria, the cytosol, and the apoplast is a typical plant response to stress. To sustain metabolic activities under PEG-induced osmotic stress, plants rely on antioxidant defense mechanism, including enzymes and secondary metabolites, which act to mitigate harmful stress factors.

The application of PEG in the culture medium of *Catharanthus roseus* cell suspension has been shown to increase terpenoid production (Azadvari *et al.*, 2022). PEG is widely used to induce water stress in plant cell suspension cultures. For instance, studies demonstrated that potato cell suspension cultures treated with 10% PEG 6000 exhibited a 50% reduction in solution production. Moreover, enzyme assay systems required PEG concentration above 5% to significantly affect the activity of three enzymes compared to untreated cells (Corcuera *et al.*, 1989).

PEG reduces water absorption by plant tissues by lowering the osmotic potential of the culture medium, thereby limiting growth and reproduction (Sarmadi *et al.*, 2019). As a flexible and non-toxic polymer can decrease the osmotic potential of plant cells. It also does not react with chemicals or biologicals, PEG decreases the osmotic potential of plant cells without reacting with chemicals or biological molecules, making it a highly

useful agent for inducing negative osmotic pressure in biochemical experiments (Tavakoli *et al.*, 2021). In a study by Ahmadi-Sakha *et al.* (2022) on the medicinal plant *Scrophularia striata* Boiss (Scrophulariaceae), HPLC-based metabolic profiling revealed significant changes in primary and secondary metabolites under PEG 6000 treatment. Notably, acteoside content was strongly induced, reaching 6.38-fold higher levels compared to the control. Similarly, in blueberry cell suspension culture, PEG application induced osmotic water stress, with samples collected at 10 and 17-day intervals. Analyzing PEG concentrations across these time points showed that the dehydrin transcript decreased by approximately 0.75 kb. In control samples, this reduction was more pronounced at 10 days than at 17 days (Parmentier-Line *et al.*, 2002).

CONCLUSION

In general, the effect of different concentrations of chitosan and various sampling times on cell growth and density were insignificant. However, the highest accumulation and production of scopolamine were observed with 50 mg/L and 25 mg/L of chitosan on the second day of sampling, respectively. The maximum accumulation and production of hyoscyamine were both obtained with 25 mg/L chitosan on the first day of sampling. It should be noted that the highest amount of hyoscyamine produced was lower than that of the control sample.

The study on the effects of different concentrations of PEG and various sampling times revealed that the highest accumulation and production of hyoscyamine and scopolamine occurred under treatment with 2.5% PEG, specifically on the third day of sampling.

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