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Alkaloid production in *Duboisia* hybrid hairy root cultures overexpressing the *pmt* gene

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Abstract

Putrescine:SAM *N*-methyltransferase (PMT) catalyses the *N*-methylation of the diamine putrescine to form *N*-methylputrescine, the first specific precursor of both tropane and pyridine-type alkaloids, which are present together in the roots of *Duboisia* plants. The *pmt* gene of *Nicotiana tabacum* was placed under the regulation of the CaMV 35S promoter and introduced into the genome of a scopolamine-rich *Duboisia* hybrid by a binary vector system using the disarmed *Agrobacterium tumefaciens* strain C58C1 carrying the rooting plasmid pRiA4. The presence of the foreign gene in kanamycin-resistant hairy roots and its overexpression were confirmed by polymerase chain reaction and Northern blot analysis respectively. The *N*-methylputrescine levels of the resulting engineered hairy roots increased (2–4-fold) compared to wild type roots, but there was no significant increase in either tropane or pyridine-type alkaloids.

Keywords: *Duboisia* hybrid; Solanaceae; Hairy roots; Hyoscyamine; Nicotine; Putrescine:SAM *N*-methyltransferase; Polyamines; Scopolamine

1. Introduction

Hairy root cultures, induced by infection with *Agrobacterium rhizogenes*, are an excellent starting material for the production of secondary metabolites by plant cells due to the stability of important characteristics, such as morphology, growth capacity and productivity of those secondary metabolites synthesized in intact parent plant roots. Since both tropane and pyridine-type alkaloids are synthesized in the roots of solanaceous plants, the application of hairy root technology has been proved beneficial. The tropane alkaloids hyoscyamine and scopolamine, widely used as anticholinergic agents that act on the parasympathetic nervous system, come exclusively from solanaceous plants such as *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia*. Moreover it has been observed that the roots of *Duboisia* species, besides producing hyoscyamine and scopolamine, also synthesize nicotine and nornicotine (Fig. 1), both of which are pyridine alkaloids normally found in tobacco plants (Endo and Yamada, 1985).

Large scale culture of hairy roots of several Solanaceae species has been reported so far (Hilton and Rhodes, 1990; Muranaka et al., 1993; Cusidó et al., 1999). However, in most cases the yields of the tropane alkaloids hyoscyamine and scopolamine are too low for commercialization. There remains a need to increase alkaloid production rates for commercial exploitation. One approach to overcoming the present limitations is the introduction of foreign genes into hairy roots in order to enhance the levels of rate-limiting intermediates. For example, the *Hyoscyamus niger* gene for hyoscyamine 6β-hydroxylase has been placed under the control of the 35S CaMV promoter and introduced into hyoscyamine-rich *Atropa belladonna* plants by a binary vector system using *A. rhizogenes*. The resulting hairy roots showed increased levels of hydroxylase activity and contained up to five-fold higher concentrations of scopolamine than wild-type hairy roots (Hashimoto et al., 1993). Due to the undesirable effects of hyoscyamine on the central

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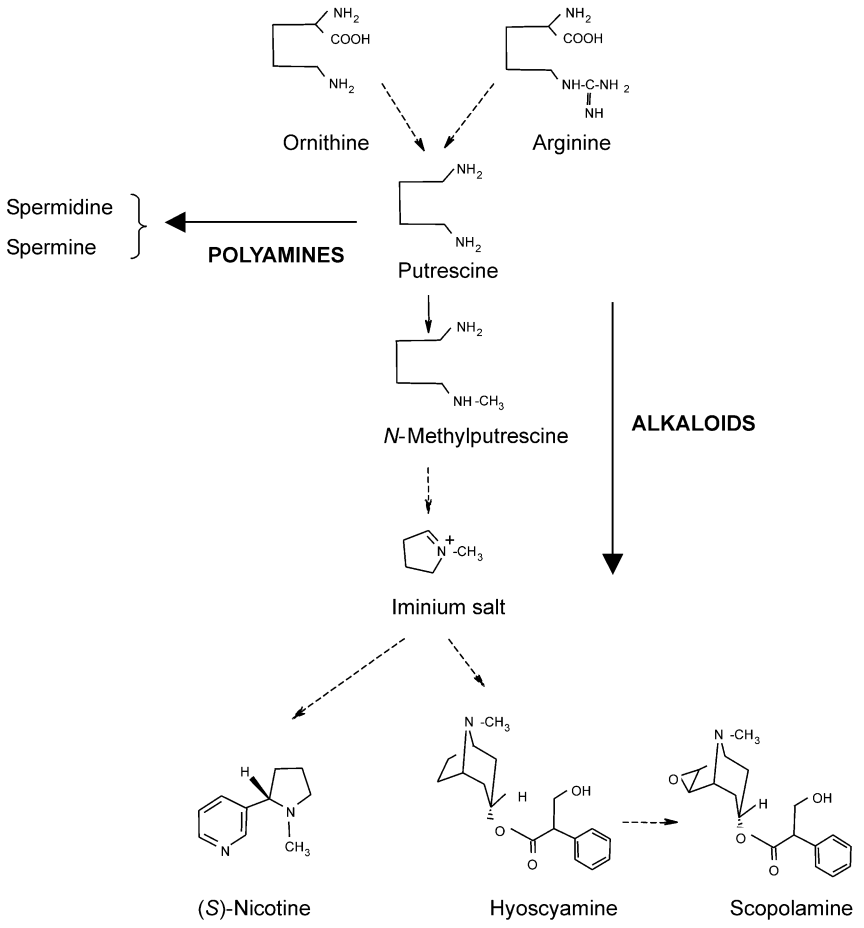

Fig. 1. Schematic biogenesis of tropane and pyridin alkaloids.

nervous system, the worldwide market for scopolamine is currently estimated to be about 10 times that of hyoscyamine and its racemic form, atropine.

A complication in terms of establishing the regulation of tropane and pyridine alkaloid biosynthesis is that the early part of these pathways, as far as putrescine, is shared with polyamine metabolism, putrescine not only being a polyamine precursor, but also of tropane and pyridine alkaloids (Guggisberg and Hesse, 1983; Hashimoto et al., 1989; Robins et al., 1991; Imanishi et al., 1998). Putrescine:SAM *N*-methyltransferase (PMT; EC.2.1.1.53) is the enzyme involved in the removal of putrescine from the polyamine pool since it catalyses the *N*-methylation of this diamine to form *N*-methylputrescine, the first specific precursor of both tropane and pyridine-type alkaloids (Fig. 1). *Atropa belladonna* and *Nicotiana sylvestris* PMT overexpressing plants have been already produced by Sato et al. (2000). These authors reported an opposite effect for the transgene expression, since no changes in *Atropa* alkaloid content were observed, while nicotine content significantly increased in *N. sylvestris* leaves.

Previous experiments with *Datura* hairy roots showed that the transcription of the gene that codes for PMT is sensitive to the culture media conditions in which the roots grow (Robins et al., 1991; Piñol et al., 1999). According to Robins et al. (1991), it seems that PMT synthesis ceases or is diminished in response to any stress that may induce an alteration in polyamine metabolism. Hence, PMT appears to be flux-limiting and consequently a very good candidate for genetic manipulation.

In this work, the *Nicotiana tabacum* PMT gene has been inserted into hairy roots of a hybrid of *Duboisia* under the control of CaMV 35S promoter, and the morphology, growth rate and alkaloid production capacity of these engineered hairy roots have been compared with the respective ones of wild-type hairy roots. Moreover, in order to contribute to the understanding of the biosynthetic processes that take place in the root, the *N*-methylputrescine and free polyamine levels have been determined and compared in both types of hairy roots considered.

2. Results and discussion

The disarmed *A. tumefaciens* C58C1 strain carrying the pRiA4 of *A. rhizogenes* and the binary vector pBMI (Fig. 2) was used to infect leaf sections of a *Duboisia*

hybrid. The binary vector pBMI contained the *pmt* gene of *N. tabacum* under the control of CaMV35S promoter and the *npt*II gene in its T-DNA region. In order to produce kanamycin-resistant hairy roots with the *pmt* transgene, co-integration of the *rol* genes from pRiA4 and the engineered genes of T-DNA from pBMI into the genome of *Duboisia* hybrid cells was carried out. Kanamycin-resistant hairy root lines were obtained and cultured in hormone-free, half strength B5 solid medium (Gamborg et al., 1968) without antibiotics. When cultured, some hairy root lines turned brown and aged considerably faster than *Duboisia* hybrid wild-type hairy root lines. These lines were discarded for further studies and the remaining hairy root lines (more than 30), named PMT lines, were subcultured for 4–6 weeks in hormone-free, half-strength B5 liquid medium.

The presence of *pmt* transgene in the genome of hairy roots was checked by polymerase chain reaction (Fig. 3). The DNA of the wild-type hairy roots (A1) did not show any amplified material. However, the putative engineered hairy roots tested (PMT), as well as the plasmid pBMI containing the tobacco PMT cDNA (A2), all gave a band of 451 bp size corresponding to the *pmt* gene fragment, which consequently confirmed the presence of the transgene in the hairy root lines considered. On the other hand, in order to verify the expression of the gene which codes for the PMT enzyme, samples of total RNA were obtained from each of the transformed root lines established at week 1, 2, 3 and 4 of the culture. These total RNA samples were analysed by means of hybridisation with specific probe, the NcoI-BamHI fragment of 1.4 kb corresponding to the cDNA for the gene which codes for tobacco PMT (Fig. 4). All root lines tested carrying 35S-*pmt* gene showed the presence of the corresponding mRNA in the Northern blot analysis. The results confirmed the capacity of the transgene to be expressed in the transformed plant cells.

Comparing growth capacity of different transformed root lines carrying *pmt* gene (more than 10). in relation to wild type transformed roots (Fig. 5a), no significant differences ($P > 0.05$) were observed. In fact, both types of roots similar growth rate (fr. wt roots/fr. wt inoculum) achieved at week four. Furthermore, transformed roots whether carrying *pmt* gene or not showed similar time course of growth and both root types achieved

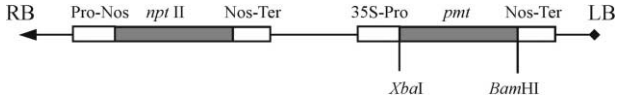

Fig. 2. Schematic representation of the pBMI plasmid.

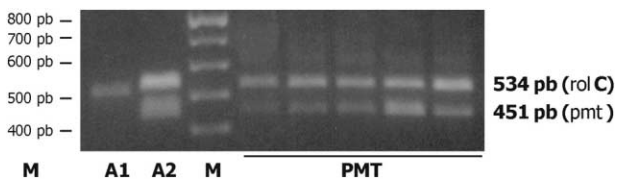

Fig. 3. PCR amplified DNA fragments of *rol*C and *pmt* genes. (A1): DNA of *Agrobacterium rhizogenes* (pRiA4). (A2): DNA of *A. tumefaciens* C58C1 (pRiA4, pBMI). M: Smartladder (400–800 pb). PMT: transformed roots induced by *A. tumefaciens* C58C1 strain (pRiA4, pBMI).

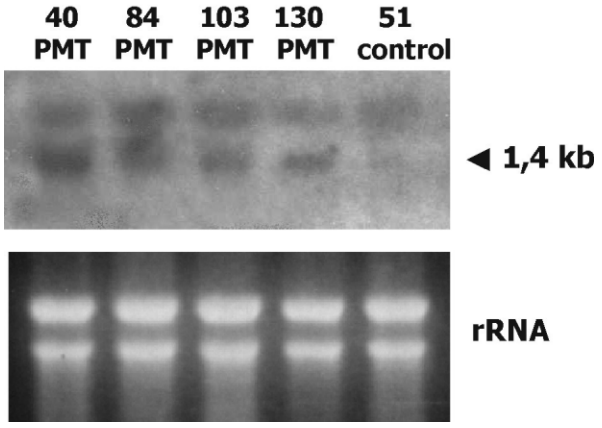

Fig. 4. Autoradiograph of Northern blot analysis using as probe a fragment of NcoI-Bam HI of 1.4 kb corresponding to the cDNA for the gene which codes for tobacco PMT. Each line contains 10 μg of the total RNA fraction. Below: ribosomic RNA fraction. PMT: transformed roots induced by *A. tumefaciens* C58C1 strain (pRiA4, pBMI). Control: transformed roots induced by *A. rhizogenes* A4 strain (pRiA4).

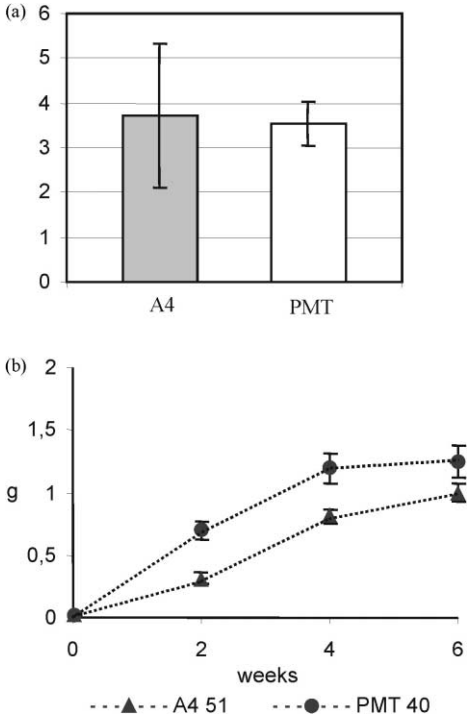

Fig. 5. (a) Growth rate (fr. wt of roots/fr. wt of inoculum) of transformed root cultures at the end of culture period (28 days). Each value is the mean of 20–30 determinations ± DS. (b) Time course of growth (fr. wt) of two randomly selected root lines (A4 51, PMT 40). Each value is the mean of 3–5 determinations ± DS.

maximum fresh weight at week six, corresponding to the end of the culture period (Fig. 5b).

Fig. 6a shows the capacity of transformed root lines to biosynthesise hyoscyamine and scopolamine, the main tropane alkaloids of *Duboisia* roots (Moyano et al., 1999). As can be deduced from our results, the presence of *pmt* tobacco gene in the plant root genome did not significantly ($P > 0.05$) modify the alkaloid content of transformed root lines. The content of the pyridine alkaloid nicotine of transformed roots was not significantly affected by tobacco *pmt* gene over-expression either (see Fig. 6a). In all cases, high standard deviation was observed due to the high variability of alkaloid production among the root lines studied (more than 10). This fact is very usual in transformed root cultures (Moyano et al., 1999; Jouhikainen et al., 1999), due to the fact that each root line is the consequence of an exclusive transformation event.

On the other hand, time course productions of *Duboisia* alkaloids of PMT and wild-type transformed roots were very similar (Fig. 6b), and both kinds of roots achieved maximum alkaloid content at the end of culture period. All these results suggest the ineffectiveness of tobacco *pmt* gene over-expression in order to increase tropane or pyridin alkaloids in transformed roots of *Duboisia* hybrid. These results are in contrast to that reported for *Hyoscyamus muticus* and *Datura metel* hairy roots over-expressing the same gene (Moyano et al., in press). In both cases hairy roots carrying *pmt* gene accumulated higher tropane alkaloids than wild type hairy roots.

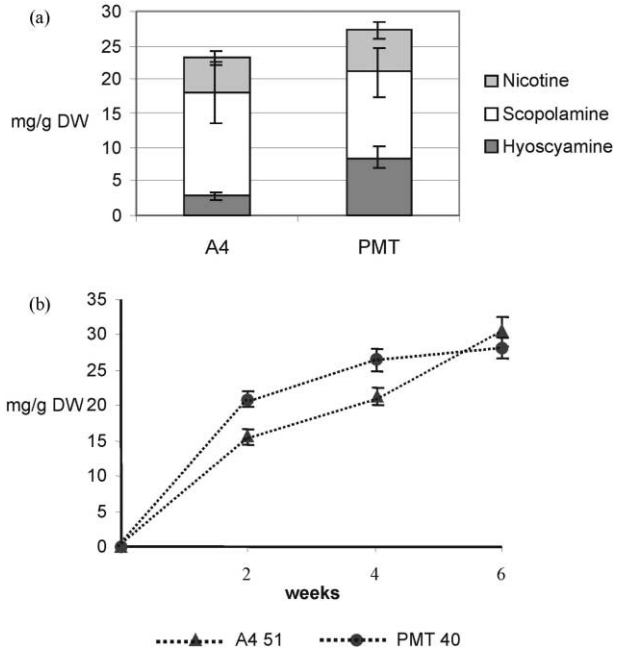

Fig. 6. (a) Alkaloid production (mg/g dry wt) of transformed root cultures at the end of culture period (28 days). Each value is the mean of 20–30 determinations ± DS. (b) Time course of total alkaloid production (mg/g dry wt) of two randomly selected root lines (A4 51, PMT 40). Each value is the mean of 3–5 determinations ± DS.

In this work we also determined *N*-methylputrescine levels of the hairy root cultures taken under consideration. This compound is directly biosynthesised by the action of the enzyme encoded by the *pmt* gene (Fig. 1), and represents the first specific precursor of tropane and pyridin alkaloids in several plant species including *Duboisia* spp. (Oksman-Caldentey and Arroo, 2000). Fig. 7 shows how in hairy roots over-expressing *pmt* tobacco gene, higher levels of *N*-methylputrescine were reached in comparison with control roots. In fact, an increase of about 2–4-fold was observed, the average content of wild type being 600 pmol/mg dry wt and that of PMT roots of 1600 pmol/mg dry wt. In order to investigate whether or not an increase in methylputrescine levels could lead to an alteration of free putrescine, spermidine and the spermine endogenous pool, we analysed the content of these polyamines. In fact, the diamine putrescine, is a common precursor for the biosynthesis of polyamines, tropane and pyridinic alkaloids and obviously, 35S-*pmt* gene overexpression could induce a deviation of polyamine metabolic flux towards methyputrescine biosynthesis (Fig. 1). In this case, no relevant changes were observed in the endogenous levels of free putrescine, spermidine and spermine. These results suggest that in *Duboisia* roots the level of putrescine was not rate-limiting in polyamine metabolism. Hence, the increase of *N*-methylputrescine level could be due to a "feed-back enhanced" de novo biosynthesis of putrescine in transformed root tissues of *Duboisia*.

As we mentioned above, the increase of *N*-methylputrescine in *Duboisia* roots carrying *pmt* transgene was not converted into the alkaloids hyoscyamine, scopolamine and nicotine. Our results showed that the increase of *pmt* gene expression did not significantly affect alkaloid production and suggested that in *Duboisia* transformed roots, alkaloid biosynthesis is downstream limited rather than at the step corresponding to the conversion of putrescine into methylputrescine. These facts are contrary

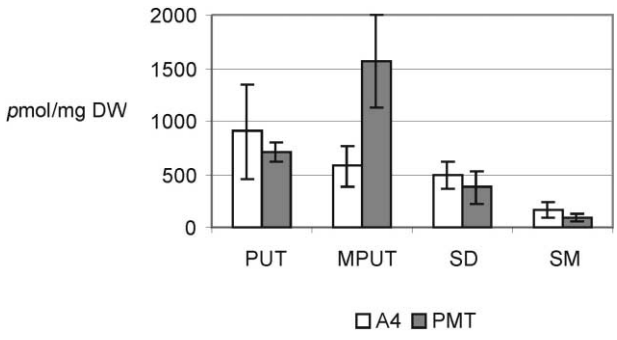

Fig. 7. Polyamine contents (pmol/mg DW) of transformed root cultures at the end of culture period (28 days) PUT: putrescine, MPUT: methylputrescine, SD: spermidine, SM: spermine. Each value is the mean of 12 determinations ± DS.

to previous results obtained by Moyano et al. (2002). These authors working with *Hyoscyamus muticus* and *Datura metel*, two plant species also known to produce tropane alkaloids, obtained transformed root cultures able to biosynthesize higher alkaloid levels. Nevertheless, Sato et al. (2000) reported an unchanged alkaloid profile, for *Atropa belladonna* PMT overexpressing roots, in addition to an increase in the endogenous polyamine pool in the whole plant. The authors suggested how PMT expression in the roots was not sufficient to boost tropane alkaloid synthesis in this Solanaceae plant. On the other hand, PMT overexpression in *N. sylvestris* led to an increase in nicotine level, followed by a slight decrease in spermidine and spermine content.

Taking together all this evidence, we suggest that the plant response to *pmt* overexpression seems to be species-related. This could be due to a different, specific post-translational regulation of the endogenous enzyme in respect to the foreign one.

3. Experimental

3.1. PTM expression vector construction

DNA manipulation was performed according to the method of Sambrook et al. (1989). We have used the cDNA encoding PMT from *N. tabacum* cloned in pTVPMT, kindly provided by T. Hashimoto (Nara Institute of Science and Technology, Kyoto, Japan). The 1400 bp *Nco*I-*Bam*HI fragment of the cDNA insert was isolated from pTVPMT and subcloned in pET-3d (Novogen). The plasmid pET-3d was used first because the strong expression vector for plants pRoc2275-C (obtained from J. Brevet, ISV-CNRS, Gif sur Ivette, Paris, France) does not have a *Nco*I site. The resulting plasmid pET-3d-PMT was introduced into competent cells of *E. coli* DH5α strain following the method described by Hanahan (1983). The 1439 bp *Xba*I-*Bam*HI fragment of the cDNA insert was isolated from pET-3d-PMT and subcloned into the plasmid pRoc2275-C between the CaMV 35S promoter and the nopaline synyhase terminator. The resulting binary vector referred to as pBMI also contained, the neomycin phosphotransferase gene (*npt*II) driven by the nopaline synthase promoter in its T-DNA region (Fig. 2). This binary vector was maintained in *E. coli* DH5α and then mobilised to disarmed *A. tumefaciens* strain C58C1 also containing de pRiA4 of A. *rhizogenes* as described by Mozo and Hooykaas (1991).

3.2. Transformation and root culture

Leaves of *Duboisia* plantlets (*Duboisia myoporoides*×*D. leichhardtii* clones) grown in vitro on MS medium (Murashige and Skoog, 1962) were used for transformation with strain A4 of *Agrobacterium rhizogenes* and the disarmed *A.tumefaciens* strain C58C1 carrying the pRiA4 of *A. rhizogenes* and the binary vector pBMI (Fig. 2). The transformed roots developed at wound sites were excised and cultured individually on solid, half-strength B5 medium (Gamborg et al., 1968) (B5/2, medium diluted to half that of normal concentration of salts) supplemented with 30 g/l sucrose. Root clones were kept in the dark at 26 °C and routinely subcultured every 25–30 days. Liquid root cultures were established from both transformed and transgenic roots. About 100 mg fr. wt of 3 cm length were inoculated into 100 ml "Baby food" flasks (SIGMA) containing 40 ml of liquid half-strength B5 medium, capped with Magenta B-cap (SIGMA) and placed in a rotary shaker (100 rpm) at 25 °C in the dark.

3.3. Polymerase chain reaction analysis

The presence of the *pmt* gene in root tissues was analysed by PCR. Genomic DNA was extracted from the putative engineered hairy root lines and wild-type hairy root lines according to Edwards et al. (1991). The oligonucleotide primers used for amplification of the *pmt* gene were 5′-GCCATTCCCATGAACGGCC-3′ (position 108–127 nt) and 5′-CCTCCGCCGATGATCAAAACC-3′ (position 569–549 nt), according to the sequence of the *pmt* gene from *Nicotiana tabacum* (Hibi et al., 1994). PCR was performed using the pre-formulated, pre-dispensed single-dose reaction beads "Ready to go™" (Pharmacia Biotech). The complete PCR mixture contained 200 ng total DNA, 25 pmol of each oligonucleotide primer, 200 μM dNTPs, 1.5 units of Taq DNA polymerase (Pharmacia Biotech.) and buffer supplied by the manufacturer (1/10 V) in a total volume of 25 μl. PCR was carried out on a MJ Research INC machine with the following conditions: 1 cycle of 5 min at 95 °C; 30 cycles of 1 min at 94 °C; 1 min at 62 °C; 1.5 min at 72 °C; 1 cycle of 5 min at 72 °C. Products (10 μl) were analysed on 1.5% agarose/TBE gel where the expected size of the amplified *pmt* fragment was 450 bp.

3.4. Polyamine analysis

Samples were extracted in 10 volumes of 4% cold perchloric acid and centrifuged at 20,000 *g* for 30 min at 4 °C. Triplicates of the supernatant were dansylated, toluene-extracted and analysed by HPLC (Jasco, Groβumstad, Germany) according to Tassoni et al. (2000). Standard polyamines were subjected to the same procedure.

3.5. Alkaloid analysis

The extraction and determination of scopolamine and hyoscyamine were carried out as reported by Piñol et al. (1996). Nicotine levels were determined according to the method described by Saunders and Blume (1981) with

minor modifications (Palazón et al., 1995). Alkaloid levels were determined both in the roots and in the culture medium.

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