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ORIGINAL RESEARCH

Expression of tropane alkaloids in the hairy root culture of *Atropa acuminata* substantiated by DART mass spectrometric technique

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ABSTRACT: *Agrobacterium rhizogenes*-mediated 'hairy root' cultures were established in *Atropa acuminata*. The chemical profiling of the hairy roots was carried out by a new mass spectrometric technique, direct analysis in real time (DART). The intact hairy roots were directly analyzed by holding them in the gap between the DART ion source and mass spectrometer. Two alkaloids, atropine and scopolamine, were characterized. The structural confirmation of the two alkaloids was made through their accurate molecular formula determinations. This is the first report of establishing hairy roots in *A. acuminata* as well as application of the DART technique for the chemical profiling of its hairy roots. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: *Atropa acuminata*; DART MS analysis; hairy root culture

INTRODUCTION

Plant-based molecules have attracted worldwide attention in recent years due to their unique therapeutic properties, and accordingly, around 40% of the currently used pharmaceuticals are either fully derived or partially semi-synthesized from plant-based molecules (Rout *et al.*, 2000; Canter *et al.*, 2005). A large number of plant-based compounds possess numerous structural/biochemical complexities, making their artificial synthesis not only difficult but also impossible. Atropine and scopolamine are pharmaceutically important tropane alkaloids, exclusively produced by plants, and the industrial demand for them is strong (Oksman-Caldentey *et al.*, 2000). Owing to their characteristic effects on the central nervous system and anticholinergic activities, they have well-established diversified therapeutic uses in the field of ophthalmology, cardiology, gastroenterology, etc. (Kursinszki *et al.*, 2005).

Atropa belladonna (Solanaceae), an endangered medicinal plant species native to Europe and Asia, normally serves as one of the main sources of these tropane alkaloids (Anonymous, 1948). *Atropa acuminata* Royle ex Lindl. is a native Asian species, which is also listed as an endangered medicinal plant due to its indiscriminate exploitation from the natural resources coupled with little or no attention towards any focused cultivation practice. *A. acuminata* closely resembles *A. belladonna* in its chemical profile and morphology, excepting the flower colour, and official recognition of the use of its alkaloids was given in the fifth addendum to the *British Pharmacopia* (Anonymous 1948). Most importantly, the major alkaloids in the two species are the same; they are synthesized in the roots and take 3–4 years of field maturation of the plant for generation of extractable root mass (Khan and Harborne, 1991). The prolonged gestation phase between planting and harvesting coupled with unpredictability in the supply of raw materials due to environmental and pathological hindrances and plant-to-plant discrepancies in the content of the active principles have led to the employment of different biotechnological tools for revealing an effectual substitute source of these important therapeutic molecules.

Modern biotechnological techniques have been employed most extensively involving *Atropa belladonna* as a model system (Banerjee *et al.*, 2002; Bonhomme *et al.*, 2000). *A. baetica*, another species of this genus, has also gained ample attention in terms of biotechnological intervention (Zarate *et al.*, 1997a,b; Zarate, 1999; Jaber-Vazdekisi *et al.*, 2006). However, in spite of the great resemblance of *A. acuminata* to *A. belladonna*, no biotechnological intervention has so far been directed towards the former.

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Abbreviations used: DART, direct analysis in real time.

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Amongst the different biotechnological tools implemented so far concerning both *A. baetica* and more particularly *A. belladonna*, *Agrobacterium rhizogenes*-mediated 'hairy root' cultures have attracted major research attention the world over in the area of *in vitro* production of plant-derived secondary products (Guillon *et al.*, 2006; Milen *et al.*, 2007). The increased importance of hairy root cultures as advantageous alternative sources of plant-based compounds is mainly due to their faster growth rate, genetic and biochemical stability over long periods in culture and accumulation of products of a type and quantity equivalent to or even higher than those of the parent plant (Hu and Du, 2006).

Isolation, separation and identification of the targeted molecule from plant-based products have always been associated with problems of tedious and intricate solvent extraction process due to the immense chemical intricacies of the extracts. Furthermore, the clonal nature of individual hairy root lines, owing to variation in copy numbers and insertion sites of Ri T-DNA, has made it crucial to screen and select the best performer amongst a wider, independently generated, heterogeneous background, as reported in earlier comparable studies (Verma *et al.*, 2007). Chemical profiling of large numbers of independently generated hairy root clones through the application of a traditional intricate solvent extraction process is not only time-consuming, but also expensive.

Recently, a new mass spectrometric ionization technique, called direct analysis in real time (DART), based on real-time source, developed by Cody *et al.* (2005), has successfully been utilized to quickly analyze the cell cultures of *Taxus wallichiana* for the accurate chemical characterization of the taxoids expressed in them (Banerjee *et al.*, 2007). The DART technique has been found to be extremely useful for direct detection of chemicals on surfaces without requiring sample preparation such as solvent extraction. DART is coupled to a time of flight mass analyzer that provides selectivity and accurate elemental composition assignment through exact mass measurement.

In consideration of the practical utility of this technique through complete elimination of the normally adopted intricate extraction processes, the DART technique has been utilized for chemical profiling of hairy root cultures of *A. acuminata.* In this communication, we report the first successful transformation of *A. acuminata* by *A. rhizogenes* strain LBA 9402 and identification of two alkaloids in a selected hairy root clone by directly analyzing the hairy roots using the DART mass spectrometric method. The structures of the two alkaloids were confirmed as atropine (**1**) and scopolamine (**2**) by exact measurements of their molecular formula (Fig. 1). To the best of our knowledge, this is the first report of establishing atropine- and scopolamine-producing hairy roots in *A. acuminata* as well as application of the DART technique for the chemical profiling of the hairy root clones of this endangered medicinal plant species.

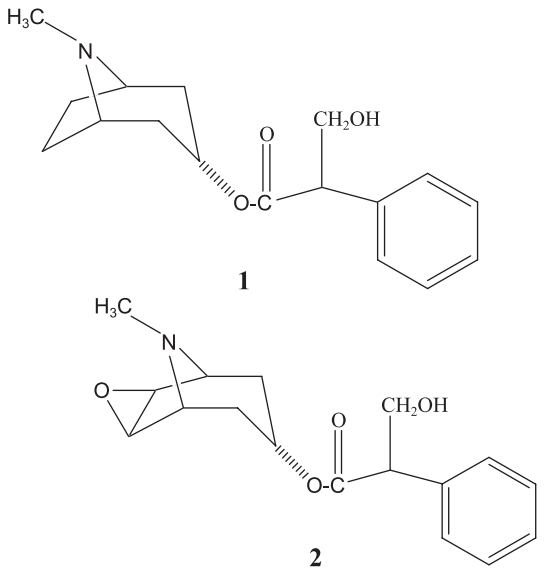

Figure 1. Chemical structures of atropine (**1**) and scopolamine (**2**).

EXPERIMENTAL

Induction and establishment of hairy roots. Seeds of *Atropa acuminata* Royle ex Lindl, were collected from Manasbel, Jammu and Kashmir and were germinated *in vitro* on Murashige and Skoog's (MS) medium (Murashige and Skoog 1962) supplemented with 2 mg/L GA_3 after initial sterilization with 0.1% $HgCl_2$ solution (w/v) for 2–3 min, followed by extensive rinsing with sterile distilled water. The germinated seedlings were subsequently maintained on hormone-free, semi-solid MS medium and served as the explant source for the genetic transformation studies.

The young leaves were directly inoculated through pricking with 48 h-old suspension culture of *A. rhizogenes* strain LBA 9402 (a gift from Professor D. Tepfer, INRA, Versailles, France), grown in liquid YMB (Hooykass *et al.*, 1977) medium ($O.D._{600}$ = 0.9–1.0) and co-cultivated on hormone-free, semi-solid MS medium at 25 ± 2°C in the dark. After 72 h of co-cultivation, the explants were transferred to the same medium supplemented with 1.0 g/L of Cephalaxin (Ranbaxy, India). Similar types of explants, pricked with a sterile needle devoid of the bacterial suspension, were cultured under uniform conditions as controls.

The emerging hairy roots were excised from several independent wound sites and were placed individually on hormone-free half-strength MS media supplemented with 3% (w/v) sucrose and 1.0 g/L of cephalaxin for their further proliferation. Once established, the individual hairy root clones were transferred to liquid half-strength MS medium with the same concentration of antibiotic and incubated on a rotary shaker in the dark at 25 ± 1°C under constant agitation (80 rpm). The antibiotic concentration was progressively

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lowered and finally completely omitted after 4 months. Control roots from non-transformed plants were also cultured under identical conditions in liquid half-strength MS medium supplemented with 3% sucrose (w/v) and 0.2 mg/L IBA to serve as control roots.

Characterization of hairy roots. In-order to confirm the transformed nature of the hairy root clones, polymerase chain reaction (PCR) was carried out to prove the transformed nature of the independently generated hairy root clones using both transformed and non-transformed roots following a published procedure (Rahman *et al.*, 2006).

DART mass spectrometry. The mass spectrometer used was a JMS-100 TLC (AccuTof) atmospheric pressure ionization time-of-flight mass spectrometer (Jeol, Tokyo, Japan) fitted with a DART ion source. The mass spectrometer was operated in positive-ion mode with a resolving power of 6000 (full-width at half-maximum). The orifice 1 potential was set to 28 V, resulting in minimal fragmentation. The ring lens and orifice 2 potentials were set to 13 and 5 V, respectively. Orifice 1 was set to a temperature of 100°C. The RF ion guide potential was 300 V. Data acquisition was from *m/z* 10 to 1050. The DART ion source was operated with helium gas flowing at approximately 4.0 L/min. The gas heater was set to 300°C. The potential on the discharge needle electrode of the DART source was set to 3000 V; electrode 1 was 100 V and the grid was at 250 V. The hairy root sample was held in the gap between the DART source and mass spectrometer for measurements. Exact mass calibration was accomplished by including a mass spectrum of neat polyethylene (PEG) glycol (1:1 mixture PEG 200 and PEG 600) in the data file. The mass calibration was accurate to within ±0.003 u. Using the Mass Center software, the elemental composition could be determined on selected peaks.

RESULTS AND DISCUSSION

Transformed roots of *A. acuminata* were established with *A. rhizogenes* strain LBA 9402 at the relative transformation frequency of 81 ± 5.8%, recorded after 12–14 days of inoculation. On the basis of maximum elongation and branching potentials, five hairy root clones were selected for chemical profiling out of 19 independently generated hairy root lines. These hairy root lines exhibited opine and *Rol-A* positive results, which indicated their transformed nature. The biosynthetic potentials of these hairy root clones were evaluated through application of the newly developed DART technique, in consideration of the practical utility of this technique through complete elimination of the normally adopted elaborate extraction processes.

Hairy root cultures of *Atropa belladonna* have been evaluated widely from different angles in terms of tropane alkaloid synthesis (Hashimoto *et al.*, 1993; Aoki *et al.*, 1997; Bonhomme *et al.*, 2000; Dimitrov *et al.*, 2005; Kursinszki *et al.*, 2005). The tropane alkaloid production potentials of the hairy root cultures of another species of this genus, i.e. *A. baetica*, have also been evaluated earlier (Zarate *et al.*, 1997a,b; Zarate, 1999; Jaber-Vazdekisi *et al.*, 2006). Most of the hairy root-related publications involving either *A. belladonna* or *A. baetica* consist of chemical analysis of tropane alkaloids through invariably employing elaborate processes of extraction and estimation.

We recently analyzed the hairy root culture of *Rauvolfia serpentine* using DART technique, in which the intact hairy root was held in front of the DART source. Two alkaloids, reserpine and vomilenine, were identified almost instantaneously (Madhusudanan *et al.*, 2007). The ease of analysis of the above study prompted us to apply the DART technique for the analysis of the compounds that were expressed in the hairy root culture of *A. acuminata*.

The intact individual hairy root clones of *A. acuminata* were held in front of the DART source. A mass spectrum was instantly obtained (Fig. 2). The peaks at *m/z* 290 and 304 were assigned to the $[M + H]^+$ of two

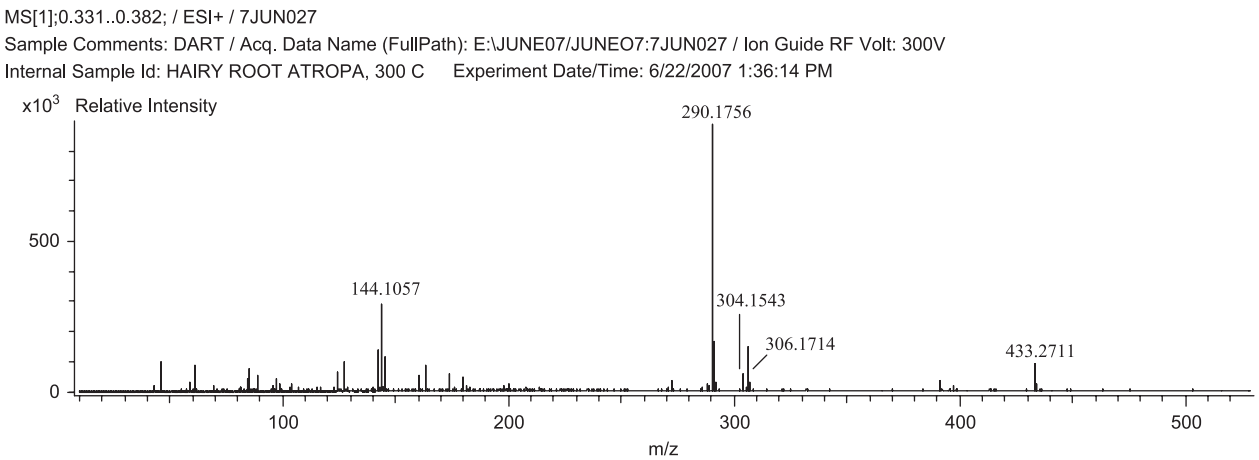

Figure 2. DART mass spectrum of the hairy roots of *Atropa acuminata*.

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Table 1. Elemental compositon of atropine (1) and scopolamine (2) in the DART mass spectrum of *Atropa acuminata*

Compound	Molecular formula (as M + H)	Calculated	Measured	Error (mmu)
1	$C_{17}H_{24}NO_3$	290.1756	290.1756	0
2	$C_{17}H_{22}NO_4$	304.1549	304.1543	−0.6

nitrogen-containing compounds. The two nitrogen-containing alkaloids were characterized as atropine (**1**) and scopolamine (**2**) on the basis of their exact mass measurement, which gave a mass difference of 0.002 between the measured and the theoretical *m/z* value of their molecular weights. The elemental compositions for the peaks at *m/z* 290 and 304 were determined using the Mass Center Software (Table 1). The molecular formula corresponding to the molecular weight calculated for the two alkaloids, atropine and scopolamine, confirmed their identity. Thus, it appears that DART would be a very useful technique for detection and characterization of atropine and scopolamine in the hairy root cultures of *A. acuminata* without the use of any solvent or sample preparation.

CONCLUSION

The DART mass spectrometric technique has been applied for the first time for profiling alkaloids expressed in the hairy root clone of *A. acuminata*. It produced an instantaneous response when the intact root was placed directly between the DART source and the mass spectrometer. Two alkaloids were detected and they were characterized as atropine and scopolamine. The results were confirmed by comparing their measured molecular formulas with the calculated ones. The expression of atropine is quite interesting as it is the first report of its occurrence in the hairy root cultures of *A. acuminata*. The DART technique can efficiently be utilized for screening and selection of the desired phytomolecule-yielding clone amongst a large number of independently generated hairy root clones of any medicinal plant species within shorter time and without sample preparation.

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