



COMPARISON OF GROWTH PROPERTIES, ALKALOID PRODUCTION AND WATER UPTAKE OF TWO SELECTED *DATURA* HAIRY ROOT LINES

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Two selected hairy root lines of *Datura*, *D. innoxia* and *D. candida* × *D. aurea* were compared for their performance in growth and tropane alkaloid productivity in shake flasks. Specific consumption rate and biomass yield on sucrose and nitrates during the phase of maximum growth were calculated versus changes in liquid medium volume during culture. Growth parameters were found to be better in *D. innoxia* hairy root cultures than in *D. candida* × *D. aurea*. Overall productivity of hyoscyamine in *D. innoxia* ($2.1 \text{ mg l}^{-1} \text{ d}^{-1}$) was double that in *D. candida* × *D. aurea* ($1 \text{ mg l}^{-1} \text{ d}^{-1}$). However, hybrid transgenic roots produced $0.5 \text{ mg l}^{-1} \text{ d}^{-1}$ of scopolamine. The relationship between water uptake and root growth was studied. During the first days of culture, water was released into the culture medium and then reabsorbed in the root tissue. After that, water uptake on a biomass basis in *D. innoxia* and *D. candida* × *D. aurea* transformed root cultures reached 0.74 and $0.53 \text{ ml g}^{-1} \text{ FW}$, respectively. The correlation between biomass accumulation and liquid medium volume could be used as a simple and inexpensive method for indirect estimation of root growth.

Keywords: *Datura*, growth, hairy roots, tropane alkaloids, water uptake.

INTRODUCTION

Plants produce a wide range of high-value chemical compounds for fragrances, flavorings, insecticides, dyes, pharmaceuticals and other products (Verpoorte et al., 2000). The production of these chemicals by plant cell and organ culture offers promising alternatives to field-grown plants or chemical synthesis (Roberts, 1998; Sato et al., 2001). Among natural products, alkaloids constitute a large group (12,000 known structures) of plant secondary metabolites (De Luca and Saint Pierre, 2000). Tropane alkaloids are antispasmodic and mydriatic (hyoscyamine), and are used in the treatment of motion sickness (scopolamine) (Oksman-Caldentey and Arroo, 2000).

Many studies have focused on establishing hairy root cultures via *Agrobacterium rhizogenes*, as a gene delivery system for the production of tropane alkaloids (Giulietti et al., 1993; Dupraz et al., 1994; Zarate, 1999; Sevon and Oksman-Caldentey, 2002). It has been shown that hairy roots are useful for the production of valuable alkaloids, especially because of their biochemical stability and fast growth in hormone-free media (Shanks and Morgan, 1999; Sevon and Oksman-Caldentey, 2002). Two transformed root lines of *Datura*, *D. innoxia* and *D. candida* × *D. aurea*, were selected for their stability, growth and good potential in producing tropane alkaloid (Nussbaumer et al., 1998; Boitel-Conti et al., 2000).

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In order to produce secondary metabolites, it is essential to characterize the plant material in terms of biomass, metabolite yield, nutrient-specific consumption rate and other parameters so that the potential for overall metabolite productivity can be evaluated and bioprocess production can be improved. In the case of hairy root growth, the average time of culture is 3–4 weeks. This relatively long period induces great changes in medium composition and also in liquid medium volume. Consequently, yields and nutrient-specific consumption have to be considered as a function of medium volume change (Ramakrishnan et al., 1999). It has been shown that in plant cell growth, water uptake is the main component of the increase of cell volume (Hsiao and Xu, 2000). Nevertheless, little is known about water uptake and water content during the growth of transgenic roots.

The objective of this study was to determine accurately the growth parameters and productivity of littorine, hyoscyamine and scopolamine in two selected hairy root lines, *D. innoxia* and *D. candida* × *D. aurea*, by incorporating changes in liquid medium volume during the culture period. Water uptake and water content of hairy roots grown in shake flasks is investigated.

MATERIALS AND METHODS

HAIRY ROOT CULTURES

Transformed root cultures of *D. innoxia* were obtained and maintained as described by Boitel-Conti et al. (2000). They were cultured in 250 ml Erlenmeyer flasks containing 100 ml of Gamborg's B5 culture medium without growth regulators (Gamborg et al., 1968) supplemented with 30 g l⁻¹ sucrose (the best concentration for this clone) and adjusted to pH 5.8 before autoclaving. Hairy roots were grown on a rotary shaker (80 rpm) at 27 ± 1°C under cool white fluorescent lamps (35 μE m⁻² s⁻¹) with a 16 h photoperiod, and were subcultured at 21-day intervals. Hairy root cultures of *D. candida* × *D. aurea* were established as described by Nussbaumer et al. (1998). They were cultured as described above with 50 g l⁻¹ sucrose (the best concentration for this clone). Hairy roots were cultured for various periods of time ranging from 0 to 21 days. Three flasks of each line were collected every 3 days to measure the biomass as fresh weight (FW) and dry weight (DW). Each point was the mean value of three replicates. Inoculum size (10 g FW l⁻¹, 20 mm long root tips) optimized

for growth rate and metabolite production was the same in all experiments (data not shown).

DETERMINATION OF SUGARS AND NITRATES

The extracellular concentrations of sugars were measured by HPLC (Waters 510 apparatus, Milford, MA) with a refractive index detector (Model 410, Waters, Milford, MA). The volume injected was 20 μl. Sucrose, glucose and fructose were resolved on a Ca²⁺ column (Rezex 8% Ca. Monos., 7.8 × 300 mm; Phenomenex, Torrance, CA) at 70°C using water as mobile phase at a flow rate of 0.5 ml min⁻¹. Total sugar levels (C_{ST}) are reported as sucrose equivalent (Sharp and Doran, 1990), considering the molecule of water incorporated during sucrose hydrolysis and given by the following equation:

$$C_{ST} = C_S + 342/360 (C_G + C_F) \quad (1)$$

where C refers to the concentration, S is sucrose, G is glucose, and F is fructose. Extracellular concentrations of NO₃⁻ were measured spectrophotometrically at 375 nm (U-2000, Hitachi, Japan) using a diagnostic kit (Nitrate Test, Merck, Germany).

DETERMINATION OF TROPANE ALKALOIDS

The roots were dried at 60°C for 3 days. The powdered dried material (40 mg) was extracted with a 4 ml mixture of methanol–chloroform–28% NH₄OH (50:49:1) for 5 h. After filtration and evaporation, the residue was dissolved in an appropriate volume of mobile phase, filtered through a Millex 0.22 μm (Millipore, MA) and analyzed by HPLC (Waters 510 apparatus). A volume of 20 μl was injected with an autosampler (AS100, Thermo-Finnigan, CA). Littorine, hyoscyamine and scopolamine were quantified with an ODS-AQ (Y.M.C, Japan) column (250 × 4.6 mm, 5 μm) at ambient temperature with UV detection at 204 nm (Waters 484 U.V detector, Milford, MA). The mobile phase was H₂O–CH₃CN–85% H₃PO₄ (89:10.8:0.2) with 1 g l⁻¹ KH₂PO₄ supplemented with triethylamine at pH 3.1. The flow rate was 0.8 ml min⁻¹. Quantification was achieved by reference to external standards of hyoscyamine, scopolamine (Sigma-Aldrich, MO). Littorine was kindly supplied by Dr. R.J. Robins (Université de Nantes, France). Maximum productivity (P_{max}) was calculated for the portion of the curve where alkaloid production was maximum. Overall productivity (P) was calculated from overall production divided by 21 days (mg l⁻¹ d⁻¹).

CALCULATIONS OF GROWTH RATE, YIELDS
AND SPECIFIC CONSUMPTION RATE

Growth rate (μ) and doubling time (t_d) of *D. innoxia* and *D. candida* \times *D. aurea* hairy root cultures were calculated by linear regression of the growth phase on a semi-logarithmic plot. Biomass yields on substrate ($Y_{X/S}$) were expressed using a biomass basis incorporating changes in liquid medium volume, that is, from residual volume, as follows:

$$Y_{X/S} = - \frac{\Delta X}{\Delta(S V_r)} \quad (2)$$

where X is hairy root biomass, S is the concentration of substrate in residual media, and V_r is the volume of residual water. $Y_{X/S}$ was estimated by plotting biomass versus substrate uptake and calculating the slope of the linear portion of the curve. Specific consumption rate (q) was calculated from growth rate (μ) and biomass yields on substrate ($Y_{X/S}$) as

$$q = \frac{\mu}{Y_{X/S}} \quad (3)$$

WATER CONTENT AND UPTAKE

After harvesting the roots, the biomass was put on filter paper. Root tissues were freely drained during 5 min, and the volume of culture medium was measured. Fresh weight (FW) was determined with a microbalance (B120S, Sartorius, Germany). Dry weight (DW) was measured after drying the biomass for 3 days at 60°C. Water content ratio (WCR) was expressed on a dry weight basis and calculated as follows:

$$\text{WCR} = (\text{FW} - \text{DW}) / \text{DW} \quad (4)$$

WCR represents the specific water content of root tissues during culture in shake flasks. The time course of this parameter can be evaluated during the 21 days of culture. WCR was chosen as a linear expression to study water exchange.

Water uptake represents the cumulative volume of water taken up by transformed root tissues grown in 250 ml shake flasks filled with 100 ml culture medium. The uptake of water by tissues was determined as follows:

$$\text{Water uptake} = V_i - (V_r + V_e + V_f) \quad (5)$$

where V_i is the initial volume in the shake flask, V_r is the residual volume, V_e is the evaporated water, and V_f is the volume retained on the filter paper. The V_f value was constant (3.5 ± 0.2 ml).

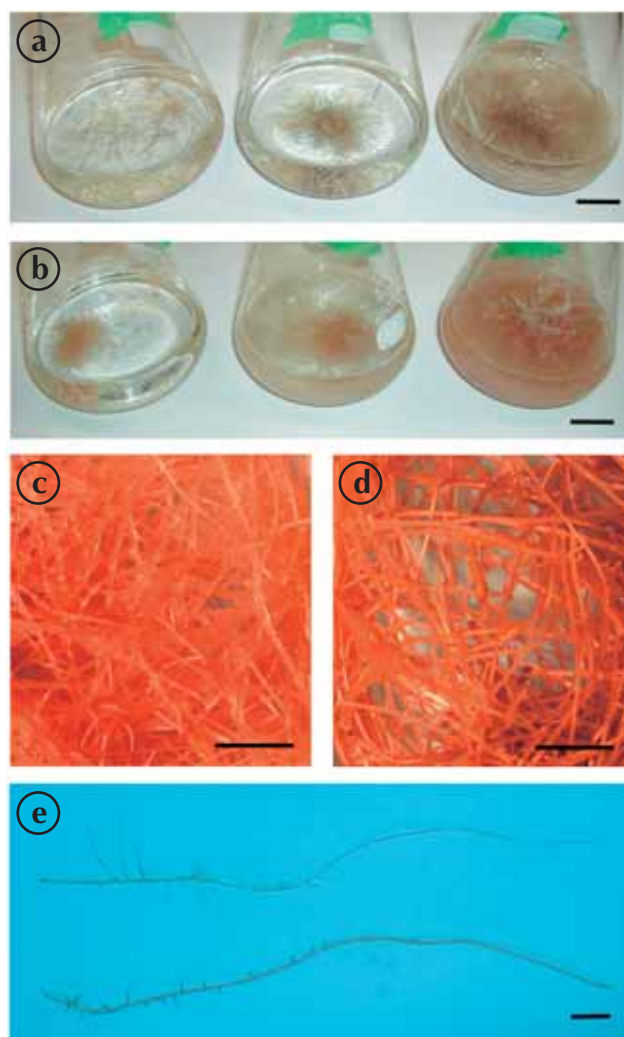


Fig. 1. Hairy root cultures of *D. innoxia* (a) and *D. candida* \times *D. aurea* (b) grown in 250 mL flasks after 2, 8 and 15 days of culture. (c,d) Density of biomass in root balls of *D. innoxia* (c) and *D. candida* \times *D. aurea* (d), (e) different phenotypes of the two hairy root species. A single root of *D. innoxia* appears thin and clear (upper root), unlike *D. candida* \times *D. aurea* which is thicker and brown (lower root). Bars in Figs. a,b = 2 cm; in Figs. c-e = 1 cm.

RESULTS AND DISCUSSION

GROWTH PARAMETERS OF HAIRY ROOTS

The growth kinetics of *D. innoxia* and *D. candida* \times *D. aurea* hairy root cultures were compared (Fig. 1). The growth phase of *D. candida* \times *D. aurea* started with a delay of 3 days, whereas no lag phase was observed in *D. innoxia* (Fig. 2). In both cases, hairy root growth was maximum between day 0 and day 15 for *D. innoxia* and between day 3 and day 15 for *D. candida* \times *D. aurea*. Growth rates were the same

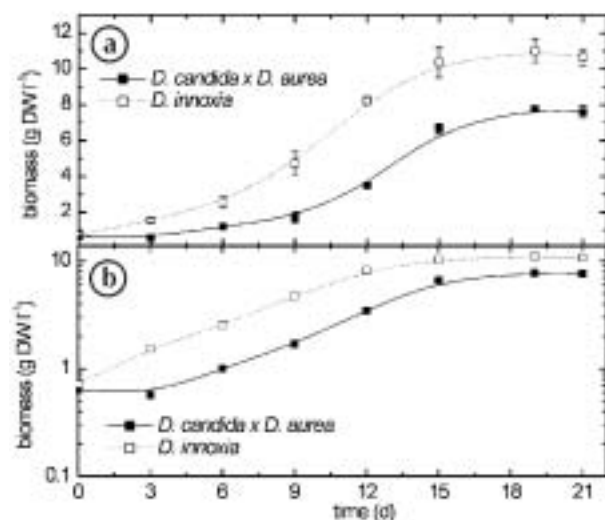


Fig. 2. Growth kinetics of *D. innoxia* and *D. candida* × *D. aurea* transformed root cultured in shake flasks. (a) Linear, (b) Logarithmic coordinates.

in both selected lines ($\mu = 0.21 \text{ d}^{-1}$ and $t_d = 3.3 \text{ d}$), and the end of accelerated growth occurred at the same time (day 15). Thus, the final biomass of *D. candida* × *D. aurea* (7.7 g DW l^{-1}) was 30% less than that of *D. innoxia* (11 g DW l^{-1}). Therefore, producing scopolamine by biotechnological processes would require reducing the lag phase and delaying the end of the growth phase.

In this study, sugars and NO_3^- , the main sources of carbon and nitrogen in hairy root cultures, were monitored (Fig. 3). Sucrose in *D. innoxia* hairy root cultures was depleted within 15 days, at a rate of $2.3 \text{ g l}^{-1} \text{ d}^{-1}$ (Fig. 3a). The medium concentrations of glucose and fructose, the products of sucrose hydrolysis, increased from day 6 to day 15, and then tended to plateau. Consumption of NO_3^- occurred mainly within the period corresponding to the maximum growth phase (days 0–15). At day 15, sucrose starvation corresponded to the end of growth (Fig. 2). No growth was observed after this period even though NO_3^- , glucose and fructose were still present in the medium. Thus, sucrose could be a growth-limiting parameter in *D. innoxia* hairy root culture supplemented with 3% sucrose. The weak consumption of glucose and fructose observed during the stationary phase could be considered as energy consumed for maintenance metabolism. Nagatome et al. (2000) reported that a sucrose threshold of 2.5 g l^{-1} was necessary for growth ability in *Nicotiana* hairy roots. Sucrose consumption in *D. candida* × *D. aurea* hairy roots started on day 8 of culture (Fig. 3b) at a

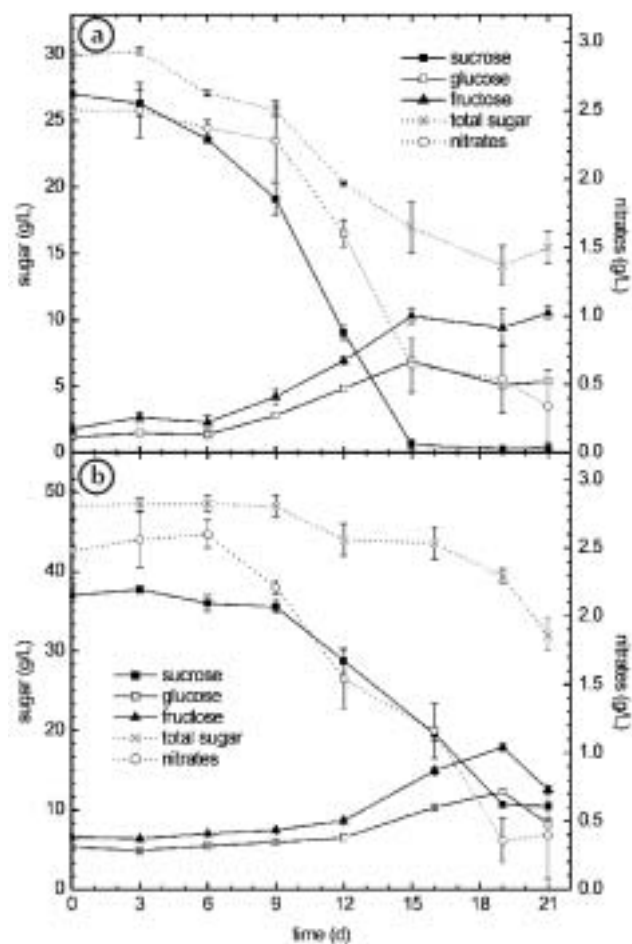


Fig. 3. Time course of sugar and nitrate extracellular concentrations of (a) *D. innoxia* and (b) *D. candida* × *D. aurea* hairy root cultures grown in shake flasks.

rate of $2.5 \text{ g l}^{-1} \text{ d}^{-1}$ up to day 19. Sugar and NO_3^- were not limiting substrates in the hybrid clone, since significant quantities remained in the culture medium during the stationary phase. In both cultures, more fructose than glucose remained on day 21.

When yield coefficients ($Y_{X/S}$) were calculated, the plots of biomass versus substrate uptake showed were linear within days 0–15 for *D. innoxia* and days 3–15 for *D. candida* × *D. aurea* (data not shown). Biomass yields on sugar ($Y_{X/\text{sugar}}$) and on nitrates (Y_{X/NO_3}) were estimated with correlation coefficients (r^2) of 0.98 and 0.96, respectively. $Y_{X/\text{sugar}}$ and Y_{X/NO_3} calculated by incorporating changes in liquid medium volumes (see Materials and Methods) and specific consumption rates of sugar and nitrates (q_{sugar} , q_{NO_3}) are presented in Table 1. $Y_{X/\text{sugar}}$ of *D. candida* × *D. aurea* (0.51) and *D. innoxia* (0.57) were higher

TABLE 1. Growth rate, doubling time, biomass yields specific consumption rate of NO_3^- and sugars within maximum growth phase of *D. innoxia* and *D. candida* \times *D. aurea* hairy root cultures grown in shake flasks

	Growth parameter			Nitrate (NO_3^-)		Sugar	
	lag phase d	μ d^{-1}	t_d d	Y_{X/NO_3^-} gDWg^{-1}	$q_{\text{NO}_3^-}$ $\text{mmol g}^{-1}\text{DWd}^{-1}$	$Y_{X/\text{sugar}}$ gDWg^{-1}	q_{sugar} $\text{mmol g}^{-1}\text{DWd}^{-1}$
<i>D. innoxia</i>	0	0.21	3.3	4.6	0.17	0.57	0.37
<i>D. candida</i> \times <i>D. aurea</i>	3	0.21	3.3	3.3	0.30	0.51	0.48

TABLE 2. Yield and production of tropane alkaloids of *D. innoxia* and *D. candida* \times *D. aurea* hairy root cultures grown in shake flasks

	Littorine			Hyoscyamine			Scopolamine		
	$Y_{l/\text{sugar}}$ mg g^{-1}	specific production $\text{mg g}^{-1}\text{DW}$	overall productivity $\text{mg l}^{-1}\text{d}^{-1}$	$Y_{h/\text{sugar}}$ mg g^{-1}	specific production $\text{mg g}^{-1}\text{DW}$	overall productivity $\text{mg l}^{-1}\text{d}^{-1}$	$Y_{s/\text{sugar}}$ mg g^{-1}	specific production $\text{mg g}^{-1}\text{DW}$	overall productivity $\text{mg l}^{-1}\text{d}^{-1}$
<i>D. innoxia</i>	4.5	8.4	4.3	2.4	4.3	2.1	–	–	–
<i>D. candida</i> \times <i>D. aurea</i>	0.8	2.2	0.9	0.9	2.9	1	0.44	1.4	0.5

than in *Atropa belladonna* hairy root cultures (Sharp and Doran, 1990). Consequently, q_{sugar} and $q_{\text{NO}_3^-}$ of *D. innoxia* were lower than in *D. candida* \times *D. aurea* hairy roots.

ACCUMULATION OF TROPANE ALKALOIDS

Hyoscyamine and scopolamine, the active molecules, and littorine, the direct precursor of hyoscyamine (Robins et al., 1994), were monitored in hairy root cultures of *D. innoxia* and *D. candida* \times *D. aurea*. In *D. innoxia*, specific littorine production was $8.4 \text{ mg g}^{-1}\text{DW}$. Specific hyoscyamine production was $4.3 \text{ mg g}^{-1}\text{DW}$ (Tab. 2), whereas scopolamine could not be detected in the extracts. In *D. candida* \times *D. aurea*, the specific production of littorine and hyoscyamine were 2.2 and $2.9 \text{ mg g}^{-1}\text{DW}$, respectively. An interesting feature of this clone was its high specific scopolamine production ($1.4 \text{ mg g}^{-1}\text{DW}$). No tropane alkaloids could be detected in the liquid medium in either culture. The time courses of littorine and hyoscyamine production in *D. innoxia* hairy root cultures are presented in Figure 4A. The major increase of littorine occurred between days 9 and 15 ($P_{\text{max}} = 8.9 \text{ mg l}^{-1}\text{d}^{-1}$), while hyoscyamine showed a regular increase during the 21 days of culture ($P_{\text{max}} = 2.1 \text{ mg l}^{-1}\text{d}^{-1}$). The difference in P_{max} between these two compounds in *D. innoxia* suggests that rearrangement of littorine into hyoscyamine could be a limiting step (Lanoue et al.,

2002). In transformed root cultures of *D. candida* \times *D. aurea*, production of littorine, hyoscyamine and scopolamine started on day 9 (Fig. 4b) and reached maximum on day 19. Overall hyoscyamine productivity in *D. innoxia* hairy roots ($2.1 \text{ mg l}^{-1}\text{d}^{-1}$) was double that in *D. candida* \times *D. aurea* ($1 \text{ mg l}^{-1}\text{d}^{-1}$); however, hybrid transgenic roots were able to produce $0.5 \text{ mg l}^{-1}\text{d}^{-1}$ of scopolamine (Tab. 2). Metabolite yields and productivity give a basis for evaluating the performance of the process. *D. innoxia* hairy root cultures had more efficient hyoscyamine yield and productivity ($Y_{h/\text{sugar}} = 2.4 \text{ mg g}^{-1}$ and $P_{\text{hyo}} = 2.1 \text{ mg l}^{-1}\text{d}^{-1}$) than *A. belladonna* ($Y_{h/\text{sugar}} = 1.4 \text{ mg g}^{-1}$ and $P_{\text{hyo}} = 0.6 \text{ mg l}^{-1}\text{d}^{-1}$) (Sharp and Doran, 1990). Further studies in a bioreactor are currently being carried out in our laboratory.

WATER UPTAKE BY ROOT TISSUES

During the experiments described above, the volume of medium decreased significantly. Growth parameters and alkaloid production were calculated versus the variation of volume. Volume decrease was investigated by studying both water uptake and water content ratio. First, the rate of evaporated water (0.28 ml d^{-1}) was measured in shake flasks containing 100 ml liquid medium without hairy roots over 21 days. Second, water uptake was calculated using equation (5). The time course of water uptake by root tissues during culture (Fig. 5) can be

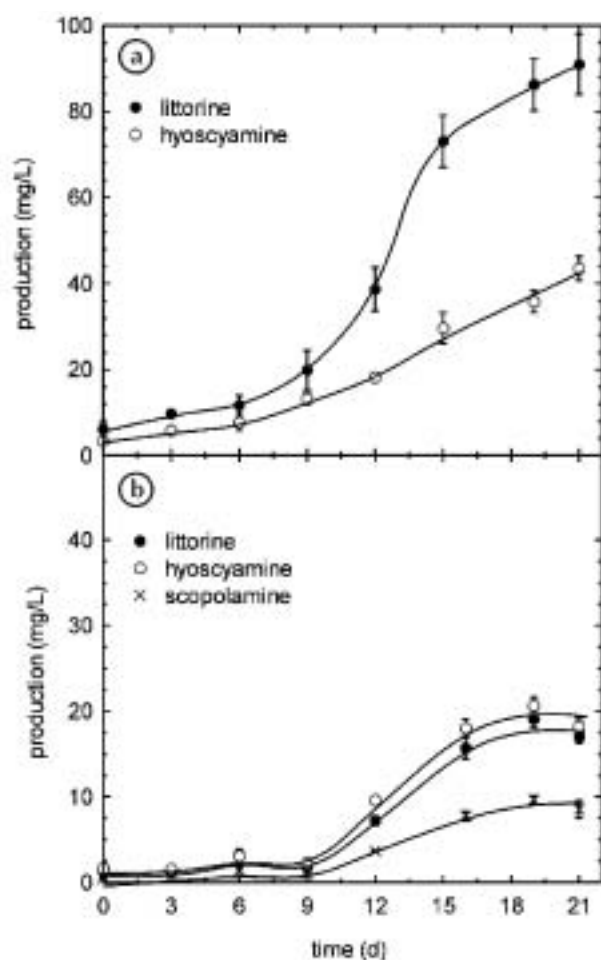


Fig. 4. Overall production of littorine, hyoscyamine and scopolamine of (a) *D. innoxia* and (b) *D. candida* × *D. aurea* hairy root cultures grown in shake flasks.

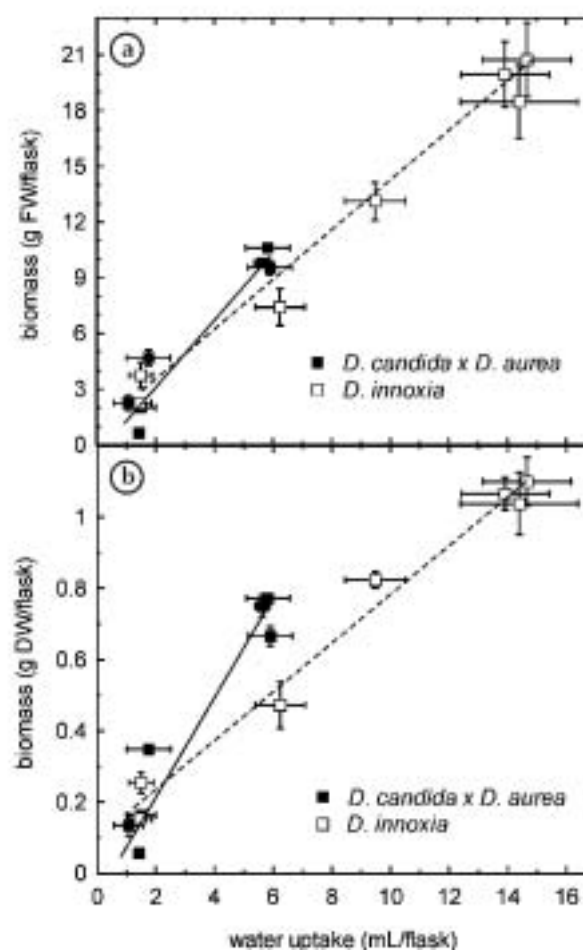


Fig. 6. Correlation of water uptake with fresh tissue biomass (a) and dry tissue biomass (b) of *D. innoxia* and *D. candida* × *D. aurea* transformed root cultures.

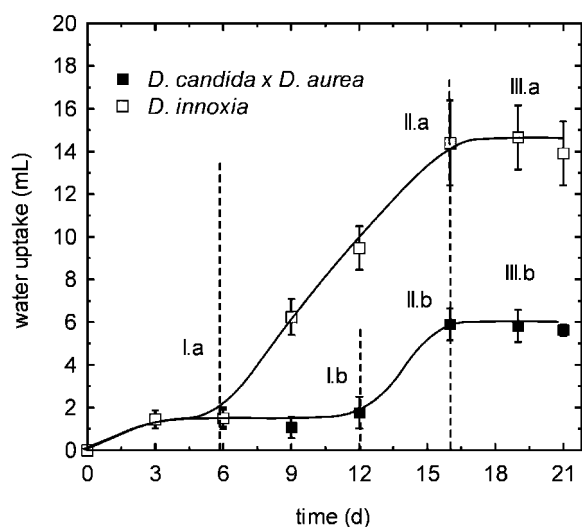


Fig. 5. Water uptake by tissues of *D. innoxia* and *D. candida* × *D. aurea* hairy roots grown in shake flasks. Intervals Ia to IIIa refer to *D. innoxia* and Ib to IIIb to *D. candida* × *D. aurea*.

separated into three phases for both species: Ia to IIIa for *D. innoxia* and Ib to IIIb for *D. candida* × *D. aurea*. Water uptake was very weak during periods Ia and Ib. These two phases correspond to the phase of low root growth of both species (Fig. 2). Almost all the water was taken up during intervals IIa and IIb, which correspond approximately to the maximum growth phase of each clone. The uptake rate in *D. innoxia* hairy roots (1.3 ml d^{-1}) was higher than in *D. candida* × *D. aurea* (0.7 ml d^{-1}). After that, no water uptake could be observed in phases IIIa and IIIb, which correspond to growth stationary phase. Root growth corresponds to a combination of cell division (meristematic region) and cell elongation (zone of elongation). Hsiao and Xu (2000) reported that cells start elongation by filling their vacuoles with water. In view of this observation, we plotted water uptake against root growth (g FW

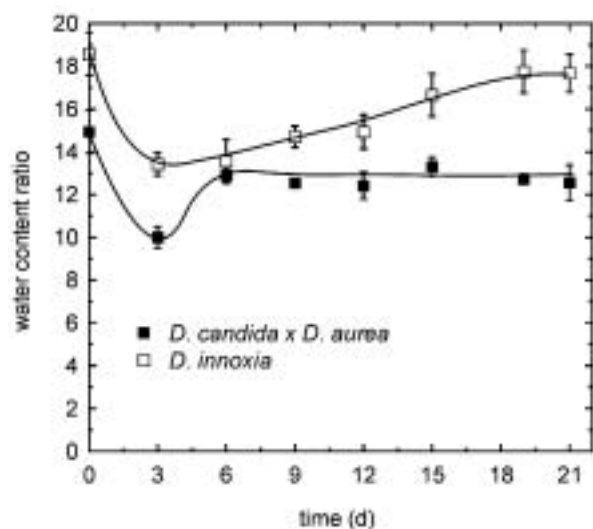


Fig. 7. Time course profiles of specific water content of *D. innoxia* and *D. candida* × *D. aurea* hairy root cultures grown in shake flasks.

flask⁻¹) (Fig. 6). Overall water uptake was proportional to biomass increase at constant rates of 0.74 ml g⁻¹ FW ($r^2 = 0.98$) for *D. innoxia* and 0.53 ml g⁻¹ FW ($r^2 = 0.94$) for *D. candida* × *D. aurea* (Fig. 6a). Similar correlations were obtained with dry tissue biomass (Fig. 6b).

The behavior of water uptake versus biomass during the first days of culture seemed to differ, so we calculated the water content ratio over the entire period of culture (Fig. 7). During the first three days, the water content ratio decreased in both hairy root species. This phenomenon could be a consequence of root subculture. Ramakrishnan et al. (1999) reported that a sudden increase in medium osmolality could activate a water exit. The initial water content in *D. candida* × *D. aurea* was partially restored after day 5 and remained constant, whereas in *D. innoxia* the water content recovered progressively until the end of the culture. After the first 5 days of culture in our experiment, the behavior of root growth versus water uptake did not match the observations described above. Although water exchange occurred during the first days following subculture, Figure 6 shows that measurements of drained liquid medium could be used to estimate the biomass of hairy roots of *D. innoxia* ($r^2 = 0.98$) and *D. candida* × *D. aurea* ($r^2 = 0.94$). Drained liquid volume is easily measured with common and inexpensive equipment.

In this study the growth and tropane alkaloid productivity of two selected hairy root lines were compared. In the studied conditions, growth par-

ameters (final biomass, biomass yields on sucrose and nitrates) were better for *D. innoxia* than for *D. candida* × *D. aurea*. Moreover, total alkaloid production in *D. innoxia* was double that in *D. candida* × *D. aurea*. However, only hybrid hairy roots produce scopolamine. It was shown that the correlation between biomass accumulation and liquid medium volume can be used as a simple and inexpensive method for indirect estimation of root growth. The description of growth kinetics, water content, nutrient consumption and specific alkaloid productivity provides valuable data for future work on metabolic engineering and scaling-up tropane alkaloid production. Reducing the lag phase and delaying the end of the growth phase without modifying the pattern of alkaloids would allow scopolamine to be produced by biotechnological processes.

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