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TROPANE ALKALOID PRODUCTION IN *DATURA STRAMONIUM* ROOT CULTURES

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SUMMARY

Tropane alkaloid production was studied in different root cultures of *Datura stramonium*. Cultured roots were obtained with 10^{-6} *M* of indolbutyric acid. Their doubling times were from 6 to 19 days. Hyoscyamine content varied from 0.17 to 0.62% dry weight, and scopolamine content from 0.08 to 0.33% dry weight, depending on the lines. A comparison of the bioproductivity of these compounds in the pot-grown plant roots showed that it was two to three orders lower than cultured roots, and it increased one order of magnitude considering the productivity on the whole plant. Bioproductivity, growth capacity and alkaloid production stability during subsequent transfers (more than 2 yr) are reported. Only one root line (N5) showed excretion of the alkaloids to the culture medium. Characterization of three selected lines (N1, N5, and N9) showed that the highest alkaloid production is reached at the stationary phase of growth, with the exception of line N9.

Key words: root culture; tropane alkaloids; *Datura stramonium*; hyoscyamine; scopolamine.

INTRODUCTION

Recently there has been an increase in the number of reports demonstrating that cell cultures of many tropane alkaloid-containing Solanaceae are capable of synthesizing them. Although hyoscyamine content in cultured cells of *Hyoscyamus niger* could be increased to comparable levels of hyoscyamine in whole plants by selecting a high-production cell line and improving the culture conditions, scopolamine content remained at about 10% of its level in intact leaves or roots (Hashimoto et al., 1982; Yamada and Hashimoto, 1982). In callus culture of different *Datura* species, only trace amounts of scopolamine have been detected by radioimmunoassay (Savary and Dougall, 1990).

Classical grafting experiments first demonstrated that the major site of tropane alkaloid biosynthesis is the root (Waller and Nowacki, 1978). Recently, different enzymes of the tropane alkaloid biosynthetic pathway, such as 6-β-hidroxyhyoscyamine hydroxylase (Hashimoto et al., 1986) and putrescine-*N*-methyl-transferase (Hashimoto et al., 1989), have been shown to be active in cultured roots but scarcely detectable in cultured cells and shoots. Tropinone reductase activity has also been found in root cultures of different Solanaceae species (Drager, 1990). Immunohistochemical localization of 6-β-hidroxyhyoscyamine hydroxylase has shown that this enzyme is present in plant roots and cultured roots, but absent in leaves, stems, and cultured cells of *H. niger* (Hashimoto et al., 1991).

Root cultures of different Solanaceae species have been established to obtain high-production cultured root lines (Endo and Yamada, 1985; Hartmann et al., 1986; Hashimoto et al., 1986), and although there has only been moderate success at this level, natural variability could be useful in selecting high-production root lines.

The aim of this paper was the screening of variability in different cultured root lines of *Datura stramonium* in terms of biomass and alkaloid production. Because of the need of information in the literature concerning stability with time, the characterization of three selected lines for alkaloid production and their stability with time are also reported.

MATERIALS AND METHODS

Plant material. Seeds of *Datura stramonium* were obtained from Dr. Bye at the Botanical Garden of the Universidad Nacional Autónoma de México. They were collected in Guadalajara, Jalisco (BYE 13372).

Plant germination. Seeds were de-coated with sandpaper, then sterilized for 3 min in 10% $Ca(OCl)_2$ and germinated over a Whatman 1 paper embedded with 3 ml of Hoagland's solution (Hoagland and Arnon, 1938), supplemented with GA_3 1×10^{-4} *M*. They were kept in a controlled environment chamber (Forma Scientific) under a 16-h light (25° C)/8-h dark (20° C) regimen.

Establishment of the root cultures. Plantlets 2- to 4-wk old were employed. Roots (ca. 5 cm in length) were separated from the stem base and cultivated in 25-ml flasks containing 10 ml of Gamborg's (B5) culture medium (Gamborg and Eveleigh, 1968) supplemented with 1×10^{-6} *M* of indolbutyric acid (IBA) and 3% sucrose. They were grown on an orbital shaker in the dark at 110 rpm and 25° C. These conditions were determined experimentally.

The plantlets were cultured for 2 wk and then transferred to 50-ml Erlenmeyer flasks with 20 ml of culture medium and finally transferred to 125-ml flasks containing 50 ml of culture medium for their maintenance.

For comparison with in vitro systems, *Datura stramonium* plants obtained from the same seed batch were pot-grown on the ground. They were irrigated daily with 500 ml of tap water and maintained under a natural light regimen in a controlled temperature room (20° C night, 30° C day). Results reported for plants are the averages of at least three determinations in different individuals made by duplications (standard error was less than 10%). The culture period was 11 mo. (from July 1989 to June 1990).

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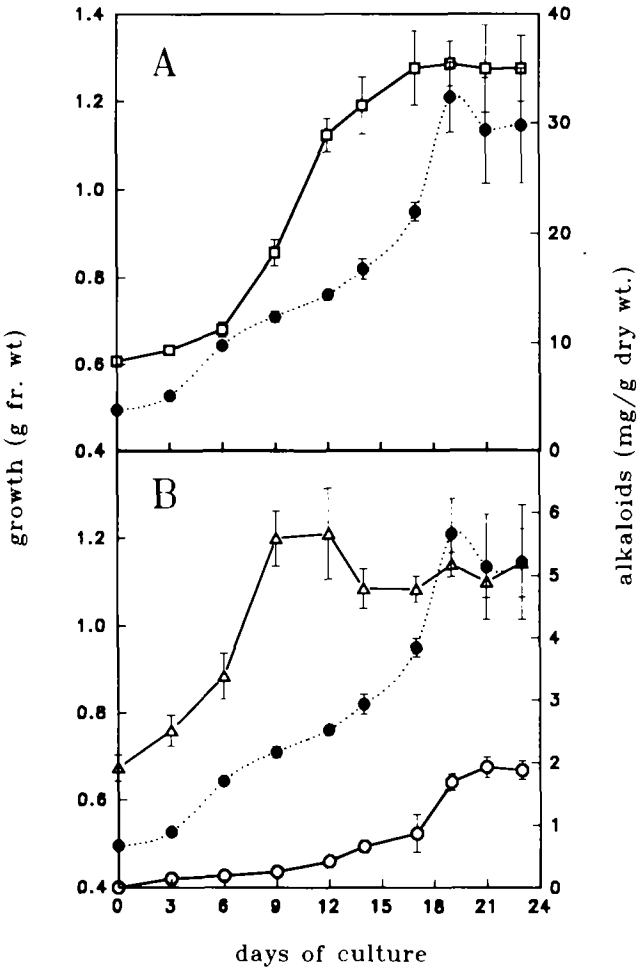

FIG. 1. Time courses of growth and alkaloid content in *D. stramonium* line N1 roots. *A*, The total fresh weight of tissue (*solid circles*) and the total alkaloid (*open squares*) per flask. *B*, Content within the tissue of hyoscyamine (*open triangles*) and scopolamine (*open circles*). Each point represents the mean ± SE for samples analyzed in sextuplicate (growth) or duplicate (alkaloids).

Subculturing of the root cultures. Cultured root lines were maintained in 125-ml flasks. The inoculum was 0.5 g fr. wt. of rapidly growing roots (1 to 2 cm in length) and the subculture period was initially of 35 days, although after some time (in the 11th subculture) a shorter subculture period (21 days) was used because longer periods induced callus formation.

Alkaloid quantification. Total alkaloids, hyoscyamine and scopolamine, were determined as follows (Monforte-González et al., unpublished data). One half gram of freeze-dried root tissue was extracted with 30 ml of MetOH for 2 min in a tissue homogenizer (Polytron). The homogenate was incubated at 50° to 55° C for 2 h and then it was filtered. The methanolic extract was evaporated to dryness and the residue was dissolved in 15 ml of H_2SO_4 2.5% (vol/vol) and washed with the same volume of EtOAc 3 times. The aqueous phase was adjusted to pH 9.5 with NH_4OH (28.4%) and this alkaline solution was extracted 3 times with 20 ml of EtOAc each time. The organic phase was recovered and evaporated to dryness, and the residue was dissolved in 0.5 ml of MeOH (analytical grade) and used for quantitative determinations. For alkaloids determination in liquid medium, 100 ml was used, it was freeze-dried and dissolved in 15 ml of H_2SO_4 2.5% (vol/vol). All other steps of the procedure were done in the same way. Total alkaloids were determined by gravimetry.

Hyoscyamine and scopolamine were determined by densitometry. Thin layer chromatography (TLC) was performed in Whatman, silica gel 60A LK 6D channeled glass plates employing the solvent system: $CHCl_3$-Me_2CO-MeOH-(28.4%) NH_4OH (75:10:15:2). To quantify these alkaloids, Dragendorff's reagent was sprayed and the area of the spots measured at 515 nm with a densitometric scanner (Shimadzu CS-930). The concentration was determined by comparing the peak area of the sample with that of a standard. The structures of hyoscyamine and scopolamine were confirmed by spectrophotometric analysis (UV/Vis, 1H NMR) as reported in Monforte-González et al., (unpublished data).

RESULTS

Selection of best-growing root lines. Forty-nine root lines were obtained and subcultured twice for 5 wk. Ten lines were selected for their high growth rates. These lines were subcultured 4 times more. However, after that time four lines had lost their growth capability (N2, N3, N4, N6). By now (after 16 subcultures) five lines have maintained their growth stability (N5, N7, N8, N9, N10), and only one (N1) had lost it.

During the present study it was observed that if subcultures are allowed up to 5 wk of growth, callus formation at the older tissue

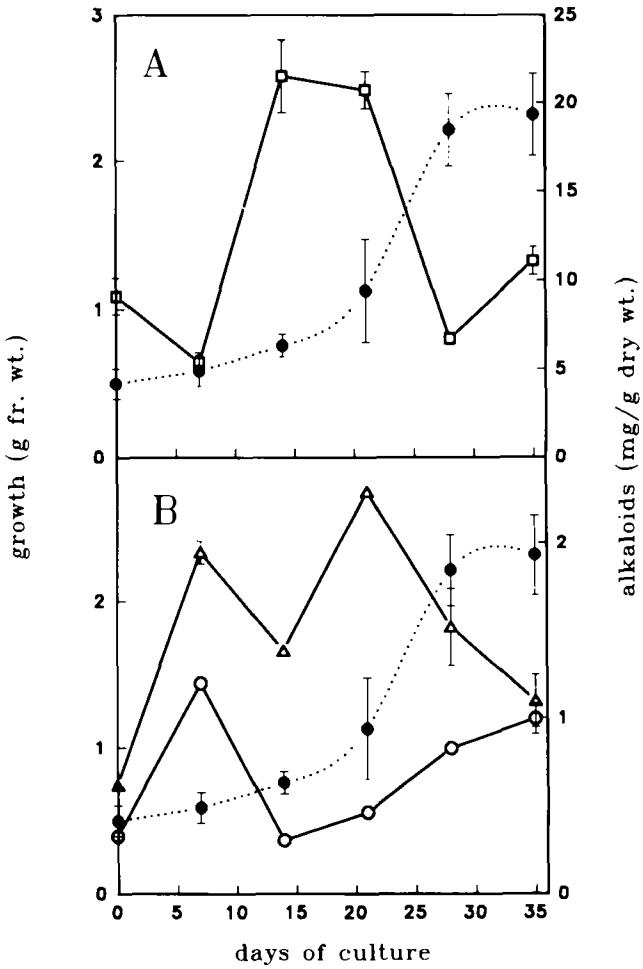

FIG. 2. Time courses of growth and alkaloid content in *D. stramonium* line N9 roots. *A*, Total fresh weight of tissue (*solid circles*) and the total alkaloid (*open squares*) per flask. *B*, Content within the tissue of hyoscyamine (*open triangles*) and scopolamine (*open circles*). Each point represents the mean ± SE for samples analyzed in sextuplicate (growth) or duplicate (alkaloids).

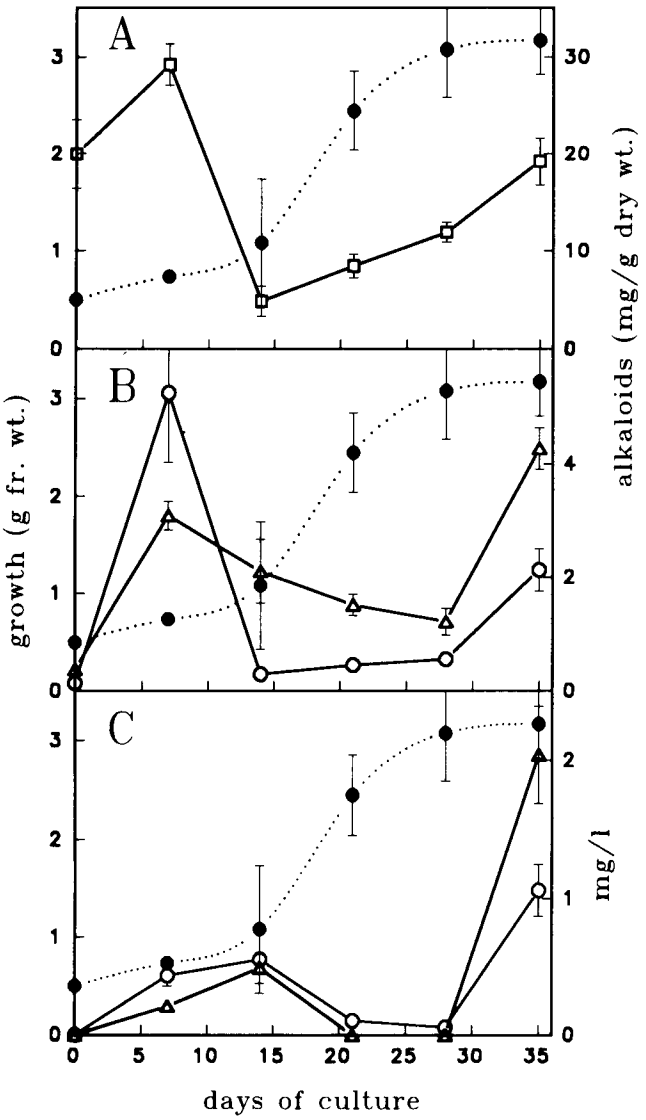

FIG. 3. Time courses of growth, alkaloid content, and the excretion of alkaloids into the extracellular medium in *D. stramonium* line N5 roots. *A*, Total fresh weight of tissue (*solid circles*) and the total alkaloid content (*open squares*) within the tissue. *B*, Content within the tissue of hyoscyamine (*open triangles*) and scopolamine (*open circles*). *C*, Content in the culture medium of hyoscyamine (*open triangles*) and scopolamine (*open circles*). Each point represents the mean ± SE for samples analyzed in sextuplicate (growth) or duplicate (alkaloids).

occurs. This was avoided by subculturing every 3 wk (during the lineal growth phase) instead of every 5 wk (late lineal growth phase or stationary phase in some cases).

Characterization of the cultures. Among root cultures, three lines with the highest hyoscyamine and scopolamine bioproductivity were chosen for further studies (N1, N5, N9). The root cultures used in the following experiments had been maintained for more than 2 yr, and in the course of the subcultures line N1 changed its growth pattern in the roots because of an extensive callusing response and a very poor growth rate (during subculture 6). This line did not show any recovery after subsequent cultures.

Figure 1 *A,B* show the time courses for growth and alkaloid content in line N1 evaluated during subculture 4. This line had a very slow growth rate (doubling time 19.4 days) and only doubled the initial inoculum, on both fresh and DW basis, by the end of the culture period. The stationary phase was reached at Day 21. Total alkaloids, hyoscyamine, and scopolamine (Fig. 1 *A,B*) followed a similar behavior as the growth pattern. Their levels were low on the early phase of culture (lag and early lineal growth phases) and increased substantially at the stationary phase. Hyoscyamine levels fluctuated between 0.2 and 0.6% dry weight (DW), whereas scopolamine content ranked from 0.01 to 0.2% DW. These alkaloids were present in the culture medium only in trace amounts.

Figure 2 *A* shows time courses of growth and alkaloid content in line N9 during subculture 8. Different from line N1, alkaloid content was not coupled with growth. At the end of this culture period it

TABLE 1

ALKALOID CONTENT IN *D. STRAMONIUM* ROOT CULTURES[a]

Line	TA, % DW	HYOS, % DW	SCOP, % DW	SCOP:HYOS
N1	0.46	0.33	0.085	0.28
N2	0.50	0.28	0.105	0.37
N3	0.92	0.17	0.166	0.88
N4	0.42	0.21	0.151	0.71
N5	0.59	0.38	0.137	0.36
N6	0.86	0.44	0.161	0.36
N7	0.60	0.34	0.139	0.41
N8	0.75	0.47	0.098	0.21
N9	1.20	0.62	0.329	0.53
N10	1.16	0.31	0.161	0.52
Plant roots	0.067	0.02	0.0056	0.28
Whole plant	0.15	0.013	0.015	1.25

[a] Measurements were made at subculture 3 after 35 days of culture. Values represent the mean for duplicate samples (root cultures) or triplicate (plant tissues). SE for each point was lower than 5%.
TA = total alkaloid; HYOS = hyoscyamine; SCOP = scopolamine.

TABLE 2

HYOSCYAMINE AND SCOPOLAMINE BIOPRODUCTIVITIES AND TIMES OF GROWTH IN *D. STRAMONIUM* ROOT CULTURES[a]

Line	Hyoscyamine, mg/g DW × Day	Scopolamine, mg/g DW × Day	Times of Growth
N1	0.018	0.0038	6.6
N2	0.006	0.0023	1.8
N3	0.011	0.0080	4.9
N4	0.004	0.0027	1.7
N5	0.018	0.0064	4.9
N6	0.008	0.0031	2.7
N7	0.006	0.0025	2.1
N8	0.013	0.0028	3.5
N9	0.011	0.0059	1.9
N10	0.007	0.0035	2.0
Plant roots	0.00006	0.000017	
Whole plant	0.00014	0.000157	

[a] Values represent the mean for duplicate samples (root cultures) or triplicate (plant tissues). SE for each point was lower than 5%.

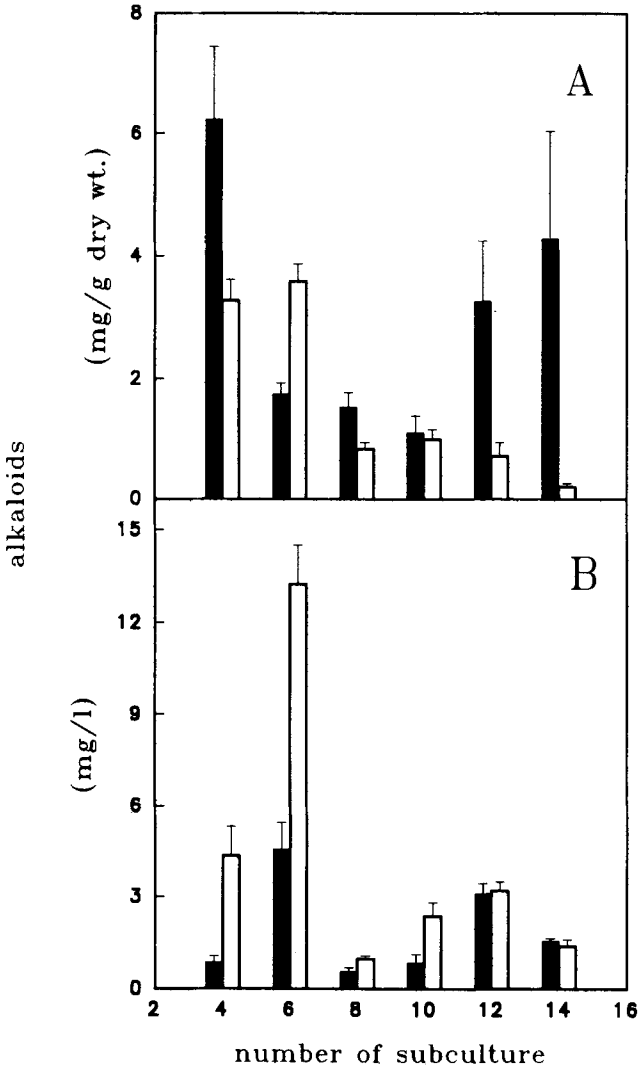

FIG. 4. Stability in hyoscyamine and scopolamine contents within the tissue or in the culture medium with time. *A*, Content within the tissue of hyoscyamine (*solid squares*) and scopolamine (*open squares*). *B*, Content in the culture medium of hyoscyamine (*solid squares*) and scopolamine (*open squares*). Each point represents the mean ± SE for samples analyzed in duplicate.

increased 4.4 times its initial biomass on a fresh and a DW basis. The doubling time for this line was 9.8 days. Total alkaloids (Fig. 2 *A*) and hyoscyamine (Fig. 2 *B*) levels showed their higher values during the first half of the exponential growth phase. Scopolamine (Fig. 2 *B*) and total alkaloid content showed a recovery at the stationary phase. Again, alkaloids in the extracellular medium were detected only in trace amounts.

Figure 3 *A* shows the time courses of growth and alkaloid content in line N5 during subculture 8. The alkaloid content was not coupled to growth as in line N1; however, it followed a different pattern from line N9. Line N5 grew faster than lines N1 and N9 with a doubling time of 5.7 days and reached a six- and fourfold increase on a fresh and DW basis, respectively. There was a peak on total alkaloids (Fig. 3 *A*), hyoscyamine, and scopolamine (Fig. 3 *B,C*) levels during the early lineal phase, which was not sustained along this phase. Alkaloid content in both the tissue and extracellular medium increased during stationary phase. This fact correlates with a decrease of the growth rate in a way similar to line 1.

One important difference in lines N1 and N9 in regard to N5 roots was that this last line could excrete higher levels of hyoscyamine and scopolamine into the culture medium along the time course of growth.

Stability of growth and alkaloid production in root lines. Tables 1 and 2 compare the data obtained for growth parameters and alkaloid production at the end of the third subculture of the different root cultures. If these data for line N1 are compared with those obtained at subculture 4 (Fig. 1) we find that the growth rate decreased to 40% in only one transfer (from 6.6 at third subculture to 2.4 times of biomass production at the 4th subculture). This line showed a continuous decline in growth capacity until transfer 6, when it showed an extensive callusing response and a lack of biomass production. Lines N5 and N9 showed a higher stability in growth which is observed in the time courses at subcultures 3 (Table 1) and 8 (Figs. 2 and 3).

During this study, alkaloid production of line N9 was monitored during subsequent transfers. We selected this line to exemplify this parameter because of its high alkaloid content in tissue and its capacity for alkaloid excretion into the culture medium (although during subculture 8 the alkaloid levels into the extracellular medium were not detectable). As we obtained our samples at Day 35 (stationary phase) of culture during the first 8 transfers, we decided to take samples at this day for subsequent transfers. Figure 4 *A,B* show high instability of this line on both alkaloid levels in the tissue and in the culture medium. Similar data were obtained for line N5 (data not shown).

Alkaloid content and bioproductivity in root cultures. Table 1 shows the alkaloid content and Table 2 the bioproductivity (defined as milligrams of alkaloid per gram of biomass (gram final DW − gram inoculum DW) − 1 produced per day − 1) of the 10 initially selected root lines which were evaluated in the 3rd subculture at Day 35. These data are comparable to those obtained by Hashimoto et al. (1986) in hyoscyamine content and growth rates. Plant roots were evaluated for alkaloid content and bioproductivity after 11 mo. in culture. Alkaloid pattern by TLC showed six to eight spots in both plants and root cultures.

The scopolamine:hyoscyamine (S:H) ratio (Table 1) obtained from the plant roots was very similar to those obtained in some root cultures, although in some lines higher S:H ratios were found (lines N10, N9, N4, N3).

Total alkaloid content (Table 1) in plants was one to two orders lower than in root cultures, and this was also true for hyoscyamine and scopolamine levels. A comparison of the bioproductivity (Table 2) of these compounds in the pot-grown plant roots shows that it was two to three orders lower than cultured roots, and it increases one order of magnitude if we consider the productivity in the whole plant for both compounds: hyoscyamine and scopolamine. The better hyoscyamine-producing root lines (N1, N9) were 300 and 128 times more productive than plant roots and whole plants, respectively. The best scopolamine-producing root line (N3) was 470 and 50 times more productive than plant roots and whole plants, respectively.

Line N9 had the higher alkaloid contents (Table 1) which were 1.2% for total alkaloid and 0.62 and 0.33% on a DW basis for

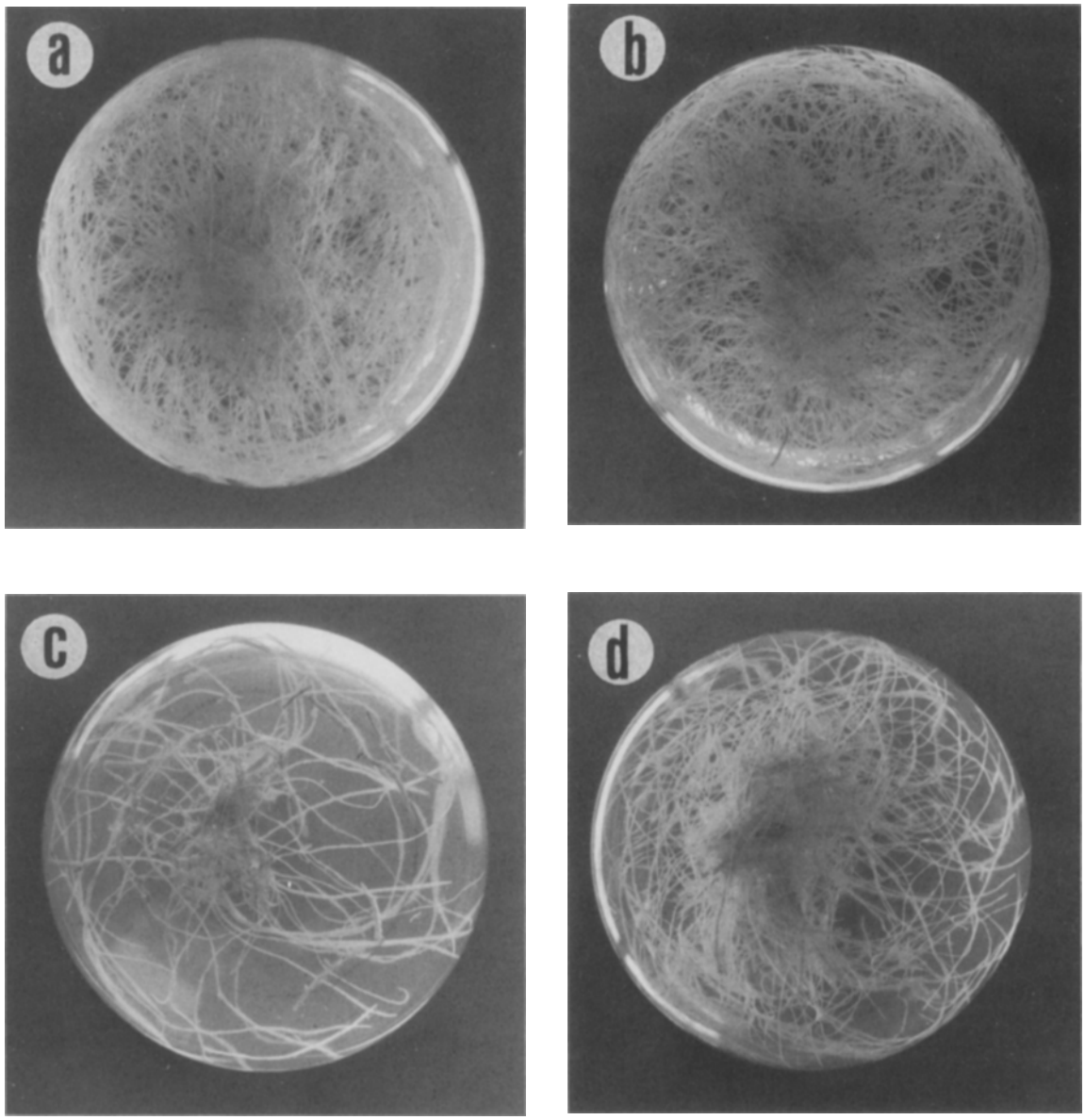

FIG. 5. Morphology of four different *D. stramonium* root lines at Day 21 of culture. *a*, line N7; *b*, line N8; *c*, line N9; *d*, line N10.

hyoscyamine and scopolamine, respectively. Hyoscyamine and scopolamine were the main alkaloids in all root cultures evaluated and their percentage of the total tropane alkaloids pool was 40 to 90%. However, other spots appeared on TLC plates. Their identification is now under way.

DISCUSSION

Alkaloid content and productivity values of our root cultures were one order of magnitude higher than *Datura stramonium* calli lines (Hashimoto et al., 1986) or even higher (Savary and Dougall, 1990). These data support the proposed hypothesis that alkaloid synthesis or accumulation (specially scopolamine) or both is associated with root differentiation as it has been shown before (Endo and Yamada, 1985; Hartmann et al., 1986; Lindsey and Yeoman, 1983).

It also has been reported for *D. stramonium* root cultures, that IBA produces a DW increase by inducing lateral root formation and alkaloid biosynthesis inhibition, affecting specifically 6-β-hydroxyhyoscyamine hydroxylase activity (Hashimoto et al., 1986). All efforts done in this work were not enough to obtain a root line able to grow in an auxin-free medium. Concentrations lower than 10^{-6} *M* (10^{-7} *M*) did not produce any growth, and at higher concentrations (10^{-4}, 10^{-5} *M*) a fast callusing response in the tissue was noticed.

The time courses for growth and alkaloid production in lines N1, N5, and N9 point out the well-known relationship that exists between lower growth rates and high levels of alkaloid accumulation in cultured tissues. However, there was an exception: line N9, in which the maximum alkaloids content was present during the lineal growth phase.

Our root cultures showed a great variability both in growth and in

alkaloid content, so it is possible to screen this variability to look for overproducing root lines and to find cultures with a superior capacity for biomass production. We also observed very different phenotypic traits (Fig. 5). which could be helpful in choosing root lines with desirable characteristics such as number of lateral roots and resistance to shear stress.

Figure 4 shows that *Datura* root cultures presented a great instability in alkaloid production. This fact represents an evident disadvantage for their use on large scale production of tropane alkaloids.

Only a few root cultures have been reported to grow well and to produce alkaloids comparable to the whole plant levels; nicotine in *Nicotiana tabacum* (Solt, 1957), nicotine and anabasine in *N. glauca* (Solt et al., 1960), atropine in *Atropa belladonna* (Mitra, 1972) are some examples. In some cases, however, higher levels of alkaloid production have been reported, as in the case of *Hyoscyamus* root cultures (Hashimoto et al., 1982). Some *Datura stramonium* root lines grew well and produced high levels of total alkaloids, hyoscyamine and scopolamine. When we considered the whole plant, we observed higher alkaloid productivity because the alkaloid content of the aerial parts were taken into account. As the evidence indicates that the biosynthetic capacity for alkaloid production is predominantly in the roots, the accumulation observed in the aerial parts must represent subsequent translocation and storage (Payne et al., 1987). If all the alkaloid production observed in plants remained in the root, the capacity of production would be as follows: total alkaloid content 2.64% DW, hyoscyamine 0.81%, and scopolamine 0.87%, these values represent a hyoscyamine and scopolamine productivity of 0.0245 and 0.02654 mg $\times g$ DW-1 Day-1, respectively, which are higher values than those obtained in the root cultures. These same observations has been pointed out before in some reports with *Hyoscyamus* and *Datura* root cultures when compared with plants (Endo and Yamada, 1985; Hashimoto et al., 1986; Payne et al., 1987).

Some of the root cultures showed higher S:H ratios compared to those of the plant roots. This could suggest that, in the case of scopolamine, the blocking of translocation in this kind of organ culture and the availability of hyoscyamine (which is converted to scopolamine by the 6-β-hydroxyhyoscyamine hydroxylase) could help the root cultures to express a higher biosynthetic potential for the production of scopolamine. One report indicates that only hyoscyamine was found in plant roots (Payne et al., 1987) whereas we found both compounds in the plant roots (Table 1). This could be due to the variability and the different environmental conditions to which pot-grown plants were exposed. Comparing scopolamine production in our root cultures with the corresponding known potential of plant roots, one can conclude that the latter has not been expressed completely in the root cultures. Nevertheless care should be taken in this respect because the alkaloid content measured in the root cultures represents the potential for alkaloid production under the present culture conditions. Therefore different approaches aimed at obtaining higher productivity should be tested.

The present data suggest that root cultures have disadvantages for tropane alkaloid production. This system, which possesses features such as great variability, changing qualitative, and quantitative pattern of alkaloids between root lines, is a good model to study alkaloid biosynthetic pathway and its regulation and, of course, it offers the possibility of obtaining a stable and high-alkaloid producing line. In this regard, we have observed recently that some of our surviving lines have started to show higher growth rates and better stability in alkaloid production (data not shown). We are following these lines to observe their evolution with time.

Acknowledgements

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