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Characteristics of growth and tropane alkaloid production in *Hyoscyamus albus* hairy roots transformed with *Agrobacterium rhizogenes* A4

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Summary. Hairy root culture of *Hyoscyamus albus* was established by transformation with *Agrobacterium rhizogenes* strain A4. The growth and production of five tropane alkaloids were investigated under various culture conditions. Among the four basal culture media tested, Woody Plant medium was the best for growth of the hairy roots, but a high amount of tropane alkaloids was obtained with Gamborg's B5 medium. Sucrose concentration in B5 medium had little effect on the growth, while 3% sucrose was suitable for the alkaloid production. Addition of KNO_3 to Woody Plant medium affected the growth, whereas the alkaloid content was not markedly improved. Supplement of some metal ions to B5 medium stimulated the alkaloid production. In particular, Cu^{2+} remarkably enhanced both the growth and the alkaloid yield. The hairy roots cultured under 16 h/day light survived for more than 32 days compared with those cultured in the dark.

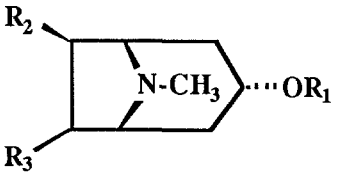
Key words: *Hyoscyamus albus* - *Agrobacterium rhizogenes* - Hairy root culture - Tropane alkaloids

Abbrevations: EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; MeOH, methanol; MS medium, Murashige and Skoog medium; WP medium, McCown's Woody Plant medium; B5 medium, Gamborg B5 medium; wt, weight.

Introduction

There is an increasing number of reports describing growth and secondary metabolite production in cultures of transformed roots. In solanaceous plants, the production of tropane alkaloids by hairy root cultures has been reported for several genera including *Atropa, Datura, Hyoscyamus, Scopolia* and *Duboisia* (Tepfer 1990 and references cited therein). The levels at which these compounds accumulated in transformed root tissues are similar to those in the roots of whole plants. Frequently, the secondary metabolite content in transformed root cultures is higher than in the parent plants (Jung and Tepfer 1987; Christen et al. 1989; Gränicher et al. 1992).

In our continuing investigation on the production of tropane alkaloids in hairy root cultures of *Hyoscyamus albus* (Ishimaru and Shimomura 1989; Shimomura et al. 1991; Sauerwein et al. 1991; Sauerwein and Shimomura 1991) we report here on the production of 7β-hydroxyhyoscyamine [1], 6β-hydroxyhyoscyamine [2], scopolamine [3], hyoscyamine [4] and littorine [5] (Fig. 1) in *H. albus* hairy root cultures transformed with *Agrobacterium rhizogenes* A4. Various culture conditions such as basal media, sucrose and nitrate concentrations, influence of inorganic cations as well as the effects of light on the root growth and the alkaloid production were investigated.



[1] R_1 = tropyl, R_2 = OH, R_3 = H
[2] R_1 = tropyl, R_2 = H, R_3 = OH
[3] R_1 = tropyl, R_2 = R_3 = --O--
[4] R_1 = tropyl, R_2 = R_3 = H
[5] R_1 = phenyllactyl, R_2 = R_3 = H

Fig.1. Tropane alkaloids simultaneously quantified in *H. albus* hairy roots transformed with *A. rhizogenes* A4.

Material and methods

Bacterial strain. *Agrobacterium rhizogenes* strain A4 harbouring the Ri plasmid (pRiA4) grown on YEB agar medium (Vervliet et al. 1975) was used in the present study.

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Induction and culture of hairy roots. The hairy roots of *Hyoscyamus albus* were established by co-culture of the leaf discs with *A. rhizogenes* A4. The excess of bacteria was eliminated on MS solid medium (Murashige and Skoog 1962) containing antibiotic (0.5 g/l Claforan, Hoechst). After elimination of the bacteria, two root tips (about 10 mg fresh wt each) were transferred to hormone-free WP liquid medium (Lloyd and McCown 1980) supplemented with 3% sucrose and cultured on a rotary shaker at 100 rpm in the dark at 25°C. They were subcultured at 4 week intervals. For all the experiments, the hairy roots were cultured in 50 ml of medium/100 ml Erlenmeyer flask. The transformation was proved by the detection of opines as described by Petit et al. (1983) using paper electrophoresis.

Alkaloid extraction and HPLC analysis. Two or three flasks of each culture were harvested and the fresh wt, and the dry wt after lyophilization, were determined individually. About 50 mg of each sample were extracted with 5 ml $CHCl_3$-MeOH-NH_4OH (15:5:1) as described by Kamada et al. (1986). The alkaloid extracts were dissolved in 150-400 µl MeOH and 5 µl were injected into HPLC, using the same system as described by Shimomura et al. (1991). For quantitative analysis the system was calibrated with authentic samples. 7β-Hydroxyhyoscyamine was isolated previously from *H. albus* hairy roots (Ishimaru and Shimomura 1989), 6β-hydroxyhyoscyamine, scopolamine and hyoscyamine were obtained from Sigma (Sigma Chemical Co., USA) and littorine was synthesized from tropine and phenyllactic acid using the same procedure described for atropine (Schmidt et al. 1967; Sauerwein et al. 1991).

Results and discussion

Effects of basal medium

The effects of various culture media containing 3 or 5% sucrose on growth and alkaloid content was examined after 19 days (Fig. 2). The media tested were half strength MS (1/2 MS), MS, B5 (Gamborg et al. 1968) and WP. The five alkaloids were detected in all the hairy root cultures analysed. Hyoscyamine was the most abundant compound throughout. The highest concentration of hyoscyamine was observed in B5 medium containing 3% sucrose, while in 1/2 MS supplemented with 3% sucrose higher content of 6β-hydroxyhyoscyamine and scopolamine were obtained. The transformed roots cultured in media with 3% sucrose showed a higher alkaloid content. In WP media (3 and 5% sucrose), the hairy roots showed the fastest growth but the alkaloid concentration was relatively low. 7β-Hydroxyhyoscyamine content remains very low in the eight culture media tested, while the concentration of littorine was found to be remarkably stable (0.08-0.09% dry wt) except in the MS media (0.04% dry wt).

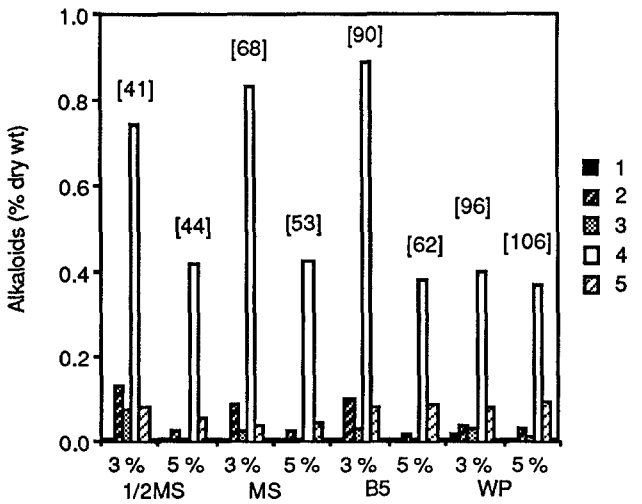

Fig. 2. Alkaloid content in *H. albus* hairy roots cultured in different liquid media for 19 days. Compounds 1-5 are shown in Fig. 1. Numbers in brackets show the fresh weight per 1 litre medium (g). Values are the mean of 2 observations.

Effects of sucrose concentration

In another experiment, the hairy roots were cultured in B5 liquid medium supplemented with different concentrations of sucrose and, after 19 days of culture, the growth and the alkaloid production were determined (Fig. 3). The fresh weight was not strongly affected by the different sucrose concentrations tested. On the other hand, the highest content of hyoscyamine and littorine was obtained with 3% sucrose (0.58 and 0.067 % dry wt, respectively). For 7β-hydroxyhyoscyamine, 6β-hydroxyhyoscyamine and scopolamine content, 2% sucrose was found to be superior to the other sucrose concentrations.

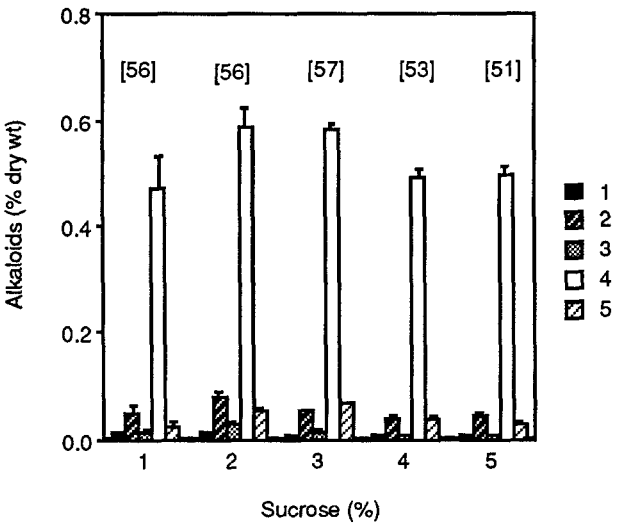

Fig. 3. Alkaloid content in *H. albus* hairy roots cultured in B5 liquid medium with different sucrose concentrations for 19 days. Compounds 1-5 are shown in Fig. 1. Numbers in brackets show the fresh weight per 1 litre medium (g). Values are the mean of 3 observations. Vertical bars denote the standard error.

Effects of nitrate concentration

Sauerwein and Shimomura (1991) reported that nitrate concentration (1-50 mM) remarkably affected growth and alkaloid yield in hairy roots of *Hyoscyamus albus* transformed with *A. rhizogenes* MAFF 03-01724. Therefore the effects of 1-50 mM KNO_3 on the growth and the alkaloid yield in hairy roots transformed with *A. rhizogenes* A4 cultured in WP liquid medium containing 3% sucrose were investigated (Fig. 4). The best growth was obtained with additional 50

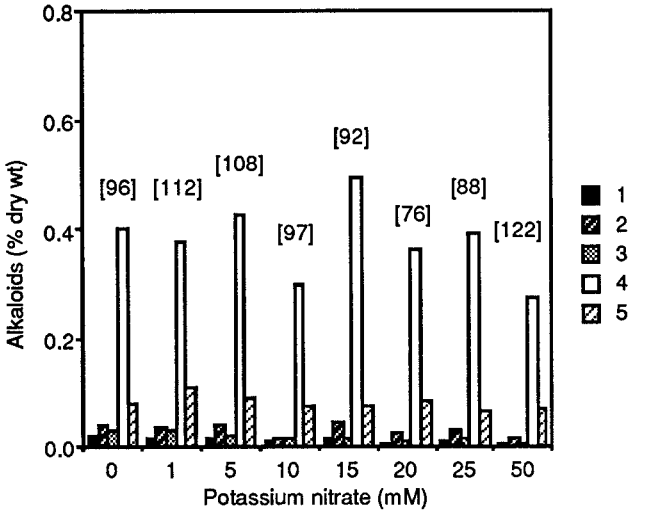

Fig. 4. Alkaloid concentration in *H. albus* hairy roots cultured in WP liquid medium with 3% sucrose and additional KNO_3 for 19 days. Compounds 1-5 are shown in Fig. 1. Numbers in brackets show the fresh weight per 1 litre medium (g). Values are the mean of 2 observations.

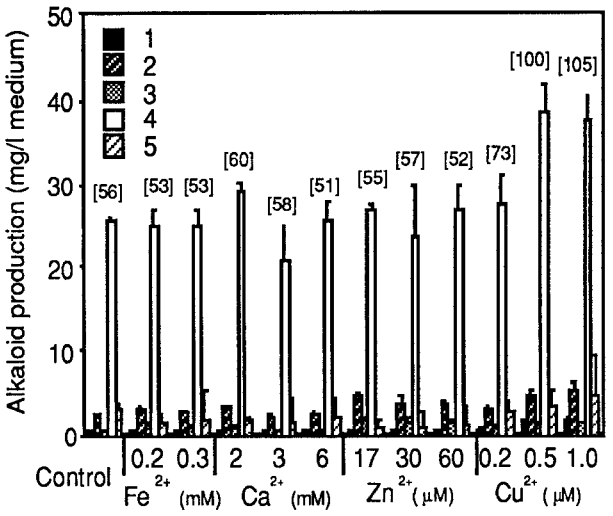

Fig. 5. Influence of different cations on growth and alkaloid concentration in *H. albus* hairy roots cultured in B5 liquid medium supplemented with 3% sucrose for 19 days. Compounds 1-5 are shown in Fig. 1. Numbers in brackets show the fresh weight per 1 litre medium (g). Values are the mean of 3 observations. Vertical bars denote the standard error.

mM nitrate after 19 days of culture but the growth varied irregularly with the increasing concentration of nitrate. On the other hand, the addition of KNO_3 to the culture medium did not strongly affect the alkaloid content in the hairy roots. The highest hyoscyamine concentration (0.50% dry wt) was obtained in WP liquid medium (3% sucrose) with additional 15 mM nitrate. The different responses observed to the addition of KNO_3 to the *H. albus* hairy roots transformed with *A. rhizogenes* MAFF 03-01724 or A4 is poorly understood and requires further investigation.

Effects of metal ions

In a further experiment, hairy root cultures were grown in B5 liquid medium supplemented with different concentrations of $FeSO_4$ (with equivalent Na_2EDTA), $Ca(NO_3)_2$, $ZnSO_4$ and $CuSO_4$. Originally the B5 medium contains Fe^{2+}, Ca^{2+}, Zn^{2+} and Cu^{2+} at 0.1 mM, 1 mM, 7 μM and 0.1 μM, respectively. After 19 days of culture, the fresh weight and the production of tropane alkaloids were determined (Fig. 5). Increasing the amount of Fe^{2+}, Ca^{2+} or Zn^{2+} had little effect on growth. On the other hand, the addition of Cu^{2+} (0.5 and 1.0 μM) had a more pronounced effect, and growth was enhanced remarkably. The addition of Ca^{2+}, Zn^{2+} and particularly Cu^{2+} yielded higher amount of tropane alkaloids than that produced in the control B5 medium. Additional 0.5 or 1.0 μM Cu^{2+} significantly stimulated the alkaloid production. Similar results were obtained in shikonin derivative production by cell suspension cultures of *Lithospermum erythrorhizon* (Fujita et al. 1981). Since we demonstrated the elicitation of alkaloid production in hairy roots of *H. albus* transformed with *A. rhizogenes* A4, further investigation is required to reveal the physiological function of Cu^{2+}.

Effects of light

During a 32-day incubation period in B5 liquid medium supplemented with 3% sucrose, the influence of light [16 h/day light (60 $\mu Em^{-2}s^{-1}$)] on growth and alkaloid production was investigated. At 5-day intervals, two flasks were harvested and growth and alkaloid production were measured. The qualitative composition of the alkaloid spectrum was not affected by the light. On the other hand, the morphology of transformed roots was different. Dark-grown cultures were pale brown as opposed to slightly greenish in color for the light-grown cultures. From an initial inoculum of 20 mg (two root tips of about 10 mg each), the fresh weight increased more than 400 times in hairy roots cultured in the light and reached 172 g/l medium (Fig. 6). On the other hand, the hairy roots cultured in the dark turned brown and died after 28 days. The highest fresh wt reached 144 g/l medium. The initial growth rate of light-grown cultures was less than that of dark-grown cultures. After 18 days, the fresh weight of hairy roots grown in the light was only a half of that cultured in the dark but increased markedly in the following weeks. Hyoscyamine was the major component of the alkaloid fraction produced by the hairy roots in the light as well as in the dark, while the production of the other alkaloids was comparably low during the entire culture period. In the light, the total amount of hyoscyamine reached about 119 mg/l medium equivalent to 1.2% (w/w) of the dry matter. In the dark, the highest hyoscyamine content was obtained after 28 days of culture (95 mg/l medium). The highest content of littorine was observed after 23 days in cultures grown in the light (6.2 mg/l medium) and after 18 days in cultures grown in the dark (4.0 mg/l medium). Afterwards, in both cultures, the production of littorine declined. Contrary to the de-

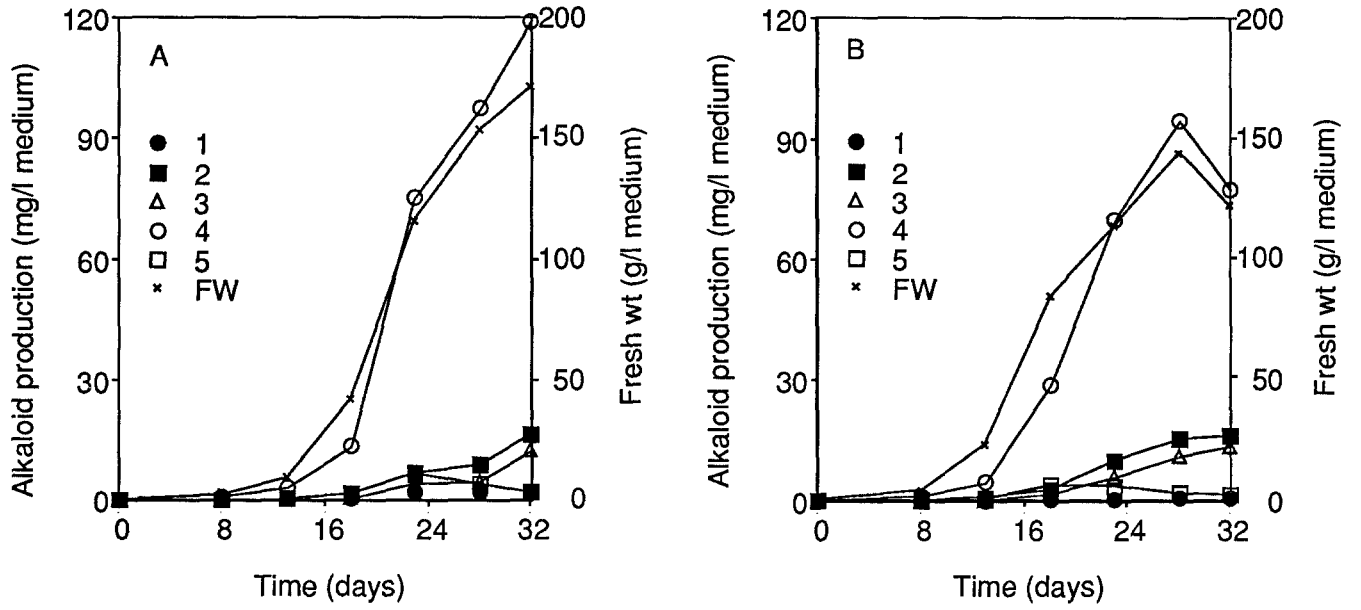

Fig. 6. Time course of growth and alkaloid production in *H. albus* hairy roots cultured in B5 medium supplemented with 3% sucrose under 16 h/day light (A) and in the dark (B). Compounds 1-5 are shown in Fig. 1. Values are the mean of 2 observations.

crease of littorine, the production of 6β-hydroxyhyoscyamine, scopolamine and hyoscyamine increased. Similar results were obtained in *H. albus* hairy roots transformed with *A. rhizogenes* MAFF 03-01724 by Sauerwein and Shimomura (1991). The production of 6β-hydroxyhyoscyamine and scopolamine in light-grown cultures (3.3 and 2.6 mg/l medium, respectively) was similar to that of cultures grown in the dark and reached a maximum after 32 days of culture in both cases.

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