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Datura stramonium Hairy Roots Tropane Alkaloid Content as a Response to Changes in Gamborg's B_5 Medium

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ABSTRACT

Hairy roots are less susceptible to manipulation by changes in medium composition than callus and cell suspension cultures. However, when the individual components of the Gamborg's B_5 medium were modified one by one, several of them modified the yield of root cultures. Nitrogen (N) source changes, the absence of phosphorus (P), calcium (Ca), and cobalt (Co), and the increase in the sucrose content of the medium increased the level of alkaloids in *Datura stramonium* hairy root cultures. The use of ammoniun as N source provoked the alkaloid excretion into the medium. The addition of different auxins inhibited the alkaloid accumulation, but the addition of abscisic acid (ABA) did not change the alkaloid content of the cultures. The use of MeJa increased the content of hyoscyamine to 100%.

Index Entries: Alkaloids; hairy roots; *Datura stramonium*.

INTRODUCTION

During the past three decades, plant cell cultures have developed into useful experimental tools to study the metabolic pathways of higher plants, in particular, those leading to natural products. However, the potential of plant cell cultures for the production of secondary metabo-

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lites is still limited by two major factors. The first one is the low productivity of the desired compounds. This problem may be partly overcome by manipulating the components of the culture medium (*1*), altering the physical environment (*2*), and screening for cell clones with high productivities (*3,4*). The use of these strategies, alone or as a combination, has resulted in over 40 examples of plant cell cultures that are able to produce secondary metabolites at higher levels than in the whole plant. However, there are many more cases in which the above strategies have failed. The second factor is the genetic instability of plant cell lines, which has been extensively documented (*5,6*).

During the last decade, the need of cell organization for the biosynthesis of secondary metabolites in plant tissue cultures has been recognized to be fundamental (*7*). In general, undifferentiated plant cell cultures do not produce secondary metabolites, but will do so when they are induced to differentiate into tissues/organs. The expression of secondary metabolic pathways in redifferentiated cell cultures is actually not surprising, because it mimics exactly what the plant does. Furthermore, in most cases the synthesis of plant natural products is under strict temporal and spatial control.

In order to overcome the problem of normal root cultures' slow growth, a system that involves the generation of fast-growing adventitious roots or hairy roots, which are the product of the infection of different tissues with *Agrobacterium rhizogenes*, has been developed. The induction of morphological changes is preceded by the stable integration of a portion of the Ri (root-inducing) plasmid into the plant genome, a characteristic that it shares with the causal agent of the crown gall tumor disease, *A. tumefaciens* (*8*). As far as we know, these hairy root cultures have the same metabolic features as normal root cultures, as well as fast-growing suspension cultures.

The effects induced by different culture media (*9–12*), as well as the changes in the medium composition observed in cell suspension cultures (*1*), have also been studied in hairy root cultures. In general, the change from one culture medium to another produces important changes in growth patterns and also in the type of secondary metabolites produced by root cultures (*11,13,14*). The best results have been obtained using simple media or by changing the original medium's ionic strength (*10, 15–19*).

The use of glucose, instead of sucrose, was deleterious not only for root growth, but also for hyoscyamine accumulation in *D. stramonium*. In contrast, an increase in sucrose concentration (from 3 to 5%) produced an increase of 32% in the hyoscyamine content, but reduced growth in 26% compared to control (*19,20*). In another *D. stramonium* line, the response to the change in the sucrose concentration was even more pronounced, i.e., the hyoscyamine content increased eightfold (*19*). The effect of reducing sucrose from 3 to 2% produced a 10-fold decrease in the scopolamine content in a hairy root line of *D. stramonium*, which does not accumulate hyoscyamine (*21*).

In *C. roseus* hairy roots, a change in sucrose concentration from 3 to 7% induced a 55% decrease in fresh weight, while the contents of ajmalicine and serpentine increased 215 and 530%, respectively (*16*). Similar results have been obtained in *Rubia tinctorum*, in which 12% sucrose resulted in maximal growth and anthraquinone production (*22*). When a two-stage process was employed, using sucrose in the first stage and fructose in the second one, there was an improvement in the volumetric yields of catharanthine of about twofold (*23*).

Changes in sucrose, phosphate, nitrate, and ammonia concentrations produced contradictory results on growth and indole alkaloid production in *C. roseus* hairy root cultures (*24*). However, when the content of individual alkaloids was analyzed, a pattern emerged. There was a preferential accumulation of ajmalicine and catharanthine when 4.5% sucrose was employed (*25*).

A decrease of the sources of both phosphate and nitrate produced an increase in hyoscyamine-synthesizing *D. stramonium* hairy roots (*20*). Similar results have been observed for the accumulation of ajmalicine and serpentine in *C. roseus* hairy roots (*16*). Addition of 1% casamino acids to the culture medium to substitute for $NaNO_3$ increased the content of scopolamine threefold in *Scopolia japonica* hairy roots (*9*).

In some species, such as *Tagetes patula* (*26*), *Rubia tinctorum* (*22*), and *Panax ginseng* (*27*), auxins, either alone or in combination with cytokinins, stimulate growth by increasing branching. In other species, auxins cause significant inhibition of root growth and indeed stimulate root structure disorganization, losing in this way the capacity for the production of secondary metabolites (SM) (*28,29*). Exogenous application of gibberellic acid (GA_3), between 10^{-8} and 10^{-3} g/L, accelerated the increase in fresh weight of *D. innoxia* hairy roots (*30*).

In *Hyoscyamus albus* (*31*), the effect of phytohormones on tropane alkaloid production varied according to the strain of *Agrobacterium rhizogenes* used for transformation; meanwhile, in root cultures of a *Dubosia* hybrid, the addition of indole acetic acid (IAA) derivatives produced an increase in the yields of scopolamine, and 50% of the total scopolamine was found in the culture medium (*32*). However, in *Dubosia myoporoides*, IAA had scarcely any effect on cell growth or in tropane alkaloids production, and indolebutric acid (IBA) only slightly increased cell growth (*33*). In contrast, napthaleneacetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) severely inhibited tropane alkaloid production (*33*).

Addition of IBA (2 mg/L) to *Panax ginseng* hairy roots produced an 84% increase in saponins accumulation; when kinetin was also added, this increase reached 179%, i.e., from 0.36 to 0.95% DW (*27*).

The addition of NAA (0.5 mg/L) to *Amsonica elliptica* hairy roots increased their growth in the light; when cultivated in the dark, there was an important decrease (*34*). In contrast, NAA increased growth in *Chaenactis douglassi*, but reduced the amount of thiarubrine almost to zero (*35*). In *Lippia dulcis*, the use of NAA (1 mg/L) induced an increase of 100% in

hernandulcin production (*36*). In *R. tinctorum*, 5 μ*M* IAA produced the highest anthraquinone production and maximal growth of all conditions tested, but kinetin had no effect at all (*22*). In *T. patula*, auxins could be playing a direct or indirect role in the regulation of thiophene levels in the root tips (*26*).

Alkaloid synthesis is also triggered in *C. roseus* and *Cinchona ledgeriana* plantlets by methyl jasmonate (MeJa) (*37*). In the presence of MeJa vapor, tabersonine, vindoline, and catharanthine content increased in a dose-response way as well as the activities enzymes involved in their synthesis (*37*). In *C. roseus* hairy roots, addition of MeJa increased the amount of ajmalicine and catharanthine by 57 and 108%, respectively, and induced their excretion into the culture medium (*38*). MeJa also increased anthocyanin accumulation in emerging soybean seedlings (*39*).

From the biotechnological point of view, the changes in the culture medium, and the different effects of the auxins can be used to increase the culture biomass, in general, or to change the content of a special metabolite. Since very little effort has been made to measure growth and alkaloid production rates under a wide range of conditions, the aim of this work is to investigate the effects of different medium components in biomass increase and alkaloid production of *D. stramonium* hairy roots.

MATERIALS AND METHODS

Plant Material

D. stramonium hairy root cultures were obtained through genetic transformation of stems with *Agrobacterium rhizogenes* strain TR-105, as reported by Maldonado-Mendoza et al. (*40*). The root line used in this work was 4 yr old at the beginning of the experiments and has been maintained through subculturing every 30 d in 100 mL Gamborg's B_5 medium (*41*), supplemented with 3% sucrose in 250 mL flasks. The root cultures were kept on orbital shakers at 100 rpm, 25°C, in the dark.

Treatments

Two different strategies were assayed in order to obtain higher alkaloid yields. The first one involved modification of the medium composition and the second one the use of different hormonal treatment with auxins and MeJa.

Modification of Gamborg's Medium

In order to develop a medium that would lead to higher alkaloid yields, some of the original mineral and organic composition of B_5 medium were modified. Regarding the major salts, $CaCl_2 2H_2O$, $MgSO_4 7H_2O$, and NaH_2PO_4 were assayed in concentrations, that ranged from total absence to a fourfold phosphate and magnesium (Mg) increase and twofold

calcium (Ca) increase. The N source was also varied, both in concentration and in the nitrate/ammonium ratio. The total N concentrations assayed ranged from 25–62.4 m*M* (from 0–60 m*M* for both nitrate and ammonium) and the nitrate/ammonium relationship ranged from 4–25. Among the minor salts, $CoCl_26H_2O$, $CuSO_45H_2O$, $FeSO_47H_2O$, $MnSO_4H_2O$, $ZnSo_4$ $7H_2O$, and H_3BO_3 were evaluated in concentrations that ranged from zero to a fourfold increase over their original ones. Since sucrose has always been the carbon source of choice, a concentration range from 2–6% was assayed.

Growth Regulators

Auxins, except IBA, were sterilized by autoclaving. IBA, ABA (mixed isomers, 90% pure) were dissolved in water at such a concentration that, when an aliquot of the filter-sterilized solution (0.22 μM millipore filter) was added to 100 mL of culture medium, the desired final concentration was obtained. MeJa was sterilized by the same procedure, except that it was dissolved in methanol (analytic grade). Control cultures were set up using aliquots of distilled water or methanol (analytical grade). Auxins were added from the beginning of the culture and hairy roots were harvested at the stationary phase (24 d). ABA and MeJa were added on the 24th culture day, and roots were harvested after 48 h.

Tropane-Derived Alkaloid Quantification

Hyoscyamine and scopolamine were extracted and quantified as reported previously by Monforte-Gonzalez et al. (*42*).

Statistical Analysis

ANOVA statistical analysis and TUKEY test (significance level: $p = 0.05$) were used for multiple comparison means from the treatments.

RESULTS

Two different strategies were tested in order to obtain higher alkaloid yields. The first one involved the modification of the medium composition and the second one the utilization of different growth regulator treatments, such as auxins and MeJa.

Growth, measured both as fresh or dry wt, was significantly promoted, from 5 g to more than 6 g per flask, when roots were cultured for 28 d in media containing 5% sucrose (data not shown). At the same time, root hyoscyamine content increased from 12.5 mg/g DW in the control (3% sucrose) to more than 32 mg/g DW in the samples cultured with 6% sucrose (Fig. 1A). Root scopolamine content was very low, and was only appreciable at low sucrose concentrations. Sucrose treatment also released alkaloids into the culture medium, from 1.5 mg of hyoscyamine per 100 mL for the control to 3.5 mg/100 mL for the treatment with 5% sucrose (Fig.

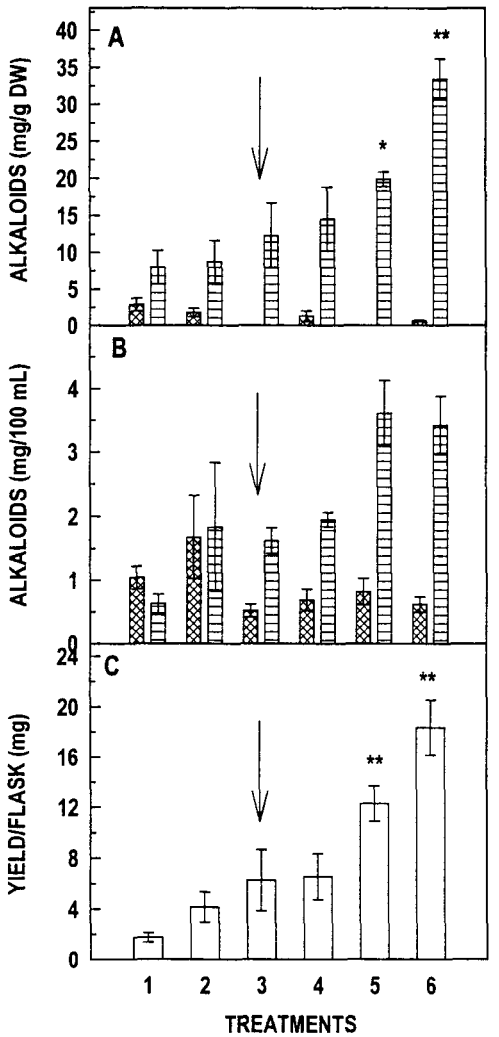

Fig. 1. Effect of different sucrose concentration on the alkaloid content (**A**), alkaloids in the culture medium (**B**), and yield per flask (**C**) of *D. stramonium* hairy roots. Treatments: 1, sucrose 1%; 2, sucrose 2%; 3, sucrose 3% (control); 4, sucrose 4%; 5, sucrose 5%; 6, sucrose 6%. (▤) hyoscyamine; (▩) scopolamine; and (□) both alkaloids. The arrow indicates the control sucrose concentration.

1B). But the most dramatic response was for scopolamine: In the presence of 2% sucrose (Fig. 1B), this compound increased up to 200% more than in the control.

It is possible that alkaloid excretion from the roots into the medium releases the feedback inhibition in the biosynthetic pathway of these two alkaloids, allowing the synthesis of more hyoscyamine. Furthermore, the mass yield of these cultures was higher, so that the total yield alkaloid per flask increased from 6 to 18 mg (Fig. 1C). This is the first time that an increase for both alkaloid content and biomass is reported for *D. stramonium* hairy roots.

Since alkaloids are nitrogen-containing molecules, much has been done in the field of modification of the nitrogen content of the culture medium; however, there is not a general consensus about the effects of the nitrogen source in the changes that are provoked in the cells, so each culture must be tested individually (*43–47*). In the present work, two different strategies were used. In the first, different concentrations and different nitrogen sources were used; in the second, a different relationship between oxidized and reduced nitrogen was used.

None of the different nitrogen sources were capable of sustaining growth as well as the control (data not shown). However, the use of ammonium, alone or in combination with di- or tricarboxylic acids, produced a significant increase in the root hyoscyamine content (Fig. 2A). By contrast, the amount of scopolamine decreases in all the treatments (Fig. 2A).

The use of ammonium as nitrogen source provoked the excretion of scopolamine into the culture medium; there was a good correlation between ammonium concentration and the amount of scopolamine released into the medium. The use of di- or tricarboxylic acids eliminated this phenomenon totally (Fig. 2B). Although it seemed that the alkaloid yield was higher in some of the N sources used, total productivity was equal to that of the control, as a result of the poor growth in these cultures (Fig. 2C).

As a result of the increment in the total nitrogen an increase in root culture biomass was produced (Fig. 3; treatments 6–9). Increase in the relationship between oxidized and reduced nitrogen provoked a growth decrease (Fig. 3; treatments 1–4). Use of 41.63 m*M* of total nitrogen, instead of 25.8 m*M* (control), significantly increased the hyoscyamine amount in the roots (Fig. 4A). The presence of higher concentrations of ammonium induced the excretion of hyoscyamine into the culture medium (Fig. 4B). As a result of better growth and hyoscyamine production, the yield per flask was around 55% bigger at higher total nitrogen (Fig. 4C).

Changes in the content of Mg, P, and Ca in the culture medium did not change the growth of the roots nor the alkaloid content. However, absence of phosphorous increased more than 130% the alkaloid excretion into the culture medium (Fig. 5); a similar response, but to a lesser extent, was provoked by the absence of calcium (Fig. 5).

A more than 100% increment in the hyoscyamine content was observed when cobalt was eliminated from the culture medium (Fig. 6A), which in turn elevated the yield per flask (Fig. 6C). None of the treatments released more alkaloids into the culture medium than in the control (Fig. 6B).

Since the role and the effect of auxins in secondary metabolism regulation in tissue culture are not clear, and since, in some cases, it is necessary to keep the culture growing, we first evaluated the effect of three different auxins at four different concentrations each on growth and alkaloid content of a *D. stramonium* hairy root line.

Addition of NAA $10^{-6}M$ and 2,4-D $5 \times 10^{-7}M$ produced the highest increase in the biomass; NAA produced 163% and 2,4-D 63% more fresh weight than the control. IBA did not have any effect on root growth.

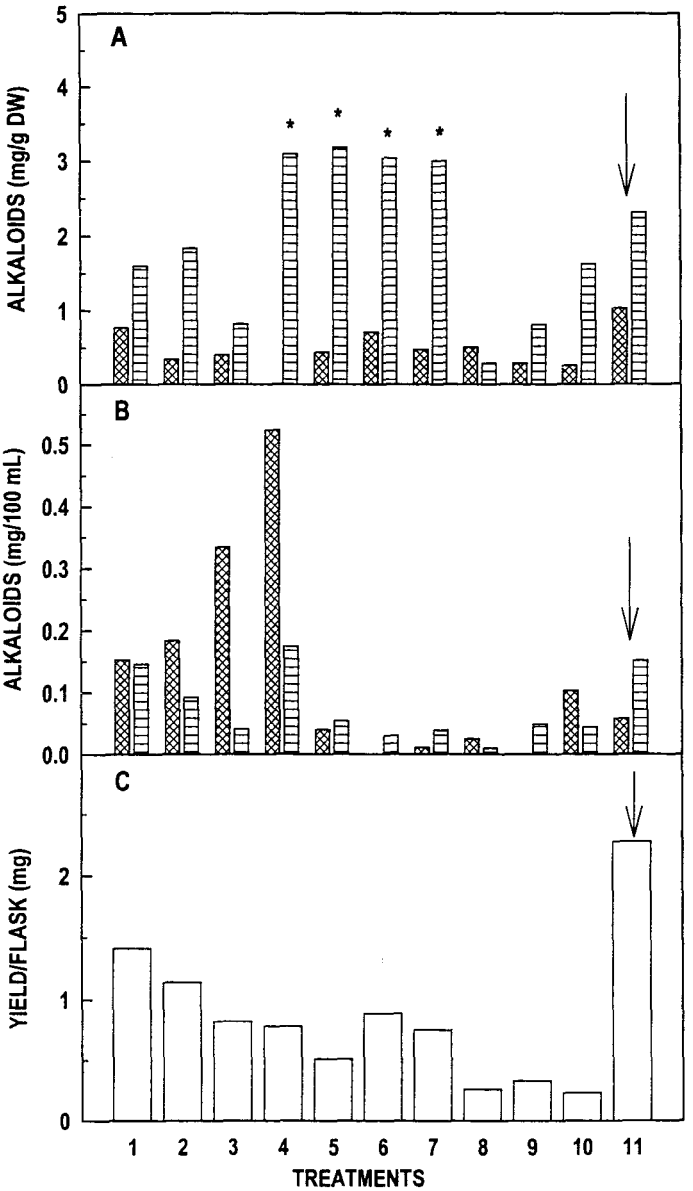

Fig. 2. Effect of different kinds and concentrations of N sources on the alkaloid content (**A**), alkaloids in the culture medium (**B**), and yield per flask (**C**) of *D. stramonium* hairy roots. Treatments: 1, 10 m*M* KNO_3; 2, 10 m*M* NH_4NO_3 + 10 m*M* KNO_3; 3, 20 m*M* NH_4NO_3 + 10 m*M* KNO_3; 4, 10 m*M* NH_4Cl; 5, 10 m*M* ammonium citrate; 6, 10 m*M* ammonium succinate; 7, 10 m*M* ammonium malate; 8, 10 m*M* aspargine; 9, 10 m*M* glutamine; 10, 10 m*M* glutamate; 11, control. (▤) hyoscyamine; (▨) scopolamine; and (□) both alkaloids. The arrow indicates the control N concentration. (*) significant.

When dry wt was evaluated instead of fresh weight, the effect was maintained, suggesting that the increase in biomass was not the effect of water incorporation into the tissues, but real growth. However, in all cases, as has been reported (*28,29*), the roots lost their morphology.

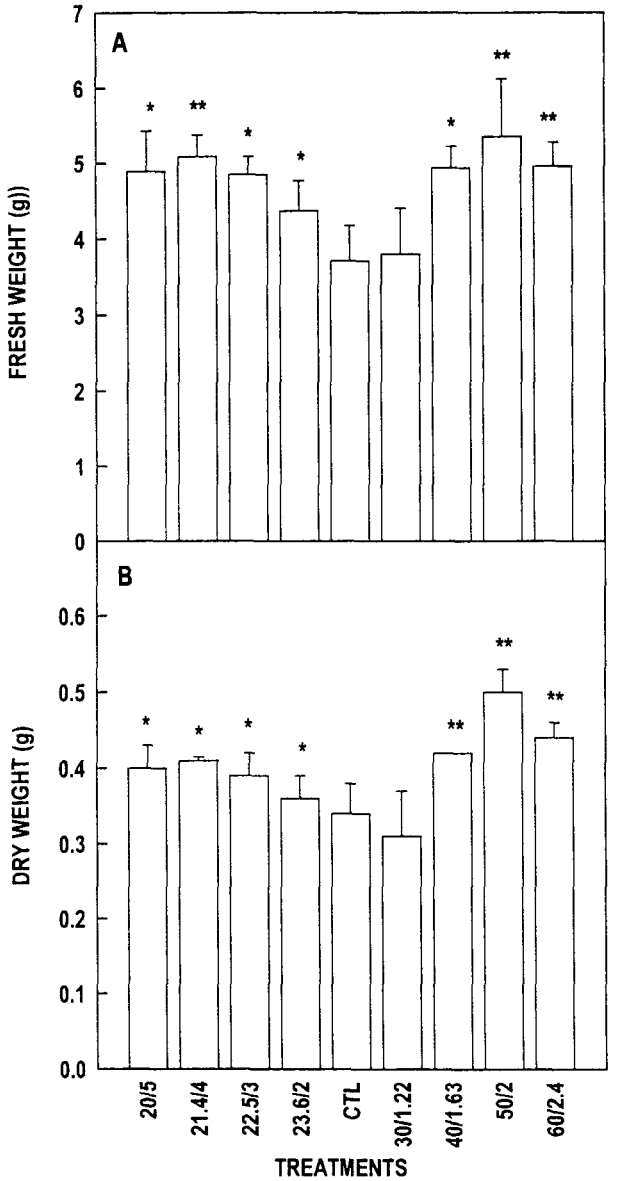

Fig. 3. Effect of the nitrate/ammonium relationship on the variation of fresh (**A**) and dry wt (**B**) of *D. stramonium* hairy roots. Treatments: 1, 20/5; 2, 21.4/4; 3, 22.5/3; 4, 23.6/2; 5, 24.7/1 (control); 6, 30/1.22; 7, 40/1.63; 8, 50/2; 9, 60/2.4. The arrow indicates the control nitrogen concentration. (*) significant; (**) highly significant.

Tropane alkaloid content was modified greatly in the presence of both 2,4-D and NAA. At higher concentration of these two auxins, the least amount of alkaloid was detected (Fig. 7A,B). 2,4-D had the major effect on alkaloid decrease, and better growth was not enough to compensate the loss of alkaloid biosynthetic capability (Fig. 8). Decrease in alkaloid content coincided with loss of root morphology.

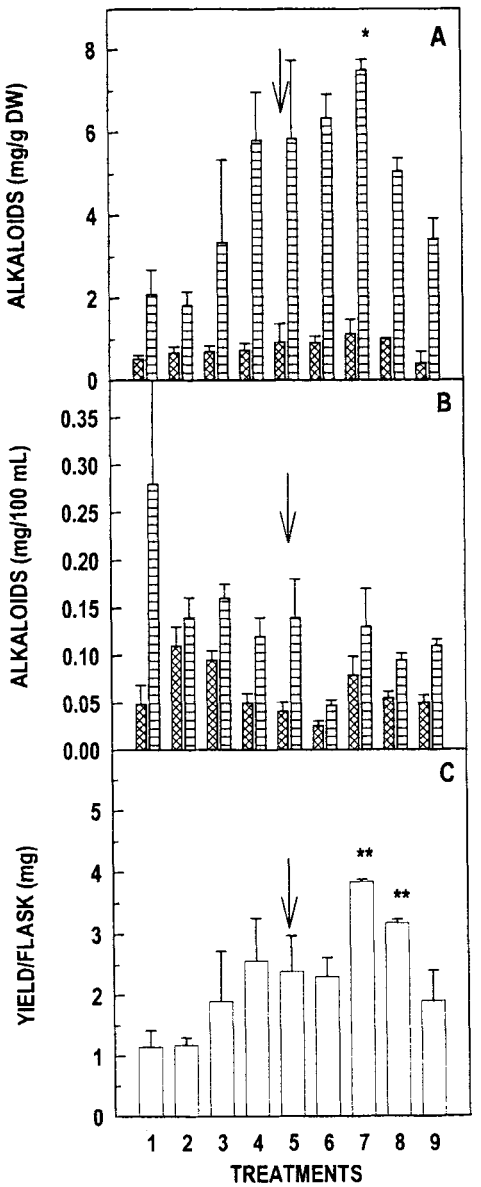

Fig. 4. Effect of the nitrate/ammonium relationship on the alkaloids content (**A**), alkaloids in the culture medium (**B**), and yield per flask (**C**) of *D. stramonium* hairy roots. Treatments are the same as in Fig. 3. (▤) hyoscyamine; (▩) scopolamine; and (□) both alkaloids. The arrow indicates the control nitrogen concentration. (*) significant; (**) highly significant.

Exposition of *D. stramonium* hairy roots to different ABA concentrations into the culture medium did not modify hyoscyamine and scopolamine content of the tissues (data not shown).

MeJa addition to the hairy root cultures had a great effect on the hyoscyamine content, showing a typical dose-response curve (Fig. 9).

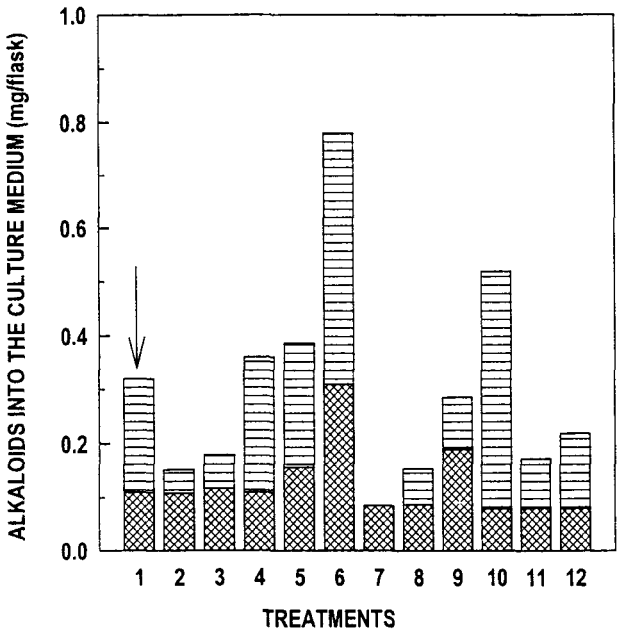

Fig. 5. Effect of different macronutrients on the alkaloids excreted into the culture medium of *D. stramonium* hairy roots. Treatments: 1, Control; 2, without Mg; 3, 0.5-fold Mg; 4, twofold Mg; 5, fourfold Mg; 6, without P; 7, 0.5-fold P; 8, twofold P; 9, fourfold P; 10, without Ca; 11, 0.5-fold Ca; 12, two-fold Ca. (▨) hyoscyamine and (▤) scopolamine. The arrow indicates the control macronutrient concentration.

Hyoscyamine content was double in relation to the control at 0.1 μM MeJa. Instead, the scopolamine content was not changed.

DISCUSSION

Previously, it has been reported that product formation is less susceptible to manipulation by changes in medium composition than callus and cell suspension cultures (*20*) and that the modification of the level of Gamborg's B_5 salts medium did not greatly affect the growth rate of *D. stramonium* transformed roots (*19*), or hyoscyamine content (*18*), except when the root cultures were grown at 20°C; however, all the salt concentrations were modified together. On the other hand, the decrease in nitrate and phosphorus slightly increased the hyoscyamine content of *D. stramonium* hairy roots (*20*).

In this work, although most of the medium components did not have any effect on root alkaloid content, several of them modified the yield of root cultures, such as the nitrogen source change (Figs. 2–4), the absence of P and Ca (Fig. 5), the absence of Co (Fig. 6), and the increase in the sucrose content of the medium (Fig. 1C). These different responses to the

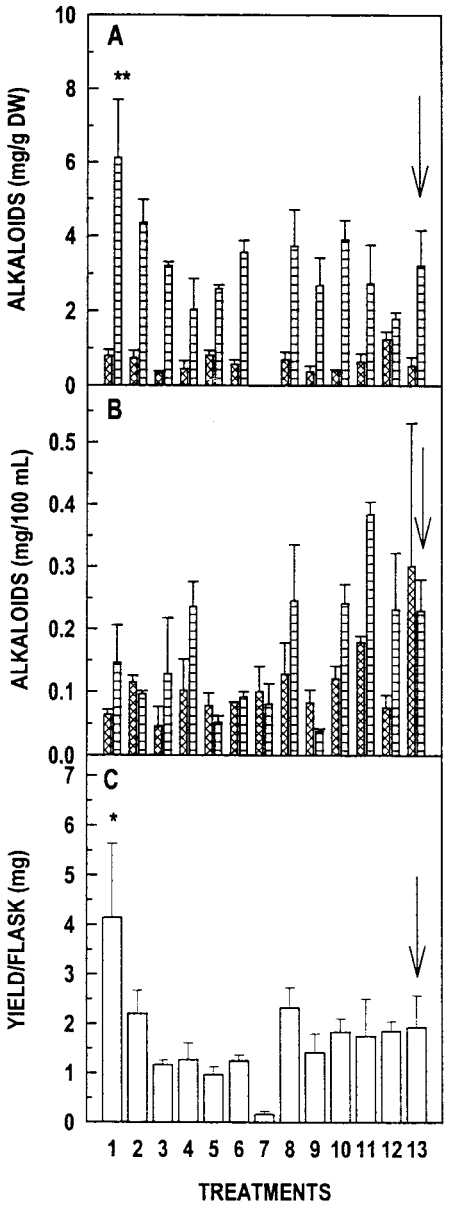

Fig. 6. Effect of different micronutrients on the alkaloid content (**A**), alkaloids in the culture meidum (**B**), and yield per flask (**C**) of *D. stramonium* hairy roots. Treatments: 1, without Co; 2, fivefold Co; 3, 10-fold Co; 4, without Cu; 5, fivefold Cu; 6, 10-fold Cu; 7, without Fe; 8, fivefold Fe; 9, 10-fold Fe; 10, without Mo; 11, fivefold Mo; 12, 10-fold Mo; 13, control. (▤) hyoscyamine; (▨) scopolamine; and (□) both alkaloids. The arrow indicates the control micronutrient concentration. (*) significant; (**) highly significant.

medium by different *D. stramonium*-transformed root cultures could be the result of different numbers of plasmid copies that could be integrated into the genome (*20*).

The yield of *D. stramonium*-transformed root cultures increased in proportion to sucrose content in the medium (Fig. 1A and C). This incre-

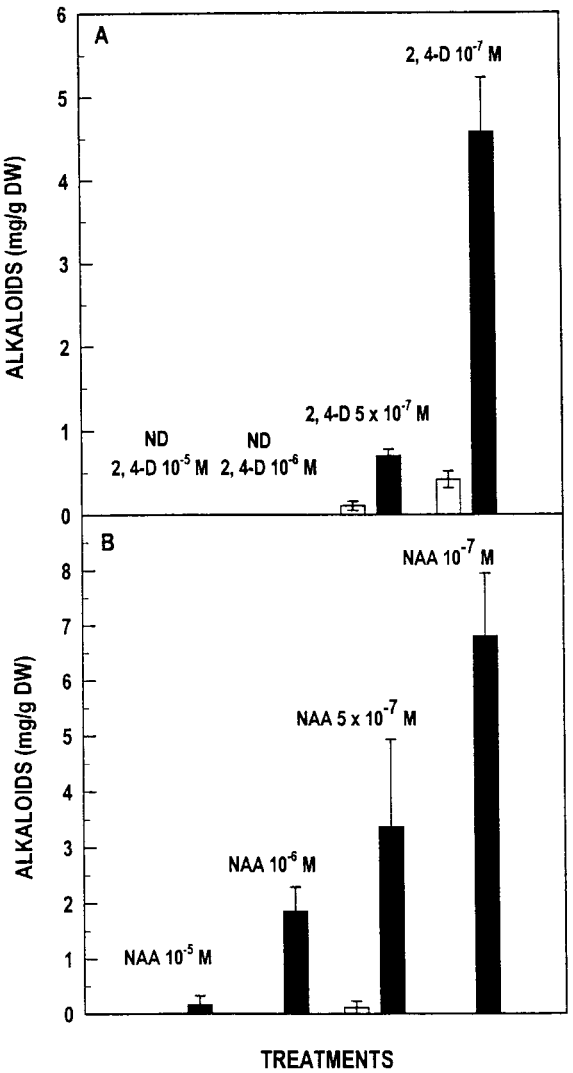

Fig. 7. Effect of different concentrations of 2,4-D (**A**) and NAA (**B**) on alkaloid content of *D. stramonium* hairy roots.

ment was not only a consequence of the major biomass but also of a higher amount of hyoscyamine inside of the cell (Fig. 1A). Similar results have been reported previously (*19*) for *D. stramonium* root cultures grown at 25°C.

Another remarkable finding was the alkaloid excretion provoked by ammonium as N source. This excretion is the consequence of a pH decrease in the medium as a consequence of ammonium ion uptake (*48*).

The addition of a single growth regulator or in combination to *Hyoscyamus albus* hairy roots obtained with *Agrobacterium rhizogenes* strain A4 reduced the alkaloid formation substantially (*31*). In contrast, transformed roots of the same species obtained with *A. rhizogenes* stain 15834 increased the alkaloid production as a result of the addition of IAA (*31*); the effect is light dependent.

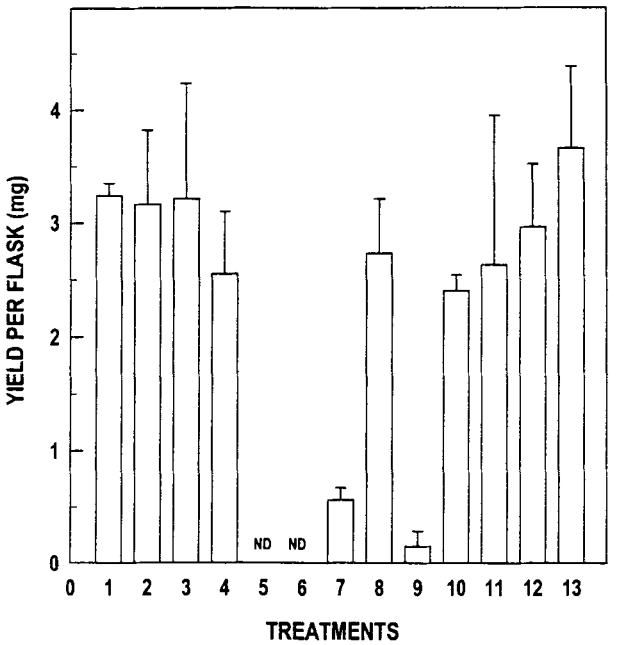

Fig. 8. Effect of different concentrations and kinds of auxins on the yield by flask of *D. stramonium* hairy roots. Treatments: 1, IBA 10^{-5}; 2, IBA 10^{-6}; 3, IBA 5×10^{-7}; 4, IBA 10^{-7}; 5, 2, 4-D 10^{-5}; 6, 2, 4-D 10^{-6}; 7, 2, 4-D 5×10^{-7}; 8, 2,4-D 10^{-7}; 9, NAA 10^{-5}; 10, NAA 10^{-6}; 11, NAA 5 X 10^{-7}; 12, NAA 10^{-7}; 13, control without auxins. ND, not detected.

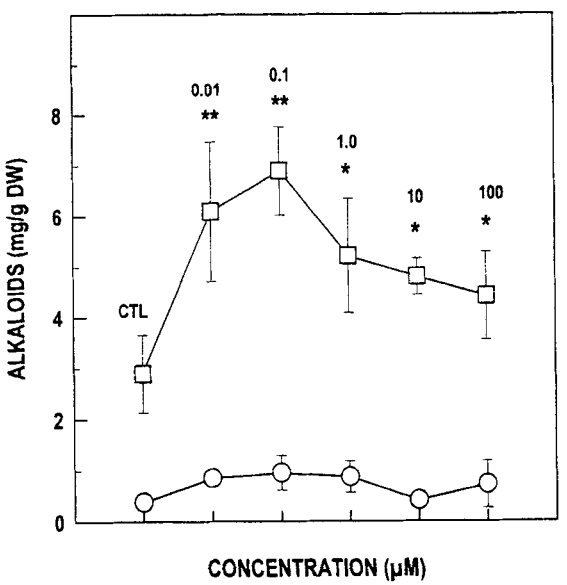

Fig. 9. Effect of MeJa on the alkaloid content of *D. stramonium* hairy roots. Hyoscyamine (□) and scopolamine (○). (*) significant; (**) highly significant.

Our data show that the phytohormones used inhibit the alkaloid accumulation as in other transformed root cultures (*28,31*). This decrease had been shown to be caused by very low levels of the enzymes involved in the early steps of hyoscyamine biosynthesis (*28*).

The fact that ABA did not modify hyoscyamine or scopolamine content is contrary to the results obtained by Smith et al. (*49*); in these studies, the addition of 31 μM ABA increased the indole alkaloid derivatives up to 268% in *C. roseus* suspension cultures in relation to the control. Similarly, in *C. roseus* plants, Sáenz et al. (*48*) have found that ABA added onto leaves increased alkaloid content by 40%. Probably the absence of effect in root cultures is because of the culture itself, since ABA receptors are probably in the leaves.

The contradictory results from the literature, together with those presented in this paper, show that we are far away from understanding the role of growth regulators in transformed root cultures, including the fact that they produce their own phytohormones. More systematic work must be done in this area of secondary metabolism before we can use it to improve the yields of transformed root cultures.

The results presented in this paper show that the growth and production of hyoscyamine by *D. stramonium*-transformed root cultures can be significantly modified by some components of the culture medium in which the cultures are grown. Since the largest effect of sucrose is on growth, the amount of hyoscyamine can be significantly increased by altering the levels of this carbohydrate first, and then by eliminating some of the minerals of the medium, i.e., using specific quelating compounds.

However, much more work must be done at the biochemical and molecular levels before the production of compounds by root cultures can be carried out scientifically instead of empirically.

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