

Improvement of atropine production by different biotic and abiotic elicitors in hairy root cultures of *Datura metel*

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Abstract: Hairy roots induced by *Agrobacterium rhizogenes* are distinguished by their high growth rate, genetic stability, and lateral branching. *Datura metel* is an important medicinal plant whose hairy roots are used for the production of atropine, a tropane alkaloid applied as an anticholinergic and parasympathetic agent. To increase the yield of atropine, biotic (*Bacillus cereus* and *Staphylococcus aureus*) and abiotic (AgNO_3 and nanosilver) elicitors were added to the hairy root cultures of *D. metel* and then analyzed through high-performance liquid chromatography. All the elicitors influenced biomass accumulation and atropine production. Among the tested elicitors, nanosilver was most effective in enhancing the hairy roots' atropine content. To the authors' knowledge, the application of nanosilver for increasing tropane alkaloid production is reported for the first time here and could improve the atropine production of pharmaceutical industries.

Key words: Hairy root, atropine, *Datura metel*, AgNO_3 , nanosilver, *Bacillus cereus*, *Staphylococcus aureus*

1. Introduction

Plant-derived drugs are important sources of various pharmaceuticals (Kim et al., 2002). Tropane alkaloids such as (-)-hyoscyamine, atropine, and scopolamine (hyoscyne) are the most well-known alkaloids, extracted from various species of Solanaceae. These compounds are widely used as anticholinergic and antispasmodic agents that affect the parasympathetic nervous system (Croteau et al., 2000; Pitta-Alvarez et al., 2000; Wu et al., 2002; Palazón et al., 2008). These alkaloids are analgesic, sedative, and narcotic and are used in asthma, Parkinson disease, and motion sickness treatment (Ajungla et al., 2009). Some alternative studies, such as chemical synthesis, normal hybridization, and plant cell and organ cultures, have been conducted for the production of these alkaloids and have found the hairy root system to be an advantageous method (Hashimoto and Yamada, 1987). *Agrobacterium rhizogenes*, which carries the Ri T-DNA plasmid, can transform plant roots to hairy roots. These roots are preferred to parent plants for their genetic and biochemical stability, high growth rate, hormone autotrophy, lateral branching, relatively low-cost culture requirements, and multienzyme biosynthetic potential (Giri and Narasu 2000; Banerjee et al., 2012).

The use of biotic and abiotic elicitors is one of the most acceptable strategies for increasing the productivity of hairy roots. Elicitors can stimulate the accumulation of phytoalexins and different types of defense responses in plants (Ebel and Scheel, 1997). Moreover, elicitors improve the release of metabolites in the medium. Examples of biotic elicitors are yeast extract, bacteria, fungi, and viruses, and examples of abiotic elicitors are inorganic components and metal ions (Pitta-Alvarez et al., 2000; Zhao et al., 2005). Elicitors are effective for the growth rate of hairy roots and the synthesis of secondary metabolites in various species of plants. For example, AgNO_3 and KH_2PO_4 are effective for the biomass growth of *Sesamum indicum* (Chun et al., 2007), and bacterial elicitors enhance the production of scopolamine in *Scopolia parviflora* (Jung et al., 2003). *Pythium aphanidermatum* and *Phytophthora parasitica* increase the coumarin in witloof chicory (Bais et al., 2000), Ca^{2+} and NO_3^- enhance the hyoscyamine in *Datura stramonium* (Amdoun et al., 2007), and fungal hyphae extract and methyl jasmonate improve the tanshinones in *Salvia miltiorrhiza* hairy roots (Zhang et al., 2011). The objective of this study is to assess the effect of biotic (*Staphylococcus aureus* and *Bacillus cereus*) and abiotic (AgNO_3 and nanosilver) elicitors on the hairy roots'

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biomass and atropine production in hairy root cultures of *Datura metel*.

2. Materials and methods

2.1. Plant transformation and hairy roots cultivation

Transformed (hairy) root cultures were acquired by infecting healthy young leaf explants of *D. metel* with *A. rhizogenes* A4 (Qing et al., 2000; Zhou et al., 2007; Eskandari-Samet et al., 2012; Pirian et al., 2012). Genomic DNA of the hairy roots was extracted using the CTAB method (Doyle et al., 1987), and genomic DNA of the plant's untransformed roots was used as negative control. The Sambrook method was adopted for plasmid DNA extraction from *A. rhizogenes* (Sambrook et al., 1989), while the extraction was used as a positive control. The presence of T-DNA in the supposedly transformed lines was displayed by polymerase chain reaction (PCR). This process was performed by PCR primers for the amplification of the *rolB* gene. The sequences of these primers were 5'-ATGGATCCCAAATTGCTATCCCCACGA-3' and 5'-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3'. The optimized PCR conditions consisted of 2 ng of plant DNA (with a 2- μ L volume), 2 μ L of 10X Taq buffer, 2 μ L of 50 mM MgCl₂, 0.4 μ L of 10 mM dNTPs, 1 μ L of 1 mM from each primer, 0.5 μ L of 5 U/ μ L Taq DNA polymerase, and 11.1 μ L of sterile ddH₂O. The total volume was 20 μ L. Thirty-five thermal cycles were performed by PCR, where each cycle included denaturation at 94 °C (1 min), primer annealing at 58 °C (1 min), and primer extension at 72 °C (1 min) (Rahnama et al., 2008). For root cultivation, 5 cm of each transformed root was cultivated for 18 days in 100 mL of hormone-free half-strength Murashige and Skoog liquid culture medium in a 200-mL Erlenmeyer flask and incubated on a rotary shaker at 120 rpm and at 27 °C.

2.2. Preparation of elicitors and elicitation

Hairy roots of *D. metel* in the exponential phase (18-day-old cultures) were exposed to the elicitors for 12, 24, and 48 h (Spollansky et al., 2000). Elicitation was obtained by adding biotic (*B. cereus* and *S. aureus*) and abiotic (AgNO₃ and nanosilver) elicitors. *B. cereus* was cultured in nutrient broth liquid medium at 27 °C and 120 rpm for 1 day, and *S. aureus* was cultured in tryptic soy broth liquid medium under the same conditions. The obtained bacterial cultures were then diluted and regulated based on an optical density of 1.0 at 600 nm. From each suspension of bacteria, 13.3 mL was added to 100 mL of 18-day-old hairy root cultures (Jung et al., 2003). The AgNO₃ was dissolved in deionized water to create a solution of a concentration of 0.3 M, which was sterilized through the leaching process. A colloidal 2000-ppm nanosilver solution of 50–60 nm in particle size, NANOCID, was obtained from Nano Nasb Pars Co., Tehran, Iran. The sterilized solutions of AgNO₃ (30 μ L) and nanosilver (1 mL) were added separately to 100

mL of 18-day-old transformed root cultures and put in a shaker at 27 °C and 120 rpm. The effects of each elicitor on biomass and atropine production were measured at 12, 24, and 48 h after elicitation (Kai et al., 2012). All experiments were repeated in triplicate.

2.3. Measurement of fresh and dry weight of hairy roots

After elicitation, the fresh weight of hairy roots was measured after 12, 24, and 48 h culture and then compared with controls. The roots were dried in the dark at room temperature for 48 h before the measurement of their dry weight.

2.4. Extraction and assessment of tropane alkaloids

Dried root samples were extracted separately after 48 h of soaking in 8 mL of ethanol containing H₂SO₄ (2%). The filtered extracts were dried and mixed with 14% ammonia at pH 8.0 in order to release alkaloids. Sodium sulfate was added to remove water, and the alkaloids were reextracted 6 times with ether (2 mL) and washed with 10% KCl solution to salt out the silver ions. The filtrate extracts were dried at room temperature and 2 mL of phosphate buffer was added to each fraction prior to the high-performance liquid chromatography (HPLC) analysis.

The HPLC analysis was conducted in a water system equipped with a 515 HPLC pump, UV-Vis detector (2487 dual absorbance) at 204 nm, and Millennium software applied for the determination of available atropine in the extract. For each sample, 20 μ L was injected into a reversed-phase HPLC column (RP-18, 250 mm \times 4.6 mm, 5- μ m particle size, Shimadzu, Japan) using NaH₂PO₄ (0.78 g/L) and H₃PO₄ (0.2 mL) in water (pH 2.6) as solvent A and acetonitrile as solvent B, with isocratic elution at 40 °C with a ratio of 90% A to 10% B at a flow rate of 2 mL/min. A standard calibration curve ranging from 10 to 500 μ g/mL was prepared for quantitative analysis using different concentrations of atropine (Sigma Aldrich, USA) as standard material (500, 250, 100, 50, 25, and 10 μ g/mL). The correlation between the concentration and the peak area of the standard was measured by adopting the minimum square method (R² value).

2.5. Statistical analysis

Each treatment was examined in triplicate, and variance was analyzed with SPSS 21 using a univariate procedure at $P < 0.05$.

3. Results

The hairy roots emerged after approximately 15–20 days from leaf explants of *D. metel* induced by *A. rhizogenes* A4. The T-DNA (TL- and TR-DNA), the virulence (*vir*) genes, and the chromosomal virulence (*chv*) genes are the 3 groups of gene families necessary for DNA transfer from *A. rhizogenes* to plant cells. The 4 *rol* genes (root loci), *rolA*, *rolB*, *rolC*, and *rolD*, are carried by TL-DNA, which differentiates the Ri plasmid from the Ti plasmid of *A. tumefaciens* (Bettini et al., 2003; Chavarri et al., 2010). The func-

tion of the *rolB* is indoxyl- β -oxidase activity and it plays a role in the secretion of active auxin (Altamura, 2003). The *rolB* gene is essential in hairy root induction, whereas *rolA*, *rolC*, *rolD*, and other open reading frames act in synergy to promote root induction (Welander and Zhu, 2006). The PCR amplification of the *rolB* gene showed that the *rolB* gene appeared in 780 bp on gel electrophoresis. Furthermore, this transformation was confirmed by positive and negative controls (Figure 1).

The results indicated that the highest level of fresh and dry weight (3107.6 and 237.23 mg, respectively) appeared after 24 h of elicitation by nanosilver. Weight was higher in hairy roots than in control samples after 12 h of elicitation. The fresh and dry weight of samples elicited by nanosilver and AgNO_3 was higher than in control samples after 48 h (Figures 2 and 3).

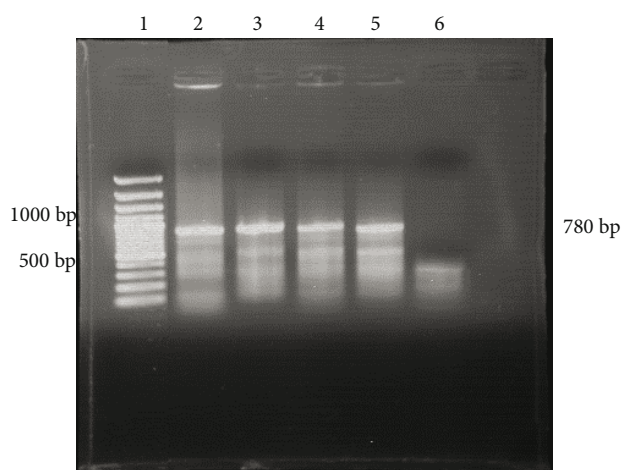


Figure 1. PCR amplification of *rolB* gene (780 bp). Lane 1: DNA marker; lanes 2, 3, and 4: *rolB* gene in hairy root DNA of *D. metel*; lane 5: positive control (plasmid DNA of *A. rhizogenes*); lane 6: negative control from nontransformed roots.

The atropine content of hairy roots was analyzed through HPLC after being treated with biotic and abiotic elicitors for 0, 24, and 48 h, respectively. The level of atropine was calculated as the percentage of the dry weight in transforming the roots of each sample. The standard samples were analyzed in 10, 25, 50, 100, 250, and 500 $\mu\text{g/mL}$ concentrations. The correlation between the concentration and the peak area of the standard was measured through the minimum square method (R^2 value). The equation obtained was $y = 13816x - 7478.9$ (Figure 4).

The level of atropine in hairy root samples treated with AgNO_3 was reduced to 0.032%, 0.042%, and 0.053% in comparison to the level of atropine in the control samples (0.116%, 0.119%, and 0.107%) at 12, 24, and 48 h, respectively. However, the yield of atropine induced with nanosilver was increased to 1.147-, 1.117-, and 2.42-fold in comparison to the control samples after 12, 24, and 48 h of treatment, respectively, where $P < 0.05$ (Figures 5 and 6a–6d).

In the hairy roots treated by *B. cereus*, the level of atropine was significantly reduced after 24 and 48 h (0.017% and 0.037%, respectively) in comparison to the control samples. In addition, after treating the hairy roots with *S. aureus*, the atropine content was significantly reduced (0.095%, 0.038%, and 0.056% after 12, 24, and 48 h, respectively) (Figure 7).

4. Discussion

4.1. Effect of biotic (*B. cereus* and *S. aureus*) elicitors on fresh and dry weight in hairy roots of *D. metel*

This study assessed the effect of elicitors on the biomass of the transformed roots. The fresh and dry weight of hairy roots that were elicited from *B. cereus* and *S. aureus* increased after 12 h (Figures 2 and 3). *B. cereus* is often

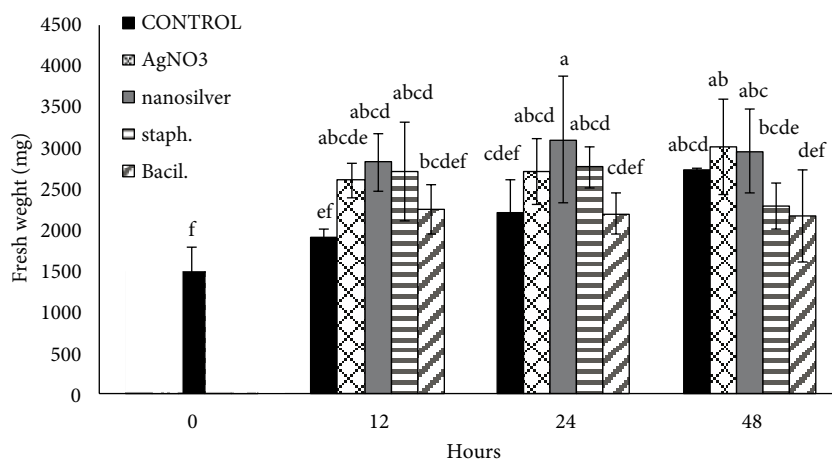


Figure 2. Effect of biotic (Bacil.: *B. cereus* and staph.: *S. aureus*) and abiotic (AgNO_3 and nanosilver) elicitors on fresh weight in hairy roots of *D. metel*. Different letters show significant differences in mean values for each parameter using Duncan's test ($P < 0.05$).

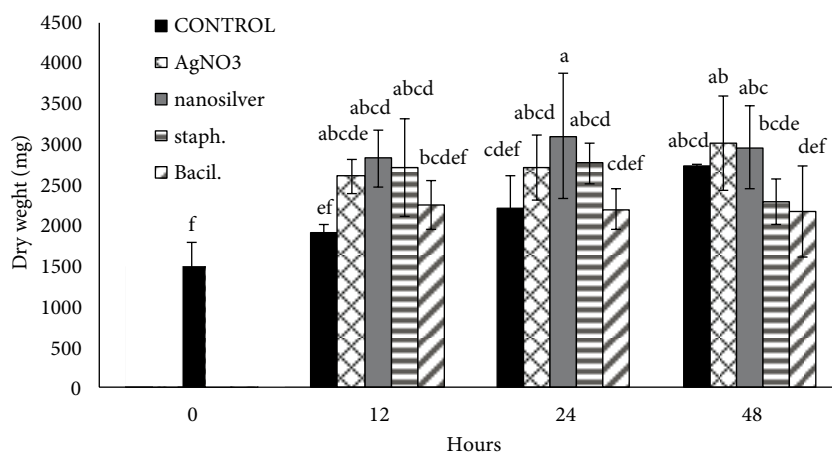


Figure 3. Effect of biotic (bacil.: *B. cereus* and staph.: *S. aureus*) and abiotic (AgNO_3 and nanosilver) elicitors on dry weight in hairy roots of *D. metel*. Different letters show significant differences in mean values for each parameter using Duncan's test ($P < 0.05$).

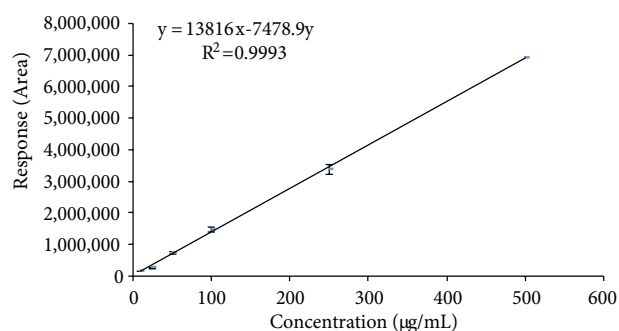


Figure 4. Calibration curve of atropine using HPLC method and acetonitrile/water (1:9) as the mobile phase with pH adjusted to 2.3 at 204 nm. Using Millennium processing software, the calibration curve was determined by linear regression in the range of 10–500 $\mu\text{g/mL}$. The regression equation was $y = 13816x - 7478.9$ with the correlation cofactor $R^2 = 0.9993$, where x was the concentration of the standard ($\mu\text{g/mL}$).

known as a plant growth promoting rhizobacterium and can produce indole acetic acid that increases the growth rate of the roots and improves the water and inorganic substance uptake (Abdul Aziz et al., 2012). Furthermore, the increase in root weight induced by *S. aureus* was higher than that of *B. cereus*. In addition, *S. aureus* can increase root weight after 24 h. Both strains of bacteria decreased the biomass of hairy roots after 48 h (Figures 2 and 3). A slight browning of the roots became evident after 24 h, which led to intense destruction of the treated roots and eventually their death 48 h later. This phenomenon could be related to the destruction or lysis of cells as a consequence of a severe attack against these bacteria (*B. cereus* and *S. aureus*). Such elicitations could lead to significant modifications in the metabolism of plant cells (Jung et al., 2003).

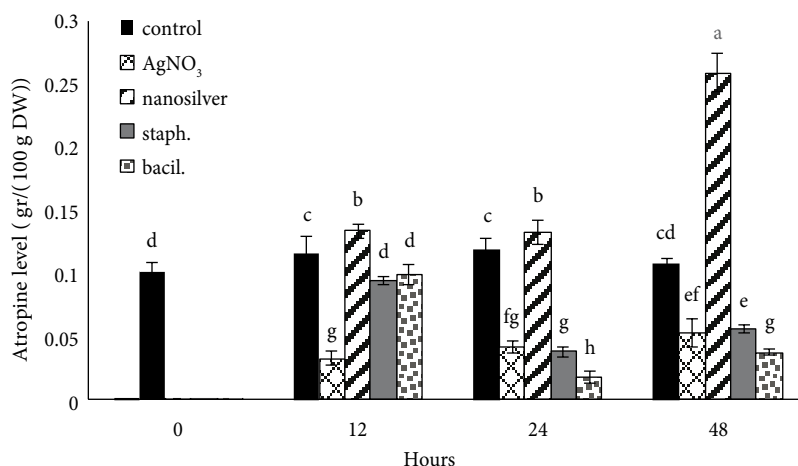


Figure 5. Effect of biotic (bacil.: *B. cereus* and staph.: *S. aureus*) and abiotic (AgNO_3 and nanosilver) elicitors on atropine level in hairy roots of *D. metel*. Different letters show significant differences in mean values for each parameter using Duncan's test ($P < 0.05$).

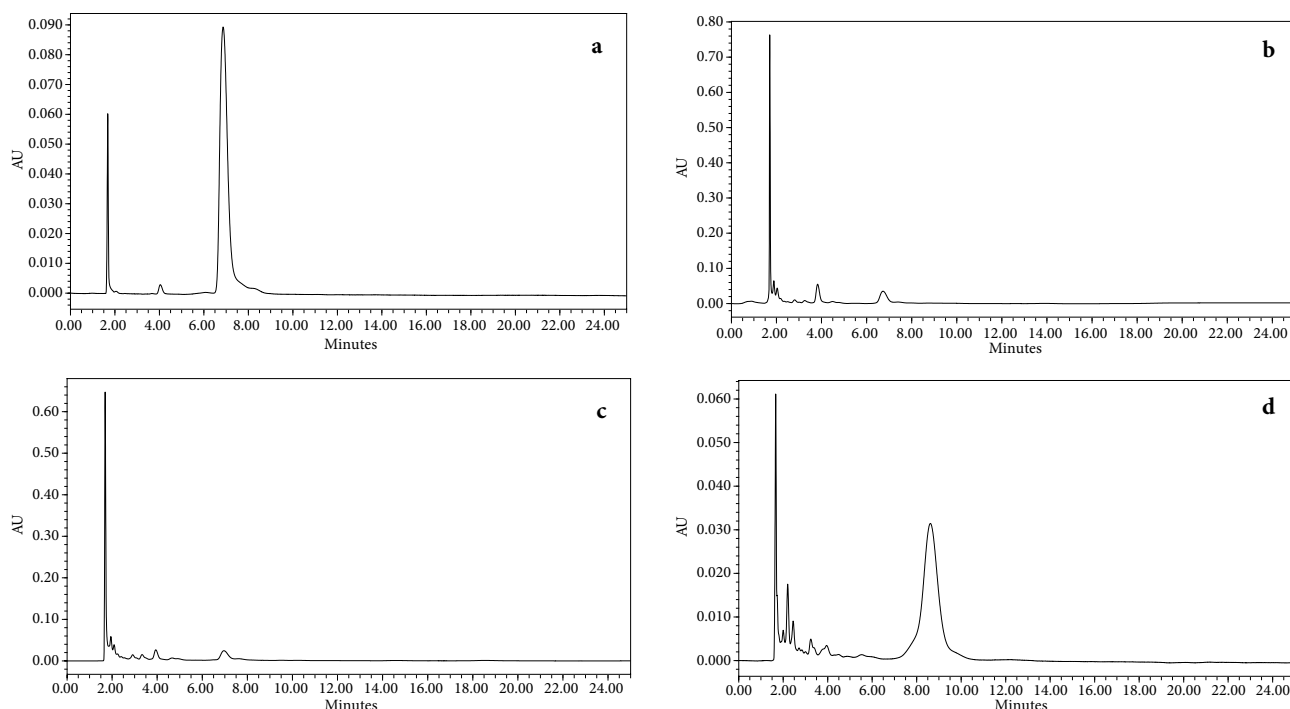


Figure 6. HPLC chromatograms of a) HPLC chromatogram of atropine standard (500 µg/mL); b) atropine from hairy roots of *D. metel* without any elicitor after 24 h; c) atropine from the hairy roots of *D. metel* elicited by AgNO_3 after 48 h; d) atropine from the hairy roots of *D. metel* elicited by nanosilver after 48 h. Chromatograms were obtained at 204 nm on a reversed-phase HPLC column (RP-18, 150 mm \times 3.9 mm, 5-µm particle size, Waters, USA) using water with H_3PO_4 (0.2%) as solvent A and acetonitrile as solvent B (9:1) at 40 °C and a flow rate of 2 mL/min.

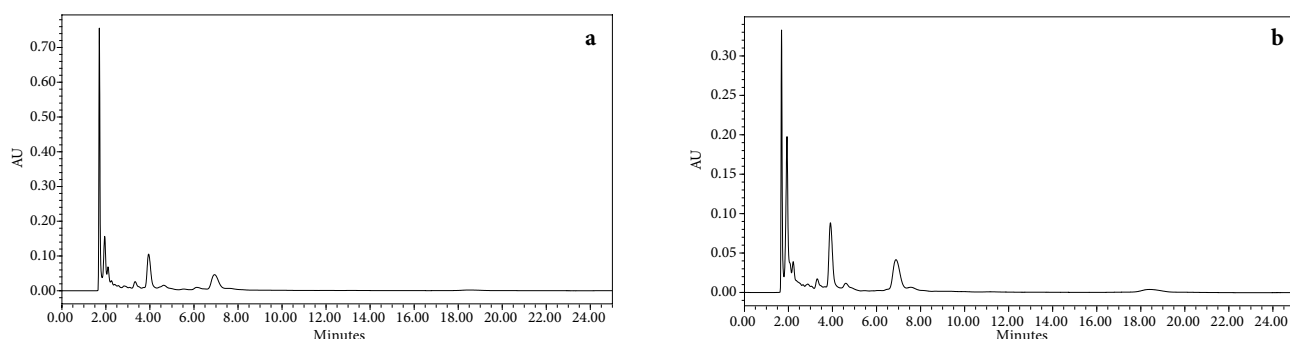


Figure 7. HPLC chromatograms of a) atropine from the hairy roots of *D. metel* elicited by *S. aureus* after 12 h; b) atropine from the hairy roots of *D. metel* elicited by *B. cereus* after 12 h. Chromatograms were obtained at 204 nm on a reversed-phase HPLC column (RP-18, 150 mm \times 3.9 mm, 5-µm particle size, Waters, USA) using water with H_3PO_4 (0.2%) as solvent A and acetonitrile as solvent B (9:1) at 40 °C and a flow rate of 2 mL/min.

4.2. Effect of abiotic (AgNO_3 and nanosilver) elicitors on fresh and dry weight in hairy roots of *D. metel*

The fresh and dry weight of hairy roots, elicited by AgNO_3 and nanosilver, increased after 12, 24, and 48 h (Figures 2 and 3). AgNO_3 is utilized for growth regulation and alteration of morphological characteristics in plants, with an abundant, specific, stable, and soluble nature in water. Silver is an ion involved in different pathways, such as

ethylene-, polyamine-, and calcium-signaling pathways. Silver nitrate can inhibit the activation of ethylene. Polyamines are involved in various important cellular procedures such as DNA replication, proliferation of cells, protein synthesis, and morphogenesis, which are related to Ca^{2+} ions in a signaling pathway. This indicates that AgNO_3 promotes polyamine synthesis and decreases ethylene production (Beyer et al., 1976; Zhao et al., 2002; Kumar et

al., 2009). Many investigations indicated that silver nitrate has a positive enhancing effect on the biomass of roots (Anantasaran and Kanchanapoom, 2008).

4.3. Effect of biotic (*B. cereus* and *S. aureus*) elicitors on atropine content in hairy roots of *D. metel*

The atropine accumulation in hairy roots infected by *B. cereus* and *S. aureus* was reduced after 12, 24, and 48 h (Figure 5).

It was shown that the live bacteria present in transformed root culture can have considerable influence on the accumulation of secondary metabolite (Wu et al., 2007). This process leads to a remarkable increase in the scale of secondary metabolite. This material is released in the medium as an antibacterial component (even though at very low scales), which may act as an inhibitor of cell division in *B. cereus* (as well as in gram-positive bacteria such as *S. aureus*) (Wu et al., 2007).

In this study, the release of secondary metabolites in the culturing medium was not measured.

The optimal pH of the LB medium for growth of bacteria is 6.5–7.0. The remarkably lower pH (about 4) of medium culture in the coculture of root-living bacteria in comparison to the optimal pH could contribute to bacterial growth prevention. The increased secondary metabolite storage in the roots may be a defense response of the transformed roots, stimulated by several elicitor components secreted from the living bacteria in the root medium cultures (Wu et al., 2007). It was reported that elicitation with biotic factors causes root death that could be due to destruction or lysis of cells as a consequence of a severe attack of these bacteria (Jung et al., 2003). As shown in Figure 5, atropine accumulation in hairy roots infected by *B. cereus* and *S. aureus* was reduced after 12, 24, and 48 h. In this study, the reduction in atropine content of *D. metel* hairy roots could be due to atropine release into the culture medium. The living bacteria may have various influences on the roots, such as cell–cell, gene–gene, and protein–protein interactions (Wu et al., 2007). These effects can similarly change the enzymatic pathways (H6H and others) to produce different tropane alkaloids in *D. metel* roots. For instance, as shown in Figures 7a and 7b, atropine was converted to scopolamine (this peak appeared in a retention time of 18.4 min) by the bacteria (*B. cereus* and *S. aureus*). In another study, gram-positive strains (*S. aureus*) were more effective in increasing the yield of tropane alkaloids in comparison to the gram-negative strains (*B. cereus*), which is in agreement with the results of the present study (Jung et al., 2003).

4.4. Effect of abiotic (AgNO_3 and nanosilver) elicitors on atropine content in hairy roots of *D. metel*

The atropine accumulation in *D. metel* hairy roots was reduced by AgNO_3 after 12, 24, and 48 h (Figure 5). It is frequently shown that AgNO_3 and CdCl_2 , being heavy

metal salts, elicit phytoalexin production and consequently increase the yield of tropane alkaloids in hairy root cultures (Angelova et al., 2006). Some metal ions, including silver, show effects on the promoter involved in expression of relative genes for some secondary metabolite production (Tang et al., 2004). The ion Ag^+ could induce the collection of tropane alkaloids due to overexpression of the AaPMT1 gene, which is involved in the first stage of the tropane alkaloid biosynthetic pathway. Upregulation of this gene enhances N-methylputrescine, which is the precursor of tropane alkaloids (Kai et al., 2012). It was reported that silver nitrate improves the secretion of tropane alkaloids 3-fold to culture medium (Pitta-Alvarez et al., 2000). Hence, it can be deduced that the atropine reduction in the hairy roots of *D. metel* caused by AgNO_3 may be due to the tropane alkaloid secretion in the medium culture.

The yields of atropine content in the *D. metel* hairy roots were increased by nanosilver as an elicitor after 12, 24, and 48 h (Figure 5). Nanosilver significantly increased atropine (2.4-fold) in the roots after 48 h (Figure 6d). This effect was not seen in AgNO_3 (Figure 6c). This could be due to the different physicochemical properties of nanosilver. The nanosilver nanoparticles have a greater adhesion to plant tissues (Seif Sahandi et al., 2011; Lee et al., 2012). Nanosilver can increase the activation of enzymatic pathways that contribute to the production of secondary metabolites (Zhang et al., 2013). These results suggest that nanosilver can be used as a powerful new elicitor to increase the yield of secondary metabolites in plant biotechnology. The same result was obtained by Zhang et al. (2013) in *Artemisia annua* hairy roots.

In conclusion, the elicitation of hairy roots was performed to increase the yield of biomass and atropine accumulation in *D. metel* by biotic and abiotic elicitors. The biotic elicitors (*B. cereus* and *S. aureus*) increased root biomass after 12 and 24 h and decreased the accumulation of atropine in the hairy root after 12, 24, and 48 h, which could be due to the secretion of atropine into the culture medium. AgNO_3 as an abiotic elicitor increased the weight of transformed roots and decreased the accumulation of atropine after 12, 24, and 48 h. Nanosilver increased the yield of biomass and the accumulation of atropine in hairy roots of *D. metel*. To our knowledge, this is the first report showing the effect of nanosilver on *D. metel* hairy roots. Regarding its influence on both biomass and tropane alkaloid production, it can be considered as a new powerful elicitor in increasing the yield of secondary metabolites in plant biotechnology.

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