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Growth and tropane alkaloid production in *Agrobacterium* transformed roots and derived callus of *Datura*

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Abstract

Small callus pieces excised from the *Agrobacterium* transformed root line D_2 of *Datura stramonium*, were cultured onto solidified MS medium supplemented with a 1.0 μM kinetin and three different concentrations (0.1, 0.5, and 1.0 μM) of 2,4-dichlorophenoxyacetic acid (2,4-D), and were examined for their alkaloid productivity in relation to organization level and growth rate. Growth of transformed roots (in a MS liquid medium without plant growth regulators) was greater than that of transformed calli excised from them and cultured separately. The addition of 1.0 μM 2,4-D to the culture medium had a positive effect on callus biomass production, while it inhibited root formation by this tissue (the lower the 2,4-D concentration in the medium the greater the number of roots which emerged from the calli). Hyoscyamine production was also higher in the transformed roots than in the transformed calli, and in these tissues the production of hyoscyamine was positively correlated with organogenesis index (*i.e.* its ability for rooting). At the same time, the epoxidation of hyoscyamine to scopolamine only took place in the transformed calli. This occurred to a greater extent at the lower concentrations of 2,4-D in the culture medium. The mode through which the 2,4-D could control the alkaloid production of transformed callus is discussed.

Key words: 2,4-dichlorophenoxyacetic acid, hyoscyamine, organogenesis index, scopolamine, Southern blot analysis

Introduction

The tropane alkaloids scopolamine and hyoscyamine are medically important, being used as anticholinergics. Commercial sources of these alkaloids are *Solanaceae* plants such as *Atropa*, *Datura*, *Hyoscyamus* and *Scopolia*, in which alkaloids are synthesized in the roots, followed by transport to and accumulation in the leaves

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(Waller and Nowacki 1977). There have been some attemps to produce tropane alkaloids by *in vitro* culture of cell and callus tissues of *Solanaceae*, however, the contents of these alkaloids in undifferentiated calli or cell suspensions were much lower than those found in the original plants (Tabata *et al.* 1972, Hiraoka and Tabata 1974). On the contrary, high hyoscyamine production by *Agrobacterium rhizogenes* transformed root cultures of several genera including *Atropa*, *Datura*, *Hyoscyamus*, *Scopolia* and *Duboisia* has recently been reported (Mano *et al.* 1986, Payne *et al.* 1987, Jung and Tepfer 1987).

We have previously reported that in non-habituated tobacco callus, in the presence of a constant amount of kinetin, it is the auxin concentration in the culture medium that controls the production of nicotine. The calli grown at lower auxin concentrations had significantly higher levels of nicotine than those grown at higher auxin concentrations (Piñol *et al.* 1984, Piñol *et al.* 1985, Palazón *et al.* 1987). Nicotine similarly as the tropane alkaloid hyoscyamine, is synthesized in the root from putrescine and accumulated in the aerial part of the plant.

With these considerations in mind, using a transformed root line of *Datura stramonium* with high ability to form callus (obtained by transformation with an A_4 strain of *A. rhizogenes*) and the callus pieces excised from it and cultured separately with different concentrations of the synthetic auxin 2,4-D in the culture medium, we have investigated their tropane alkaloids production in relation to organization level and growth rate.

Materials and methods

Establishment and culture of transformed root lines and callus: Sections from the surface-sterilized stems of 3-month-old *Datura stramonium* plants were inoculated with *Agrobacterium rhizogenes* agropine type strain A_4 grown at 28 °C in the medium described by Petit and Tempé (1978). The explants were subsequently cultured on Murashige and Skoog's (MS) medium (Murashige and Skoog 1962) solidified with 0.8 % *Bacto-agar* (*Difco Laboratories*, Detroit, USA) at 25 °C in the dark. Three to four weeks after inoculation, the roots which appeared on the inoculated sites were removed individually and transferred to hormone-free MS agar medium containing 0.5 g dm^{-3} carbenicillin. Rapidly growing roots without bacterial contamination were used to establish the cultures of transformed root lines. These were grown in 40 cm^3 of hormone-free MS liquid medium without antibiotics and maintained in the dark at 26 ± 2 °C on an orbital shaker at 80 rpm. One transformed root line (D_2) was selected for its high and stable capacity to produce small pieces of callus, and subcultured each 6 weeks in the indicated conditions. At the same time, the small pieces of callus were excised from these roots and cultured as separate lines on MS basal medium solidified with 0.8 % agar and supplemented with 1.0 μM kinetin and 3 different concentrations of 2,4-D (0.1, 0.5, and 1.0 μM). Six pieces of callus tissue each weighing 130 ± 1 mg were inoculated onto each of the three Petri dishes (100 × 25 mm) containing 20 cm^3 of MS medium and grown at 25 °C in the dark for 6 weeks.

DNA analysis: DNA was extracted from root and callus tissues according to Dellaporta *et al.* (1983). DNA was digested with Eco RI, and then fractionated on 1 % agarose gel, transferred to nylon membrane and hybridized to a radioactive probe, a 4.3 kb fragment (E15) of the pRi of *Agrobacterium rhizogenes* obtained from Prof. J.J. Hooykas (Leiden University, The Netherlands) and labelled using the Boehringer random-priming kit. Southern blot hybridization was carried out in 1.5 × SSPE (0.27 M NaCl, 15 mM $NaH_2PO_4.H_2O$, pH 7.7, and 1.5 mM EDTA): 1 % (m/v) SDS; 0.5 % (v/v) (10 % non fat dried milk, 0.2 % sodium azide and 0.5 mg cm^{-3} of herring sperm DNA). Washing was done in 2 × SSC (0.36 M NaCl; 20 mM $NaH_2PO_4.7H_2O$; 2 mM EDTA) four times for 15 min each, followed by 2 × SSC; 0.5 × SSC and 0.1 × SSC, each of them with 0.1 % SDS. Another wash in 0.1 × SSC with 0.1 % SDS for 30 min at 50 °C, and finally in 0.1 × SSC without SDS at room temperature. Membrane was autoradiographed on *Kodak RP-X Omat* film with a *Dupont Lighting Plus* intensification screen at -70 °C for 12 to 24 h.

Extraction and determination of alkaloids: Extraction of alkaloids was based essentially on the method described by Yamada and Hashimoto (1982). Transformed root and callus tissue were oven-dried to constant mass and powdered and *ca.* 100 mg of dry mass was extracted in a *Soxhlet* with MeOH. The metanolic extract was dried and the residue was dissolved in 5 cm^3 of 200 mM NH_4Cl, pH 9.8. This alkaline aqueous solution was applied to a glass column (20 × 1cm) filled with 4.5 g of *Extrelut-20* (*Merck*) and the column eluted with 3 × 10 cm^3 $CHCl_3$ saturated with NH_3 (Duez *et al.* 1985). The eluate was dried at 40 °C under a warm air flow. The residue was dissolved in 1 cm^3 of HPLC mobile phase [17 % (v/v) acetonitrile in 50 mM KH_2PO_4 and H_3PO_4, pH 3.0] , filtered through a millipore filter (0.45 μm) and stored at -20 °C.

A sensitive and selective method for the determination of tropane alkaloids in complex samples of plant extracts has been developed, based essentially on the method described by Collinge and Yeoman (1986). The *Applied Biosystems Liquid Chromatograph* was equipped with two pumps (*400 Solvent Delivery System*), gradient control (*1000s Gradient Program*), a UV detector (*1000s Diodo Array Detector*) operating at wavelengths from 190 to 555 nm, a PC integrating system (*PE Nelson, 3000 Series Chromatography Data System, Model 2600, Revision S.1*), a 15 × 0.4 cm *Spherisorb C8* 5 mm column (*Tracer Analitica*, Barcelona, Spain) and a 2 cm × 2 mm inner diameter precolumn (*2C - 13OB, Tracer Analitica*) packed with *Pelicul C18*. Samples 0.02 cm^3 were separated at constant ambient temperature by a 5-step gradient program consisting of acetonitrile and a buffer containing 50 mM KH_2PO_4 adjusted to pH 3.0 with orthophosphoric acid. Chromatograms were monitored at 216 nm.

Results

Growth and morphogenesis: When the purifed 4.3 kb Eco RI-I5 fragment of pRi was used as a radioactive probe, the Eco RI-digested DNA from transformed roots and

callus tissues showed the presence of a strong hybridizing band on the autoradiogram proving the transformation of the analysed material (Fig. 1). Taking together the data

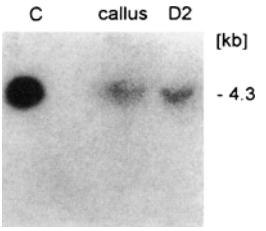

Fig. 1. Southern blot analysis of total DNA from transformed root D_2 and calli derived from it. 5 μg of total DNA from each sample were digested with Eco RI, denatured, transferred onto nylon membrane and hybridized against the Eco RI-15 fragment (TL-DNA) used as a probe and labelled with ^{32}P. C - 100 ng of Eco RI-15 fragment used as positive control.

shown in Fig. 2, it can be deduced a positive correlation between the callus growth and the 2,4-D concentration in the culture medium. The growth rate (measured as an increase in fresh and dry mass) of calli grown in the presence of 1.0 μM 2,4-D for 6 weeks was 1.3 and 1.2 times greater than that of calli grown at 0.1 μM 2,4-D in the culture medium. The growth index (sample fresh mass *per inoculo* fresh mass) at week 6, when the experimental period ended, indicates that the transformed root line D_2 is able to increase its biomass to a much greater extent than that of the transformed callus tissue, independently of the 2,4-D amount added to the culture medium. During the period considered, the fresh mass of transformed roots increased about 23.6 times (from the inoculum of 150 ± 1 mg reached the fresh mass of 3548 ± 268 mg), while the transformed calli grown at 1.0 μM 2,4-D (the optimal concentration for transformed callus growth) increased only about 9.3 times (from the inoculum of 130 ± 1 mg reached the fresh mass of 1217 ± 38 mg).

The organogenesis process of transformed calli was also highly affected by the presence of 2,4-D in the culture medium. The organogenesis index (calli with roots/total calli ratio × 100) of transformed calli showed that the older the culture and the lower the 2,4-D concentration in the culture medium, the more roots appeared in the calli (Fig. 2). At the same time comparing the different treatments carried out, we observed that the higher the 2,4-D concentration, the lower was the transformed calli organogenesis index.

Tropane alkaloid production: HPLC analysis of the alkaloid fraction from culture extracts revealed scopolamine and hyoscyamine presence with retention times of 7.2 and 9.3 min respectively, as the only tropane alkaloids identified. The other alkaloid components present in culture extracts are as yet unidentified.

After 2, 4, and 6 weeks of culture hyoscyamine and scopolamine content in transformed roots of D_2 line and calli excised from them and cultured separately (Table 1) showed the high capacity of transformed roots to biosynthesize hyoscyamine in comparison to transformed calli, since after 6 weeks hyoscyamine

content was about 60 times higher in roots than in the most productive calli. During the first weeks, the calli had very low alkaloid content and production was always higher in calli grown in the culture medium supplemented with 0.1 μM 2,4-D than in

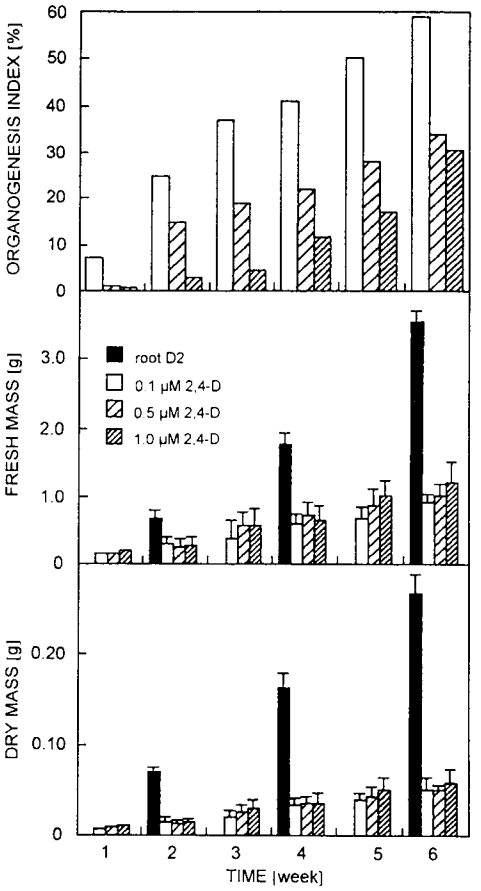

Fig. 2. Changes in fresh mass, dry mass and organogenesis index (calli with roots/total calli harvested ratio × 100) of transformed roots line D_2 and calli during the 6-weeks period [MS culture medium without plant growth regulators (roots) or with 1 μM kinetin and 0.1, 0.5 or 1 μM 2,4-D]. Values are the means of 18 replicates. Bars represent SE.

these grown with 0.5 and 1.0 μM 2,4-D. The lowest alkaloid content was reached by the calli grown with the highest 2,4-D concentration in the culture medium. Comparing the organogenesis index and the capacity to synthesize tropane alkaloids in all the calli (Table 1 and Fig. 2), it is possible to conclude that there is a positive relationship between these two parameters, and it is also apparent that the presence of 2,4-D inhibits to a larger extent the calli tissue capacity to form roots and to synthesize tropane alkaloids the greater concentration of 2,4-D in the culture medium was used. It is very important to emphasize the scopolamine presence in the

transformed calli after 5 weeks of culture, since this alkaloid has not been found in the tissues of transformed root line D_2. The scopolamine content in the transformed calli grown for 5 weeks in the culture medium with 0.1 μM 2,4-D was 36- and 31-fold higher than that of the calli grown with 0.5 and 1.0 μM 2,4-D, respectively. In the following week, when the experimental period finished (week 6) the levels of scopolamine increased significantly ($P < 0.001$) in all the calli, at the same time the hyoscyamine levels decreased; this fact suggested a quick epoxidation of this alkaloid to transform itself into scopolamine.

Table 1. Alkaloid content [μg g^{-1}(d.m.)] of transformed roots of D_2 line and calli excised from them and cultured separately. Means of 6 replicates ± SE. (n.d.: not determined, -: not detected). Hyoscyamine content in roots of the original plant was 2501.3 μg g^{-1}(d.m.).

Time [weeks]	Alkaloid	Root D_2	Callus 0.1 μM 2,4-D	0.5 μM 2,4-D	1.0 μM 2,4-D
2	hyoscyamine	1622.7 ± 283.3	18.5 ± 0.7	-	-
	scopolamine	-	-	-	-
3	hyoscyamine	n.d.	23.9 ± 0.9	-	-
	scopolamine	n.d.	-	-	-
4	hyoscyamine	1855.2 ± 83.5	23.0 ± 0.8	-	2.2 ± 0.1
	scopolamine	-	-	-	-
5	hyoscyamine	n.d.	92.3 ± 3.8	10.3 ± 0.7	6.2 ± 0.1
	scopolamine	n.d.	49.9 ± 2.0	1.4 ± 0.2	1.6 ± 0.3
6	hyoscyamine	5577.9 ± 381.9	63.0 ± 2.3	7.9 ± 0.1	-
	scopolamine	-	127.3 ± 5.2	3.0 ± 0.1	6.3 ± 0.1

Discussion

Our aim was to determine tropane alkaloid production in relation to organization level and growth rate in both the transformed root line of *D. stramonium* with high ability to form small mass of callus and the small mass excised from it and cultured separately with different concentrations of 2,4-D (0.1, 0.5, and 1.0 μM). In our experiment, it is apparent that low 2,4-D concentrations inhibited root formation in the callus tissues, which demonstrates the well known high sensitivity of tissues transformed with *A. rhizogenes* to auxin (Shen *et al.* 1988). In comparison with 0.1 μM 2,4-D, the 1.0 μM 2,4-D inhibited to a larger degree organogenesis and the ability of transformed calli to biosynthesize tropanic alkaloids; this result agrees with those of Robins *et al.* (1991), which prove that high concentrations of 2,4-D added to *D. stramonium* transformed roots caused the disorganisation of the roots and hyoscyamine biosynthesis inhibition. Moreover, in relation to transformed root cultures it has been demonstrated a dramatic inhibition of alkaloid production in tumor cultures of *Atropa belladona* which contain high concentrations of IAA (Ondřej and Protiva 1987).

Ri T-DNA stimulated hyoscyamine production in root line D_2 (the improvement was of 2.2-fold relative to normal roots, see Table 1), however, the significant difference in the level of alkaloid production of transformed root line and the calli excised from it and cultured separately, seems to indicate that the presence of T-DNA in the cells genome does not activate the hyoscyamine biosynthesis unless the organization of tissue cells as root occurs.

Regarding the effect of 2,4-D presence in the culture medium upon hyoscyamine biosynthesis of transformed calli, our results indicate that the presence of this synthetic auxin did not directly affect the biochemical differentiation. The inhibition in the presence of 1.0 μM 2,4-D was mainly due to an increase in callus biomass production, while the stimulation of hyoscyamine production in the presence of 0.1 μM 2,4-D was mainly due to morfological differentiation; all this was most likely caused by having affected the intracellular hormonal balance. Auxins are required to stimulate growth in many normal cultures and in some species and inverse relationship between growth and alkaloid content has been observed (Piñol *et al.* 1984, Palazon *et al.* 1987, Hashimoto *et al.* 1986). With respect to scopolamine production, the presence of T-DNA could induce the conversion of hyoscyamine to scopolamine only when the levels of the former are adequate. The precursor hyoscyamine would be biosynthesized by the roots emergent from the callus tissues and accumulated in their parts without organogenesis. In *D. stramonium*, hyoscyamine is normally synthesized in plant roots, and its conversion to scopolamine only takes place in shoot tissue from which transport back to the roots probably accounts for reports of this alkaloid in root tissue (Evans 1979, Hashimoto *et al.* 1986).

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