Differential Production of Tropane Alkaloids in Hairy Roots and *in vitro* Cultured Two Accessions of *Atropa belladonna* L. under Nitrate Treatments

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Plants are a potential source of a large number of valuable secondary metabolites. *In vitro* cultures are being considered as an alternative to agricultural processes for studying valuable secondary metabolites. In this way, nutritive factors are important parameters influencing the production of these compounds in plants. Effects of nitrate concentrations (KNO_3) on the production of two tropane alkaloids, hyoscyamine and scopolamine, and the growth of aerial parts and roots of two *in vitro* propagated accessions of *Atropa belladonna* and hairy roots were investigated. As hairy roots cultures are able to keep a stable production of alkaloids over long periods of subculturing, they are considered as an interesting option for the study of alkaloid biosynthesis. A hairy roots culture of *Atropa belladonna* was established by transformation with *Agrobacterium rhizogenes* strain *AR15834*. The results of our study showed that a rise in KNO_3 concentration caused a decline in hairy roots growth, and had a remarkable effect on the alkaloid content. The alkaloid concentrations obtained in the hairy roots were 3–20 times higher than that in the plants at 35 mM of KNO_3. Increasing the nitrate concentration in the medium of hairy roots also improved the hyoscyamine/scopolamine ratio, while it increased the scopolamine/hyoscyamine ratio in the studied plants.

Key words: Agrobacterium rhizogenes, *Atropa belladonna*, Hairy Roots, Tropane Alkaloids

Introduction

Plant alkaloids are one of the largest groups of natural products; they are pharmacologically important compounds. The Solanaceae plants such as *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopodia* produce a range of biologically active alkaloids including hyoscyamine (atropine) and scopolamine (hyoscine) that act on the parasympatic nervous system and are widely used as anticholinergic agents (Takizawa *et al.*, 2007; Palazon *et al.*, 2003; Pinol *et al.*, 1999). Plants synthesize L-isomers of hyoscyamine and scopolamine. Atropine, a tropane alkaloid of medicinal interest, the racemic form of hyoscyamine, has been isolated from some solanaceous species; usually racemization occurs during its isolation from plants (Hashimoto and Yamada, 1986). Although the market volume for scopolamine and hyoscyamine is not very large, there are no other classes of compounds that could substitute these plant-derived drugs. Therefore the demand for them will continue (Palazon *et al.*, 2003). Alkaloids are formed in the roots and transported to the aerial parts of the plant (Rothe *et al.*, 2003). Based on previous studies, the concentrations of scopolamine and hyoscyamine in cultured cells and in unorganized *in vitro* cultures of Solanaceae are generally very low (Hashimoto and Yamada, 1983). It seems that the alkaloid pathway requires root organization in order to be developed completely. The production of tropane alkaloids by hairy roots cultures has been reported for genera such as *Atropa*, *Datura*, *Duboisia*, and *Hyoscyamus* (Dechaux and Boitel-Conti, 2005; Mateus *et al.*, 2000). These roots often grow faster than plant cell cultures and do not require hormones in the medium (Kim *et al.*, 2002). The greatest advantage of hairy roots is that their cultures often exhibit the same or greater biosyn-

thetic capacity for secondary metabolite production compared to their mother plants.

Elicitation strategies are compounds or treatments that induce plants or different organ cultures to synthesize secondary metabolites. The nitrogen concentration and the carbon/nitrogen ratio of the culture medium often influence the synthesis of alkaloids (Payne *et al.*, 1987). *In vitro* Solanaceae cultures are usually grown on media containing nitrate and ammonium as nitrogen sources (Bensaddek *et al.*, 2001). Although NO_3^- is often the preferred form, both have advantageous and disadvantageous properties, depending on soil and climatic conditions and on the plant species considered. Since hyoscyamine and scopolamine are synthesized in the roots of *Atropa belladonna* plants, from the amino acid precursors phenylalanine, ornitine, and possibly arginine (Demeyer and Dejaegere, 1992), also amino acids in the roots are built from nitrogen; we found it worth investigating whether nitrate in the mineral nutrition of the plant could influence its alkaloid production.

To our knowledge there has not been any report so far on the study of tropane alkaloid levels after using a nitrate elicitor in intact plants. For this reason, in the present work, the production of atropine and scopolamine in two *in vitro* culture systems, hairy roots and tissue culture-derived intact plants, was analyzed after introducing a nitrate elicitor to the medium. Our aim was to investigate whether the roots of intact plants could produce more scopolamine and atropine in comparison to hairy roots when exposed to exceeded amounts of nitrate. Atropine and scopolamine contents were compared in the aerial parts and roots of these new plants and hairy roots, obtained from Iranian *Atropa belladonna*, under the effect of different nitrate concentrations.

Material and Methods

Plant tissue cultures and effects of the nitrate concentrations

Seeds of *Atropa belladonna* were collected from Vaz and Garmestan regions (N: 52º 7', E: 36º 20' and N: 53º 9', E: 36º 14', respectively) of Iran. The seeds were surface-sterilized with 70% (v/v) ethanol for 70 s and 10% (v/v) sodium hypochlorite for 20 min, and then were washed with sterile distilled water more than five times. They were placed on MS solid medium for germination (Murashige and Skoog, 1962). The obtained plantlets, that were 5–7 cm high (Figs. 1a, b), slabed uniformly into segments with one internode, two nodes, and 2–3 leaves. Then they were subcultured on MS solid medium, supplemented with 3% (w/v) sucrose, 0.1 mg L^{-1} indole acetic acid (IAA) and 1 mg L^{-1} benzyladenine (BA). These cultures were maintained at 28 ºC with a daily 16-h photoperiod. The achieved shoots were cultured on MS medium plus 0.2 mg L^{-1} IAA (for rooting) supplemented with different concentration of KNO_3 (0, 15, 35, and 95 mM). The cultures were incubated at 28 ºC with a 16-h photoperiod for 28 d.

The whole plants were separated from the medium, and the fresh weight of shoots and roots, shoot height and root length of plants were measured. All experiments were conducted in triplicate.

Plant transformation

Sterile leave explants of 5-week-old *A. belladonna* plants were infected with the *Agrobacterium rhizogenes* strain *AR15834* and grown at 28 ºC in hormone-free MS medium containing 200 mg L^{-1} cefotaxime antibiotic (Pinol *et al.*, 1999). Sterile leave explants without infection were grown in the medium as control. Generation of hairy roots was initiated two weeks after inoculation. The roots which appeared on the explants were removed and transferred to the medium. The transgenic nature of the hairy roots was confirmed by PCR using *rol*B specific primers. The growth rate of the hairy roots and untransformed control was measured up to 14 d on hormone-free MS medium. The hairy roots were treated with different concentrations of nitrate (0, 15, 35, and 95 mM) in 100-mL flasks containing 30 mL of hormone-free MS medium. All root cultures were initiated from 2.5 g FW (fresh weight) and maintained in the dark at 27 ºC on an orbital shaker at 110 rpm for two weeks. After that time, the roots of different treatments were weighed and saved for tropane alkaloids extraction.

PCR analysis

Extraction of DNA from the roots was done using a simplified CTAB (cetyltrimethyl ammonium bromide) method (Khan *et al.*, 2007). PCR analysis was conducted with *rol*B with a predicted product size of 700 bp. Amplification conditions

for *rol*B were 35 cycles in which first denaturation was carried out at 94 °C for 5 min, then segment denaturation at 94 °C for 1 min, annealing at 54 °C for 50 s, extension at 72 °C for 1.5 min, and final extension for 10 min at 72 °C. PCR products were analysed by electrophoresis in 0.8% (w/v) agarose-ethidium bromide gel. Meanwhile the Ri-plasmid of *Agrobacterium rhizogenes* was used as positive control (Wang *et al.*, 2006).

Extraction of tropane alkaloids

Hairy roots and root and aerial parts of intact plants (1.5 g FW) were extracted with 30 mL of 96% EtOH under reflux. Extracts were filtered through No. 1 filter paper, and then dried by evaporation using a rotary pump. The dry residue was dissolved in 60 mL of 5% H_2SO_4 and diethyl ether (1:1). Then the colourless aqueous phase was collected, and the pH value was adjusted to 10 using 10 mM NaOH. Thereafter, 60 mL of $CHCl_3$ were added to the decanter and shaken. Then the $CHCl_3$ phase was collected and evaporated. The

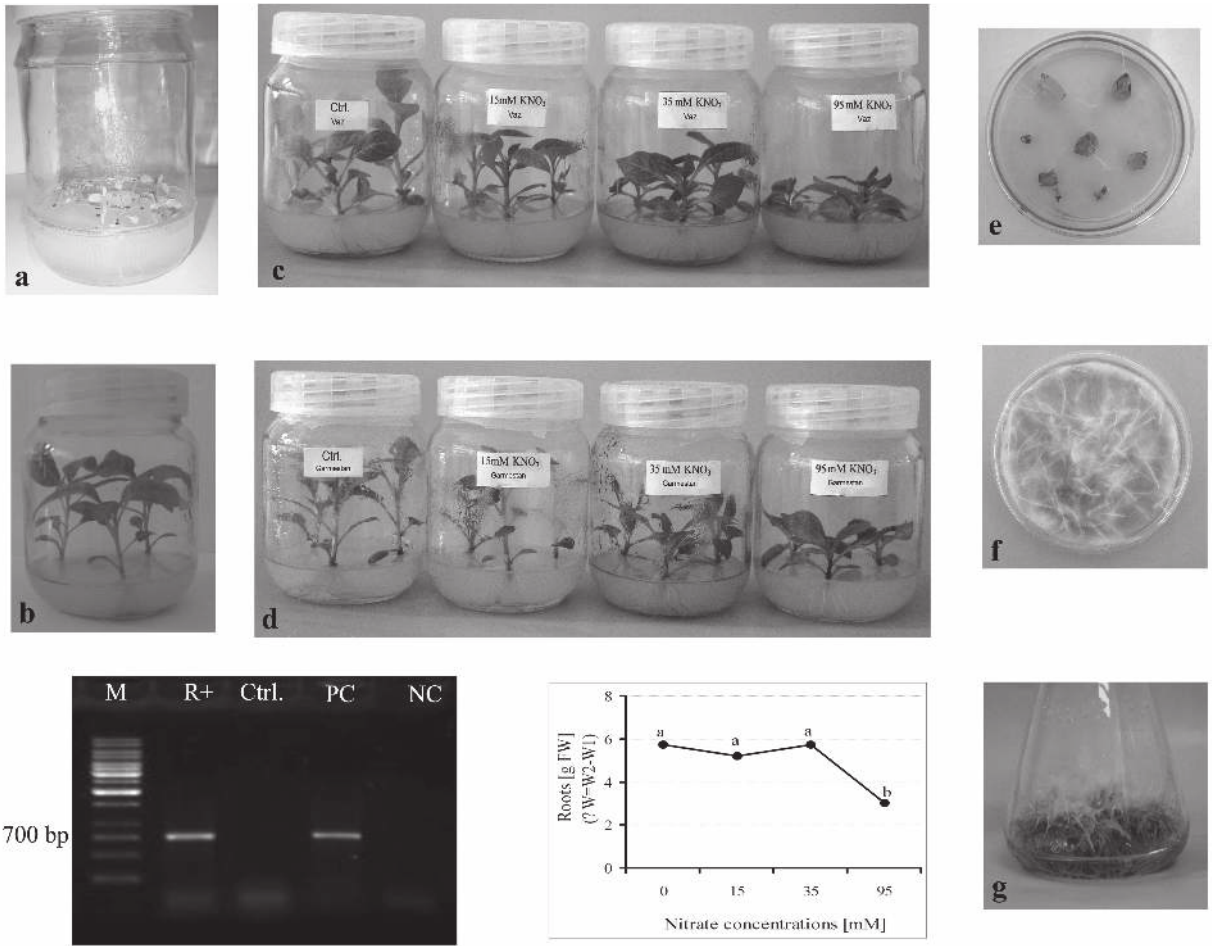

Fig. 1. (a) *In vitro* germinated seedlings of *Atropa belladonna* plants. (b) *In vitro* plants. (c, d) Growth of Garmestan and Vaz plants under different nitrate concentrations, respectively. (e) Induction of hairy roots from leave explants. (f, g) Establishment of hairy roots in MS medium. (h) Electrophoretogram showing the presence of *rol*B gene (700-bp band) in transformed hairy roots; M, molecular weight marker; R^+, transformed root lines; Ctrl, non-transformed root; PC, positive control; NC, negative control. (i) Growth rate of hairy roots under different nitrate concentrations; W1, root FW before treatment; W2, root FW after 14 d.

Table I. Means of shoot length (L.SH), root length (L.R), shoot fresh weight (W.SH), root fresh weight (W.R), chlorophyll and anthocyanine contents in Vaz and Garmestan plants. Different letters show significant differences between the means ($P \leq 0.05$).

Variety	KNO_3 [mM]	L.SH [cm]	W.SH [g FW]	L.R [cm]	W.R [g FW]	Chlorophyll a [mg g^{-1} FW]	Chlorophyll b [mg g^{-1} FW]	Anthocyanine [mmol g^{-1} FW]
Vaz	0	2.7 ab	0.28 abc	6.998 a	0.2236 a	0.455 d	0.1520 c	0.071 bc
	15	2.4 bc	0.22 c	7.430 a	0.1490bcd	0.685 bc	0.2311 bc	0.046 c
	35	2.1 bc	0.29 ab	7.130 a	0.2022 ab	0.700 bc	0.2540 abc	0.067 bc
	95	1.6 c	0.31 a	6.740 ab	0.1684 abc	0.678 bc	0.2257 bc	0.062 bc
Garmestan	0	3.4 a	0.28 abc	6.730 ab	0.1498 bcd	0.786 ab	0.3093 ab	0.110 a
	15	3.2 a	0.24 bc	6.833 ab	0.1348 cd	0.896 a	0.3449 a	0.081 b
	35	3.2 a	0.25 bc	6.564ab	0.1058 cd	0.749 ab	0.2760 ab	0.070 bc
	95	1.8 c	0.27abc	5.432 b	0.0958 d	0.537 cd	0.2122 bc	0.067bc

dry residue, for analyzing, was dissolved in 500 μL MeOH (Dashek, 1997).

Analysis of alkaloids

Atropine and scopolamine contents were analyzed using quantitative HPLC (Roos and Lau-Cam, 1986). The stationary phase was a C_{18}-S5ODS1 column (250 × 4.6 mm). The elution solvent was composed of water, MeOH, triethylamine and acetic acid (83:15:0.5:1.5, v/v). Spectrophotometric UV detection was used (λ = 230 nm). The calibration was made with standard scopolamine hydrobromide [RT = (5.5 ± 0.5) min] and atropine sulfate [RT = (11.5 ± 0.5) min]. Every sample was assayed in triplicate. The alkaloid production was determined using a calibration curve prepared with standards and a co-chromatogram of the standards and samples.

Chlorophyll and anthocyanin assay

Chlorophyll was extracted with 80% acetone from the leaf samples (Arnon, 1949). Extracts were filtered and the content of chlorophylls a and b were determined by spectrophotometry at 645 and 663 nm. The content of chlorophyll was expressed as mg g^{-1} FW. Anthocyanin extraction was carried out according to the procedure of Krizek *et al.* (1998). Leaf samples were homogenized in a mortar and pestle with 3 mL of 1% HCl/methanol (1:99, v/v). The homogenate was centrifuged at 15000 × *g* for 30 min at 4 °C, and then the supernatant was filtered through No.1 Whatman paper to remove particulate matter and stored in the darkness at 3 °C overnight. The amount of anthocyanin was determined from the absorbance at 550 nm using the extinction coefficient of anthocyanin (ε = 33000 cm^{-2} mol^{-1}).

Statistical analysis

All data were analysed using MSTAT-C software. Duncan's multiple range test was used to measure statistical differences between treatment methods and controls. $P \leq 0.05$ or $P \leq 0.01$ was considered significantly different.

Results

Hairy roots formation

Hairy roots were induced in 66% of the explants 14 days after infection (Figs. 1c, d). The transgenic nature of hairy roots was confirmed throuth the presence of *rol*B gene in the hairy roots that was detected by PCR analysis. The transformant showed the presence of diagnostic 700-bp *rol*B product amplification (Fig. 1 h). The DNA of the normal roots did not show any amplified material. The growth rate of the hairy roots in liquid MS medium was measured up to 14 days. In this period the growth rate for the transgenic roots was 0.68 g d^{-1}, while it was 0.09 g d^{-1} for the control (cuted root of plantlet). It is concluded that infected roots of the plant had grown 7.6 times faster than the non-infected control.

Effects of the nitrate concentrations on growth

The nitrate treatments affected the hairy roots growth of *A. belladonna* (Fig. 1i). When the cultures were treated with 15 and 35 mM of nitrate, during the two weeks of exposure, the hairy root dry weight (DW) had no significant differences

Table II. Comparison of means of scopolamine and atropine contents after different treatments. Different letters show significant differences between the means ($P \leq 0.05$).

Alkaloid	KNO_3 [mM]	Hairy roots	Vaz		Garmestan	
			Leaf	Root	Leaf	Root
Scopolamine [mg g^{-1} FW]	0	0.1240 a	0.0385 hi	0.0346 hij	0.0178 jk	0.0708 e
	15	0.1172 ab	0.0506 gh	0.0732 de	0.0687 ef	0.0888 cd
	35	0.1160 ab	0.0577 efg	0.0097 k	0.0526 fgh	0.1044 bc
	95	0.0927 c	0.0403 ghi	0.0171 jk	0.0255 ijk	0.0390 hi
Atropine [mg g^{-1} FW]	0	0.1314 c	0.0125 j	0.0120 j	0.0246 hij	0.0651 d
	15	0.1855 b	0.0461 efg	0.0196 ij	0.0329 ghi	0.0628 de
	35	0.2294 a	0.0241 hij	0.0089 j	0.0539 def	0.0408 fgh
	95	0.1408 c	0.0066 j	0.0072 j	0.0168 ij	0.0147 ij

to the control, while the hairy roots DW was significantly declined when the nitrate concentration was raised to 95 mM. To investigate the effects of nitrate treatments on the growth of intact plants of *A. belladonna*, we employed the same concentrations as used for the hairy roots. Table I shows the effect of nitrate on the growth of roots and shoots of *in vitro* propagated *A. belladonna* plants originated from two populations. When the cultures were treated with 95 mM nitrate, the shoot length was significantly decreased compared to the control in both studied populations (Figs. 1c, d), but the shoot biomass was not affected. However, the various concentrations of exogenous nitrate did not have any significant effects on root length and weight of both populations. The means calculated for weight and length of roots of plants originated from the Vaz region were slightly higher than those from the Garmestan region.

Analysis of alkaloids

The highest amount of atropine in transformed roots was observed in 35 mM KNO_3 (0.23 mg g^{-1} FW) (Table II). However, there were no differences in the amount of scopolamine at different treatments except for 95 mM nitrate, where the scopolamine content was significantly decreased. In addition, the atropine/scopolamine ratio was enhanced by increasing the nitrate concentrations. In the aerial parts of Vaz plants, the scopolamine contents increased by increasing the nitrate concentration to 35 mM, but in the roots of these plants, the highest scopolamine contents were observed with low concentrations of nitrate (15 mM). The atropine content in the different parts of Vaz plants showed no significant difference by increasing the nitrate concentrations. In general, the scopolamine/atropine ratio in the aerial parts of these plants increased with application of 35 mM nitrate. On the other hand, in the roots of these plants the scopolamine/atropine ratio decreased with raised nitrate concentration. The results of scopolamine analysis in the Garmestan plants showed that in the aerial parts and roots of these plants, the scopolamine content enhanced by increasing the nitrate concentration (except for 95 mM KNO_3). Similar results were obtained for atropine in the aerial parts of these plants, but in the roots, the atropine content decreased while rising the nitrate concentration. Higher concentrations of KNO_3 (95 mM) had an inhibitory effect on the alkaloid production in both plant groups. Generally, the tropane alkaloid contents in hairy roots were considerably high compared to those in plants. The amount of atropine was significantly higher in hairy roots compared to plant leaves and roots under the nitrate treatments. The scopolamine content in the hairy roots was 10 times higher than in organs of intact plants. The highest levels of these alkaloids between the studied plant groups were observed in the roots of Garmestan plants. It could be said that these treatments have positive effects on the production of alkaloids in the roots.

Chlorophyll and anthocyanin

The results showed no considerable alteration in the chlorophylls a and b contents for both populations. Generally, the content of chlorophylls a and b in the aerial part of Garmestan plants was higher than in Vaz plants. The anthocyanin content was not different in the populations (Table I).

Discussion

Growth and alkaloid accumulation were influenced by nitrate concentrations. Rising the nitrate concentration to 95 mM had same effects on the growth rate of hairy roots and the shoot length of plants. In Vaz and Garmestan plants, the fresh weights of the aerial parts were nearly similar at the different levels of nitrate. Our results are consistent with those of Yamamoto and Kamura (1997) who reported that ammonium salts strongly inhibited the growth of *Bupleurum falcatum* L. roots. The effect of nitrate concentrations on the hairy roots was more evident regarding the level of the alkaloid content. Application of low concentrations of nitrate in the culture medium led to increased alkaloid contents in the hairy roots of *A. belladonna*. The atropine/scopolamine ratio was also modulated by the composition of the culture medium. It was increased 1.1–1.5 times compared to the control roots when the concentrations of nitrate were raised. Bonhomme *et al.* (2000) similarly reported that the hyoscyamine/scopolamine ratio was predominant in hairy roots of *A. belladonna*, but these results were in contrast with reports of Bensaddek *et al.* (2001). In hairy roots, in most of the treatments, the atropine/scopolamine ratio was increased. This could mean that the epoxidation of hyoscyamine to scopolamine was not related to the hyoscyamine content of the roots, but was rather due to a more or less hyoscyamine-6β-hydroxylase (H6H) activity (Hashimoto *et al.*, 1991). Palazon *et al.* (2003) concluded that adding H6H in *Duboisia* plants may lead to better conversion of hyoscyamine to scopolamine, and consequently that hyoscyamine may be a feedback regulation signal in alkaloid accumulation. In the transformed lines they obtained that this inhibition was removed by the production of scopolamine.

When scopolamine and atropine accumulation were analyzed in different organs of *Atropa belladonna* plants from Vaz, a gradual increase in scopolamine/atropine ratios from roots to leaves was noticed (Table II). The results suggest that there is an increase in the accumulation of scopolamine and atropine at low concentrations of nitrate, and this could probably be due to an increased activity in the biosynthesis pathway of these compounds, or to a reduced degradation rate. In Garmestan plants the contents of the two alkaloids was higher than in Vaz plants; on the other hand, the chlorophyll content of Garmestan plants was higher than that of Vaz plants. Mendoza and Vargas (1995) reported that probably it is a correlation between scopolamine production and photosynthetic activity and suggested that scopolamine production parallels an increase in chlorophyll levels and chlorophyll a/chlorophyll b ratios. In the whole plant, tropane alkaloids are biosynthesized in the root cells and translocated to the aerial parts for storage (Rothe *et al.*, 2003). It seems that a loss in scopolamine/hyoscyamine ratio in roots and its enhancement in leaves under nitrate treatments are due to transportation of these components from roots to aerial parts. The scopolamine content on the other hand seems to be more affected by the developmental stages of the plant (Demeyer and Dejaegere, 1992). It is worth noticing that the highest growth was always associated with the highest alkaloid productivity. In contrast, Mantell *et al.* (1983) have shown that nicotine appeared to be synthesized in *Nicotiana tabacum* cells, which were not in the division phase.

In conclusion, quantitative and qualitative changes in FW and tropane alkaloid yields of *A. belladonna* plants and hairy roots were achieved by modification of the nitrate concentrations in the medium. In general, the amount of alkaloids in plants is very small compared to transformed lines whereas hairy roots have rapid growth compared to a common culture of these plants. Nitrate had a clear influence on the alkaloid content and on the atropine/scopolamine ratio.

Arnon D. I. (1949), Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24**, 1–15.

Bensaddek L., Gillet F., Nava Saucedo J. E., and Fliniaux M. (2001), The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots. J. Biotechnol. **85**, 35–40.

Bonhomme V., Laurain-Mattar D., Lacoux J., Fliniaux M., and Jacquin-Dubreuil A. (2000), Tropane alkaloid production by hairy roots of *Atropa belladonna* obtained after transformation with *Agrobacterium rhizogenes* 15834 and *Agrobacterium tumefaciens* containing *rol* A, B, C genes only. J. Biotechnol. **81**, 151–158.

Dashek W. V. (1997), Methods in Plants Biochemistry and Molecular Biology. CRC Press, Boca Raton, USA, pp. 185–189.

Dechaux C. and Boitel-Conti M. (2005), A strategy for overaccumulation of scopolamine in *Datura innoxia* hairy root cultures. Acta Biol. Cracov. Ser. Bot. **47/1**, 101–107.

Demeyer K. and Dejaegere R. (1992), Effect of the nitrogen form used in the growth medium (NO_3^-, NH_4^+) on alkaloid production in *Datura stramonium* L. Plant Soil **147**, 79–86.

Dupraz J., Christen P., and Kapetanidis I. (1993), Tropane alkaloids in transformed roots of *Datura quercifolia*. Planta Med. **60**, 158–162.

Hashimoto T. and Yamada Y. (1983), Scopolamine production in suspension cultures and redifferentiated roots of *Hyoscyamus niger*. Planta Med. **47**, 121–141.

Hashimoto T. and Yamada Y. (1986), Hyoscyamine 6*β*-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, in alkaloid-producing root cultures. Plant Physiol. **81**, 619–625.

Hashimoto T., Hayashi A., Amano Y., Kohno J., Iwanari H., Usuda S., and Yamada Y. (1991), Hyoscyamine 6*β*-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. J. Biol. Chem. **266/7**, 4648–4653.

Khan S., Irfan Qureshi M., Kamaluddin Alam T., and Abdin M. Z. (2007), Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. Afr. J. Biotechnol. **6**, 175–178.

Kim Y., Wyslouzil E. B., and Pamela J. (2002), Secondary metabolism of hairy root cultures in bioreactors. In vitro Cell. Dev. Biol. Plant. **38**, 1–10.

Krizek D. T., Brita S. J., and Miewcki R. M. (1998), Inhibitory effects of ambient levels of solar UV-A and UV-B on growth of cv. New Red Fire lettuce. Physiol. Plant. **103**, 1–7.

Mantell S. H., Pearson D. W., Hazell L. P., and Smith H. (1983), The effect of initial phosphate and sucrose levels on nicotine accumulation in batch suspension cultures of *Nicotiana tabacum*. Plant Cell Rep. **2**, 73–77.

Mateus L., Cherkaoui S., Christen P., and Veuthey J. (2000), Enantioseparation of atropine by capillary electrophoresis using sulfated b-cyclodextrin: application to a plant extract. J. Chromatogr. A **868**, 285–294.

Mendoza M. I. E. and Vargas L. V. M. (1995), Establishment and characterization of photosynthetic hairy root cultures of *Datura stramonium*. Plant Cell Tiss. Organ Cult. **40**, 197–208.

Murashige T. and Skoog F. (1962), A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. **15**, 473–497.

Palazon J., Moyano E., Cusidó R. M., Bonfill M., Oksman-Caldentey K. M., and Pinol M. T. (2003), Alkaloid production in *Duboisia* hybrid hairy roots and plants overexpressing the *h6h* gene. Plant Sci. **165**, 1289–1295.

Payne J., Hamill J. D., Robins R. J., and Rhodes J. C. (1987), Production of hyoscyamine by hairy root cultures of *Datura stramonium*. Planta Med. **53**, 474–478.

Pinol M. T., Palazon J., Cusido M. R., and Ribo M. (1999), Influence of calcium ion-concentration in the medium on tropane alkaloid accumulation in *Datura stramonium* hairy roots. Plant Sci. **141**, 41–49.

Roos R. W. and Lau-Cam C. (1986), General reversed-phase HPLC method for the separation of drugs using triethylamine as a competing base. J. Chromatogr. **370**, 403–418.

Rothe G., Hachiya A., Yamada Y., Hashimoto T., and Èger B. (2003), Alkaloids in plants and root cultures of *Atropa belladonna* overexpressing putrescine *N*-methyltransferase. J. Exp. Bot. **54**, 2065–2070.

Takizawa M., Hori K., Inai K., Takase H., Hashimoto T., and Watanabe Y. (2007), A virus-induced gene silencing approach for the suppression of nicotine content in *Nicotiana benthamiana*. Plant Biotechnol. **24**, 295–300.

Wang B., Zhang G., Zhu L., Chen L., and Zhang Y. (2006), Genetic transformation of *Echinacea purpurea* with *Agrobacterium rhizogenes* and bioactive ingredient analysis in transformed cultures. Colloids Surf. B **53**, 101–104.

Yamamoto O. and Kamura K. (1997), Production of saikosaponin in cultured roots of *Bupleurum falcatum* L. Plant Tissue Cult. Biotechnol. **3**, 138–147.