



Elicitor-mediated enhancement of tropane alkaloid production in callus cultures of *Datura stramonium* L.

Šarlota Kaňuková^{1,2} · Sofia Mikulášková¹ · Jennifer Scherrens¹ · Ján Kraic^{1,2}

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Abstract

Callus cultures provide an alternative in vitro platform for the production of secondary metabolites, although their potential remains underexploited in many plant species. This study evaluated the capacity of elicited callus cultures of *Datura stramonium* L. to produce the tropane alkaloids hyoscyamine and scopolamine. Callus cultures were induced from root, stem, and leaf explants on media supplemented with several combinations of auxins and cytokinins. Callus biomass gain was assessed as fresh weight over a 16-week cultivation period. Several elicitors significantly promoted callus biomass accumulation, as reflected by increased fresh weight. For alkaloid production and elicitation experiments, roots-derived callus cultures were used. Untreated calli produced relatively low levels of hyoscyamine and scopolamine. However, specific elicitors substantially enhanced alkaloid accumulation. Yeast extract at 300 mg/L was the most effective, increasing hyoscyamine content up to 24.7 mg/g dry weight (DW), representing a 38.8-fold increase compared over the untreated control. A plant extract containing cucumber mosaic virus (CMV) also exhibited strong elicitation potential. Both yeast extract (300 mg/L) and the CMV-containing extract increased scopolamine levels by more than 30-fold. These findings demonstrate that root-derived callus cultures of *D. stramonium* offer a promising in vitro system for tropane alkaloid production and highlight the critical role of optimizing plant growth regulator combinations and elicitor treatments to enhance secondary metabolite biosynthesis.

Key message

Root-derived callus cultures of *Datura stramonium* L. produced significant amounts of hyoscyamine and scopolamine following treatment with selected elicitors.

Keywords Thornapple · In vitro · Callus · Elicitation · Hyoscyamine · Scopolamine

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid	DIECA	immuno sorbent assay
2iP	6-(γ,γ -Dimethylallylamino)purine	DW	Sodium diethyldithiocarbamate trihydrate
ANOVA	Analysis of variance	EDTA	Dry weight
CMV	Cucumber mosaic virus	FW	Ethylenediaminetetraacetic acid
DAS-ELISA	Double antibody sandwich-Enzyme-linked	GC-MS	Fresh weight
		KIN	Gas chromatography-mass spectrometry
		MS	Kinetin
		NAA	Murashige and Skoog medium
		PGR	Naphtaleneacetic acid
		TDZ	Plant growth regulator
		ToMV	1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea
			Tomato mosaic virus

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✉ Šarlota Kaňuková
sarlota.kanukova@ucm.sk

¹ Faculty of Natural Sciences, Department of Biotechnology,
University of Ss. Cyril and Methodius, Trnava
91701, Slovakia

² National Agricultural and Food Center, Research Institute of
Plant Production, Piešťany 92168, Slovakia

Introduction

Historically, the genus *Datura* (family *Solanaceae*) was valued by ancient civilizations for its narcotic, hallucinogenic, and medicinal properties, as well as for its cultural significance. Its use extended to religious rituals, shamanistic practices, superstitions, and the treatment of various physical and mental ailments across both the New and Old Worlds (Geeta and Gharaibeh 2007). Today, *Datura* continues to attract significant interest in ethnopharmacology, folk medicine, and modern pharmacological research (Soni et al. 2012; Sayyed and Shah 2014; Kadam et al. 2018; Ibrahim et al. 2018). Among its species, *D. stramonium* L. (commonly known as thornapple, jimsonweed, or devil's trumpet) is also notable for its production of tropane alkaloids hyoscyamine and scopolamine. Atropine, a racemic mixture of (–)-hyoscyamine and (+)-hyoscyamine, is a highly valuable tropane alkaloid in the pharmaceutical industry, with global demand projected to grow at an annual rate of approximately 2.5%. Similarly, scopolamine (also known as hyoscyne) is a prominent pharmacological agent used to manage nausea, vomiting, motion sickness, and smooth muscle spasms. Beyond their traditional ethnomedicinal applications, tropane alkaloids such as atropine and scopolamine are widely utilized in modern medicine for the treatment of respiratory, cardiovascular, neurological, ophthalmological, and renal disorders, as well as in the management of Parkinson's disease, wound healing, and various other therapeutic applications (Soni et al. 2012; Sharma et al. 2021; Pandey et al. 2022). Extracts from *Datura* species, or their constituents, has also biopesticidal potential. In *D. stramonium*, extracts obtained from various plant parts have demonstrated insecticidal, larvicidal, herbicidal, acaricidal, antifungal, and antibacterial activities against agricultural pests and pathogens (Iranbakhsh et al. 2010; Awulachew 2022; Céspedes-Méndez et al. 2021). Furthermore, hyoscyamine and scopolamine exhibit significant allelopathic effects (Levitt and Lovett 1984; Yorulmaz and Yergin Özkan 2020), which could be harnessed to support the conservation of native plant species in protected ecosystems and biodiversity hotspots (Elisante and Ndakidemi 2014), as well as to enhance sustainable crop production.

Although hyoscyamine and scopolamine can be chemically synthesized, they are traditionally obtained from plant material through various extraction and purification methods (Śramska et al. 2016). Natural sources of these tropane alkaloids include members of the *Solanaceae* family, particularly the genera *Atropa*, *Datura*, *Hyoscyamus*, *Scopolia*, *Duboisia*, *Anisodus*, among others. Along with *D. innoxia* and *D. metel*, *D. stramonium* is a significant source of both hyoscyamine and scopolamine, with these alkaloids together accounting for more than three-quarters of the total alkaloid

content in this species (Berkov et al. 2006; El Bazaoui et al. 2011). The content of these tropane alkaloids in plants is highly variable and influenced by numerous factors, including genus, species, ploidy level, cultivation practices, environmental conditions, geographical location, temperature, humidity, and storage conditions. Significant variation in alkaloid concentrations can be observed within a single species, occurring among different plant organs, individual plants, and growing locations. While both alkaloids are distributed throughout the plant, their concentrations differ depending on the plant organ type and developmental stage. Jakabová et al. (2012) reported the highest concentration of hyoscyamine and scopolamine in the stems. Miraldi et al. (2001) found that young plants (<30 cm) contained higher alkaloid levels than mature ones. In younger plants, the stem was the primary site of accumulation, while in older plants, the seeds became the predominant reservoir. Similarly, Iranbakhsh et al. (2006) observed the highest alkaloid concentration in leaves and stems during the vegetative phase. Despite this variation, total alkaloid levels remain relatively high even in mature seeds (Friedman and Levin 1989). This *in vivo* variation suggests that all parts of *D. stramonium* serve as potential sources of alkaloids, a characteristic that could be translated under *in vitro* conditions. Moreover, alkaloid levels in soil-grown plants can be enhanced through direct *in planta* elicitation (Mihálik et al. 2022).

Research on tropane alkaloids is advancing rapidly, with several alternative production strategies emerging to improve or replace conventional plant-based extraction methods. These include genetic engineering of alkaloid biosynthesis pathways in *Solanaceae* species (Gong et al. 2022), gene editing using CRISPR/Cas9 technology (Zeng et al. 2021), and heterologous production in recombinant microorganisms engineered with key plant biosynthetic genes (Pramod et al. 2010; Li et al. 2012; Srinivasan and Smolke 2020).

Increasing attention is also being given to the development of efficient production strategies using *in vitro* plant cultivation systems (Wang et al. 2024). For optimal secondary metabolite production, it is preferable to initiate cultures from plant organs, tissues, or cells known to naturally synthesize and accumulate high levels of the target alkaloids. Several *Solanaceae* species have traditionally served as *in vitro* producers of hyoscyamine and scopolamine, with *D. innoxia* L. and *D. metel* L. being among the most commonly used within the genus *Datura*. Among these approaches, the cultivation of hairy roots is the most widely applied *in vitro* method for alkaloid production (Gong et al. 2022). Hairy root cultures, typically established through genetic transformation using *Agrobacterium rhizogenes*, represent the most widely used *in vitro* system for producing hyoscyamine and scopolamine from *D. stramonium* (Payne et al. 1987; Hilton

and Rhodes 1990; Marchev et al. 2012; Harfi et al. 2015). Cell suspension cultures derived from calli have been used far less frequently (Ballica et al. 1993; Iranbakhsh et al. 2007). Similarly, the use of in vitro cultures of non-transformed (true) roots and adventitious roots of *D. stramonium* for tropane alkaloid production remains limited, with only a few older studies available (Maldonado-Mendoza et al. 1992; Baiza et al. 1998).

Information on the use of callus cultures for the production of tropane alkaloids in *D. metel*, *D. innoxia*, or *D. stramonium* remains scarce, despite the fact that callus induction is often a preliminary and essential step in the development of other in vitro plant production systems. However, emerging evidence suggests that callus cultures may have the potential to synthesize hyoscyamine, scopolamine, and other tropane alkaloids. Idioblasts, specialized cells involved in alkaloid biosynthesis, have been identified in callus tissues derived from the basal part of *D. stramonium* leaves (Iranbakhsh et al. 2006). Moreover, transformed calli derived from hairy roots were capable of converting hyoscyamine to scopolamine via epoxidation, a transformation not observed in the hairy roots themselves (Palazón et al. 1995). If in vitro production of secondary metabolites through callus cultures proves feasible, at least for specific metabolites and plant species, then the combination of such systems with biotic or abiotic elicitation could represent a relatively simple, scalable, and competitive alternative to field-grown plants for metabolite production (Rao and Ravishankar 2002; Ramirez-Estrada et al. 2016).

This study aimed to establish callus cultures of *D. stramonium* L. from various explants, to characterize fresh callus biomass accumulation during extended cultivation, and to enhance production of hyoscyamine and scopolamine using abiotic and biotic elicitors.

Materials and methods

Plant material

Seeds of *D. stramonium* L. were obtained from SemenaOnline s.r.o. (Jeneč, Czechia). For scarification, the seeds were treated with concentrated sulfuric acid and then thoroughly rinsed with sterile distilled water. Surface sterilization was performed by immersing the seeds in 96% (v/v) ethanol for 30 s, followed by treatment with a 4.7% sodium hypochlorite solution containing 0.2% (v/v) Tween 20 under constant stirring for 15 min. After several rinses with sterile distilled water, the seeds were transferred to culture vessels containing half-strength Murashige and Skoog medium (Murashige and Skoog 1962; Duchefa Biochemie B.V., Haarlem, Netherlands), solidified with 0.8% (w/v) agar and adjusted to pH

5.7–5.8. The resulting seedlings were cultivated in a controlled growth chamber at 23 ± 2 °C under a 16/8 h light/dark photoperiod for four weeks.

Callus initiation, growth, and elicitation

Callus cultures were initiated from leaf, stem, and root segments excised from 4–5-week-old aseptic *D. stramonium* L. plants. Explants were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with vitamins, 30 g/L sucrose (w/v), and solidified with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.7–5.8.

Six distinct combinations of plant growth regulators (PGRs) were tested for callus induction (Table 1), each containing 2 mg/L of an auxin (2,4-D or NAA) and 0.5 mg/L of a cytokinin (KIN, 2iP, or TDZ). All PGRs were obtained from Duchefa Biochemie B.V. (Haarlem, Netherlands). Explants were cultivated in the dark at 23 ± 2 °C and subcultured onto fresh medium of the same composition every four weeks.

Callus fresh weight (FW) was recorded at each subculture interval – weeks 4, 8, 12, and 16. The effects of PGR combinations on callus induction and biomass accumulation were evaluated using five biological replicates per explant type (leaf, stem, root), with each replicate consisting of five explants. Stem and root segments had a length of ≈ 1.0 cm, leaf segments had an area of ≈ 0.5 cm².

Elicitation experiments were performed on callus cultures derived from root explants, previously established on MS medium supplemented with one of two plant growth regulator (PGR) combinations: 2 mg/L 2,4-D+0.5 mg/L KIN or 2 mg/L 2,4-D+0.5 mg/L 2iP. Calli used for elicitation were subcultured for 4 weeks, followed by an additional 20 days of growth until reaching the exponential phase. They were then transferred to elicitor-containing medium and, after 21 days of elicitation, harvested for tropane alkaloid analysis. Biotic elicitors included yeast extract (Sigma Chemical Co., St. Louis, USA) at concentrations of 100, 300, and 500 mg/L, chitosan (Merck KGaA, Darmstadt, Germany) at 300 and 500 mg/L, and crude extracts from plants infected with Tomato mosaic virus (ToMV, family *Virgaviridae*) or Cucumber mosaic virus (CMV, family *Bromoviridae*). Abiotic elicitors included jasmonic acid and methyl jasmonate (both from Duchefa Biochemie B.V., Haarlem, The Netherlands), applied at concentrations of 50 and 100 mg/L. A Norit buffer, composed of 0.05 M sodium/potassium phosphate buffer (pH 7.0), 1 mM EDTA, 5 mM DIECA, and 5 mM thioglycolic acid, was also used. All elicitors were incorporated into the culture medium prior to solidification. For virus treatments, plant extracts prepared in Norit buffer from ToMV- or CMV-infected plant tissue were applied

Table 1 Induction and characterization root-, stem-, and leaf-derived calli after 4, 8, 12, and 16 weeks of in vitro culture

Explant type	Callus induction [%]			Callus fresh weight [g]			
	Auxin [2 mg/L]	Cytokinin [0.5 mg/L]	4 w	4 w	8 w	12 w	16 w
<i>Root</i>	2,4-D	2iP	100	0.825±0.305 ^A	1.785±0.581 ^A	8.401±3.678 ^A	14.462±6.947 ^A
		KIN	100	0.133±0.064 ^B	0.358±0.149 ^C	5.101±1.401 ^{ABC}	11.233±4.243 ^{AB}
		TDZ	100	0.290±0.090 ^B	0.496±0.163 ^{BC}	1.701±0.122 ^C	3.183±0.667 ^C
	NAA	2iP	100	0.306±0.122 ^B	0.672±0.222 ^{BC}	8.563±1.206 ^A	15.498±2.892 ^A
		KIN	100	0.421±0.143 ^B	1.080±0.295 ^B	6.628±3.245 ^{AB}	11.035±7.765 ^{AB}
		TDZ	100	0.291±0.327 ^B	0.769±0.750 ^{BC}	3.946±2.670 ^{BC}	6.264±2.720 ^{BC}
<i>Stem</i>	2,4-D	2iP	100	1.114±0.761 ^A	4.400±3.469 ^A	13.353±5.200 ^A	22.512±7.276 ^{AB}
		KIN	100	1.063±0.782 ^A	4.292±3.151 ^A	11.479±6.188 ^A	21.565±5.171 ^{AB}
		TDZ	100	1.194±0.566 ^A	4.584±2.429 ^A	11.799±5.385 ^A	19.769±4.643 ^{AB}
	NAA	2iP	100	0.665±0.395 ^A	3.630±1.481 ^A	14.309±5.984 ^A	27.222±8.775 ^A
		KIN	100	0.784±0.398 ^A	2.805±0.920 ^A	12.388±4.139 ^A	24.494±7.667 ^{AB}
		TDZ	100	0.552±0.339 ^A	2.299±0.790 ^A	8.379±1.530 ^A	17.245±1.815 ^B
<i>Leaf</i>	2,4-D	2iP	100	1.855±0.789 ^{AB}	5.049±1.478 ^{AB}	10.693±5.568 ^{AB}	18.072±7.571 ^A
		KIN	100	0.900±0.424 ^C	2.851±1.839 ^B	7.931±4.509 ^{AB}	15.882±4.481 ^A
		TDZ	100	1.011±0.427 ^{BC}	2.904±1.327 ^B	7.309±3.879 ^{AB}	15.104±4.503 ^A
	NAA	2iP	100	0.875±0.305 ^{BC}	2.875±0.468 ^{AB}	6.151±0.715 ^{AB}	11.781±4.694 ^A
		KIN	100	0.595±0.163 ^C	2.497±0.714 ^B	4.844±1.354 ^B	14.405±3.463 ^A
		TDZ	100	1.993±1.036 ^A	5.784±3.405 ^A	12.056±5.243 ^A	18.536±3.612 ^A

4 w, 8 w, 12 w, 16 w refer to 4, 8, 12, 16 weeks of in vitro culture, respectively. Superscripts (^{A, B, C}) indicate statistically different differences between mean values as determined by one-way ANOVA followed by the Least Significant Difference (LSD) test at a significance level $p \leq 0.05$. Statistical differences were evaluated between treatments within the same time point

directly to the surface of the callus after transfer to the culture medium. The presence of ToMV and CMV in the extracts was confirmed by double antibody sandwich-linked immunosorbent assay (DAS-ELISA, Clark and Adams 1977) using ELISA Reagent Sets (Agdia, Inc., Elkhart, USA).

Tropane alkaloids

Tropane alkaloids were extracted from callus tissues following the modified protocol described by Harfi et al. (2018). The modification involved omitting the initial hexane defatting step and extending the acid contact time from 10 min to 20 min. Callus tissue (50 mg) was dried for 3–4 h at 50 °C, powdered, and extracted with 12 mL of 0.1N HCl for 20 min. After centrifugation, the pH of the supernatant was adjusted to 10 using 28% NH₄OH. The aqueous phase was filtered and extracted three times with an equal volume of chloroform. The combined organic phases were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in 1 mL of dichloromethane and passed through a 0.2 µm PTFE filter. Alkaloid analysis was conducted using gas chromatography-mass spectrometry (GC-MS) according to the method of Nguyen et al. (2015). The GC-MS system comprised an Agilent 7890B gas chromatograph coupled with an Agilent 5977 A Series Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA), equipped with a VF-5 MS low-flow capillary

column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies, Santa Clara, CA, USA).

A 1 µL aliquot of each sample was injected in splitless mode, with the injector temperature maintained isothermally at 250 °C. The GC oven temperature program was as follows: initial hold at 40 °C for 1 min, ramp at 30 °C min⁻¹ to 130 °C, followed by a ramp of 10 °C min⁻¹ to 280 °C, and a final hold at 280 °C for 5 min. The transfer line and ion source temperatures were set at 300 °C and 200 °C, respectively. Electron ionization (EI) was performed at 70 eV, and mass spectra were acquired over a range of 30–600 m/z at a rate of 4.7 scans s⁻¹.

Hyoscyamine and scopolamine were identified and quantified using external calibration with reference standards of hyoscyamine sulfate (PhytoLab GmbH & Co. KG, Vertenbergsgruth, Germany) and scopolamine hydrobromide (Merck KGaA, Darmstadt, Germany), both dissolved in dichloromethane (Fig. 1). Calibration curves were constructed using standard solutions with concentrations up to 1.6 mg/mL. Compound identification was confirmed by comparing sample spectra with entries from the NIST 2007 mass spectral library.

Data analysis

Data were analyzed by analysis of variance (ANOVA) using Statgraphics software, version 19.2.01 (Statgraphics Technologies, Inc., The Plains, VA, USA). Post-hoc comparisons

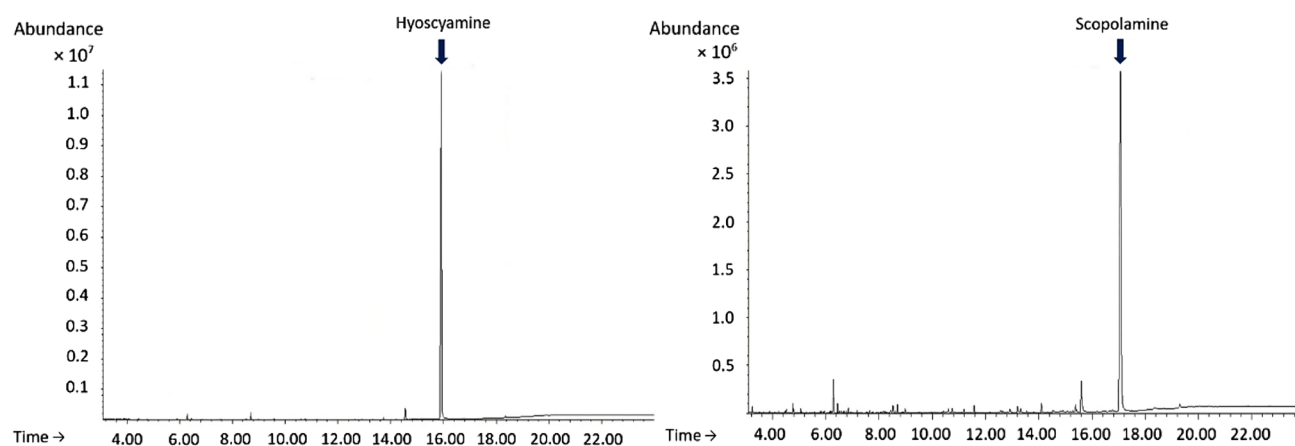


Fig. 1 GC chromatograms of hyoscyamine (left) and scopolamine (right) reference standards

were performed using the least significant differences (LSD) test at a significance level of $p < 0.05$. Statistically significant differences between control and elicited groups ($p < 0.05$) are indicated by different superscript letters in the bar charts.

Results

Callus culture

Callus formation was successfully initiated from all three explant types—root, stem, and leaf segments—on all tested combinations of plant growth regulators (PGRs) in MS callus induction medium (Table 1). However, callus morphology varied according to the explant source. Calli derived from root and leaf segments were consistently firm and compact across all PGR combinations, whereas stem-derived calli exhibited a softer texture throughout the 16-week cultivation period. Callus coloration was also variable and non-uniform, ranging from white to shades of yellow, green, gray, and brown, with colors changing over time during cultivation.

Callus fresh weight (FW) accumulation was used as a key indicator of callogenic response and potential for downstream applications. Initial FW measurements at four weeks revealed explant- and PGR-specific trends. Root-derived calli showed the highest FW on medium supplemented with 2,4-D+2iP, stem-derived calli performed best on all three 2,4-D+cytokinin combinations, and leaf-derived calli exhibited the greatest FW on NAA+TDZ. These trends persisted through the eighth week. Following subculturing at weeks 12 and 16, a shift in FW dominance was observed. Root- and stem-derived calli reached the highest FW on NAA+2iP, whereas leaf-derived calli exhibited comparable FW on both NAA+TDZ and 2,4-D+2iP. By week 16, the

overall highest callus FW (exceeding 27 g) was recorded for stem-derived calli cultured on NAA+2iP (Table 1). However, it was significantly affected by an undesired organogenic response, specifically the formation of adventitious roots and, in some cases, shoot regeneration. Such regeneration consistently occurred in media containing NAA as the auxin (Fig. 2d, e, f). In contrast, cultures grown with 2,4-D showed no visible organogenesis, allowing for a more accurate assessment of true callus biomass (Fig. 2a, b, c). Callus formation was absent or minimal on control medium without PGRs (Fig. 2g).

Among all tested PGR combinations, 2,4-D+2iP consistently supported the highest accumulation of non-regenerating callus biomass across all explant types. The maximum fresh weight (FW) of calli (22.5 g) was recorded from stem-derived explants after 16 weeks of cultivation (Table 1). This value represents a 20.2-fold increase from the initial biomass and corresponds to an average daily FW gain of 0.255 g between weeks 4 and 16.

Elicited callus biomass

Roots are the primary site of tropane alkaloid biosynthesis in *Datura* species. Accordingly, experiments aimed at enhancing callus fresh weight (FW) through elicitation were conducted using non-organogenic root-derived calli cultured on MS media supplemented with either 2 mg/L 2,4-D+0.5 mg/L KIN or 2 mg/L 2,4-D+0.5 mg/L 2iP. After a four-week treatment period, callus FW varied depending on the elicitor applied and the plant growth regulator (PGR) combination used in the culture medium (Fig. 3).

In calli cultured on medium containing 2,4-D+KIN, methyl jasmonate at 100 mg/L exerted the most pronounced and statistically significant ($p \leq 0.05$) positive effect, increasing callus fresh weight (FW) by 1.28-fold compared to the untreated control. Additional positive effects on FW were

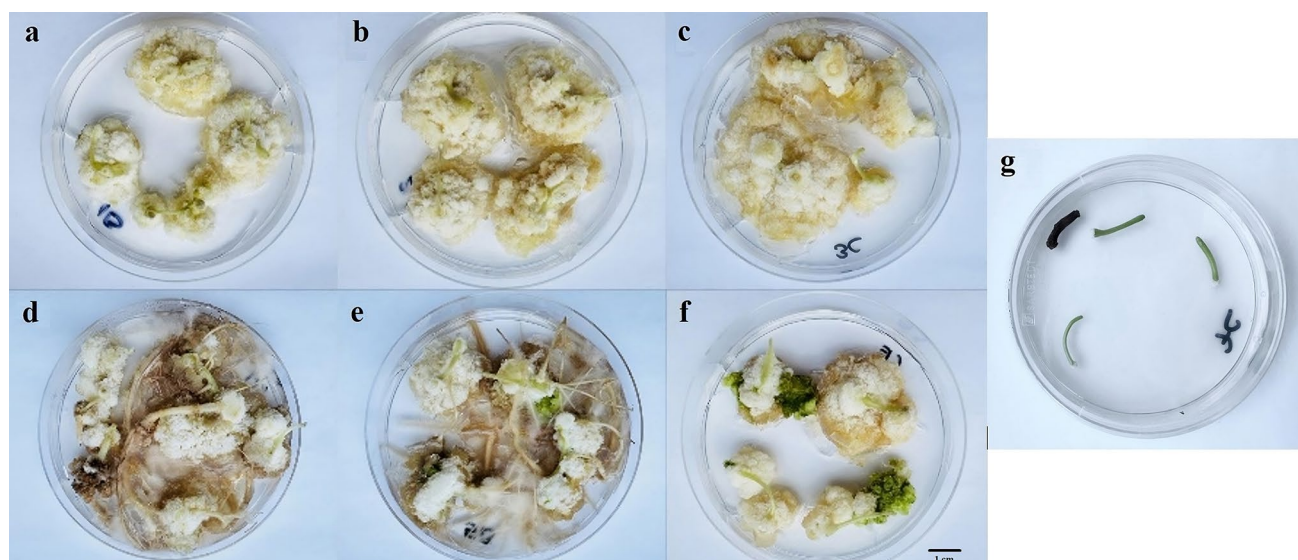
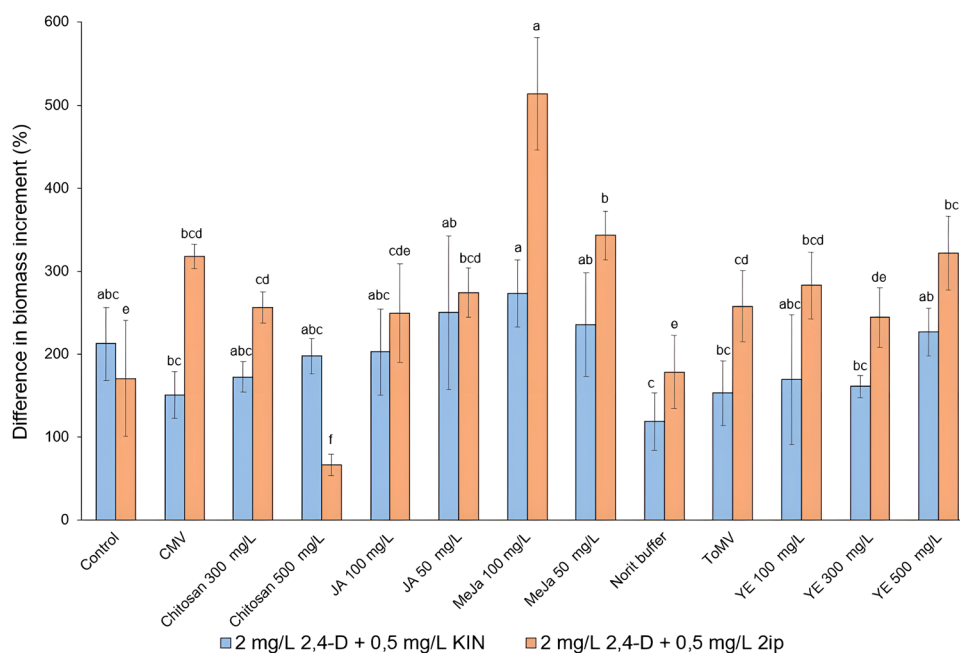


Fig. 2 Structure and organogenic responses of calli derived from *D. stramonium* stem explants after 16 weeks of cultivation on medium supplemented with various plant growth regulator combinations: (a) 2 mg/L 2,4-D+0.5 mg/L KIN; (b) 2 mg/L 2,4-D+0.5 mg/L 2iP; (c)

2 mg/L 2,4-D+0.5 mg/L TDZ; (d) 2 mg/L NAA+0.5 mg/L KIN; (e) 2 mg/L NAA+0.5 mg/L 2iP; (f) 2 mg/L NAA+0.5 mg/L TDZ; (g) Control medium without PGRs

Fig. 3 Effect of elicitors on biomass increment (%) based on fresh weight (FW) gain in root-derived *D. stramonium* callus cultures. Elicitors tested included CMV (plant extract containing Cucurbit mosaic virus), JA (jasmonic acid), MeJa (methyl jasmonate), ToMV (plant extract containing Tomato mosaic virus), and YE (yeast extract). Superscript letters above the bars indicate statistically significant differences between treatments within the same plant growth regulator combination ($p < 0.05$; LSD test)



also observed with methyl jasmonate at 50 mg/L, jasmonic acid at 50 mg/L, and yeast extract at 500 mg/L however, these increases were not statistically significant. The use of other elicitors resulted in a reduction in callus FW, though the differences were likewise not significant. In contrast, calli grown on medium supplemented with 2,4-D+2iP exhibited the highest FW increase (3.01-fold) when treated with 100 mg/L methyl jasmonate. Yeast extract at concentration of 500 mg/L also promoted biomass accumulation, resulting in a 1.88-fold increase in FW. Most other elicitors

had either positive or neutral effects however, chitosan at 500 mg/L caused a statistically significant decrease in FW.

Elicitation of hyoscyamine and scopolamine production

In untreated control calli, hyoscyamine content was 0.324 ± 0.100 mg/g DW on medium supplemented with 2,4-D+KIN and 0.637 ± 0.077 mg/g DW on medium with 2,4-D+2iP. Elicitation significantly influenced hyoscyamine

Fig. 4 Hyoscyamine levels (mg/g DW) in root-derived *D. stramonium* calli treated with various elicitors: CMV (plant extract containing Cucumber mosaic virus), JA (jasmonic acid), MeJa (methyl jasmonate), ToMV (plant extract containing Tomato mosaic virus), and YE (yeast extract). Super-scripts above the bars indicate statistically significant differences between treatments within the same PGR combination ($p < 0.05$; LSD test)

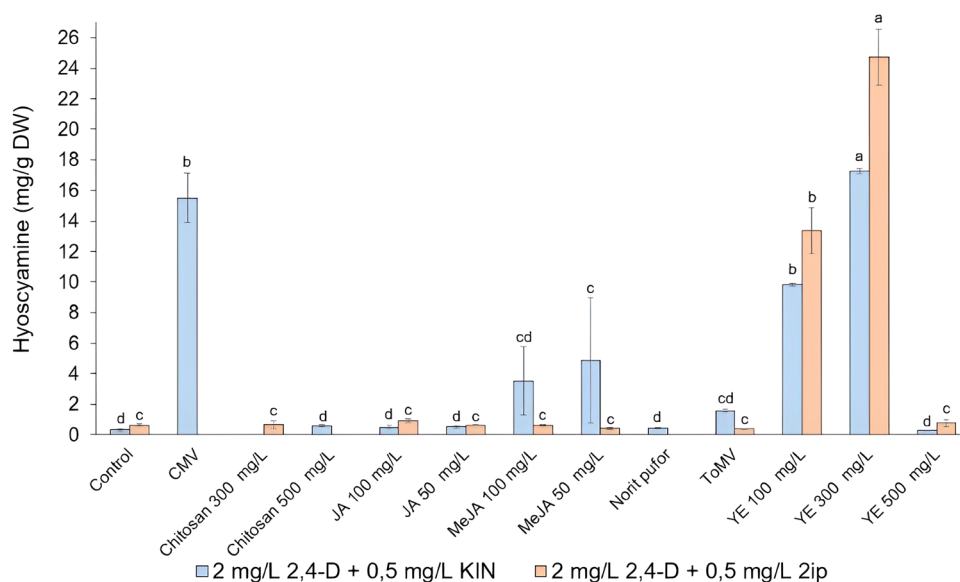
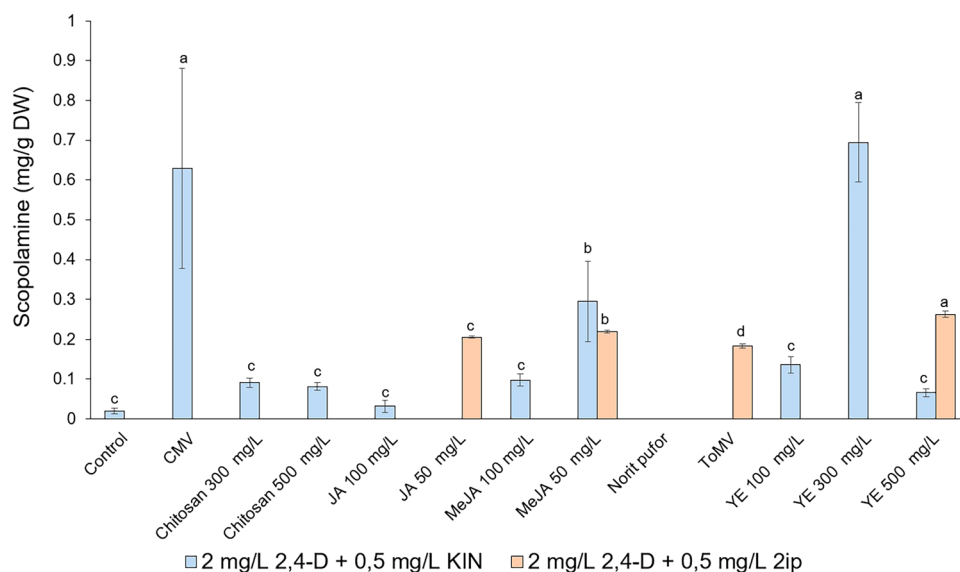


Fig. 5 Scopolamine content (mg/g DW) in root-derived *D. stramonium* calli treated with elicitors: CMV (plant extract containing Cucumber mosaic virus), JA (jasmonic acid), MeJa (methyl jasmonate), ToMV (plant extract containing Tomato mosaic virus), and YE (yeast extract). Super-scripts above the bars indicate a statistically significant differences between treatments within the same PGR combination ($p < 0.05$; LSD test)



accumulation, with specific elicitors and plant growth regulator (PGR) combinations producing distinct effects (Fig. 4). Chitosan, jasmonic acid, methyl jasmonate, Norit buffer, yeast extract at 500 mg/L, and the plant extract containing ToMV did not significantly influence hyoscyamine production. In contrast, yeast extract at concentrations of 100 and 300 mg/L and the CMV-containing plant extract were effective in enhancing hyoscyamine levels. Notably, the stimulation effect of the CMV-containing extract was limited to calli grown on 2,4-D+KIN medium, where it increased hyoscyamine concentration to 15.508 ± 1.633 mg/g DW. Yeast extract at 300 mg/L yielded the highest alkaloid accumulation, raising hyoscyamine levels to 17.247 ± 0.152 mg/g DW on 2,4-D+KIN medium and 24.726 ± 1.819 mg/g DW on 2,4-D+2iP medium. These values represent 38.8-fold

and 53.2-fold increases, respectively, compared to the corresponding untreated controls.

Scopolamine content in both control and elicited calli was significantly lower than that of hyoscyamine, differing by several orders of magnitude (Fig. 5). In control calli, scopolamine was detected only when cultured on medium supplemented with 2,4-D+KIN, and even then, at a minimal concentration of 0.020 ± 0.007 mg/g DW. Among the elicitors tested, yeast extract at 300 mg/L (0.694 ± 0.100 mg/g DW) and plant extract containing CMV (0.630 ± 0.252 mg/g DW) significantly enhanced scopolamine production, but only in the calli cultured on medium containing 2,4-D+KIN. These levels represent 34.7-fold and 31.5-fold increases, respectively, compared to untreated control calli.

The results shown demonstrate that root-derived callus cultures of *D. stramonium* constitute a suitable in vitro

system for the production of tropane alkaloids. Both hyoscyamine and scopolamine were detected in the untreated calli, albeit at relatively low concentrations (Fig. 6a). The levels of both alkaloids were significantly influenced by the specific combination of plant growth regulators (PGRs) used during callus induction and cultivation, as well as by the type and concentration of elicitors applied. Among the elicitors tested, the plant extract containing Cucumber mosaic virus (CMV) and yeast extract at 300 mg/L were the most effective. These elicitors exhibited a dual effect, significantly enhancing the accumulation of both hyoscyamine and scopolamine (Fig. 6b, c). Statistically significant differences were observed in callus fresh weight, alkaloid content, and responses to elicitor treatments.

Discussion

The callus induction phase demonstrated that all three explant types (root, stem, leaf) were capable of initiating callus formation in response to various combinations of auxins and cytokinins in the induction medium. However, significant differences were observed among explant types with respect to undesired organogenesis (i.e., shoot and root formation), callus morphology, coloration, and fresh weight (FW) accumulation. These differences were influenced both by the source of the explant and the specific plant growth regulator (PGR) combinations used. The auxin 2,4-D was the most effective in inducing long-term cultivable callus cultures and was therefore used in elicitation experiments. However, for *in vitro* systems producing metabolites for human use, 2,4-D should ideally be replaced by auxins of lower toxicity due to its documented adverse effects on living organisms (Kim et al. 2005; Garabrant and Philbert 2002) and the environment (Islam et al. 2018; Magnoli et al. 2020). Unfortunately, most alternative auxins are also classified as hazardous substances. Potential substitutes include IBA or callus induction without growth regulators (Pérez-Francés et al. 1995), although these approaches are unlikely to support both long-term callus maintenance and metabolite production in *D. stramonium*. The second auxin tested, NAA, was unsuitable for callus induction and sustained biomass growth, as it promoted the formation of regenerating structures rather than undifferentiated tissue. This is consistent with previous studies reporting the organogenic activity of NAA in *D. stramonium* callus (Amiri et al. 2011) *D. innoxia* (Twaij et al. 2019), confirming its suitability for regeneration protocols rather than for callus maintenance.

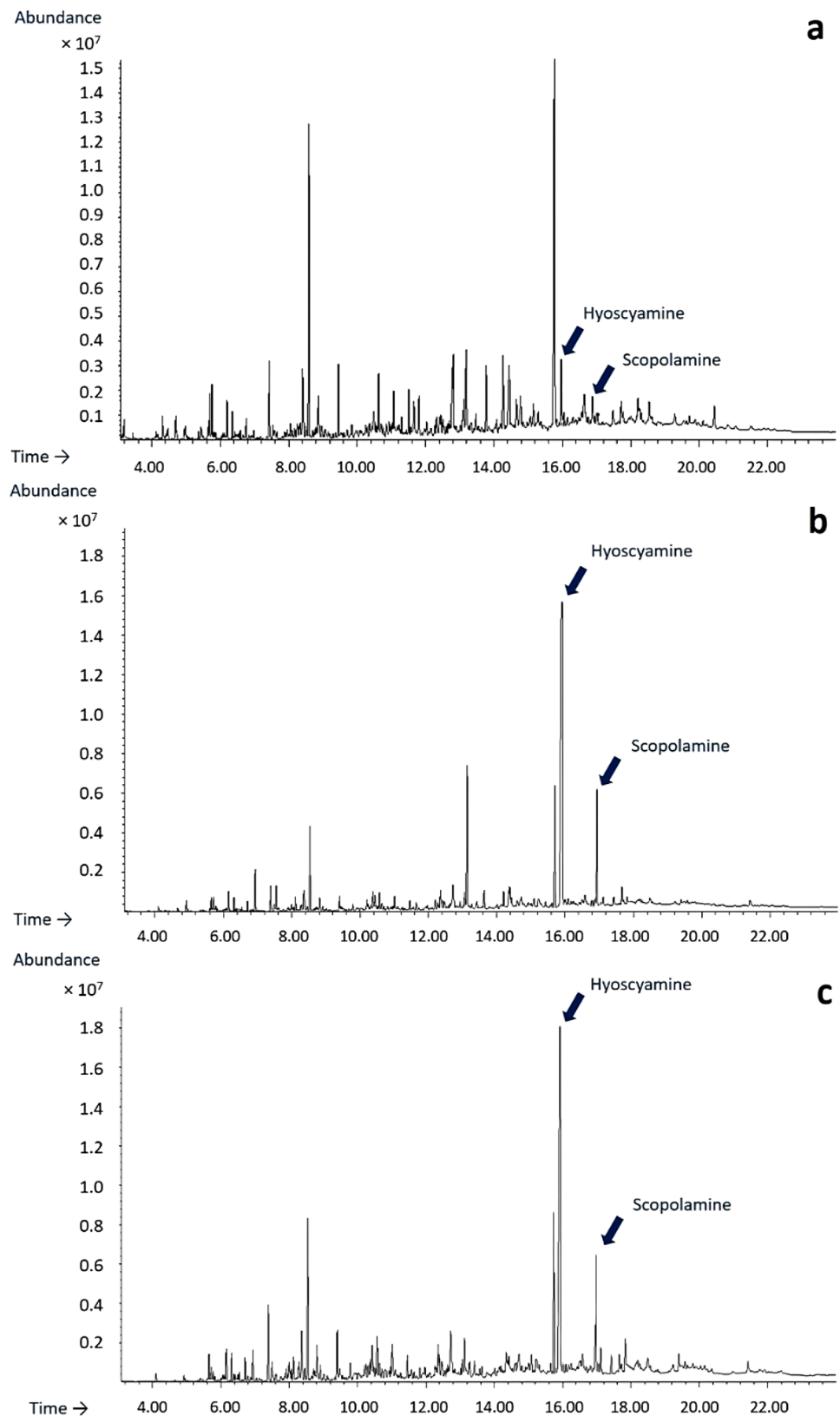
Initial evaluation after four weeks of cultivation revealed that calli exhibited heterogeneous coloration, ranging from yellow to green, gray, and brown, regardless of explant type or PGR combination. This variegated pigmentation appears

to be characteristic of *D. stramonium* calli (Iranbakhsh et al. 2006; Kanani and Mohammed 2021). Calli derived from root and leaf explants had a firm and compact structure, suggesting strong cellular cohesion likely influenced by differences in cell wall properties and water retention capacity. In contrast, stem-derived calli were consistently softer, potentially reflecting a looser cellular organization. Similar observations were made by Gatz et al. (2017), who noted that callus texture and growth in tobacco were affected by 2,4-D concentration, with friable calli and lower auxin levels supporting increased callus biomass. Furthermore, the endogenous hormonal balance modulates these responses through interactions with exogenous PGRs (Gaspar et al. 1996). Consistent with these findings, our experiments indicated that a friable callus texture was generally associated with enhanced growth and higher fresh weight (FW) accumulation.

The fresh weight (FW) of callus biomass could be an indicator of hyoscyamine and scopolamine production. However, in this study, FW was not a decisive factor in the accumulation of these secondary metabolites in callus tissue. Notably, the average daily FW gain (g/day) observed in the callus cultures was comparable to values reported for *Datura metel* (El-Rahman et al. 2008), a species known for its high tropane alkaloid content. Importantly, the absence of undesired shoot regeneration and adventitious roots formation was essential for maintaining callus quality. These developmental responses were influenced by both the explant type and, more significantly, the specific auxin applied. The use of 2,4-dichlorophenoxyacetic acid (2,4-D) effectively maintained the callus in an undifferentiated state, consistent with its role in suppressing organogenesis and promoting callus proliferation. In contrast, naphthaleneacetic acid (NAA) demonstrated a strong capacity to induce organogenesis by stimulating the division and differentiation of embryogenic cells into regenerative structures (Li et al. 2010).

In species of the *Solanaceae* family, tropane alkaloid biosynthesis primarily occurs in the roots, followed by translocation to the aerial parts of the plant for storage (Kohnen Johanssen and Kayser 2019). Consequently, *in vitro* production of tropane alkaloids has been frequently targeted and successfully achieved using hairy root cultures (Hilton and Rhodes 1990; Maldonado-Mendoza et al. 1992; Maldonado-Mendoza and Loyola-Vargas 1995; Pavlov et al. 2009; Marchev et al. 2012; Harfi et al. 2018). Elicitation strategies have been shown to enhance the expression of key biosynthetic genes predominantly in roots rather than leaves (Rasi et al. 2024). In contrast, studies on tropane alkaloid production using cell suspension cultures of *D. stramonium* remain limited (Stohs 1969; Balica et al. 1993; Iranbakhsh et al. 2007; Khater et al. 2018).

Fig. 6 Profiles of tropane alkaloids in *D. stramonium* callus cultures. Hyoscyamine and scopolamine content in (a) non-elicited (control) callus, (b) callus elicited with plant extract containing Cucumber mosaic virus (CMV), and (c) callus elicited with yeast extracts at 300 mg/L



Our experiments focused on the *in vitro* production of tropane alkaloids using callus cultures of *D. stramonium*, an approach that remains largely underexplored, with only one study published to date (Rahman et al. 2013). Another related study was conducted on the related species *D. innoxia* (Zayed et al. 2006), which limits the scope for direct comparison. Zayed et al. (2006) reported that unorganized calli of *D. innoxia* contained only trace amounts of hyoscyamine and scopolamine, with total alkaloid content below 0.5 mg/g fresh weight (FW), a level considered negligible due to the high water content of callus tissue. Similarly, Rahman et al. (2013) reported that untreated *D. stramonium* calli produced both alkaloids at concentrations below 0.5 mg/g dry weight (DW). Upon elicitation with 125 mM NaCl, alkaloid production increased to 1.331 mg/g dry weight (DW) for hyoscyamine and 1.187 mg/g dry weight (DW) for scopolamine, despite a reduction in callus dry weight. Comparable trends were observed in *D. metel* calli, which yielded 2.788 mg/g DW of hyoscyamine and 5.279 mg/g DW of scopolamine following NaCl elicitation, with fresh weight accumulation also significantly suppressed. The authors concluded that *in vitro* callus culture represents a more efficient and sustainable source of tropane alkaloids than field cultivation and extraction from seeds of *D. stramonium* or *D. metel*. Our findings support this conclusion and further demonstrate that an optimized *in vitro* production system, characterized by high callus induction efficiency and biomass accumulation, combined with an effective elicitor (in our case mainly yeast extract), can yield substantial amounts of hyoscyamine and scopolamine in *D. stramonium* callus cultures. However, the most appropriate combinations of PGRs and elicitors must be carefully selected, as the PGRs used for induction and cultivation result in significant differences not only in callus biomass accumulation (Fig. 3) but also in the production of both tropane alkaloids following elicitation. Similar conclusions were reported for callus cultures of *Hyoscyamus aureus*, where PGR choice influenced hyoscyamine and scopolamine production (Beshar et al. 2014). When cultures are subjected to elicitation, the combination of PGRs with elicitors becomes even more critical than PGRs themselves (Figs. 4 and 5).

Plants possess a remarkable capacity to synthesize a diverse range of secondary metabolites. Plant *in vitro* cultures provide a valuable alternative platform for phytochemical production, independent of environmental variability and climatic fluctuations. Over the past two decades, numerous strategies have been developed to enhance the biosynthesis and recovery of phytochemicals from plant *in vitro* cultures, often yielding highly promising results (Nielsen et al. 2019). Our findings on tropane alkaloid production by *D. stramonium* calli further reinforce the potential of *in vitro*

culture systems as efficient and controllable platforms for production of valuable secondary metabolites.

Conclusions

This study successfully demonstrated the induction and sustained *in vitro* cultivation of *D. stramonium* callus derived from root, stem, and leaf explants using various combinations of plant growth regulators (PGRs). Callus fresh weight (FW) varied significantly depending on both the explant source and the applied PGR combination. The highest overall FW was observed in stem-derived calli cultured on medium supplemented with NAA+2iP, although these cultures also exhibited regenerating structures. The highest FW of non-organogenic calli was likewise recorded in stem-derived calli grown on medium containing 2,4-D+2iP. However, elicitation experiments aimed at enhancing both callus biomass accumulation and tropane alkaloid production (hyoscyamine and scopolamine) were performed on root-derived calli induced with either 2,4-D+KIN or 2,4-D+2iP. Both biotic and abiotic elicitors significantly influenced callus biomass and alkaloid production. Methyl jasmonate (100 mg/L) proved to be the most effective elicitor for increasing callus biomass. In contrast, the most potent elicitor for hyoscyamine production was yeast extract at 300 mg/L, which led to a significant increase in hyoscyamine levels, up to 25 mg/g dry weight (DW), representing a 38.8-fold enhancement in cultures grown on 2,4-D+2iP medium. Yeast extract (300 mg/L) also increased scopolamine production approximately 30-fold in calli cultured on 2,4-D+KIN medium. A plant extract containing cucumber mosaic virus (CMV) showed a comparable stimulatory effect on alkaloid accumulation. Our findings confirm that root-derived callus of *D. stramonium* represents a viable and efficient *in vitro* platform for the production of valuable tropane alkaloids. Optimization of PGR combinations and elicitor treatments is critical for maximizing hyoscyamine and scopolamine biosynthesis. Future research should focus on elucidating the underlying metabolic pathways and regulatory mechanisms to further enhance alkaloid yields and support the development of scalable production systems.

Author contributions ŠK and JK conceptualized and designed the experiments. ŠK, SF and JS conducted the experimental work. ŠK and JK validated data and results. ŠK and JK created figures and tables and drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

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Data availability All data obtained during this study are original and included in this published article. We are open to discussing them with colleagues who are interested.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals.

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