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Alkaloid Production in Diploid and Autotetraploid Plants of *Datura stramonium*

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Abstract

A comparative investigation of alkaloid production and accumulation in the roots and leaves of diploid (2n = 24), and C_4 generation of induced autotetraploid (4n = 48) *Datura stramonium* L. plants was performed. Fifteen alkaloids have been determined in the roots and two in the leaves, at a level of 1% or more of the crude alkaloid fractions in both ploidy levels. Two 3-tigloyloxy-6-isovaleryloxy-7-hydroxytropane isomers were detected for the first time in genus *Datura*. In the conditions of the prolonged photoperiod, hyoscyamine was the main alkaloid in the roots whereas scopolamine was the main alkaloid in the leaves of both ploid forms. In comparison to diploids, the roots and leaves of tetraploids had a higher alkaloid content and scopolamine/hyoscyamine ratio.

Keywords: *Datura stramonium*, induced polyploidy, tropane alkaloids.

Introduction

Polyploidy is a widespread phenomenon in nature (Stebbins, 1971). Compared to diploids, polyploid plants have different morphological, physiological and biochemical parameters. The first (C_1) and subsequent generations of induced polyploids are sources of variability and new genotypes, subjected to various manipulations aimed at plant improvement (Eigsty, 1957; Beamish et al., 1957; Jan, 1988; Hussain et al., 1997). Observed differences in the quantity and spectrum of active substances in diploids, plants with various ploidy obtained *in vitro* (Hiraoka & Tabata, 1973; Bajaj et al., 1980), and induced polyploids (Haskell, 1968; Wold et al., 1992; Hiraoka, 1998) have provoked an interest in polyploidization of medicinal plants.

The alkaloids of *Datura* species have been extensively investigated (Lette, 1979; Petri & Bajaj, 1989), but data concerning the effects of genetic factors, particularly of the chromosome number on biosynthesis and accumulation of tropane alkaloids are still insufficient and vague. *Datura* diploids have a higher alkaloid content than haploids (Mechler & Kohlenbach, 1978). With respect to the alkaloids in induced *Datura* tetraploids, several research groups have found a higher concentration of alkaloids in the leaves, as compared with diploids (Karnick & Saxena, 1970; Hiraoka & Tabata, 1973; Djurmanski & Jankulov, 1978). To our knowledge, there are no data available about the alkaloid content and composition in the roots (the place of tropane alkaloid biosynthesis) of tetraploid *Datura* plants.

Different genotypes behave differently when cultivated under invariable conditions. In order to study the influence of a single factor – the ploidy level on alkaloid production, we conducted our experiments under controlled conditions (Gottshalk, 1988).

The present study deals with a comparison of biosynthetic ability of roots and alkaloid accumulation in leaves between diploid plants and the fourth (C_4) autotetraploid generation of *Datura stramonium* L. (Solanaceae).

Materials and methods

Plant material

The initial diploid form was collected from natural habitats in the vicinities of Lovech, Bulgaria, in 1997. Autotetraploid (2n = 4x = 48) plants of *D. stramonium* L. were obtained by treatment of diploid seedlings (2n = 2x = 24) with 0.05% aqueous colchicine solution for 48 h (Pundir et al., 1983). The ploidy of plants was determined by chromosome counting in

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squashed root tips (Melander & Wingstrand, 1953) or by measuring the length of stomatal guard cells after preliminary determining the correlation between the stomata length and ploidy level (Letchamo & Vomel, 1989). The examination was conducted with diploid plants as a control group and a line of C_4 generation obtained from C_1 plant with the highest reproductive ability. For simultaneous germination, diploid and tetraploid seeds were husked and incubated in Petry dishes at 22 ± 0.1 °C on wet filter paper. The obtained seedlings were placed in flower pots filled with 500 cm^3 mixture of soil/sand (3 : 1) and cultivated in a growth chamber at 25 ± 2 °C, with a 16-h illumination daily. In the first flower phase, the leaves and roots of plants from the tested two ploidy levels were collected and dried at 45 °C.

Alkaloid extraction

Root and leaf samples (0.2–0.5 g) were ground up with sea sand and macerated in 10 ml 3% H_2SO_4 for 6 h at room temperature. After filtering and washing of the plant residue with 5 ml of distilled water, the solutions were adjusted to pH 9–10 with 25% NH_4OH and triple extracted with 10 ml $CHCl_3$. The combined chloroform extracts were dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo* to give the crude alkaloid fractions. Thus obtained residues were resolved in CH_3OH for further analysis.

Gas chromatography (GC)

GC was performed on a Hewlett Packard 5890 equipped with a HP-1 column (30 m × 0.25 mm × 0.25 µm). The flow rate of carrier gas (N_2) was 0.8 ml/min^{-1} and the splitting ratio was 1 : 100. The temperature program was 150–270 °C at 6 °/min^{-1} and held at the final temperature for 15 min. A flame ionization detector was used at 300 °C and the injector temperature was 280 °C.

Gas chromatography/mass spectra (GC/MS)

The GC/MS were recorded on a Hewlett Packard 6890/MSD 5972A, operating in EI mode at 70 eV. A HP-5 MS column (30 m × 0.25 mm × 0.25 µm) was used. The temperature program was 80–280 °C at 10 °/min^{-1} and 10 min hold at 280 °. Injector and detector temperatures were 280 °C. The flow rate of carrier gas was 0.8 ml/min^{-1}. Identification was accomplished with data from the library Wiley 275, as well as literature data (Evans & Major, 1968; Witte et al., 1987; Parr et al., 1990; Ionkova et al., 1994). In some cases, when no identical spectra were found, the structural type of the corresponding component was suggested only on the basis of its mass spectral fragmentation and retention time.

Thin-layer chromatography (TLC)

TLC was carried out on Merck aluminum sheets silica gel 60 F_{254}. Silica gel 60 PF_{254} was used for preparative TLC. The mobile phase was chloroform/methanol/25% ammonia (85 : 15 : 0.7, v/v/v). Hyoscyamine and scopolamine were compared by R_f values with authentic samples. Compounds were visualized after spraying with Dragendorff's reagent.

Statistical test

For each series of tested plants, we carried out a variance analysis of the obtained alkaloid contents, in order to determine if there was any difference between the ploidy levels (*t*-test; Lidanski, 1988).

Results and discussion

The GC/MS data of alkaloid extracts prepared from *D. stramonium* roots and leaves showed more than 25 tropane alkaloids, but only 15 were observed at levels of 1% or more of the crude alkaloid fractions (Table 1). Four of them had minimal values under the 1% barrier and were not included in the variance analysis. Three alkaloids, named A, B and C, have not been identified, but according their MS fragmentation patterns they belonged to the tropane alkaloid series (see Table 1). Alkaloid A had identical MS fragmentation as 3-tigloyloxy-6-isovaleryloxy-7-hydroxytropane, detected by us in the same alkaloid extract (for detailed fragmentation, see Table 1). In addition, the both alkaloids had very adjacent retention time. According to these data, the alkaloids are isomeric tropine and pseudotropine esters (Witte et al., 1987). The 3-tigloyloxy-6-isovaleryloxy-7-hydroxytropane isomers are reported for the first time for genus *Datura*. Previously, these alkaloids had been found in *D. sanguinea* and *D. candida* × *D. aurea* hybrid (Evans & Major, 1968; Robins et al., 1990), species belonging to genus *Brugmansia* (D'Arcy, 1973; Lockwood, 1973; Griffin & Lin, 2000). Alkaloid B showed a $[M]^+$ at *m/z* 331 and base peak at *m/z* 124 and its retention time was between the retention times of hyoscyamine and scopolamine and alkaloid C- a $[M]^+$ at *m/z* 329, base peak at *m/z* 124 and retention time after 6 β-hydroxyhyoscyamine. In the related literature, we could not find alkaloids with such characteristics. According to the review article of Lounasmaa & Tamminen (1993), there are no alkaloids with $[M]^+$ at *m/z* 329 and 331 in Solanaceae. Therefore, alkaloids B and C may be new compounds.

Alkaloids in roots

We tested the influence of ploidy levels on alkaloid biosynthesis by cultivating of plants under controlled conditions (Gottschalk, 1985, 1988). The results have shown that diploid and tetraploid forms of *D. stramonium* have different ability to produce alkaloids. No differences in the alkaloid spectrum were found between the roots of diploid and tetraploid plants (Table 1). The main alkaloids (calculated in % of the sum of alkaloids studied) in the roots were hyoscyamine, followed by 3,6-dityglyloxy-7-hydroxytroplane. In amount, scopo-

Table 1. The alkaloid content in roots of diploid (2n) and tetraploid (4n) *Datura stramonium* plants presented as a % of DW.

	retention time	2n		4n		4n/2n ratio
		mean ± sd	%[4]	mean ± sd	%[4]	
3-Tigloiloxy-6-hydroxytropane	4.88	0.0237 ± 0.0085	4.56	0.0297 ± 0.0107	4.71	1.25[5]
Meteloidine	6.65	up to 0.0119		up to 0.0264		
3-Hydroxy-6-tigloiloxytropane	6.82	up to 0.0049		up to 0.0129		
Apoatropine	6.99	–		up to 0.0169		
3-Tigloiloxy-6-methylbutyryl-oxytropane	7.53	up to 0.0096		up to 0.0106		
Alkaloid 325	8.14	0.0695 ± 0.0182	13.38	0.0517 ± 0.0310	8.21	0.74[5]
Hyoscyamine	8.65	0.1722 ± 0.0723	33.15	0.2132 ± 0.1203	34.28	1.24[5]
3,6-Ditigloiloxytropane	8.93	0.0208 ± 0.0075	4.00	0.0295 ± 0.0159	4.68	1.42[5]
3-Tigloyloxy-6-isovaleroxy-7-hydroxytropane	9.08	0.0231 ± 0.0091	4.45	0.0347 ± 0.0144	5.51	1.50[5]
Alkaloid A[1]	9.26	0.0124 ± 0.0047	2.29	0.0223 ± 0.0086	3.54	1.80[5]
Alkaloid B[2]	9.62	0.0116 ± 0.0052	2.23	0.0129 ± 0.0047	2.05	1.11[5]
Scopolamine	10.06	0.0481 ± 0.0279	9.26	0.0648 ± 0.0192	10.28	1.35[5]
3,6-Ditigloiloxy-7-hydroxy-tropane	10.52	0.0978 ± 0.0279	18.83	0.1219 ± 0.0500	19.35	1.25[5]
7-Hydroxytropane	10.85	0.0251 ± 0.0101	4.83	0.0274 ± 0.0093	4.35	1.06
Alkaloid C[3]	15.64	0.0152 ± 0.0034	2.93	0.0192 ± 0.0059	3.05	1.26[5]

The values indicate the mean ± standard deviation (sd) and limits of alkaloids which are more than 1% of the total alkaloids.
MS m/z (rel. int.):
[1)] 339(2), 240(3), 237(3), 222(2), 220(2), 210(3), 154(6), 139(8),138(64), 137(40), 120(6), 93(44), 94(100), 85(4), 83(12), 82(4), 81(4), 60(2), 55(16), 42(10).
[2)] 331(6), 272(2), 147(2), 140(5), 131(4), 124(100), 103(4), 96(8), 94(18), 82(17), 67(8).
[3)] 329(3), 328(12), 281(2), 207(4), 140(5), 130(28), 124(100), 103(4), 96(8), 94(16), 82(15), 67(8).
[4)] Alkaloid percentage in total alkaloid mixture.
[5)] Significant at P > 0.01%.

lamine rated fourth for diploids and third for tetraploids. With an exception of alkaloid 325, all other alkaloids on tetraploid level had higher mean concentrations than diploids. The greatest increase was observed for alkaloid A (1.80-fold) and the lowest for 6-hydroxyhyoscyamine (1.06-fold). Compared to diploid level, the increase of hyoscyamine on tetraploid level was 1.24-fold and 1.35-fold for scopolamine. The scopolamine/hyoscyamine ratio slightly increased from 0.28 in diploids to 0.30 in tetraploids. The amplitudes (min–max values) of alkaloids were greater on tetraploid level. These results demonstrated that an increased biosynthetic ability of tetraploid roots lead to a higher alkaloid accumulation in the leaves of tetraploid plants.

Table 2. Hyoscyamine and scopolamine content presented as a % of DW and scopolamine/hyoscyamine ratio in leaves of diploid (2n) and tetraploid (4n) *D. stramonium* plants.

	2n mean ± sd	4n mean ± sd	4n/2n ratio
Hyos	0.0543 ± 0.0258	0.0767 ± 0.0468	1.41
Sco	0.1528 ± 0.0331	0.4026 ± 0.0864	2.63
Sco/Hyos	3.65 ± 2.19	6.58 ± 2.98	1.18

The values indicate the mean ± standard deviation (sd) of alkaloids.
[1)] Significant at P > 0.01%.

Alkaloids in leaves

Only two alkaloids exceeded 1% of the total alkaloids in leaves: hyoscyamine and scopolamine. The concentration of alkaloids in the leaves was considerably higher than in the roots (Table 2). The mean value of scopolamine was 0.40% in tetraploid and 0.15% in diploid leaves, demonstrating a 2.63-fold increase, whereas hyoscyamine recorded 0.08% in tetraploid and 0.05% in diploid leaves, showing a 1.41-fold increase. The mean scopolamine/hyoscyamine ratio altered from 3.65 for diploids to 6.58 for tetraploids. The variation of alkaloids, and scopolamine/hyoscyamine ratio (from 1.39 to 7.09 for diploids and from 2.55 to 13.59 for tetraploids) were markedly higher at the tetraploid level. In contrast to the roots, the leaves accumulated scopolamine as a main alkaloid on both ploidy levels. In the literature, *D. stramonium* is generally considered to be a plant that accumulates

mainly hyoscyamine in leaves, scopolamine being a minor alkaloid under natural growth conditions (Spurina et al., 1981). Quite probably, in our experiments the closely related hormonal balance, enzyme activity (particularly of hyoscyamine-6β-hydroxylase) and alkaloid formation were influenced by the artificial conditions of growth. This led to intensive transformation of hyoscyamine into scopolamine, with the latter being accumulated in leaves. Our results have confirmed the findings of Cosson (1969), who was the first to point out the great influence of day-length and light intensity on the formation of tropane alkaloids in *D. metel*.

Thus, we support the idea that epoxidation of hyoscyamine to scopolamine in *D. stramonium* begins in roots, but mainly takes place in the aerial parts. The process is genetically determined but could be controlled by external factors. Because of the great interest in manipulation of the biosynthetic pathway for scopolamine production, this phenomenon needs further elucidation of the causes for such an intensive transformation of hyoscyamine to scopolamine. The higher mean concentration of alkaloids and variation of scopolamine/hyoscyamine ratio in the leaves of tetraploids indicates that on tetraploid level, scopolamine synthesis is more intensive than on diploid level. Furthermore, the increased content and variation of alkaloids in the leaves of tetraploid plants provide a good opportunity for selection of alkaloid-rich plants by inducing polyploidy.

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