Alkaloid Spectrum in Diploid and Tetraploid Hairy Root Cultures of *Datura stramonium*

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Hairy root cultures were obtained from diploid and induced tetraploid plants of *Datura stramonium* and analyzed by gas chromatography/mass spectrometry. Twenty alkaloids (19 for diploid and 9 for tetraploid hairy root cultures) were identified. A new tropane ester 3-tigloyloxy-6-propionyloxy-7-hydroxytropane was identified on the basis of mass spectral data. Hyoscyamine was the main alkaloid in both diploid and tetraploid cultures. In contrast to diploid hairy roots, the percentage contributions of the alkaloids, with exceptions for hyoscyamine and apoatropine, were higher in the total alkaloid mixture of tetraploid hairy roots.

Key words: Alkaloids, Polyploidy, Hairy Roots

Introduction

The genus *Datura* produce a great range of tropane alkaloids (Lounasmaa and Tamminen, 1993). Two of them – hyoscyamine and scopolamine are important for pharmaceutical industry.

Recently, there has been considerable interest in the production of active compounds by genetically transformed root cultures, so called hairy roots. Many factors have been investigated with respect to biosynthesis and accumulation of tropane alkaloids in hairy root cultures of *Datura* plants (Payne *et al.*, 1987; Robins *et al.*, 1990; Hilton and Rhodes, 1993; Zabetakis *et al.*, 1999). To our knowledge, there are no data about the influence of genome (ploidy level) on biosynthesis of secondary metabolites in genetically transformed root cultures. It is well known that polyploid plants have altered morphological, physiological and biochemical features than their diploid progenitors (Stebbins, 1971).

Present study is a part of investigations on the effects of chromosome number on alkaloid biosynthesis in hairy root cultures. We report our results on alkaloid spectrum of diploid and tetraploid genetically transformed root cultures of *Datura stramonium* L.

Material and Methods

Plant material

The initial diploid cytotype (2n = 2x = 24), collected from natural habitats in the vicinities of Lovech – Bulgaria, was polyploidized with colchicine solution after the method of Pundir *et al.* (1983). The ploidy of plants was determined by chromosome counting in squashed root tips stained with haematoxylin after Gomori (Melander and Wingstrad, 1953). An autotetraploid line (2n = 4x = 48) with high reproductive abilities was isolated from the first colchiploid generation. This line was reproduced for five generations before the induction of tetraploid hairy root cultures.

Bacteria

Agrobacterium rhizogenes ATCC 15834 was grown on YEB medium: 5.0 g/l Difco Bacto beef extract, 5.0 g/l peptone, 1.0 g/l yeast extract, 5.0 g/l sucrose, 0.5 g/l MgSO4, (Chilton *et al.*, 1974) and subcultured at 1-month intervals. Before transformation, the bacteria were cultivated in liquid YEB in flasks (100 ml) with 25 ml medium on a shaker (13 rad/s) at 20 °C for 16 h.

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Establishment of hairy root cultures

Leaves from 6–8 weeks old diploid and tetraploid *D. stramonium* plants were sterilized (15 min) with sodium hypochlorite (2% available chlorine) and 2–3 drops Tween 20, thoroughly rinsed with sterile distilled water and dried on sterile filter paper. The surface sterilized leaves were transferred on hormone-free MS nutrient medium (Dixon, 1985), supplemented with 3% sucrose and 0.8% agar. Leaves were directly infected with bacterial suspension with sterile needle and cultivated at 26 °C under 16 h light and 8 h dark photoperiod. After three days infected leaves were transferred on hormone-free MS nutrient medium, supplemented with 3% sucrose, 0.8% agar and 0.25 g/l claforan (Hoechst A. S., Istanbul/Turkey) and cultivated in the same conditions. Hairy roots appeared four weeks after infection. 20 mm long segments were cut of and transferred on above mentioned medium at 26 °C in the dark and subcultured for 15-day intervals (the first three subcultured were carried out with addition of 0.25 g/l claforan). The obtained *D. stramonium* diploid and tetraploid hairy root cultures were subcultured more than one year before the present investigation. Their chromosome numbers were checked in squashed root tips.

Cultivation of hairy root

D. stramonium hairy roots used in the experiments were cultivated in hormone free liquid MS nutrient medium (Dixon, 1985), supplemented with 3% sucrose. The cultivation was carried out in Erlenmeyer flasks (500 ml) with ⅕ net volume on a shaker (11.6 rad/s) at 26 °C in the dark. For inoculation 1–1.5 g (for each flask) fresh hairy roots, grown on the solid MS medium for 7 days, were used. After a period of 15 days, the hairy roots were collected and dried at 50 °C.

Alkaloid extraction and determination

The root samples of diploid and tetraploid hairy roots, collected from 6 flasks per ploidy level, were dried, ground up with sea sand and macerated in 3% H_2SO_4 for 2 h at room temperature. After filtering and washing of the plant residues with distilled water, the solutions were made basic with 25% NH_4OH (pH 9–10) and applied on Extrelut (Merck) columns. The alkaloids were eluted with CH_2Cl_2 (6 ml/1 g Exrelut) and the solvents were evaporated under reduced pressure. Thus obtained residues were resolved in CH_3OH for further analysis. The tropane alkaloids were analyzed by GC/MS as described in Philipov and Berkov (2002).

Results and Discussion

Two hairy root clones, diploid and tetraploid, with stabile growth and morphological characteristics were isolated. To our knowledge this is the first time that autotetraploid hairy roots of medicinal plants has been reported. The tetraploid hairy roots showed cell aggregate formation on solid MS medium and relatively slower growth in liquid MS medium. Both diploid and tetraploid clones reached stationary phase within 15 days in liquid MS medium during the experiment.

Identification of alkaloids

Capillary gas chromatography/mass spectrometry (GC/MS) is the most suitable and powerful method for rapid separation and identification of complex mixtures of tropane alkaloids (Witte *et al.*, 1987; Christen *et al.*, 1990; Ionkova *et al.*, 1994). Diploid and tetraploid hairy root clones showed different alkaloid composition. Nineteen tropane alkaloids were recognized for diploid and nine for tetraploid roots on the basis of their mass-spectral fragmentation (Table I).

Two alkaloids, D and E were left unidentified. Alkaloid **4** shows M^+ at *m/z* 199. Four tropane alkaloids have the same molecular ion (Lounasmaa and Tamminen, 1993). One of them is C-2 substituted while the rest are C-3, C-3 and C-7 substituted tropanes. The ions at *m/z* 82, 83, 96 and 97 are characteristic for tropine and ecgonine (Blossey *et al.*, 1964). The base peak (at *m/z* 82) indicates C-1–C-2 cleavage of the tropane ring suggesting a C-2 substitution. The presence of ions at *m/z* 182 (M^+-OH), 168 (M^+-OCH_3) and 140 ($M^+-COOCH_3$) indicate that alkaloid **4** is 2-methoxycarbonyl-3-hydroxytropane (methylecgonine, Fig. 1) – a constituent of the aerial parts of *Erythroxylon coca* and *E. novogranatense* (Lounasmaa and Tamminen, 1993). The structure of this compound is confirmed by mass spectrum of the reference compound from database NIST 98.

Table I. Alkaloids identified in diploid (2n) and tetraploid (4n) hairy roots cultures of *Datura stramonium*.

	Rt	M^+	% of the alkaloid mixture		MS Ref.
	[min]		2n	4n	
Hygrine (**1**)	4.14	141	0.79	2.76	Witte *et al.*, 1983
Tropinone (**2**)	5.18	139	0.15	0.34	Ionkova *et al.*, 1994
3-Acetoxytropane (**3**)	7.02	183	2.47	28.43	Witte *et al.*, 1983
Methylecgonine[1] (**4**)	9.10	199	0.48	–	–
3-Hydroxy-6-acetoxytropane (**5**)	9.54	199	0.40	–	Parr *et al.*, 1990
3-Acetoxy-6-hydroxytropane (**6**)	9.62	199	–	1.55	Witte *et al.*, 1983
3,6-Diacetoxytropane (**7**)	10.38	241	1.22	2.54	Parr *et al.*, 1990
3-Tigloyloxytropane (**8**)	11.03	223	0.36	1.18	Witte *et al.*, 1983
3-Tigloyloxy-6-hydroxytropane (**9**)	13.12	239	0.34	–	Witte *et al.*, 1983
3-Hydroxy-6-tigloyloxytropane (**10**)	13.23	239	0.22	–	Witte *et al.*, 1983
3-Tigloyloxy-6-propionyloxy-7-hydroxy-tropane[2] (**11**)	14.02	311	1.04	–	–
Phenylacetoxytropane (**12**)	14.29	259	0.51	–	Ionkova *et al.*, 1994
Apoatropine (**13**)	15.11	271	7.19	5.73	Witte *et al.*, 1983
Alkaloid D (**14**)	16.05	–	0.64	–	–
Hyoscyamine (**15**)	16.53	289	78.75	56.60	Witte *et al.*, 1983
3,6-Ditigloyloxytropane (**16**)	16.68	321	1.14	–	Witte *et al.*, 1983
3-(3′-Acetoxytropoyloxy)tropane (**17**)	17.24	331	0.43	0.87	Philipov and Berkov, 2002
Alkaloid E (**18**)	17.88	319	2.17	–	–
3-Tropoyloxy-6-acetoxytropane[3] (**19**)	18.51	347	0.82	–	–
6-Tigloyloxyhyoscyamine (**20**)	18.80	387	0.89	–	Witte *et al.*, 1983

Mass spectral data of new or unusual alkaloids. GC-MS 70 eV, *m/z* (rel. int.):
[1]M^+ 199 (20), 182 (8), 168 (12), 155 (6), 152 (7), 140 (6), 122 (4), 112 (14), 96 (63), 83 (69), 82 (100), 68 (10), 55 (12), 42 (21).
[2]M^+ 311 (18), 296 (5), 228 (2), 218 (11), 194 (5), 195 (5), 155 (6), 141 (4), 127 (10), 113 (8), 95 (59), 94 (100), 85 (21), 83 (5), 71 (24), 57(18), 55 (12), 43 (12).
[3]M^+ 347 (8), 261 (6), 182 (43), 122 (56), 103 (12), 95 (70), 94 (100), 82 (16), 55 (24), 43 (28).

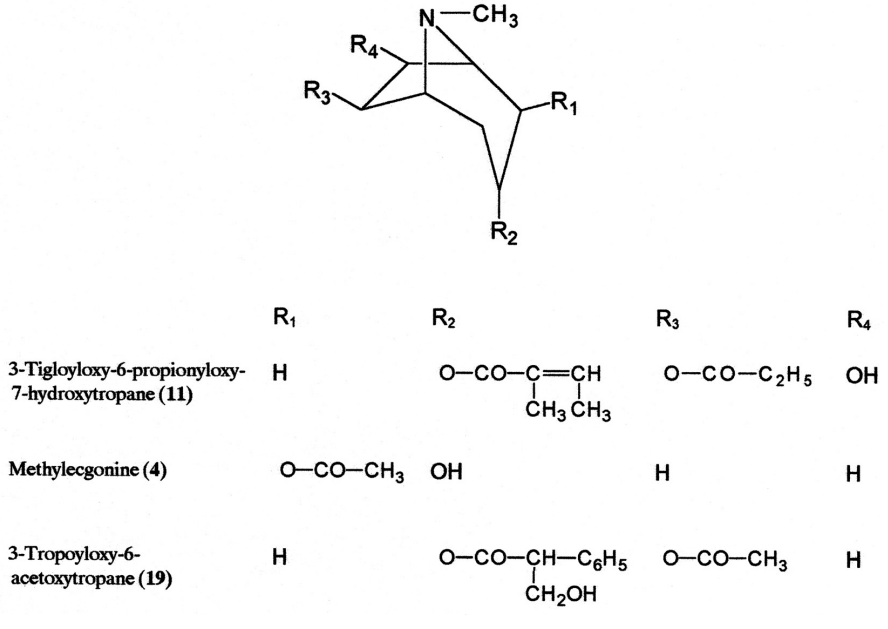

Fig. 1.

This alkaloid is reported for the first time for the family *Solanaceae.* C-2 substituted tropanes are chemotaxonomic markers for family *Erythroxylaceae* (Griffin and Lin, 2000).

The mass spectrum of alkaloid **11** displayed a characteristic fragmentation pattern of the tropane-3,6,7-triol ester. The molecular ion at *m/z* 311 corresponds to molecular formula $C_{16}H_{25}NO_5$. This ion is with 16 mass units higher than 3-tigloyloxy-6-propionyloxytropane (M^+ 295) reported for roots of *D. innoxia* (Witte *et al.*, 1987). It can be ascribed to an additional hydroxyl group in the molecule of alkaloid **4**. As in 3-tigloyloxy-6-propionyloxytropane, the ion at *m/z* 228 ($M^+ - 83$) indicates a tigloyl group at C-3. The tigloyl moiety is confirmed by ions at *m/z* 55 and 83. Ions at *m/z* 228 and 212 corresponds to a 6-propionyloxy-7-hydroxy substituted tropane nucleus. The peak at *m/z* 57 confirms the propiolyl moiety. On the basis of this data alkaloid **11** has been identified as 3-tigloyloxy-6-propionyloxy-7-hydroxytropane – a new tropane ester (Fig. 1).

Alkaloid **19** has a mass spectrum characteristic for a disubstituted tropane nucleus and retention time after hyoscyamine. The M^+ at *m/z* 347 corresponds to molecular formula $C_{19}H_{25}NO_5$. The ion at *m/z* 261 indicates tropoyl group at C-3 whereas ion at 182 is indicative of an acetyl group at C-6. Alkaloid **19** is identified as 3-tropoyloxy-6-acetoxytropane which is new for genus *Datura* (Fig. 1). Previously it has been reported for *Anthocercis angustifolia* (Evans and Ramsey, 1983).

The rest of alkaloids have been identified according their MS fragmentation pattern reported in the literature (Witte *et al.*, 1987; Christen *et al.*, 1990; Ionkova *et al.*, 1994; Philipov and Berkov, 2002).

Alkaloids in diploid and tetraploid hairy roots

The two ploidy levels of hairy roots showed significant differences in alkaloid spectrum and accumulation. In contrast to diploid, tetraploid root cultures have nine compounds in the alkaloid mixture. One of them, alkaloid **6**, was not detected in the alkaloid mixture of the diploid roots. Hyoscyamine was the main compound in the alkaloid mixtures of both diploid and tetraploid hairy roots, 78.8% and 56.6% respectively of the total alkaloids. In comparison to diploid hairy roots which accumulated apoatropine (7.2%), tetraploid hairy roots accumulated 3-acetoxytropane (28.4%) as a second compound of the alkaloid mixture. C-3 monosubstituted tropanes dominated in the alkaloid mixture of the tetraploid clone while disubstituted tropanes dominated in the alkaloid mixture of the diploid clone. The formation of cell aggregates on tetraploid level could be a probable reason for the differences between the diploid and tetraploid clones. The relationship between hairy root morphology and tropane alkaloid synthesis has been discussed by other authors (Robins *et al.*, 1991).

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