Induction of Tropane Alkaloid Formation in Transformed Root Cultures of *Brugmansia suaveolens* (Solanaceae)

Rawia Zayed[b] and Michael Wink[a,*]

[a] Institut für Pharmazie und Molekulare Biotechnologie, Universität Heidelberg, INF 364, D-69120 Heidelberg, Germany. Fax: 06221-544884. E-mail: wink@uni-hd.de
[b] Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig 44519, Egypt

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c**, 863–867 (2004); received June 16/July 8, 2004

Hairy root cultures of *Brugmansia suaveolens* were set up by infection of root tips with *Agrobacterium rhizogenes*. The successful transformation was confirmed by analysing rolC and virC genes using polymerase chain reaction (PCR). Hairy root cultures were employed to study the formation of tropane alkaloids, such as hyoscyamine. The transformed cultures were incubated with potential elicitors, such as methyljasmonate, quercetin and salicylic acid in order to stimulate the biosynthesis of tropane alkaloids. Profile and amounts of tropane alkaloids were analysed using capillary GLC-MS. At least 18 different tropane alkaloids could be identified. Treatment of the cultures with 200 μM methyljasmonate increased the alkaloid accumulation 25-fold up to a level of 1 mg/g fresh weight as compared to untreated controls. Quercetin enhanced the alkaloid production 10 fold (0.4 mg/g fresh weight) within 24 h. In contrast 100 μM salicylic acid decreased alkaloids to a level of 1 μg/g fresh weight.

Key words: Brugmansia suaveolens, Tropane Alkaloids, Elicitation

Introduction

Datura and *Brugmansia* are closely related genera that produce tropane alkaloids (Gemeinholzer and Wink, 2001). Perennial and often woody plants are included in the genus *Brugmansia* whereas herbaceous annual plants belong to the genus *Datura*. *Datura* and *Brugmansia* produce tropane alkaloids, such as hyoscyamine and scopolamine as main defence compounds. Tropane alkaloids are also typical secondary metabolites of several other solanaceous plants of the genera *Hyoscyamus, Atropa, Scopolia, Mandragora*, and *Duboisia*. Tropane alkaloids inhibit the muscarinergic acetylcholine receptor and show parasympatholytic properties. As such they are used in medicine to treat spasms, to sedate patients and for dilatation (mydriasis) of the pupil. Furthermore, tropane alkaloids affect neuronal activities and are known hallucinogens (for review: Roberts and Wink, 1998; Wink, 2001). *Brugmansia* has been mainly employed externally to treat rheumatic and arthritic pains, swelling, scalds, inflammations, skin rashes, haemorrhoids and wounds. Its extracts exhibit spasmolytic, antiasthmatic, anticholinergic, narcotic and anaesthetic properties.

Most alkaloids are important for the fitness and survival of the plants producing them since they help to protect against herbivores and/or infecting microorganisms (Harborne, 1993; Wink, 1988, 1999a, b). The alkaloid formation is not static but under the regulation of internal and external factors. Upon attack or infections, secondary metabolism is enhanced in many plants; either the biosynthesis of new compounds (phytoalexins) takes place or the concentration of already existing compounds is increased (reviews in Wink, 1999a, b).

Jasmonic acid and its ester methyljasmonate have been found to be elements of a signal pathway leading to the induction of secondary metabolites involved in defence against herbivores and microorganisms. Fungal cell-wall elicitors and methyljasmonate can induce secondary metabolism in soybean cell cultures by different mechanisms (Enyedi *et al.*, 1992). Jasmonates have several biological activities, including promotion of the stomata closure (Horton, 1991), acceleration of leaf senescence in oats and barley, pericarp senescence in soybean fruit, as a potent inducer of tendril coiling in *Bryonia*, and the stimulation of stem length and differentiated root system in potato plantlets (Weidhase *et al.*, 1987; Lopez *et al.*, 1987; Falkenstein *et al.*, 1991; Ravnikar *et al.*, 1992; Reinbothe *et al.*, 1992). Some of these effects are

0939–5075/2004/1100–0863 $ 06.00

apparently mediated by controlling gene expression (Rickauer *et al.*, 1997). Intracellular jasmonates transiently accumulate in cell suspension cultures that have been treated with elicitors implicating a complex physiological role for jasmonates, possibly in the signal transduction system of the defence response (Mueller-Uri *et al.,* 1988; Gundlach *et al.*, 1992). It has been established that treatment of *in vitro* cultures with exogenous methyljasmonate can elicit the accumulation of several classes of alkaloids (Gundlach *et al.*, 1992; Aerts *et al.*, 1996; Zabetakis *et al.*, 1999; Baldwin, 1999).

The alkaloid formation is often tissue-specific. In *Datura, Brugmansia, Atropa, Hyoscyamus* and other plants with tropane alkaloids the site of alkaloid formation is the roots (reviews in Roberts and Wink, 1998; Robins, 1998; Suzuki *et al.*, 1999). Tropane alkaloids are exported from the roots to other plant organs via the xylem. In the sink tissues they are often stored in epidermal cells (reviews in Roberts and Wink, 1998; Wink, 1999a, b).

In this communication we report the establishment of stable hairy root cultures of *Brugmansia suaveolens* via transformation with *Agrobacterium rhizogenes.* These transformed root cultures are valuable and versatile systems for the study of secondary metabolism. Furthermore, the production of tropane alkaloids in hairy root cultures of *Brugmansia suaveolens* was analysed by capillary GLC-MS after treatment with potential inducing compounds, such as methyljasmonate, quercetin and salicylic acid.

Materials and Methods

Plant material

Establishment of transformed root cultures

Hairy root cultures (HRC) of *Brugmansia suaveolens* were initiated by infecting the root tips with *Agrobacterium rhizogenes* strains 15834, TR 105 or LBA (kindly provided by Prof. Dr. W. Alfermann, Düsseldorf). Transformed root cultures were cultivated in hormone-free WP liquid-medium on a gyratory shaker at 110 rpm at 25 °C in an illuminated culture room. Cultures were placed into fresh medium every two weeks. Liquid hormone-free WP medium (Lloyd and McCown, 1980) was employed for subculture, growth and alkaloid production.

Elicitation

HRC were treated by different inducers such as methyljasmonate, quercetin to stimulate the accumulation of tropane alkaloids. Dose-dependant induction experiments were repeated with different concentrations (10, 50, 100, and 200 μM) of methyljasmonate (MJ) in MeOH, quercetin (Q) in DMSO and salicylic acid (SA) in H_2O (Sigma-Aldrich-Chemie, Germany). After different incubation times HRC were harvested by vacuum filtration, weighed and kept at −20 °C until the extraction and analysis by GLC-MS.

Analysis of rolC and virC genes by polymerase chain reaction (PCR)

In order to confirm the transformed nature of the cultures, total DNA was extracted from the root cultures of *Brugmansia suaveolens.* PCR (in a DNA thermal cycler; Biometra TGradient) was employed to show the presence or absence of rolC and virC genes. PCR conditions: 96 °C for 2 min; followed by 36 cycles of 94 °C for 30 s, 60 °C for 30 s; 72 °C for 2 min; and finally 15 min extension at 72 °C. The primers for rolC DNA were 5′-ATGGCTGAAGACGACCTGTGTT-3′ and 5′-TTAGCCGATTGCAAACTT G CAC-3′ (Oono *et al.*, 1993). The primers for virC DNA were 5′-ATCATTTGTAGC GACT-3′ and 5′-AGCTCAA ACCTGCTT C-3′ (Sawada *et al.*, 1995).

Gel-electrophoresis of PCR products from transformed and non-transformed root cultures as well as *from A. rhizogenes* was carried out to show the presence or absence of rolC and virC genes. The results from agarose gel electrophoresis showed that the rolC gene was present in HRC but not in non-transformed root cultures whereas a PCR product corresponding to a fragment of the virC gene occurred only in DNA from *A. rhizogenes.* This finding indicates that the hairy root cultures were not contaminated with *A. rhizogenes* and were actually transformed.

Alkaloid extraction, separation, GLC and GLC-MS

Plant material was homogenized in 1 M HCl, filtered, and the acid aqueous solution was then basified with concentrated NH_4OH and extracted with CH_2Cl_2. The organic phase was filtered, dried with anhydrous Na_2SO_4, filtered again and finally concentrated *in vacuo*. The acid base purification pro-

cedure was repeated three times to give a dark brown semi-solid extract of alkaloids.

The total alkaloid extract was analyzed by capillary GLC and GLC-MS. GLC was performed on a Varian gas chromatograph (3300), equipped with a FID detector and a Spectra Physics Integrator SP4290. Conditions: OV-1 fused silica capillary column (15 m × 0.25 mm), carrier gas He; detection temperature 300 °C; injection temperature 250 °C; split 1:20; oven temperature program: initial temperature 120 °C, 2 min isothermal, increased 10 °C min^{-1} to 300 °C. For GLC-MS a OV-1 fused silica capillary column (30 m × 0.25 mm) was used coupled to a quadropole Finnigan Mat 4515 mass spectrometer. EI-MS were recorded at 40 eV and evaluated with the INCOS DATA SYSTEM. Conditions: carrier gas He; splitless injection; temperature 250 °C; oven temperature program: initial temperature 120 °C, 3 min isothermal, 8 °C min^{-1} to 300 °C. CIMS was performed using NH_3 as a reactant gas under the following conditions: split 1:5, oven temperature program: 120 °C, 3 min isothermal, 120 °C–138 °C, 6 °C min^{-1}, 138 °C–300 °C, 6 °C min^{-1}. The Kovats retention indices (RI) were calculated by co-injection with standard hydrocarbons (C_{12}–C_{28}) and compared with reference RI values stored in a data base of the Institut für Pharmazie und Molekulare Biotechnologie, Heidelberg.

Table I. GLC-MS analysis of tropane alkaloids in hairy root cultures of *Brugmansia suaveolens*. Base peaks are printed in bold.

No.	Compound	RI*	M^+ (%)	Characteristic ions (rel. int.%)
1	Tropine	1167	141(26)	124(22), 113(14), 96(64), 83(52), **82(100)**, 67(2), 55(18), 42(68)
2	Pseudotropine	1185	141(26)	124(23), 113(15), 96(62), 83(52), **82(100)**, 67(2), 55(18), 42(68)
3	Scopoline	1255	155(36)	126(12), 110(3), 96(64), 81(20), 70(14), 57(28), 42(100)
4	Scopine	1285	155(25)	110(13), 94(17), 82(20), 68(18), 57(10), **42(100)**
5	3α-Acetoxy tropane	1303	183(28)	140(10), **124(100)**, 96(26), 94(42), 83(24), 82(56), 67(15), 42(52)
6	3-Acetoxy-6-hydroxytropane	1495	199(22)	155(4), 140(20), 122(2), 95(54), **94(100)**, 82(8), 43(2), 42(60)
7	3α-Tigloyloxytropane	1643	223(20)	208(4), 140(9), **124(100)**, 96(18), 94(34), 83(32), 82(52), 42(50)
8	Cuscohygrine	1650	224(,4)	223(2), 209(6), 141(12), 140(23), 127(8), 98(10), **84(100)**
9	3-Hydroxy-6-(2-methyl butyryloxy)-tropane	1725	241(14)	140(11), 122(8), **113(100)**, 96(31), 94(22), 82(16), 57(28), 42(39)
10	3-Tigloyloxy-6-hydroxytropane	1825	239(12)	195(4), 140(26), 112(4), 95(64), **94(100)**, 83(2), 55(21), 42(30)
11	3-Hydroxy-6-tigloyloxytropane	1830	239(20)	156(8), 122(10), **113(100)**, 96(38), 94(28), 83(11), 55(28), 42(26)
12	Apoatropine	2028	271(44)	140(10), **124(100)**, 103(28), 95(39), 94(28), 83(4), 82(59), 42(28)
13	3-Tigloyloxy-6-(2-methylbutyryloxy)-tropane	2093	323(5)	240(3), 224(8), 138(5), 122(18), **95(100)**, 94(95), 83(4)
14	Aposcopolamine	2130	285(78)	154(30), 138(76), 136(32), 108(44), 103(74), **94(100)**, 81(36), 42(95)
15	Hyoscyamine	2170	289(22)	140(8), **124(100)**, 96(20), 94(14), 83(16), 82(28), 67(12), 42(24)
16	3α,6β-Ditigloyloxytropane	2220	321(2)	238(1), 222(2), 122(8), **94(100)**, 83(4), 55(10)
17	7β-Hydroxyhyoscyamine	2335	305(16)	261(11), 140(32), 95(78), **94(100)**, 82(5), 57(4), 42(30)
18	6β-Hydroxyhyoscyamine	2355	305(9)	261(8), 140(18), 95(5), **94(100)**, 82(4), 57(2), 42(18)

* Kovats retention indices.

Results and Discussion

Analysis of alkaloid profiles of hairy root cultures by GLC-MS

The tropane alkaloid profiles of hairy root cultures of *Brugmansia suaveolens* were investigated by capillary GLC and GLC-MS. The alkaloids were identified in comparison with the reported data (Evans and Major, 1968; Evans and Lampard, 1972; Witte *et al.*, 1987; Ionkova *et al.*, 1994; Philipov and Berkov, 2002; Berkov *et al.*, 2003). The root cultures of *Brugmansia suaveolens* contain a complex mixture of more than 18 alkaloids. Hyoscyamine and tropine figure as the major alkaloids followed by hygrine, cuscohygrine, homatropine and many other alkaloids as minor alkaloids (Table I).

Induction of alkaloid accumulation

Methyljasmonate and salicylic acid are involved in signal transduction and induce the transcription of biosynthetic enzymes involved in the formation of defence compounds in plants (review in Baldwin, 1999). Since MJ can induce the formation of secondary metabolites in other systems, we have tried to stimulate the accumulation of tropane alkaloids in hairy root culture of *Brugmansia suaveolens* by adding MJ and SA.

The hairy root cultures of *Brugmansia suaveolens* were treated with MJ. After 24 h of incubation the alkaloids were extracted and determined quantitatively by GLC. MJ (at a concentration of 200 μM) induced the accumulation of hyoscyamine by approximately 25-fold as compared to the untreated control. The highest stimulation resulted in a hyoscyamine level of 1 mg/g fresh weight (FW) after 24 h of incubation (Table II). There was little or no diffusion of the alkaloids into the medium (data not shown). The induction can be suppressed by pre-incubation of the cells with salicylic acid (Fig. 1). Also SA alone led to a decrease of alkaloid contents after incubation for 24 h (Table II). Also SA alone (100 μM SA) reduced hyoscyamine to 1 μg/g FW (Table II). SA has been reported to interfere with the jasmonic acid pathway in other plants (Pena-Cortes *et al.*, 1993; Doares *et al.*, 1995; O'Donnell *et al.*, 1996).

Also the flavonoid quercetin, that had been used as an inductor in *Sanguinaria canadensis* cultures (Mahady and Beecher, 1994), enhanced hyoscyamine accumulation approximately 10-fold (Table II). The maximum level (with 100 μM quercetin) was 0.3–0.4 mg/g FW hyoscyamine. If cultures were pretreated with SA, this induction was significantly suppressed (Fig. 1). We assume that SA inhibits oxidation steps of the jasmonic acid pathway. This observation indicates that quercetin probably triggers the tropane alkaloid formation via the jasmonate induction pathway. Our experiments with *Brugmansia suaveolens* are another example for the power of methyljasmonate to induce the formation of alkaloids. Since the production of tropane alkaloids in *in vitro* cultures is of biotechnological importance, the elicitation via MJ offers a chance to improve yields.

Table II. Hyoscyamine concentration in hairy root cultures of *Brugmansia suaveolens* after incubation for 24 h with different concentrations of methyljasmonate (MJ), quercetin (Q) and salicylic acid (SA). The values are given as the mean ± SD.

Elicitor concentration	Hyoscyamine content [μg/g FW] MJ	Q	SA
Control	35.8 ± 6.96	38.2 ± 5.1	45.9 ± 11.2
10 μM	874.3 ± 83.4	3.73 ± 0.2	48.8 ± 10.8
50 μM	347.6 ± 22	10.9 ± 1.7	1.67 ± 0.7
100 μM	757 ± 60.1	393.3 ± 23.2	0.9 ± 0.7
200 μM	1047.6 ± 187	2.67 ± 0.5	1.83 ± 0.2

Acknowledgements

We gratefully acknowledge Mrs A. Backhaus for GLC-MS measurements and Mrs H. Staudter for maintaining the tissue cultures. The first author thanks Prof. Dr. M. El-Dakakny, Prof. Dr. T. Sarg and Prof. Dr. A. Deiab for kindly reading of the manuscript.

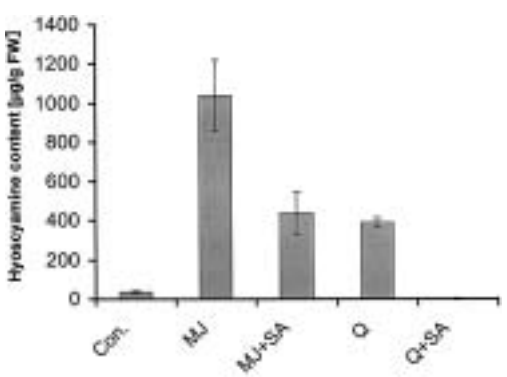

Fig. 1. Effect of salicylic acid (SA) on the elicitation of hyoscyamine by methyljasmonate and quercetin. SA (100 μM) was added to the culture 1 h before the addition of methyljasmonate (MJ) or quercetin (Q). Con. = untreated control.; means ± SD.

Aerts R. J., Shafer A., Hesse M., Baumann T. W., and Slusarenko A. (1996), Signaling molecules and the synthesis of alkaloids in *Catharanthus roseus* seedlings. Phytochemistry **42**, 417–422.

Baldwin I. (1999), The jasmonate cascade and complexity of induced defence against herbivore attack. In: Function of Plant Secondary Metabolites and their Exploitation in Biotechnology. Annual Plant Reviews, 3, (Wink, M., ed.). Sheffield Academic Press, Sheffield, pp. 155–186.

Berkov S., Pavlov A., Kovatcheva P., Stanimirova P., and Philipov S. (2003), Alkaloid spectrum in diploid and tetraploid hairy root cultures of *Datura stramonium*. Z. Naturforsch. **58c**, 42–46.

Doares S. H., Narvaez-Vasquez J., Conconi A., and Ryan C. A. (1995), Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol. **108**, 1741–1746.

Enyedi A. J., Yalpani N., Silverman P., and Raskin I. (1992), Signal molecules in systemic plant resistance to pathogens and pests. Cell **70**, 879–886.

Evans W. and Major V. (1968), The tropane alkaloids of genus *Datura*, section *Brugmansia*. Part V. Alkaloids of *D. sanguinea* R., and P., and related esters of tropane-3α,6β,7β-triol. J. Chem. Soc. 2775–2778.

Evans W. and Lampard J. (1972), Alkaloids of *Datura suaveolens*. Phytochemistry **11**, 3293–3298.

Falkenstein E., Groth B., Mithofer A., and Weiler E. W. (1991), Methyljasmonate and linolenic acid are potent inducers of tendril coiling. Planta **185**, 316–322.

Gemeinholzer B. and Wink M. (2001), Solanaceae: Occurrence of secondary compounds versus molecular phylogeny. In Solanaceae V: Advances in Taxonomy and Utilization. (van den Berg R. G., Barendse G. W. M., van der Weerden G. M., and Mariani C., eds.). Nijmegen University Press, pp. 165–178.

Gundlach H., Muller M. J., Kutchan T. M., and Zenk M. H. (1992), Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA **89**, 2389–2393.

Harborne J. (1993), Introduction to Ecological Biochemistry. Academic Press, London.

Horton R. F. (1991), Methyljasmonate and transpiration in barley. Plant Physiol. **96**, 1376–1378.

Ionkova I., Witte L., and Alfermann A. W. (1994), Spectrum of tropane alkaloids in transformed roots of *Datura innoxia* and *Hyoscyamus* × *gyorffyi* cultivated *in vitro*. Planta Med. **60**, 382–384.

Lloyd G. and McCown B. (1980), Commercially feasible micropropagation of mountain Laurel, *Kalmia latifolia*, by use of shoot tip culture. Intl. Plant Prop. Soc. Proc. **30**, 42–427.

Lopez R., Dathe W., Bruckner C., Miersch O., and Sembdner G. (1987), Jasmonic acid in different parts of the developing soyabean fruit. Biochem. Physiol. Pflanzen **182**, 195–201.

Mahady G. and Beecher C. (1994), Quercetin-induced benzophenanthridine alkaloid production in cell suspension cultures of *Sanguinaria canadensis*. Planta Med. **60**, 553–557.

McCrown B. H. and Lloyd G. B. (1982), A survey of the response of Rhododendron to *in vitro* culture. Plant Cell Tissue and Organ Culture **2**, 77–85.

Mueller-Uri F., Parthier B., and Nover L. (1988), Jasmonate-induced alteration of gene expression in barley leaf segments analysed by *in vivo* and *in vitro* protein synthesis. Planta **176**, 241–247.

O'Donnell P. J., Calvert C., Atzorn R., Wasternack C., Leyser H. M., and Bowles D. J. (1996), Ethylene as a signal mediating the wound response of tomato plants. Science **274**, 1914–1917.

Oono Y., Suzuki T., Toki S., and Uenimtya H. (1993), Effects of the over-expression of the rolC gene on leaf development in transgenic periclinal chimeric plants. Plant Cell Physiol. **34**, 745–752.

Pena-Cortes H., Albrecht T., Prat S., Weiler E., and Willmitzer L. (1993), Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta **191**, 123–128.

Philipov S. and Berkov S. (2002), GC-MS Investigation of tropane alkaloids in *Datura stramonium*. Z. Naturforsch. **57c**, 559–561.

Ravnikar M., Vilhar B., and Gogala N. (1992), Stimulatory effects of jasmonic acid on potato stem node and protoplast culture. J. Plant Growth Regul. **11**, 29–33.

Reinbothe S., Reinbothe C., Lehmann J., and Parthier B. (1992), Differential accumulation of methyljasmonate-induced mRNAs in response to abscisic acid and desiccation in barley (*Hordeum vulgare*). Physiol. Plant. **86**, 49–56.

Rickauer M., Brodschelm V., Bottin A., Véronési C., Grimal H., and Esquerré-Tugayé T. (1997), The jasmonate pathway is involved differentially in the regulation of different defence responses in tobacco cells. Planta **202**, 155–162.

Roberts M. F. and Wink M. (1998), Alkaloids: Biochemistry, Ecology and Medicinal Applications. Plenum Press, New York.

Robins R. (1998), The biosynthesis of alkaloids in root cultures. In: Alkaloids (Roberts M. and Wink M., eds.). Plenum Press, New York, pp. 199–217.

Sawada H., Leki H., and Matsuda L. (1995), PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. Appl. Environ. Microbiol. **61**, 828–831.

Suzuki K.-I., Yun D.-J., Chen X.-Y., Yamada Y., and Hashimoto T. (1999), An *Atropa belladonna* hyoscyamine 6β-hydroxylase gene is differentially expressed in root pericycle and anthers. Plant Mol. Biol. **40**, 141–152.

Weidhase R. A., Kramell H.-M., Lehmann J., Liebisch W., Lerbs W., and Parthier B. (1987), Methyljasmonate-induced changes in the polypeptide pattern of senescing barley leaf segments. Plant Sci. **51**, 177–186.

Wink M. (1988), Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivors. Theor. Appl. Gen. **75**, 225–233.

Wink M. (1999a), Biochemistry of Plant Secondary Metabolism. Annual Plant Reviews, 2. Sheffield Academic Press, Sheffield.

Wink M. (1999b), Function of Plant Secondary Metabolites and their Exploitation in Biotechnology. Annual Plant Reviews, 3. Sheffield Academic Press, Sheffield.

Wink M. (2001), Interference of alkaloids with neuroreceptors and ion channels. In: Bioactive Natural Products (Atta-ur-Rahman, ed.). Elsevier, Amsterdam, pp. 1–127.

Witte L., Müller K., and Alfermann W.-A. (1987), Investigation of the alkaloid pattern of *Datura innoxia* plants by capillary gas-liquid-chromatography-mass-spectrometry. Planta Med. **53**, 192–197.

Zabetakis I., Edwards R., and O'Hagan D. (1999), Elicitation of tropane alkaloid biosynthesis in transformed root cultures of *Datura stramonium*. Phytochemistry **50**, 53–56.