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LONG-TERM EVALUATION OF BIOMASS AND TROPANE ALKALOID BIOSYNTHESIS IN *Datura* HAIRY ROOT CULTURES ELICITED WITH $AgNO_3$

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ABSTRACT. This study evaluated the growth kinetics and hyoscyamine biosynthesis of transgenic hairy roots from *Datura innoxia* (DI), *Datura stramonium* var. stramonium (DS) and *Datura stramonium* var. tatula (DT) induced by *Agrobacterium rhizogenes* strain A4 and cultured in vitro for five years after their induction using liquid chromatography–tandem mass spectrometry for alkaloid analysis. The lines were elicited with $AgNO_3$ for 24 and 48 h to determine its effects on the biomass, hyoscyamine and scopolamine yield of hairy roots. DT grew better, resulting in 0.295 g of dry mass after 21 d of culture on B5 medium in the first year, compared to 0.229 g for DI and 0.205 g for DS. Over five years, DT grew better than DS and DI. Furthermore, the hyoscyamine yield was higher in DT than in DI and DS with the best yield occurring in the first year of culture (8.575 mg g^{-1} DW). After chemical elicitation, the biomass, expressed as the dry weight of DS and DT, decreased under all $AgNO_3$ treatments. However, the DI biomass increased after $AgNO_3$ treatment for 24 and 48 h (74%) of the control. Only two treatments promoted hyoscyamine biosynthesis, and the best yield was obtained with 7.89 mg g^{-1} DW of hyoscyamine after 48 h of elicitation. However, the scopolamine yield was improved by $AgNO_3$ elicitation for 24 h with the best result obtained for DT hairy roots (0.539 mg g^{-1} DW).

Keywords: alkaloids; biomass; *Datura*; hairy roots; in vitro.

INTRODUCTION

Plants from the Solanaceae family, such as *Datura*, are attractive ornamental

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and medicinal species that produce hyoscyamine and scopolamine, which are tropane alkaloids, synthesized in small amounts (Lian *et al.*, 2022). Hyoscyamine and scopolamine have economic value for their large-scale use as pharmaceutical agents (Amdoun *et al.*, 2022). Hyoscyamine is a muscarinic antagonist frequently used to prevent the cholinergic side effects of pyridostigmine without affecting the effectiveness of the drug at the neuromuscular junction (Gehi *et al.*, 2008). Scopolamine is a para-sympatholytic agent that is often used to treat motion sickness and neural diseases (Santos *et al.*, 2024), explaining the increasing demand for such molecules (Gong *et al.*, 2022). Climate conditions, parasitic insects and phytopathogenic fungi make field cultivation unstable and insufficient. Therefore, many modern methods are available for producing molecules of interest. Moreover, the production of such molecules through biotechnological methods provides stable productivity due to the control over growth conditions (Amdoun *et al.*, 2022; Khelifi *et al.*, 2011). Hyoscyamine is produced in plant roots, which makes the cultivation of roots in vitro an interesting method for achieving intensive production (Mihálik *et al.*, 2022; Moussous *et al*., 2025). Hairy roots of *Datura*, which are commonly induced using *Agrobacteium rhizogenes* via the transfer of their *rol* genes into plant cells, may have important industrial production implications for hyoscyamine (Moussous *et al.*, 2018). Elicitation is an important process by which a chemical compound or part of a microorganism, such as salicylic acid, jasmonic acid, and proline, is used to trigger plant resistance mechanisms, increasing the plant's synthesis of the targeted molecules (Amdoun *et al.*, 2010; Swiatek *et al.*, 2002). In this study, we evaluated the evolution of biomass and hyoscyamine production in hairy roots in *Datura stramonium* Var *Stramonium*, *Datura stramonium* Var *tatula* and *Datura innoxia* induced by *Agrobacterium rhizogenes* strain A4. Additionally, in this paper, we reported the effect of silver nitrate, which is used as an elicitor, after two different elicitation exposure times of 24 h and 48 h on the growth and biosynthesis of tropane alkaloids hyoscyamine and scopolamine.

MATERIALS AND METHODS

Chemicals

All chemicals used in the present study, including methanol, chloroform, atropine, the scopolamine standard and ammonium, were purchased from Sigma–Aldrich (Germany).

Plant material

Datura seeds were collected from Algiers, Algeria, identified by professors from the botanical department of the Algerian National Higher School of Agronomy, and conserved in the seed collection of the department's herbarium since 2007. The seeds were scarified, and the thick teguments were removed using glass paper (80 points), washed in a solution of 70% ethanol (v/v) for 30 s and 12% sodium hypochlorite (v/v) for 10 min, rinsed three times with distilled water, and dried on filter paper. The seeds were placed in test tubes containing 20 mL of Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 20 g/L sucrose and 7 g agar. In vitro culture was established in a culture chamber at 26±1°C with a

photoperiod of 16 h and a light intensity of 490 µmol m^{-2} s^{-1}.

Transgenic roots of *Datura innoxia*, *Datura stramonium* and *Datura stramonium* Var *tatula* were induced by the inoculation of fragments (1 cm of the stem) of 60-day-cultured explants with a solution of *Agrobacterium rhizogenes* strain A4 previously activated and incubated in YEM liquid medium for 72 h following the Amdoun transformation protocol described by Moussous *et al.* (2018). A total of 300 seedling test tubes were prepared with 100 for each line. The confirmation of root genetic transformation occurred after genomic DNA extraction from hairy roots via amplification of the r*olB* gene using PCR with the program described by Moussous *et al.* (2018). Following root induction, A4 was eliminated using 250 mg L^{-1} cefotaxime, and the three lines were transferred to B5 medium supplemented with 5 g agar.

MS medium was used for seedling and root induction using A4, and Gamborg B5 medium supplemented with 5 g agar was used to conserve hairy roots in culture. The growth kinetics of hairy roots were determined in liquid B5.

To study the biomass evolution and hyoscyamine biosynthesis, a B5 liquid culture of hairy roots was used throughout the experiment, and 0.1 g of hairy roots was cultivated in a fresh medium for 20 d after which the biomass and hyoscyamine yield were recorded. After 21 days, 0.1 g was transferred to new media, and the operation was repeated every 21 d. For this study, the middle value of three representative cultures was calculated every six months. A total of 10 values were considered for the whole culture period.

Silver nitrate elicitation

In this study, triplicate runs of each experiment were performed. A liquid culture of 100 mg of fresh hairy roots was prepared in 50 mL of liquid B5 medium and cultivated under dark conditions in a shaker at 100 rpm/min. To determine the effects of $AgNO_3$ on biomass and tropane alkaloid yield of the hairy roots, 100 mL of 1 mM $AgNO_3$ liquid solution was prepared in a new flask and autoclaved for 20 min. The pH of the $AgNO_3$ solution was adjusted to 5.6–6.8, and 5 mL of the sterilized solution was added to the culture twice at 24 and 48 h of elicitation. The controls were cultivated for the same period of 20 days and not treated with $AgNO_3$.

Tropane alkaloid extraction and chromatographic analysis

After 20 days of culture in liquid medium, the hairy roots were harvested and dried at 60°C for 24 h, after which the dry weight was recorded. For hyoscyamine extraction, we followed the Kartal protocol adapted by Amdoun *et al.* (2005). The dry extract of the alkaloids was diluted in 1 mL of methanol and analysed using a liquid chromatography–tandem mass spectrometry (LC–MS-MS) system based on a pump connected to a photodiode array detector and a linear trap quadrupole Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific®, Courtaboeuf Cedex, France) with an atmospheric pressure ionisation interface working in electrospray ionisation mode. After dilution of the extract in 1 mL of MeOH, 10 µL of each sample was separated on a C18 (150 × 2.1 mm) column (Grace®-Alltech®, Columbia, MD, USA) using a gradient of mobile phase A (1% v/v) in water

acidified with formic acid and mobile phase B composed of acetonitrile. A flow rate of 0.3 mL min^{-1} was used.

The biomass and hyoscyamine biosynthesis of hairy roots were studied over 5 years by taking the average value to determine the possibility of industrially producing hyoscyamine from transgenic hairy roots for more than three years.

Data analysis

One-way analysis of variance (ANOVA) and Tukey's test (classification into homogeneous groups) at the 5% significance level were used to analyse the data in Statistica software 6.0 version. Means followed by the same letter(s) are not significantly different. The bars on the graphs represent the standard deviation (SD) at a 5% significance level.

RESULTS

Morphological comparison between hairy roots and natural untransformed roots of *Datura* showed that their ramifications were shorter and more abundant than in natural case, as illustrated in *Figure 1*.

DNA from *Datura* hairy root lines was extracted, and the *rolB* from *A. rhizogenes* was amplified via PCR for comparison with normal untransformed roots after revelation on 1% agarose gel. This confirmed the presence of the *Agrobacterium rolB* gene in the three lines, as previously described by Moussous *et al.* (2018). The transgenic hairy roots of DT, DS and DI were maintained in B5 medium, which was refreshed after 21 d (*Figure 2*).

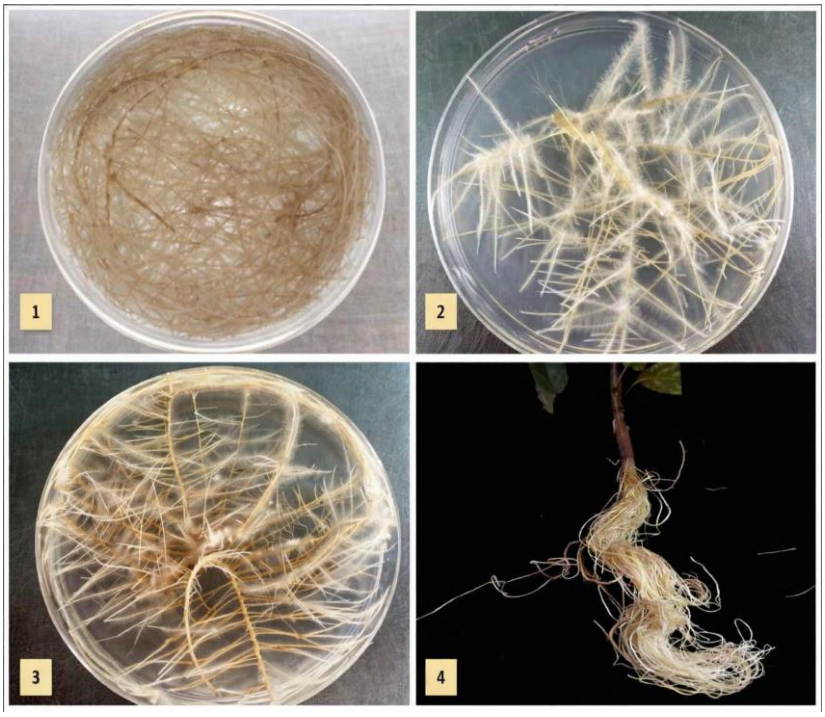


Figure 1 – Morphology of hairy root lines cultivated in B5 media for 20 days and comparison with natural plant roots; 1: DS, 2: DI, 3: DT, 4: D. stramonium var. tatula roots

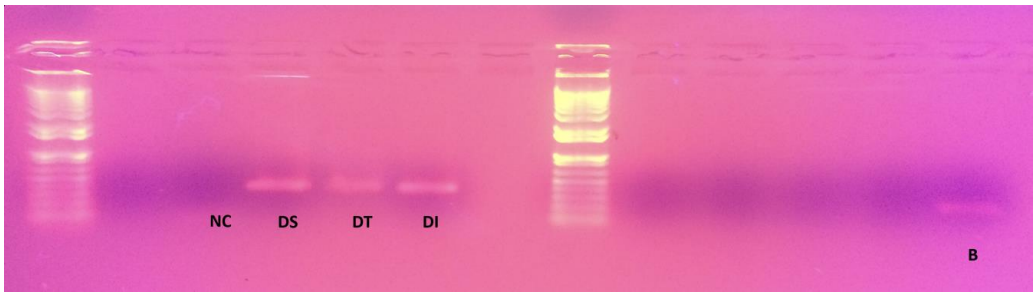


Figure 2 – DNA from hairy roots; NC: negative control, B: bacterial DNA. DS, DT and DI are the hairy root DNA. The marker is 1 k bp, and the bands are at 400 bp

Main alkaloids in hairy roots

The analysis of tropane alkaloids illustrated in figure 3 showed that hyoscyamine was the main alkaloid biosynthesized in all *Datura* transgenic hairy roots. All lines produced more hyoscyamine than scopolamine, with a middle value of 8.575 mg g^{-1} DW obtained in DT hairy roots. Similarly, the scopolamine yield was 175% lower than that of hyoscyamine, which was 0.049 mg g^{-1} DW. DS and DI produced 8.16 and 3.15 mg g^{-1} DW of hyoscyamine, respectively. DI produced the most scopolamine, with a moderate value of 0.179 mg g^{-1} DW compared to 0.098 mg g^{-1} DW for DS and 0.049 mg g^{-1} DW for DT.

ANOVA revealed a highly significant effect of the root line on the hyoscyamine and scopolamine yields. A comparison using Tukey's test revealed the presence of three homogeneous groups. Group A represented DT, which was the best in terms of hyoscyamine yield.

Biomass evolution during hairy root culture on B5 media

Biomass analysis indicated that the hairy roots of DT, DS and DI grew differently. The dry weight depended on the line and year of evaluation. The hairy roots of DT exhibited the greatest growth performance after full culture. However, the biomass of each line decreased from one year to another. The best results were obtained during the first year, in which the DT hairy roots produced 0.295 g DW, and DS and DI produced 0.205 and 0.229 g DW, respectively.

All lines showed decreased growth during the second year of cultivation. For DS, DT and DI, weight reductions of 42, 12 and 62%, respectively, were observed compared to the first-year results. The growth of all three lines was slower than that in the year of induction (*Figure 4*).

The decrease in root growth was maintained for the third, fourth and fifth years of cultivation, with lower rates of 0.200 g DW for DT, 0.088 g for DI, and 0.076 g DW for DS, representing losses of 47, 195 and 172%, respectively. Thus, the growth of the lines slowed with age.

Hyoscyamine biosynthesis by hairy roots during culture

In the first analysis of tropane alkaloids produced by hairy roots, hyoscyamine was the main alkaloid produced by the three lines (*Figure 3*). In addition, depending on the line and cultivation year, the average hyoscyamine yield of the selected root lines varied. In the first three years, DT produced more hyoscyamine, reaching a

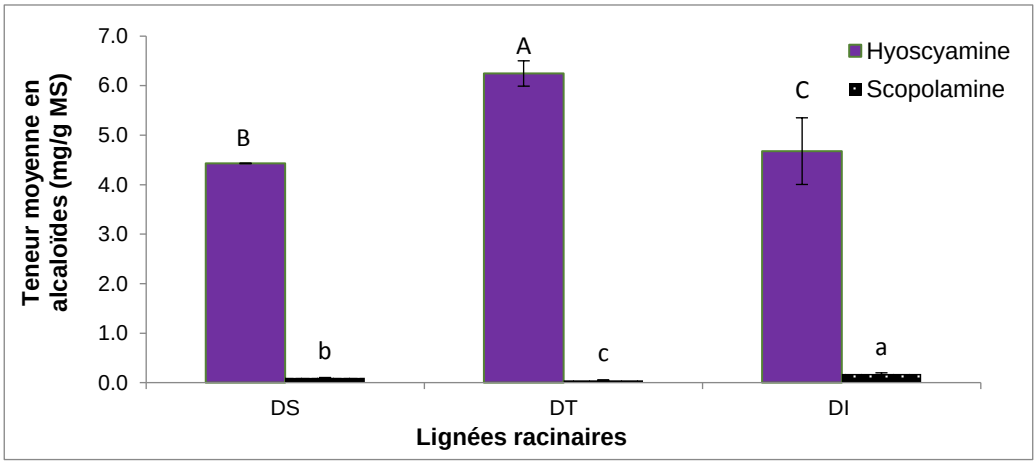
peak of 8.575 mg g^{-1} DW in the first year. This outcome was comparable to the 8.159 mg g^{-1} DW produced by DS. DI had the lowest average hyoscyamine yield (3.153 mg g^{-1} DW) in its first year. Hyoscyamine synthesis from both the DS and DT root lines decreased in the second year, with losses of 36 and 4%, respectively. However, hyoscyamine synthesis increased in DI, with the hyoscyamine yield increasing from 3.153 mg g^{-1} DW.

In all root lines, hyoscyamine synthesis continued to decrease during the third year of root cultivation, with losses of 11% in DS, 12% in DT and 12% in DI. Despite decreasing from the previous year, the average hyoscyamine yield in DI was still 92% greater than that in the first production year. The average hyoscyamine yield decreased by 31% in DS, 102% in DT and 30% in DI after four years of culture. In the fifth year of production, the hyoscyamine yield in DT stabilised. However, the yield decreased much more in DS and DI, dropping to 1.137 mg g^{-1} DW in DS and 3.594 mg g^{-1} DW in DI, representing losses of 64 and 14%, respectively (*Figure 5*).

Effect of silver nitrates on hairy root biomass

Treatment of root lines with the chemical elicitor $AgNO_3$ inhibited the growth of DT and DS. DS produced the same results for the two elicitation times at 24 and 48 h with 0.015 g DW and a biomass loss of 57% compared to the control (*Figure 6*). The mean biomass loss of DT was 4% lower for the 24-h elicitation interval and 12% lower for the 48-h interval. DI responded well to the same elicitor for 24 and 48 h. The biomass increased, showing the highest average biomass of 0.174 g for DW after 48 h of interaction, representing 74% improvement over the control.

Furthermore, the average biomass of DI improved by 25% after 24 h of silver nitrate treatment. ANOVA demonstrated that silver nitrate had a significant effect on the average biomass of the transgenic root lines.



Different lowercase letters above the bars indicate significant differences between the means according to Tukey's test ($p \leq 0.05$)

Figure 3 – Hyoscyamine and scopolamine production in transgenic hairy roots of *Datura* induced by *Agrobacterium rhizogenes*

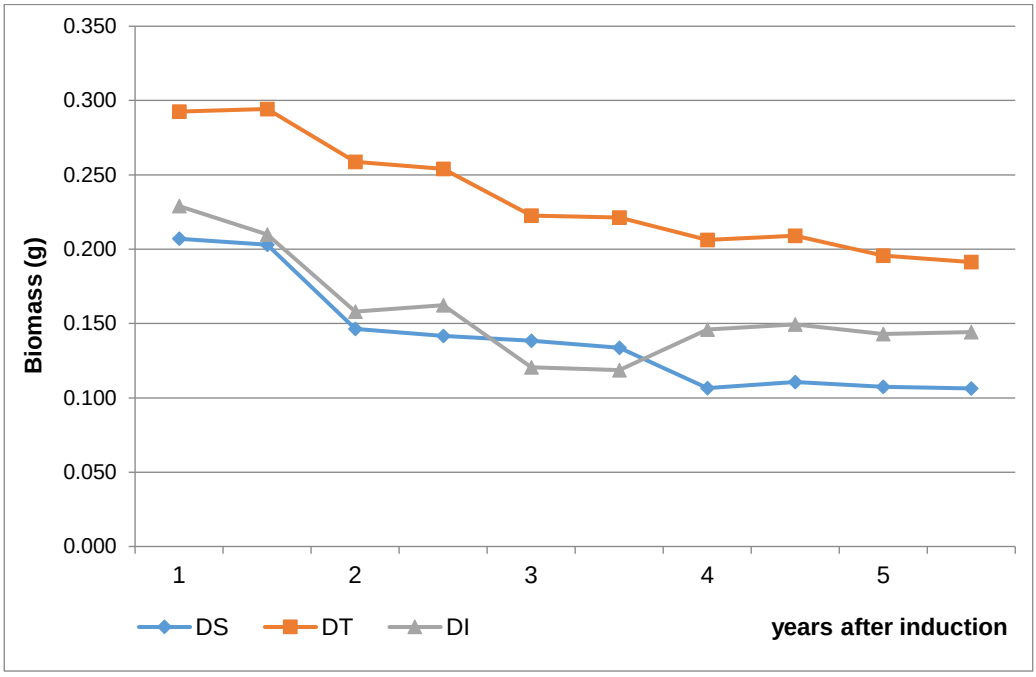


Figure 4 – Evolution of hairy root biomass induced by *Agrobacterium rhizogenes* strain A4

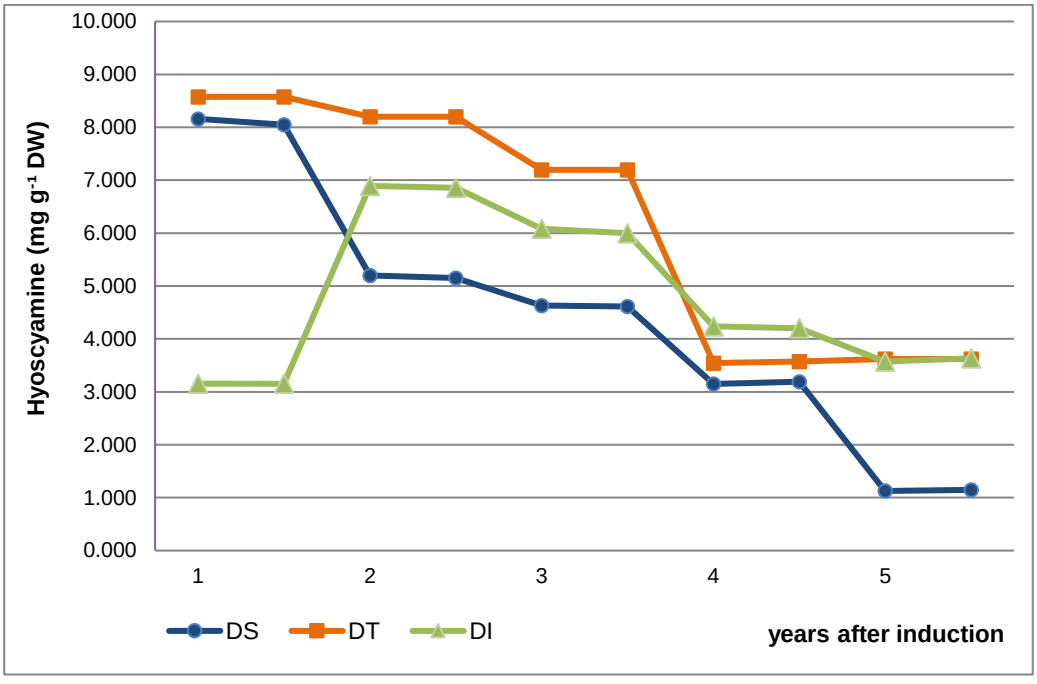
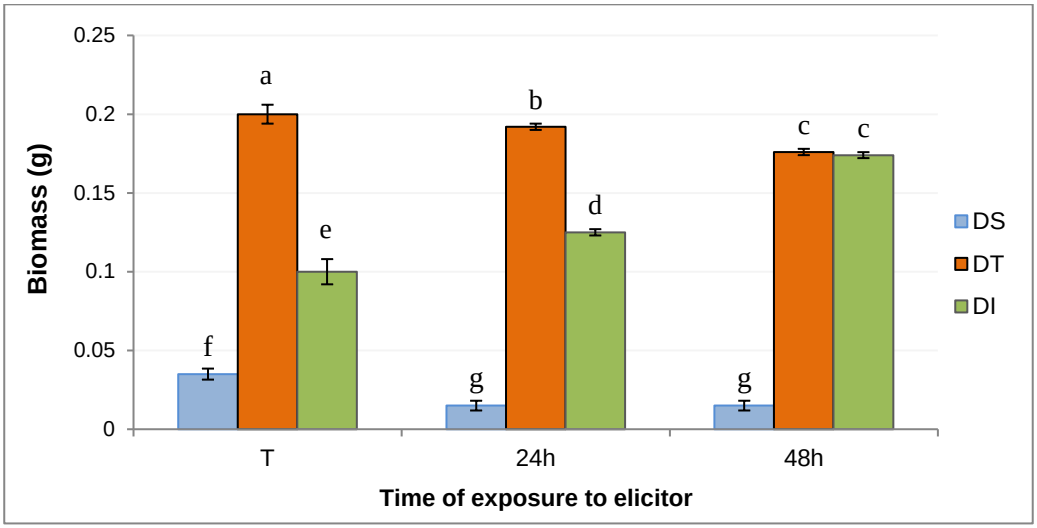


Figure 5 – Evolution of hyoscyamine production in the hairy roots of DS, DT and DI induced by *Agrobacterium rhizogenes* strain A4



T represents the control. Different lowercase letters above the bars indicate significant differences between the means according to Tukey's test ($p \leq 0.05$)

Figure 6 – Biomass of DS, DI and DT hairy roots after 24 and 48 h of elicitation with $AgNO_3$

Effect of silver nitrate on hyoscyamine biosynthesis

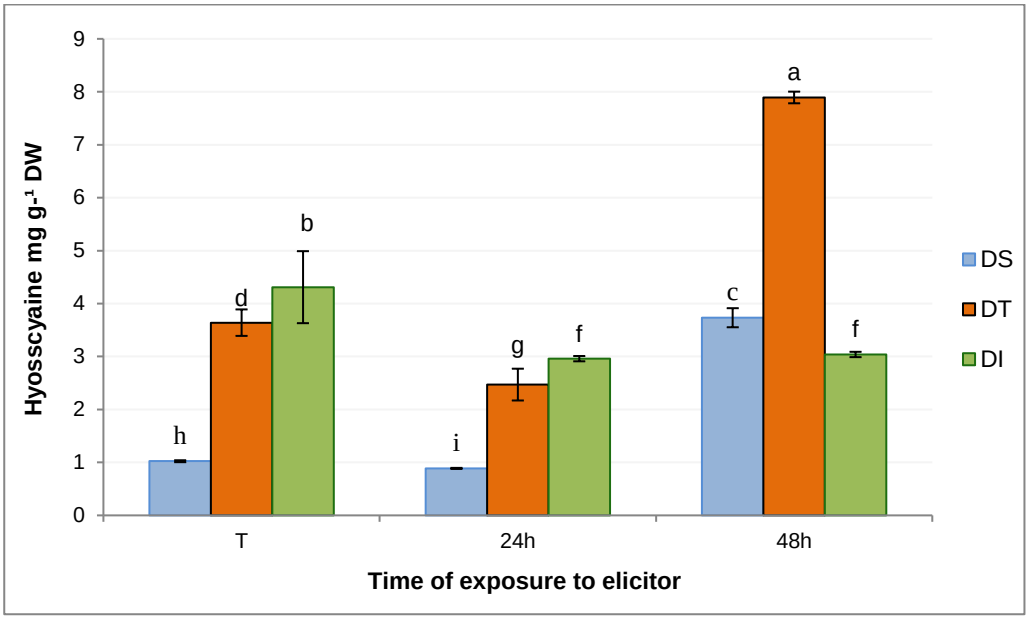
The mean hyoscyamine yield of the root lines elicited by $AgNO_3$ at the two contact times, 24 and 48 h, ranged from 0.887 to 7.89 mg g^{-1} DW. For all DS and DT line treatments, a 48-h contact period produced the best results. DT produced the highest results, reaching 7.89 mg g^{-1} DW, an increase of 117% compared to the non-elicited control (3.64 mg g^{-1} DW). The average hyoscyamine yield in the three root lines did not improve with 24 h of elicitation, with losses of 13, 32 and 31% compared to those in the DS, DT and DI controls, respectively (*Figure 7*).

However, we observed increases of 196 and 117% for DS and DT, respectively, when contact with the elicitor was longer (48 h).

Effect of silver nitrate on scopolamine biosynthesis

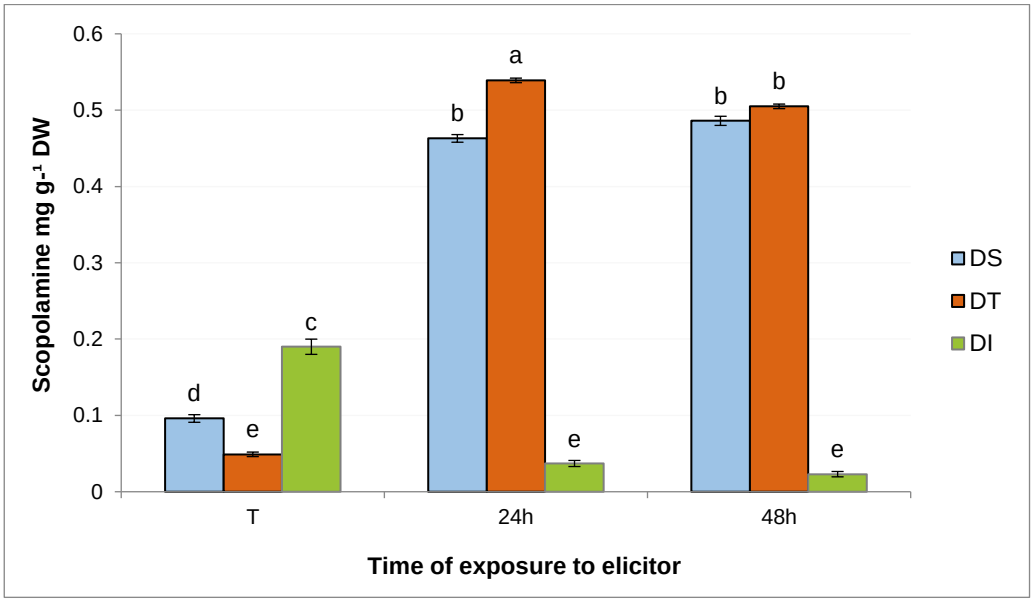
Elicitation with 1 mM $AgNO_3$ enhanced scopolamine synthesis in DS and DT, regardless of the contact time (24 or 48 h). In fact, the increase in DT reached up to 1000% compared to the control. The best performance of all treatments was achieved with an average scopolamine yield of 0.539mg g^{-1} DW after 24 h of contact. This outcome was comparable to that achieved with a 48-h contact time. The best elicitation for DS line was found 48 h after contact, when the average scopolamine yield reached 0.463 mg g^{-1} DW, representing a 406% improvement over the control.

Furthermore, with a contact time of 24 h, the improvement was 382%. In the DI line, silver nitrate treatment decreased the average scopolamine yield compared to that in the control group. The scopolamine yield was similar to that of the control at both elicitation times. After 24 and 48 h, reductions of 81 and 88%, respectively, were achieved (figure 8). ANOVA demonstrated that silver nitrate had a significant effect on the average scopolamine yield in the transgenic root lines.



T represents the control. Different lowercase letters above the bars indicate significant differences between the means according to Tukey's test ($p \leq 0.05$)

Figure 7 – Hyoscyamine yield of DI and DT hairy roots after 24 and 48 h of elicitation using $AgNO_3$



T represents the control. Different lowercase letters above the bars indicate significant differences between the means according to Tukey's test ($p \leq 0.05$)

Figure 8 – Scopolamine extracts of DI and DT hairy roots after 24 and 48 h of elicitation using $AgNO_3$

DISCUSSION

Culture of transgenic roots for the long-term production of tropane alkaloids

Hairy root culture is considered as biotechnological method for producing natural products designated for the pharmaceutical industry (Lanoue *et al.*, 2004).

Transgenic hairy root culture is often used to engineer highly hyoscyamine, and scopolamine which are naturally synthesised in plant roots but in small concentrations (Mishra and Ranjan, 2008).

The genetic and metabolic stability of this type of tissue and abundant secondary metabolite production make it commercially profitable. Secondary metabolites have a high economic value (Ono and Li, 2011; Oksman-Caldentey, 2007).

According to Primorse (2003), root hairs generated by *A. rhizogenes* can be maintained in culture with the potential to create secondary metabolites at a higher level than cell suspension cultures and entire plant cultures.

Furthermore, this type of culture is regarded as a crucial factor when combined with different biomass production types and the build-up of tropane alkaloids.

In our study, even five years after their induction, hairy roots of DS, DT and DI still produce an important amount of hyoscyamine and scopolamine, this includes liquid culture of the plant tissues without considering the elicitation using a chemical molecule or biotic organisms. This makes them a model for production of such molecules for long term production of tropane alkaloids.

Effect of $AGNO_3$ on root biomass

In a previous study with the same lines, Moussous *et al.* (2018) demonstrated that the elicitation of hairy roots using bacterial strains had the ability to improve hyoscyamine yield to 16.63 mg g^{-1} DW produced by DT when exposed to the biotic elicitor Pseudomonas ssp. In the same axis of research and to compare the effect of chemical elicitation using $AGNO_3$ in different exposure time with hairy roots on biomass and tropane alkaloid content considering that $AGNO_3$ is an important source of nitrogen. Indeed, nitrogen is a crucial element that plays an important role in plant growth and development. The ammonium-to-nitrate ratio (NO_3^-/NH_4^+) of the medium influences the productivity of hairy root culture (Shinde *et al.*, 2010). A high biomass of *Anisodus acutangulus* hairy root cultures was generated using 90 mM nitrogen at a 4:1 ratio of NH_4^+/NO_3^- (Liu *et al.*, 2013). However, Sharafi *et al.* (2013) suggested that the same ratio of 20:10 mM optimised biomass accumulation in liquid MS media of hairy root cultures. $AGNO_3$ is used as a growth regulator to prevent plant morphological alterations (Pitta-Alvarez *et al.*, 2000**)**. In our study, $AgNO_3$ stimulated the root growth of DI plants but inhibited the root growth of DS and DT, indicating that genetic factors in *Datura* species are involved in cellular reactions to elicitors.

Effect of $AGNO_3$ on alkaloids

Plant tissues react to chemical elicitors in different ways. Many elicitors modify the metabolic pathways of some bioactive molecules, increasing cell production. The time that plant tissues are exposed to the elicitor significantly

influences their reaction. Amdoun *et al.* (2010) demonstrated that 0.06 mM of jasmonic acid optimised the hyoscyamine yield of DS after 24 h of elicitation when the culture conditions of NO_3^- and Ca^{2+} were optimised for a 28-d culture period on B5 medium. In the present study, the use of 1 mM $AgNO_3$ for 24 h of elicitation decreased hyoscyamine and scopolamine production. This decrease is attributed to the liberation of alkaloids in the medium due to osmotic stress induced by Ag^+ ions (Pitta-Alvarez *et al.*, 2000). Shakeran *et al*. (2015) suggested that the decrease in hyoscyamine is due to cell lysis by Ag^+, which is known to be toxic to plant cells. However, the increase in alkaloid production after treatment with $AgNO_3$ is due to the inhibition of ethylene biosynthesis caused by Ag^+ cations, which results in phytoalexins production as a reaction to toxicity, eliciting the overproduction of tropane alkaloids hyoscyamine and scopolamine in hairy root cultures of *Brugmansia candida* (Angelova *et al.*, 2006). In addition, Ag^+ is involved in the overexpression of the putrescin methyltransferase gene, which regulates the last step of the tropane alkaloid biosynthesis pathway (Kai *et al.*, 2012). In our study, $AgNO_3$ increased the hyoscyamine yield in DS by up to 117 and 1000% compared to the non-elicited control group.

CONCLUSIONS

Study of the biomass and hyoscyamine synthesis in three *Datura* transgenic hairy root lines indicated that these plant tissues grew quickly and produced a high amount of hyoscyamines compared to normal roots. However, a decrease in growth was observed after the first year of culture. Silver nitrate elicitation at two different exposure times (24 and 48 h) showed that this chemical treatment did not optimise the biomass of DS or DT. However, it increased DI growth after 48 h of treatment. The average hyoscyamine yield of DS and DT was optimised after 48 h of elicitation with silver nitrate. However, the scopolamine yield increased in both lines compared to that in the control after 24 and 48 h of silver nitrate elicitation. This study initiated the possibility of bioreactor culture for hairy root culture of tropane alkaloids, suggesting the use of chemical elicitation for optimisation without inducing new lines each time. Furthermore, based on the targeted molecule, we described the elicitor. $AgNO_3$ was adequate for enhancing the scopolamine yield in DI hairy roots.

Author Contributions: Conceptualisation: KL, MA; Methodology: MA; Software: MW; Formal analysis: MW; Investigation: MW; Resources, Data curation: MW; Writing: MA; Original draft: MA; Writing - Review and editing: MA, MW; Visualisation, Supervision: KL, MA; Project administration: MA; Funding acquisition: MA; English improvement: MA, KL. All authors declare that they have read and approved the publication of the manuscript in this present form.

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Data availability statement: The data presented in this study are available upon request from the corresponding authors.

Conflicts of interest: The authors declare no conflicts of interest.

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