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ALKALOID PRODUCTION BY PLANTS REGENERATED FROM CULTURED CELLS OF *DATURA INNOXIA*

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Key Word Index—*Datura innoxia*; Solanaceae; plant tissue culture; tropane alkaloids; differentiation; chromosome variation.

Abstract—Cellular aggregates in *Datura innoxia* suspension cultures give rise to large numbers of shoots when such aggregates are cultured in the light on an auxin-free agar medium supplemented with kinetin. These shoots form roots on a kinetin-free medium to develop into complete plants. Most of the regenerated plants are diploid, and the frequencies of aneuploid or polyploid plants are much lower than might be expected from the distribution of chromosome number in the cultured cells. During root differentiation and plant development, scopolamine synthesis is initiated and there is a progressive increase in the alkaloid content. Consequently, the general pattern of alkaloid composition is restored to a normal state in the majority of the regenerated plants including aneuploids or polyploids. Nevertheless, some of the plants show an abnormal expression in alkaloid metabolism, such as the complete hydrolysis of scopolamine in the dried leaves.

INTRODUCTION

THE FORMATION of tropane alkaloids in the callus cultures of *Datura* plants has been described by various workers, but their alkaloid contents are generally much lower than those of the original plants.[1–8] We have suggested that the low production rate of alkaloids found in undifferentiated cultures of *Datura* as well as *Scopolia* is caused mainly by a strong repression in the biosynthesis of tropic acid, the acidic moiety of hyoscyamine and scopolamine.[8,9] However, it has been demonstrated in both *Atropa*[10] and *Scopolia*[11] cultures that the synthesis of tropane alkaloids is promoted significantly in the roots differentiated from the callus tissues, showing a tendency to recovery of normal chemical pattern. Such a relationship between organogenesis and alkaloid formation has not so far been studied in any *Datura* cultures. Furthermore, it remains to be seen whether or not the cultured cells still possess the intact genetic potentiality to synthesize specific alkaloids in normal quantities. The importance of this question is realized when one considers the chromo-

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[10] BHANDARY, S. B. R., COLLIN, H. A., THOMAS, E. and STREET, H. E. (1969) *Ann. Botany* **33,** 647.
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somal instability of cultured cells. An experimental approach to this problem is to induce plant development in cultured cells so as to follow changes in alkaloid pattern during the developmental stages and to examine the cytology in individual plants derived from those cells. The results of such experiments with the cultured cells of *D. innoxia* are described in this paper.

RESULTS AND DISCUSSION

Induction of organogenesis and plant development

In attempts to induce shoot differentiation, cellular aggregates of *D. innoxia* proliferated in a liquid medium[12] supplemented with 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) were plated on agar media containing 1-naphthaleneacetic acid (NAA) and kinetin in various concentrations, and incubated under light from fluorescent lamps. As shown in Table 1, the aggregates of cells initiated shoots at high kinetin/NAA ratios. Shoots were most frequent in the medium containing no NAA but kinetin (10^{-4} M). This result is in accordance with that obtained by Engvild[13] recently that *D. innoxia* callus initiates shoots at high cytokinin/auxin ratios, showing no synergism between cytokinin and auxin. Despite their conspicuous effects on organogenesis, the growth regulators hardly affected alkaloid formation in *D. innoxia* cultures and no significant differences in alkaloid content were observed between the shoot-forming and the undifferentiated cultures (Table 1). Apparently, shoot formation is not so intimately associated with the promotion of alkaloid synthesis as it is for nicotine synthesis in the tobacco callus.[14]

TABLE 1. EFFECTS OF NAA AND KINETIN ON SHOOT FORMATION AND ALKALOID CONTENT IN CALLUS TISSUES CULTURED ON THE BASAL MEDIUM FOR 10 WEEKS

Conc NAA (M)	Conc Kinetin (M)	Fresh wt of callus (g/flask)	Number of shoots per flask	Alkaloid content (% of dry wt)
0	0	10·4	0	0·019
	10^{-7}	10·5	0	0·020
	10^{-6}	9·9	0	0·020
	10^{-5}	10·8	2·3	0·021
	10^{-4}	8·9	30·3	0·013
10^{-8}	0	11·1	0	0·018
	10^{-7}	11·1	0·3	0·025
	10^{-6}	10·9	0	0·033
	10^{-5}	11·2	0·6	0·024
	10^{-4}	10·1	17·5	0·011
10^{-6}	0	12·0	0	0·011
	10^{-7}	11·2	0	0·011
	10^{-6}	9·9	0	0·011
	10^{-5}	11·3	0	0·012

Large numbers of shoots isolated from callus tissues were transferred individually to various kinds of test-media to induce root formation. Although no roots were formed in the presence of kinetin, 32% of the shoots (9/28) grown on the basal medium without any

[12] LINSMAIER, E. M. and SKOOG, F. (1965) *Physiol. Plant.* **18**, 100.
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growth regulators initiated roots within 7 weeks. Unexpectedly the addition of either 3-indole-acetic acid (10^{-7}–10^{-5} M) or casein hydrolysate (0·2% w/v) failed to stimulate root formation. Moreover, the frequency of root-forming shoots was reduced to only 6% (9/140) by supplying yeast extract (0·2% w/v) to the basal medium. These results indicate that the shoots obtained from cellular aggregates on an auxin-free medium containing kinetin should be transferred to a basal medium without kinetin in order to initiate roots. This procedure has made it possible to obtain whole plants readily from *D. innoxia* suspension cultures.

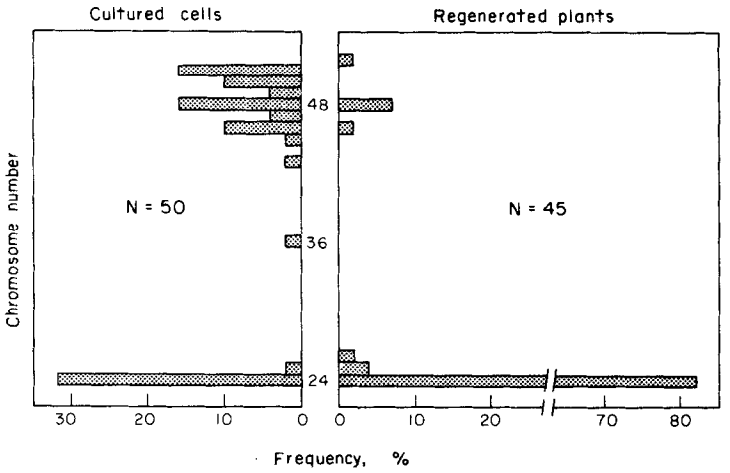

FIG. 1. DISTRIBUTION OF CHROMOSOME NUMBER IN THE CULTURED CELLS (LEFT) AND THE CHROMOSOME NUMBERS OF THE REGENERATED PLANTS (RIGHT).
N represents the number of cells or plants observed.

Chromosome variations in cultured cells and regenerated plants

A total of 45 plantlets, which formed roots successfully from the basal parts of the shoots placed on the kinetin-free medium, were examined cytologically for chromosome number in the root tip and the results were compared with the distribution of chromosome number in the cultured cells used for the induction of shoots. The data in Fig. 1 show that only 32% of the dividing cells in suspension culture were diploid ($2n = 24$), whereas most of the remaining cells had chromosome numbers of tetraploid level ranging from $4n - 5$ to $4n + 3$. On the other hand, examination of chromosome numbers in the plants derived from this heterogeneous population of cultured cells revealed that 82% of the plants (37/45) were normal diploids, the rest consisting of tetraploids and aneuploids ($2n + 1$, $2n + 2$, $4n - 2$ and $4n + 4$). Each of these plants must have originated from a single cell, but not from a genetically heterogeneous mass of cultured cells, since no chromosomal differences were observed among different root tips of the same plants. Interestingly, the frequency of diploid plants was much greater than might have been expected from the frequency of diploid cells in the original culture. This discrepancy apparently indicates the selective advantage of diploid cells over tetraploid and aneuploid cells in the course of differentiation.

However, it is not known whether the chromosome numbers vary from one subculture to another. Furthermore, there is a strong possibility that tetraploid and aneuploid shoots

would have been found more frequently in those shoots which were not cytologically examined due to a complete failure of root formation. The distribution of chromosome number in cultured cells varied with the culture strain of callus; e.g. a strain of stem origin contained no diploid cells but two kinds of cells having 46 and 44 chromosomes in the proportion 4:1, none of which was capable of developing into plants on any test-medium.

TABLE 2. CHANGE IN THE ALKALOID PATTERN DURING ORGANOGENESIS AND PLANT DEVELOPMENT DERIVED FROM CULTURED CELLS PLATED ON SYNTHETIC AGAR MEDIUM

Time after plating (month)	Material analyzed	Alkaloid composition*					Alkaloid content (% of dry wt)
		A	B	C	D	E	
1	Unorganized callus	+	−	+	+	−	1×10^{-2}
2	Shoot-forming callus	+	−	−	+	−	$1{\cdot}5 \times 10^{-2}$
3	Developing shoots	+	−	−	+	−	2×10^{-2}
5	Root-forming shoots	+	+	−	+	−	3×10^{-2}
6	Leaf of young plant	+	+	−	+	+	1×10^{-1}
7	Leaf of mature plant	+	+	−	+	+	3×10^{-1}

* A = hyoscyamine; B = scopolamine; C, D and E = unidentified minor alkaloids.
+ = Presence, − = Absence.

Change in alkaloid pattern during development

Table 2 shows the change in the pattern and content of tropane alkaloids at consecutive stages of development from callus tissue to mature plant. Although hyoscyamine was detected throughout the course of development, scopolamine appeared first at the stage of root formation and eventually superseded hyoscyamine in quantity to become the main alkaloid in the mature plants. This suggests that the formation of scopolamine, the epoxide of hyoscyamine, is associated with root formation, which is in agreement with the earlier results of Romeike.[15] The total alkaloid content, which was very low in callus tissues, increased progressively with organization and plant growth until it reached a maximum in the leaves of plants just before flowering.

Alkaloid composition of regenerated plants

Although 45 plantlets derived from cultured cells were transplanted to pots in the greenhouse, only 19 plants ($2n:2n + 1:4n - 2 = 17:1:1$) grew to maturity. For analysis of alkaloids, leaf samples were collected at the flowering stage from the individual plants derived from cultured cells. The results of TLC analyses showed that most of 19 plants examined had the same alkaloid content as normal plants, containing scopolamine and at least 9 minor alkaloids. The toal alkaloid content was 0·33% on average, which is comparable to that of normal plants. The alkaloid contents of the aneuploid plants with 25 and 46 chromosomes were 0·36 and 0·25%, respectively, though the latter was a dwarf plant that was 1/4 as tall as the diploid plant at the fruit stage. It is considered therefore that the genetic potentiality for synthesizing alkaloids has generally been retained by the cultured cells of *D. innoxia* during the cycle of "de-differentiation" and "re-differentiation", as in the case of tobacco callus cultures.[16] Not all the plants, however, had the same alkaloid pattern after "re-differentiation", since some of the diploids with normal growth habit were

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[16] TABATA, M., YAMAMOTO, H. and HIRAOKA, N. (1968) *Japan. J. Genet.* **43**, 319.

unusual in their alkaloid metabolism. One plant completely lacked both scopolamine and hyoscyamine in spite of the presence of other minor alkaloids; this, however, was observed only when the leaf samples were collected from the plant at the flowering stage, since both scopolamine and hyoscyamine were found to be produced after flowering. Six other plants were peculiar in that nearly all the scopolamine present in the leaves collected at flowering decomposed to yield a large amount of scopine when the leaf samples were dried at 50°, while the other alkaloids including hyoscyamine remained unaffected. On the other hand, no scopolamine was hydrolyzed at all when fresh leaves of the same plants were treated with MeOH immediately after harvest. Thus a hydrolytic enzyme specific to scopolamine is activated in the leaves of these particular plants during the process of drying. Such metabolic abnormalities suggest that certain modifications of the regulatory mechanism have occurred in some of the cultured cells, and have been carried over into the regenerated plants.

EXPERIMENTAL

Plant material and culture method. Suspension cultures of *Datura innoxia* Mill., which were originally derived from the embryo callus, have been maintained for 18 months in the Linsmaier–Skoog basal medium[12] (250 ml) containing 10^{-6} M 2,4-D in 500 ml flasks agitated by a reciprocal shaker at a speed of 100 rev/min. Cell suspensions were transferred to fresh medium every 2–3 weeks and incubated usually in the dark at 25°. For induction of shoot differentiation, the cellular aggregates consisting of single cells and multiple cells were plated on the agar medium containing 10^{-4} M kinetin in 100 ml flasks and incubated at 27 ± 2° under illumination (*ca* 10000 lx, 12 hr/day) from fluorescent lamps. The shoots developing from callus tissues were transferred to the basal medium without growth hormones to induce roots. After the development of roots, the plantlets (4–7 cm high) were taken out of the flasks and cultivated in pots in the greenhouse.

Analysis of tropane alkaloids. The quantitative estimation of tropane alkaloids in callus tissues or leaves was done spectrophotometrically after the Vitali–Morin reaction modified by Freeman[17] and the alkaloid content was calculated as the amount of hyoscyamine on the basis of dry wt of the material. For TLC analysis of alkaloids, the test solutions were applied on silica gel G plates and developed with $CHCl_3$–EtOH – 28% NH_4OH (85:14:1). The alkaloidal spots were detected with Dragendorff's reagent.[8]

Determination of chromosome number. For cytological observation of chromosomes, cultured cells or root tips of the regenerated plants were treated with a saturated soln of 8-hydroxyquinoline for 2–3 hr, washed twice with H_2O, and then fixed with the Carnoy fluid (EtOH–$CHCl_3$–AcOH = 6:3:1). The microscopic slides were prepared by the Feulgen squash method.[18]

Acknowledgement—This work was supported partly by the Grant No. 66-001 from the Ministry of Education.

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