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ENHANCED HYOSCYAMINE PRODUCTION IN *DATURA STRAMONIUM* L. CALLUS CULTURES ELICITED BY EXTRACTS FROM VIRUS-INFECTED PLANTS

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ABSTRACT

This study investigated the effects of two plant viruses as biotic elicitors on hyoscyamine production in *Datura stramonium* L. callus cultures. Their effects were compared with those of acetylsalicylic acid, Norit buffer, and mechanical callus injury. The results demonstrated varying effects of the elicitors and differences in hyoscyamine production over time, with some acting more as inhibitors than elicitors. Eighteen days after the start of elicitation, the highest hyoscyamine production was observed in calli treated with tomato mosaic virus, reaching 4.06 ± 0.50 mg per gram dry weight of callus. Elicitation with cucumber mosaic virus increased hyoscyamine levels to 2.55 ± 0.68 mg/g of dry weight, while the application of 0.1 mM ASA resulted in 2.35 ± 0.18 mg/g of dry weight. In untreated control callus, hyoscyamine production was 1.02 ± 0.01 mg/g of dry weight. Mechanical damage to callus tissue led to hyoscyamine production of 2.76 ± 0.7 mg/g of dry weight after 23 days of elicitation, compared to 1.71 ± 0.17 mg/g of dry weight in untreated callus. In contrast, treatment with Norit buffer and 0.5 mM ASA reduced hyoscyamine production. The use of extracts from virus-infected plants as elicitors in *in vitro* plant cultures has not been reported to date. Both viruses in extracts demonstrated a positive effect on hyoscyamine production, particularly tomato mosaic virus. These findings clearly indicate that plant viruses are effective elicitors of secondary metabolite production in *in vitro* cultures.

Keywords: thornapple, callus, elicitor, ToMV, CMV, hyoscyamine

INTRODUCTION

Datura stramonium L., a species of flowering plant, belongs to the genus *Datura* and the family *Solanaceae*. It is commonly known by various names, including jimsonweed, devil's trumpet, thornapple, devil's weed, locoweed, and Jamestown weed. These names reflect its toxicity, as all parts of the plant, particularly the seeds and flowers, are poisonous due to the presence of tropane alkaloids. *D. stramonium* naturally produces tropane alkaloids, including hyoscyamine (a precursor of atropine, which forms through racemization during extraction and purification) and scopolamine. Traditionally, it has been used in various cultures for pain relief, asthma treatment, sedation, and psychoactive applications, including the induction of delirium and hallucinations. In modern medicine, hyoscyamine and scopolamine serve as essential precursors in the synthesis of various pharmaceuticals (**Gaire and Subedi, 2013**). Atropine sulfate, a key component in numerous medications, is used to induce mydriasis, regulate cardiac rhythm, and counteract organophosphate poisoning. Scopolamine hydrobromide, another significant alkaloid, is primarily employed to prevent motion sickness, particularly in transdermal patches, and, in some cases, as a pre-anesthetic agent. The extraction and isolation of tropane alkaloids from *D. stramonium* plants involve the use of organic solvents such as methanol, ethanol, and chloroform, followed by separation and purification using chromatographic techniques. The identification and quantification of these alkaloids are performed using chromatographic methods (GC-MS, LC-MS), as well as colorimetric, densitometric, and NMR techniques (**Cinelli and Jones, 2021**). The significance of tropane alkaloids in the study of *D. stramonium* and related species, as well as their role in various biotechnological applications, is well-documented. Optimizing their production through *in vitro* cultures of *D. stramonium* is essential, utilizing controlled cultivation of cells, tissues, or organs in a laboratory setting. *In vitro* systems include callus and cell suspension cultures, as well as true, adventitious, and hairy root cultures. *In vitro* callus cultures are generally easy to establish from various species and explant sources. The type and concentration of auxins and cytokinins, as well as their combinations, play a crucial role in inducing callogenesis. However, only a few studies have investigated *D. stramonium* callus cultures for their potential in secondary metabolite production and whole-plant regeneration (**Amiri *et al.*, 2011**; **ElNour *et al.*, 2012**). If *D. stramonium* callus cultures can produce tropane alkaloids, then long-term *in vitro* callus cultures could serve as a viable production system for these secondary metabolites. However, information on such a culture is currently lacking.

An effective strategy for increasing the production of secondary metabolites, including tropane alkaloids, in *in vitro* plant cultures is elicitation, i.e. modulating the biosynthetic pathways of secondary metabolites (**Ramirez-Estrada *et al.*, 2016**). Elicitation can be achieved using abiotic elicitors such as methyl jasmonate, jasmonic acid, salicylic acid, heavy metal ions, UV radiation, osmotic stress, and temperature stress, or biotic elicitors, including yeast extract, chitosan, and fungal or bacterial extracts. These are standard elicitors used across various plant species. *D. stramonium* is susceptible to multiple plant viruses that can influence its growth, development, and potentially the production of secondary metabolites, including tropane alkaloids. Plant viruses known to infect *D. stramonium* include tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), potato virus Y (PVY), tomato spotted wilt virus (TSWV), potato leafroll virus (PLRV) (**Massumi *et al.*, 2009; Singh, 2016**), but also many others (**Thornberry, 1966**). Plant viruses or their components have been shown to act as elicitors in plants, impacting plant defense, disease resistance, yield and quality of production, and even biotechnological applications (**Culver and Dawson, 1989**; **Li et al., 2023**; **Ortiz-Martínez *et al.*, 2024**). In *in vivo* conditions, plant viruses naturally infecting a host can trigger the plant's immune system, a process that essentially functions as elicitation (**Tomitaka *et al.*, 2024**). Additionally, the application of attenuated or avirulent virus strains as elicitors has been utilized in in vivo cross-protection strategies to enhance plant resistance.

One method of *in vivo* viral infection in plants involves spraying an extract containing the virus onto the plant's leaves. Several studies have examined the impact of viral infections on secondary metabolite production in plants *in vivo*, as summarized by **Mishra *et al.* (2020)**. These studies suggest that plant viruses, which regulate the accumulation of various secondary metabolites, could also be exploited at an industrial level. This assumption is supported by findings showing an increase in tropane alkaloid content in *D. stramonium* plants cultivated and infected *in vivo* with tobamoviruses, including pepper mild mottle virus (PMMoV), tomato mosaic virus (ToMV), and tobacco mosaic virus (TMV) (**Mihálik *et al.*, 2022**). Despite lacking the complex, spatially organized defence responses exhibited by whole plants, such as stomatal closure, localized hypersensitive reactions in leaves, and systemic responses, the callus response may be simplified and primarily cellular (**Nagy *et al.*, 2005**). Plant viruses have been shown to act as

biotic elicitors, capable of inducing a variety of responses in *in vitro* cultures, including callus cultures (**Chen *et al*., 2004**; **Al Abdallat *et al*., 2010**). Although undifferentiated, callus tissue retains the fundamental cellular machinery of plants, including the ability to recognize and respond to elicitors such as viruses. The mechanisms by which viruses function as elicitors in callus cultures are similar to their actions in whole plants. Callus cells detect viruses, specifically their nucleic acids, capsid proteins, and movement proteins, which activate various defence responses, biochemical and physiological changes including the production of secondary metabolites via the salicylic acid and jasmonic acid pathways (**Mauck *et al*., 2014**; **Huang *et al*., 2023**). Viral elicitation could alter the biosynthesis of tropane alkaloids in *D. stramonium* callus, with the types of alkaloids produced depending on the virus strain, callus line, and other conditions. Because all cells in the callus mass are potentially exposed to the virus as an elicitor, the virus can be delivered by simply inoculating the callus with extracts from infected plants. To date, elicitation using plant viruses, applied in some living form, to enhance secondary metabolite production in callus cultures has not been reported. It is hypothesized that plant viruses could efficiently elicit the production of tropane alkaloids in *D. stramonium* callus cultures. Therefore, the aim of this study was to test this possibility using two plant viruses.

MATERIAL AND METHODS

Plant material

Seeds of *Datura stramonium* L. were obtained from SemenaOnline s.r.o. (Jeneč, Czechia). To enhance germination, they were scarified with concentrated sulfuric acid, thoroughly washed, and surface-sterilized by immersion in 96% ethanol (v/v) for 30 seconds, followed by 15 minutes in a 4.7% sodium hypochlorite solution with two drops of Tween 20 under continuous stirring. After multiple rinses with sterile water, the seeds were germinated in culture vessels containing Murashige and Skoog (MS) medium (**Murashige and Skoog, 1962**) (Duchefa Biochemie B.V., Haarlem, Netherlands), but only with half the concentration of its nutrients and vitamins (½MS), solidified with 0.8% agar (w/v), and adjusted to a pH 5.7–5.8. The seedlings were cultivated in a growth chamber at 23 ± 2°C under a 16-hour light/8-hour dark photoperiod.

Callus induction and elicitation

Callus cultures were initiated from root segments of 5-week-old aseptic *Datura stramonium* L. plants. Explants were placed on MS medium supplemented with vitamins, 30 g/L sucrose (w/v), and 0.8% agar (w/v), pH 5.7–5.8. The medium contained a combination of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and the cytokinin kinetin (KIN) in a ratio of 2 mg/L auxin to 0.5 mg/L cytokinin. All plant growth regulators (PGRs) were purchased from Duchefa Biochemie B.V. (Haarlem, Netherlands). The cultures were incubated in darkness at 23 ± 2°C. Elicitations were performed of calli four weeks after the beginning of their induction and after transfer to fresh culture medium of the same composition.
Elicitation was performed on calli cultured on callus induction MS medium. Biotic elicitors included two plant viruses, tomato mosaic virus (ToMV) and cucumber mosaic virus (CMV), as well as acetylsalicylic acid (ASA) (Merck KGaA, Darmstadt, Germany) at concentrations 0.1 mM and 0.5 mM. The abiotic elicitor was Norit buffer, containing 0.05 M sodium/potassium phosphate buffer (pH 7.0), 1 mM EDTA, 5 mM DIECA, and 5 mM thioglycolic acid. Virus elicitation was performed using extracts from symptomatic leaves of tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativum* L.) plants infected with ToMV and CMV, respectively. The presence of CMV and ToMV antigens in infectious extracts was analyzed using a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) according to **Clark and Adams (1977)**, using ELISA reagent sets specific to CMV and ToMV (Bioreba AG, Reinach, Switzerland). Leaves were homogenized using a TissueLyzer II (Qiagen N.V., Hilden, Germany) in Norit buffer. Resulting supernatants were filtered through 0.22 µm Millex® Syringe Filters (Merck KGaA, Darmstadt, Germany) Mechanically wounded calli were immersed in the virus-containing plant extract, transferred to solid MS medium supplemented with PGRs, and cultivated in darkness at 23 ± 2°C. All elicitors were applied to the calli in this way. Unelicited and undamaged calli were negative controls. Elicitations were initiated on 4-week-old calli, and hyoscyamine content in the calli was monitored after 3, 10, 18, and 23 days.

Analysis of hyoscyamine

The hyoscyamine content was determined in the dry matter of calli. Calli were dried for 120 min at 55 °C. Subsequently, the samples were processed for the extraction of tropane alkaloids according to the method of **Harfi *et al.* (2018)**. Alkaloid analysis was performed by gas chromatography-mass spectrometry (GC-MS) as described **Nguyen *et al.* (2015)** using an Agilent 7890B GC system equipped with an Agilent 5977A Series Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA). A VF-5 MS low-flow column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies) was employed for separation. One microliter of the sample was injected in splitless mode at an injector temperature of 250 °C, which was held isothermal. The oven temperature was programmed as follows: initial hold at 40 °C for 1 min, ramp at 30 °C/min to 130 °C, followed by an increase at 10 °C/min to 280 °C, with a final hold at 280 °C for 5 min. The transfer line and ion source were maintained at 300 °C and 200 °C, respectively. Electron ionization (EI) was conducted at 70 eV, with mass spectra recorded in the range of 30–600 m/z at a scan rate of 4.7 scans/s (**Nguyen *et al*., 2015**). The tropane alkaloid hyoscyamine was identified and quantified using standard reference compound hyoscyamine sulfate (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany). Standard solutions were prepared in CH_2Cl_2 at concentrations of 1.6 mg/mL to generate a calibration curve. Identification was based on a comparison of mass spectra with the NIST 2007 database.

Data Analysis

Statistical analyses were conducted using analysis of variance (ANOVA) in Statgraphics software version 19.2.01 (Statgraphics Technologies, Inc., The Plains, VA, USA). Significant differences among treatments were determined using the least significant difference (LSD) test at a 5% significance level ($p < 0.05$). Variables with statistically significant differences ($p < 0.05$) between the control and treatment groups were denoted using alphabetic letters.

RESULTS AND DISCUSSION

The callus induction medium MS supplemented with the PGRs combination 2,4-D+KIN, was suitable for callus induction and the subsequent cultivation and growth of calli. The color of the calli varied in shades of yellow, with lighter areas. During and after the application of elicitors, the treated calli did not differ in color, texture, or growth intensity from the control calli (Figure 1).

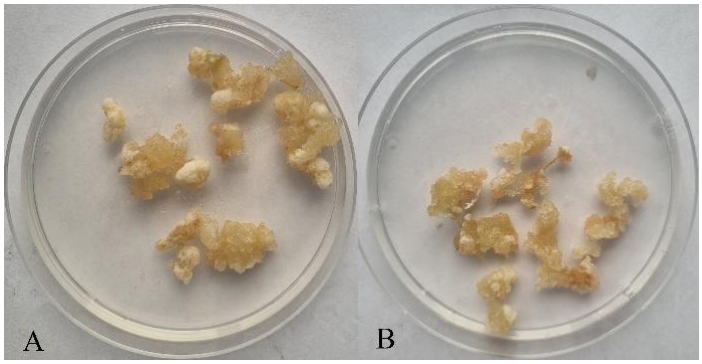

Figure 1 Untreated, control calli (A) and calli elicited with extract from ToMV-infected tomato plants (B).

However, effects were observed at the metabolic level, by increasing the biosynthesis of tropane alkaloids. The subject of interest was hyoscyamine, determined qualitatively and quantitatively by GC-MS (Figure 2). Hyoscyamine was present in all samples analyzed, in control as well as in all elicited calli.

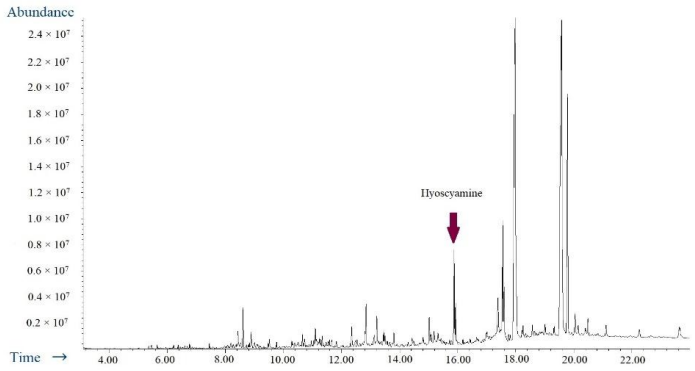

Figure 2 Chromatographic analysis of hyoscyamine produced in callus cultures of *D. stramonium*.

The hyoscyamine contents in the calli was statistically significantly different at all monitored intervals from the beginning of elicitation. In individual intervals from the time of elicitation, its content depended on the elicitor used. (Figure 3).

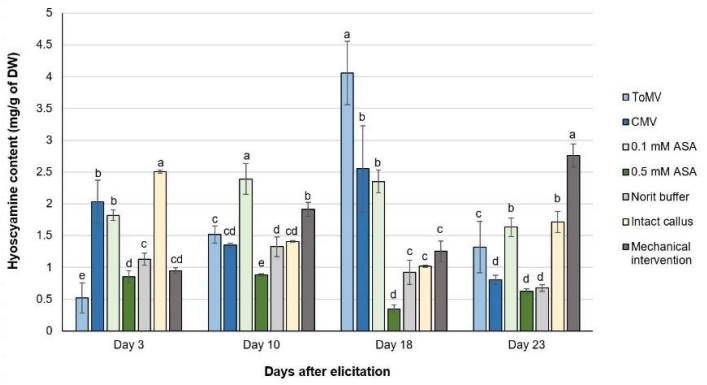

Figure 3 Elicitor-induced changes in hyoscyamine content in *D. stramonium* callus cultures. Different letters above the bars indicate a statistically significant difference at $p<0.05$.

After three days of elicitation, the hyoscyamine level, calculated per callus dry weight, increased the most in intact (control) calli, reaching a value of 2.51 ± 0.02 mg/g of dry weight (DW). However, a three-day period is not sufficient for callus increase growth and potential increase in hyoscyamine content, as there is practically no observable increase in callus weight over such a short period. After 10 days, the application of 0.1 mM ASA proved to be an effective elicitor, with the hyoscyamine level reaching 2.39 ± 0.24 mg/g DW. From a production perspective, the most significant results were observed 18 and 23 days after the beginning of elicitation. After 18 days, hyoscyamine production in calli was induced by both viruses (ToMV and CMV), with hyoscyamine levels reaching 4.06 ± 0.50 mg/g DW after elicitation with ToMV and 2.55 ± 0.68 mg/g DW with CMV. At this time, the highest hyoscyamine content was observed in calli treated with ToMV. Norit buffer alone was used as a control for both viruses, as it served as the extraction solution in which the viruses were applied. Norite buffer, as well as 0.5 mM ASA, did not function as elicitors but rather acted as inhibitors, reducing hyoscyamine production at each observed time point. After 23 days of treatment, mechanical damage to the callus proved to be the most effective elicitation method, yielding hyoscyamine level of 2.76 ± 0.18 mg/g DW, which was, however, lower than the level obtained with ToMV, after 18 days of elicitation.

Acetylsalicylic acid (ASA), an analogue of salicylic acid, is frequently used as a biotic elicitor in plant *in vitro* cultures. Studies have demonstrated increased expression of the hyoscyamine-6-beta-hydroxylase (*h6h*) gene and enhanced production of the tropane alkaloids hyoscyamine and scopolamine in hairy root cultures of *Hyoscyamus reticulatus* L. (**Norozi *et al*., 2018**) and in three *Datura* species (**Harfi *et al*., 2018**). There have been no reports to date on the other biotic elicitors used in our study. However, plants continuously interact in the environment with various biological entities, including plant viruses that have an impact on their metabolism. Virus-plant interactions alter the expression of many genes encoding plant hormones, modulate their levels, and trigger various plant defense mechanisms, including of the production of secondary metabolites (**Mishra *et al.*, 2020**). Consequently, viruses can manipulate the biosynthesis of these compounds. The selection of tomato mosaic virus (ToMV) from the *Virgaviridae* family was based on its exceptionally high stability *in vitro* and its ability to retain active for several months (**Brunt, 1986**). Its host range includes *D. stramonium* and other *Solanaceae* species, with *D. stramonium* even serving as an indicator plant for this virus (**Hamdi *et al*., 2020**). Previous research has shown that ToMV can enhance the synthesis and accumulation of tropane alkaloids in *D. stramonium* plants infected (elicited) *in vivo* (**Mihálik et al., 2022**). Cucumber mosaic virus (CMV) from the *Bromoviridae* family is another well-known multi-host pathogen, capable of infecting over 1,200 species across more than 100 plant families (**Sacristán *et al*., 2004**). Consistently high levels of CMV infection have also been observed in species of the genus *Datura*, including *D. stramonium* (**Ormeño *et al*., 2006**). These findings prompted an investigation into the potential use of both viruses as elicitors in *in vitro* culture. The results of this study confirm that plant extracts from virus-infected plant can be used as biotic elicitors to enhance hyoscyamine production in *D. stramonium* callus cultures. This suggest that viral elicitation could also be applied to induce the production of valuable secondary metabolites in cultivation systems *in vitro* of other plant species.

CONCLUSION

Two plant viruses, ToMV and CMV, used for the treatment of *Datura stramonium* L. callus cultures, proved to be effective elicitors for enhancing hyoscyamine production. Their efficacy exceeded than of the commonly used elicitor, acetylsalicylic acid (ASA). Eighteen days after the beginning of elicitation, hyoscyamine levels increased 3.98-fold in ToMV-treated calli and 2.50-fold in CMV-treated calli compared to untreated controls. These results represent the first documented use of plant viruses, applied in extract from virus-infected plants, as elicitors in *in vitro* culture and highlight their potential for enhancing secondary metabolite production in plant cultivation systems *in vitro*.

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