Effects of AgNO3 on Alkaloids and Key Enzyme Gene Expression in Atropa belladonna Hairy Roots (Postprint)

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Abstract

This study investigated the effects of different concentrations of AgNO3 on tropane alkaloids and the expression of key enzyme genes involved in their synthesis during the growth of Atropa belladonna hairy roots. It is well established that the accumulation of plant secondary metabolites is directly related to certain primary metabolites or the activity of key enzymes in secondary metabolism. As a heavy metal, AgNO3 can induce oxidative stress in plants, thereby exerting significant effects on plant growth and key metabolic enzymes. Therefore, this study treated Atropa belladonna hairy roots cultured in darkness for 12 days with five different concentrations of AgNO3, and after 2 days, collected the hairy roots to determine their fresh and dry weights, tropane alkaloid content, selected physiological indicators (MDA, Pro, soluble sugars, soluble proteins), and the expression levels of key enzyme genes (pmt, trI, trII). The results showed that although AgNO3 inhibited the growth of Atropa belladonna hairy roots, it promoted the accumulation of tropane alkaloids. Compared with the control, treatments with 50, 100, and 150 μmol · L-1 significantly increased the yield of tropane alkaloids. Meanwhile, under the 150 μmol · L-1 treatment, MDA content in the hairy root metabolic pathway showed a significant increase compared with the control. Proline content also showed a significant increase under 150 μmol · L-1, reaching 5.92 g · g-1 FM, which was 2.91 times that of the control. The contents of soluble sugars and soluble proteins under treatment at 150 μmol · L-1 concentration were 1.55 and 1.67 times that of the control, respectively. The expression levels of key enzyme genes (pmt, trI, trII) involved in tropane alkaloid synthesis were enhanced to varying degrees under different concentrations of AgNO3 treatment. Therefore, it can be inferred that under 150 μmol · L-1 treatment, AgNO3 may affect the synthesis of tropane alkaloids in Atropa belladonna hairy roots by regulating the expression of these primary metabolites such as proline, soluble proteins, and soluble sugars, or certain key enzyme genes. This study provides a theoretical basis for future research on

the synthesis mechanism of tropane alkaloids in Atropa belladonna, and also offers a reference method for increasing the medicinal components in Atropa belladonna hairy roots in production.

Full Text

Effects of AgNO on Alkaloids and Key Enzyme Gene Expression in Hairy Roots of *Atropa belladonna* L.

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Abstract

This study investigated the effects of different concentrations of silver nitrate (AgNO) on tropane alkaloid accumulation and the expression of key biosynthetic enzyme genes in hairy roots of *Atropa belladonna* during culture. The accumulation of plant secondary metabolites is directly related to the availability of primary metabolites and the activity of key enzymes in metabolic pathways. As a heavy metal, AgNO can induce oxidative stress in plants, thereby significantly affecting plant growth and key metabolic enzymes. To explore this relationship, five concentrations of AgNO were applied to 12-day-old *A. belladonna* hairy roots cultured in darkness. After two days of treatment, the hairy roots were harvested and analyzed for fresh and dry weight, tropane alkaloid content, selected physiological indices (malondialdehyde, proline, soluble sugars, and soluble protein), and the expression levels of key enzyme genes (*pmt*, *trI*, *trII*).

The results demonstrated that although AgNO inhibited hairy root growth, it promoted tropane alkaloid accumulation. Compared with the control, treatments of 50, 100, and 150 μmol·L ^{1} AgNO significantly increased total tropane alkaloid yield. Under the 150 μmol·L ^{1} treatment, hairy roots showed significant increases in MDA content and proline accumulation, with proline reaching 5.92 g·g ^{1} fresh mass—2.91 times that of the control. Soluble sugar and soluble protein contents at 150 μmol·L ^{1} were 1.55- and 1.67-fold higher than the control, respectively. Furthermore, the expression of key enzyme genes (*pmt*, *trI*, *trII*) in the tropane alkaloid biosynthetic pathway was upregulated to varying degrees across all AgNO treatments. These findings suggest that treatment with 150 μmol·L ^{1} AgNO may influence tropane alkaloid synthesis in *A. belladonna* hairy roots by regulating primary metabolites such as proline, soluble protein, and soluble sugars, and/or by modulating the expression of specific key enzyme genes. This study provides a theoretical foundation for future investigations into the synthesis mechanisms of tropane alkaloids in *A. belladonna* and offers

a practical approach for enhancing the production of medicinal compounds in hairy root cultures.

Keywords: *Atropa belladonna* L.; silver nitrate; tropane alkaloids; hairy roots; gene expression

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Introduction

Atropa belladonna L., commonly known as deadly nightshade or belladonna, is a perennial herbaceous plant in the Solanaceae family and an important medicinal plant documented in the Chinese Pharmacopoeia (2015). The entire plant is used medicinally, with leaves and roots containing hyoscyamine and scopolamine–compounds widely used in clinical preparations for anesthesia, analgesia, and anti-motion sickness applications (Xiao, 2002). Growing evidence indicates that medicinal plants contain numerous bioactive compounds essential for human health and development (Verpoorte et al., 2002). However, increasing market demand, coupled with unsustainable harvesting practices and environmental degradation, has led to declining concentrations of active compounds in medicinal plants, creating a major bottleneck for their entry into international markets (Namdeo et al., 2007).

In recent years, hairy root culture technology has been extensively and effectively applied in the production of plant secondary metabolites, cultivar improvement, and specialized plant cultivation (Georgiev et al., 2007). Additionally, the addition of elicitors during plant growth represents an effective strategy for enhancing secondary metabolite content in various plant species. Elicitors can trigger the synthesis of specific secondary metabolites, making them valuable tools for improving production in hairy root cultures (Savitha et al., 2008).

Silver nitrate is a heavy metal that induces oxidative stress in plants, significantly impacting growth and metabolic processes (Koehler, 2007). Generally, the accumulation of plant secondary metabolites is directly or indirectly associated with the activity of key enzymes in metabolic pathways. For instance, Huang et al. (2013) reported that lead nitrate could promote the growth of

Sorbus aucuparia suspension cells while stimulating secondary metabolite accumulation. Previous research on *A. belladonna* has primarily focused on cloning key enzyme genes in metabolic pathways (Qiang, 2012) and enhancing tropane alkaloid content through overexpression of these genes (Li et al., 2013). However, such genetic approaches are costly and unsuitable for large-scale production, and gene overexpression may cause chimerism and genetic instability. To date, no studies have investigated the effects of elicitor addition on alkaloid content and key enzyme gene expression in *A. belladonna* hairy roots.

This study employed *A. belladonna* hairy roots as experimental material to investigate how the elicitor AgNO affects growth and the expression of key metabolic enzyme genes. The objective was to provide a reference for improving the production of medicinal compounds in *A. belladonna* and to establish a theoretical foundation for understanding the regulatory mechanisms of tropane alkaloid synthesis. The findings will support the scale-up cultivation of *A. belladonna* hairy roots and advance research into the molecular regulation of tropane alkaloid biosynthesis.

Materials and Methods

1.2.1 AgNO Preparation

Silver nitrate was dissolved in distilled water and diluted to a final concentration of 100 μmol · L ^{1} as a stock solution. The solution was sterilized by filtration through a 0.22 μm membrane filter and stored for later use.

1.2.2 Elicitor Addition and Culture Conditions

Fresh *A. belladonna* hairy roots (0.5 g) were inoculated into 250 mL Erlenmeyer flasks containing 150 mL of B5 liquid medium. The cultures were maintained on a rotary shaker at 110 rpm at (25±1)°C in darkness for 12 days. After this period, the original medium was removed and replaced with fresh B5 liquid medium supplemented with AgNO at final concentrations of 25, 50, 100, 150, or 200 μmol · L ^{1}. The hairy roots were cultured for an additional 2 days under the same conditions.

1.2.3 Determination of Fresh and Dry Weight

After treatment, hairy roots were collected and rinsed with distilled water to remove residual culture medium. Surface water was absorbed with filter paper before weighing to obtain fresh weight. For dry weight determination, the fresh hairy roots were dried in an oven at 60°C to constant weight before weighing.

1.2.4 Extraction of Tropane Alkaloids from Hairy Roots

Tropane alkaloids were extracted using a modified method from ZÁRATE et al. (1997). Hairy roots were dried at 60°C to constant weight and ground into a fine powder, which was passed through a 50-mesh sieve. A 0.1000 g sample was mixed with 10 mL of extraction solution (chloroform:methanol:ammonium hydroxide, 15:5:1) and sonicated for 30 minutes, then left overnight at room temperature. The extract was filtered, and the residue was washed with 2 mL of chloroform. All filtrates were combined and concentrated to dryness under vacuum at 40°C. The residue was dissolved in 5 mL of chloroform and 2 mL of 1 mol · L ^{1} sulfuric acid. After phase separation, the sulfuric acid phase was collected and its pH adjusted to 10 with concentrated ammonia water. The alkaloids were extracted twice with 4 mL of chloroform each time. The lower phase was collected, concentrated under vacuum at 40°C, and the final residue was dissolved in 1 mL of methanol as the sample solution. The solution was filtered through a 0.22 μm membrane and stored at -4°C for subsequent analysis.

1.2.5 Chromatographic Conditions

Chromatographic separation was performed on an Ultimate XB-C18 column (5 μm, 4.6×250 mm). The mobile phase consisted of methanol:ammonium acetate (pH 4.6) at a ratio of 58:42. The flow rate was 1.0 mL · min ^{1}, detection wavelength was 215 nm, column temperature was 40°C, and injection volume was 10 μL.

1.2.6 Determination of Physiological Indices

Proline and soluble protein contents were determined according to the methods described by Gao (2006). Malondialdehyde (MDA) content was measured using the thiobarbituric acid (TBA) colorimetric method (Zhao et al., 2006), and soluble sugar content was determined by the anthrone colorimetric method (Li, 1994).

1.2.7 Quantification of Key Enzyme Gene Expression

Total RNA was extracted using the Biospin Plant Total RNA Extraction Kit. cDNA was synthesized from the extracted RNA using the Promega GoScript Reverse Transcription Kit. Using the synthesized cDNA as template and *pgk* as the reference gene, quantitative real-time PCR was performed with the Promega GoTaq® qPCR Master Mix. After amplification, melting curves, amplification curves, and standard curves were analyzed to determine the expression levels of target genes. The primer sequences used for PCR are listed below:

Primer	Sequence (5’ -3’)
F-qpgk	TCGCTCTTGGAGAAGGTTGAC
R-qpgk	CTTGTCCGCAATCACTACATCAG

Primer	Sequence (5’ -3’)
F-Abpmt	CCTACTTACCCTACTGGTGTTATC
R-Abpmt	GCGAAAGATGGCAAAATAAAAGC
F-Abh6h	TTCCACTTGAGCAGAAAGCAAAGC
R-Abh6h	CCTCATGGTCAACTTCCTCACTTCC
F-AbtrI	TTCTTTGCTTCCCTGCTGCTTC
R-AbtrI	GAGGCCAACCTTAGTATCACACAG

1.3 Data Analysis

Experimental data were processed and analyzed using Microsoft Excel 2007 and SPSS 22.0 software for statistical analysis and one-way ANOVA. Graphs were prepared using Origin 8 software. All data are presented as mean ± standard deviation (x ± s).

Results

2.1 Effects of AgNO on Hairy Root Growth and Tropane Alkaloid Content

The effects of AgNO on hairy root growth and alkaloid accumulation are summarized in Table 1 . Silver nitrate significantly inhibited the growth of *A. belladonna* hairy roots. Fresh weight decreased progressively with increasing AgNO concentration, with significant inhibition observed at concentrations above 100 μmol · L ^{1} compared to the control. Dry weight was also significantly suppressed across all treatment groups, with the most pronounced inhibition occurring at the highest concentration (200 μmol · L ^{1}), where dry weight was only 73.64% of the control value.

Despite the growth inhibition, AgNO treatment enhanced tropane alkaloid content. Scopolamine content showed significant increases at 50, 100, and 150 μmol · L ^{1}, reaching 2.5 times the control level at 100 μmol · L ^{1}. Hyoscyamine content was significantly elevated by all AgNO treatments. Consequently, the total tropane alkaloid yield was significantly improved in the 50, 100, and 150 μmol · L ^{1} treatment groups compared to the control.

2.2 Effects of AgNO on Physiological Indices During Hairy Root Growth

The effects of AgNO on selected physiological indices are illustrated in Figure 1 [Figure 1: see original paper]. With the exception of the 25 μmol · L ^{1} treatment, all AgNO concentrations significantly increased MDA content, with the 150 μmol · L ^{1} treatment showing the most pronounced effect (1.24 times the control). Proline content was significantly elevated by all AgNO treatments, reaching

5.92 g · g ¹ fresh mass at 150 µmol · L ¹—2.91 times higher than the control and significantly different from the 25, 50, and 200 µmol · L ¹ treatments.

Both soluble sugar and soluble protein contents were significantly increased compared to the control. Soluble sugar content at 150 and 200 µmol · L ¹ was 1.55 and 1.52 times the control value, respectively, and significantly different from the 25 and 50 µmol · L ¹ treatments. Soluble protein content showed notable increases at 50 and 150 µmol · L ¹, reaching 10.228 and 9.949 mg · g ¹ fresh mass, respectively—representing 20.08% and 16.78% increases over the control. These two treatment groups were significantly different from the 25 and 50 µmol · L ¹ treatments.

2.3 Effects of AgNO on Expression of Key Genes in Tropane Alkaloid Biosynthesis

The effects of AgNO on the expression of key biosynthetic genes are presented in Figure 2 [Figure 2: see original paper]. All five AgNO concentrations significantly stimulated *pmt* gene expression, with the 50 and 100 µmol · L ¹ treatments inducing 3.45- and 3.32-fold increases over the control, respectively—significantly higher than the 25 and 200 µmol · L ¹ treatments. Similarly, *trI* gene expression was significantly upregulated by all concentrations except 200 µmol · L ¹, with the 50 and 100 µmol · L ¹ treatments showing the strongest induction and differing significantly from the other three groups.

For the *h6h* gene, significant expression increases were observed at all concentrations except 200 µmol · L ¹. The 100 µmol · L ¹ treatment produced the most potent induction effect, increasing *h6h* expression by 1.16-fold compared to the control.

Discussion

Silver nitrate, as an abiotic elicitor, induces oxidative stress in plants, thereby affecting growth and metabolic processes (Koehler et al., 2007). Consequently, its application has become increasingly common for enhancing secondary metabolite production in recent years. The present study found that various concentrations of AgNO inhibited *A. belladonna* hairy root growth, likely by interfering with essential biological processes such as nutrient uptake, respiration, and photosynthesis (Clemens et al., 2006). Despite this growth inhibition, scopolamine content increased significantly at 50, 100, and 150 µmol · L ¹ AgNO . This enhancement may be attributed to two complementary mechanisms: first, the stress response triggered by AgNO leads to the production of soluble sugars and proteins that protect against stress, and excess primary metabolites may be shunted into secondary metabolic pathways; second, AgNO may directly induce the expression of key enzyme genes in the tropane alkaloid biosynthetic pathway, similar to the observation by Xiao et al. (2010) that Ag treatment of *Salvia miltiorrhiza* hairy roots induced peak PAL transcription at day 6.

During elicitor-plant interactions, alterations in cell structure and membrane oxidation occur, while plants synthesize small molecules to enhance osmotic adjustment capacity. Malondialdehyde serves as an indicator of membrane oxidation and reflects structural damage induced by elicitor stress. Proline, soluble sugars, and soluble protein function as effective osmotic regulators and nutrients; their accumulation significantly improves cellular osmotic adjustment, enabling cells to maintain normal function under stress conditions. Sun (2014) reported that 200 μmol · L ^{1} methyl jasmonate treatment of *Datura stramonium* hairy roots significantly increased proline content compared to controls. The present study similarly demonstrated that AgNO addition elevated MDA, proline, soluble sugar, and soluble protein contents in hairy roots. The osmotic capacity of soluble sugars and proteins prevents excessive cellular dehydration, maintains water balance, and mitigates the negative effects of elicitors on growth (Zhang, 2014). Additionally, increased soluble protein content may reflect the synthesis of defense proteins as the plant' s defense system becomes activated. These factors collectively enhance the regulatory capacity of *A. belladonna* hairy root cells and promote secondary metabolite accumulation.

Elicitors can be recognized by receptors on the plant cell membrane, triggering a cascade of intracellular reactions that either synthesize enzymes related to secondary metabolism or alter their activities, thereby modifying gene expression and leading to secondary metabolite synthesis and accumulation. Elicitor-plant interactions can selectively, rapidly, and specifically induce the expression of particular genes, resulting in the accumulation of specific secondary metabolites (Zhai, 2011). Peter et al. (2006) demonstrated that fungal elicitor addition to poppy suspension cultures significantly induced transcription of seven out of eight alkaloid biosynthesis genes. In the current study, AgNO treatment at 25–150 μmol · L ^{1} significantly stimulated expression of the key tropane alkaloid biosynthetic genes *pmt*, *trI*, and *h6h*, indicating that upregulation of these genes effectively enhances tropane alkaloid content in *A. belladonna* hairy roots.

In summary, this study demonstrates that while AgNO treatment inhibits *A. belladonna* hairy root growth, it significantly increases tropane alkaloid yield at concentrations of 50, 100, and 150 μmol · L ^{1}. These concentrations also significantly elevated MDA, proline, soluble protein, and soluble sugar contents, while upregulating the expression of key enzyme genes in the tropane alkaloid biosynthetic pathway. We therefore hypothesize that AgNO stress influences primary metabolism through changes in these physiological indices, thereby affecting alkaloid accumulation, while simultaneously modulating secondary metabolism via effects on key enzyme gene expression. However, whether the observed physiological changes directly influence enzyme expression remains to be elucidated in future studies.

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Note: Figure translations are in progress. See original paper for figures.