HAIRY ROOT CULTURE OF *LACTUCA VIROSA* L.

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ABSTRACT

A hairy root culture of *Lactuca virosa* L. (Asteraceae) was initiated by infection of the leaf explants of the aseptically plants with *Agrobacterium rhizogenes* – strain LBA 9402. One of the obtained clones which showed the most favourable growth rate was used for further investigation. An influence of different nutrient media and sucrose concentrations on the biomass accumulation was studied. The most advantageous for the biomass increment were media: MS and MS containing macronutrients reduced to half concentration, supplemented with 5% sucrose. A preliminary chromatographic analysis of the tissue extract revealed the presence of sesquiterpene lactones characteristic of the roots of the intact plant.

KEY WORDS: *Lactuca virosa* L., *Agrobacterium rhizogenes*, hairy roots, sesquiterpene lactones.

INTRODUCTION

In spite of the great amount of studies devoted to sesquiterpene lactones, only a few papers concerning accumulation of the compounds in tissue cultures were published (Goleniowski et al. 1990, 1992, Pestchanker et al. 1990, Fulzele et al. 1991, Woerdenbag et al. 1993).

In a recent pharmacological study (Gromek et al. 1992) some crude sesquiterpene lactone preparations obtained from aerial parts and roots of *Lactuca virosa* as well as the pure guaianolide lactucin showed analgesic and sedative properties in mice. We examined (Stojakowska et al. 1994) callus and cell suspension cultures of the plant which contained small amounts of sesquiterpene lactones but no lactucin-like guaianolides – the most characteristic compounds of the intact plant.

The results prompted us to investigate a hairy root culture of *Lactuca virosa*. Such cultures turned out to be a good and stable source of many valuable natural products (Hamill et al. 1987, Flores and Curtis 1992) due to their genetic stability and high level of differentiation, comparing with callus and suspension cultures. Thus the aim of the present study was to initiate a transformed culture of *Lactuca virosa* and to establish the most favourable conditions for the biomass production, as well as to examine in a preliminary screening the capability to produce sesquiterpene lactones characteristic of the plant.

MATERIAL AND METHODS

Plant material

Seeds of *Lactuca virosa* L. were provided by Botanical Garden in Oberholz (Germany). After immersing in 70% ethanol for 30 seconds, the seeds were surface sterilized with 5% solution of Dimanin C (Bayer). Afterwards, the seeds

were rinsed 4 times with sterile distilled water and aseptically germinated on a hormone-free agar medium containing 1/4 of MS (Murashige and Skoog 1962) macro- and micronutrients, at 25°C and continuous light (2000 lx). After two weeks, the seedlings were transferred to the MS medium without hormones and cultivated under the previous conditions for four weeks.

Bacterial strain

Agrobacterium rhizogenes – strain LBA 9402, rifampicine resistant, synthesizing both agropine and mannopine, was maintained on a solid (1% w/v of agar) YMB (Hooymaas et al. 1977) containing 100 mg/l of rifampicin, at 5°C, in the dark. The bacteria were transferred to the liquid YMB, at 28°C, 48 hours before infection of the leaves.

Induction and culture of hairy roots

The leaf fragments of the aseptic plantlets were cut off and placed, the upper surface down, onto the agar (0.8% w/v) MS medium. The veins of the leaves were wounded with a scalpel immersed previously either in the bacterial suspension (10 explants) or in the liquid YMB (10 explants – control). Each of the explants was wounded in 2-4 sites. The cultures were kept at 28°C in the light. After one week, roots developed from the each site of infection. In contrast, non-infected leaves did not produce neither roots nor callus, and wounded tissues became necrotic after 2 weeks in culture. The apical parts of twelve individual roots, from the different explants, were excised and transferred to the liquid MS medium (1/2 of macronutrients), hormone-free, containing 500 mg/l of cefotaxime (Sigma). Six of the clones were further successfully subcultured. After 10 passages in cefotaxime-containing medium, the roots proved to be bacteria-free, and were further cultivated in the liquid MS medium with reduced concentration of macronutrients, at 25°C, in the dark, on a gyrotory shaker (110 r.p.m.). The cultures were subcultured every two weeks. One of the

obtained clones was used to investigate the effect of culture medium on the biomass production and to check the capability of the culture to synthesize sesquiterpene lactones.

Opine analysis

Extraction and detection of opines was performed as described by Morgan et al. (1987).

Rifampicin resistance of the transformed tissues

Small portions of calli: untransformed and obtained from the hairy root, growing on the solidified MS medium containing 0.1 mg/l of 2,4-D, in the dark, were placed on the surface of the MS medium to which rifampicin (100 mg/l) was added, and cultivated for four weeks at 10°C, in the dark.

Growth studies of hairy roots

Culture media tested: MS – (A), MS containing macronutrients reduced to half concentration – (B), 1/2 MS – (C), 1/4 MS – (D), MS supplemented with 10 mg/l of GA₃ – (E), MS containing 10 mg/l of GA₃ and 10 mg/l of BAP – (F), MS with 10 mg/l of GA₃ and 2.25 mg/l of BAP added – (G), MS – NH₄-free formulation (Sigma) – (H), B5 (Gamborg et al. 1968) – (I). All the media contained 100 mg/l of myo-inositol and 30 g/l of sucrose, pH was adjusted to 5.8, before autoclaving.

To investigate the effect of sucrose content on the biomass accumulation, the MS medium with reduced concentration of macronutrients (B) was used. A series of the media containing: 2%, 3%, 4% and 5% of sucrose was prepared.

Inoculum weight was established as 1 g of the fresh weight of the roots. Inoculations were made in triplicate. Each experiment was repeated.

Measurement of the growth rate: a ratio of the final weight of the culture, after three weeks of growth, to the weight of inoculum (G.I. – growth index) was calculated for both dry (DW) and fresh (FW) weights of the tissue.

Preliminary phytochemical analysis

Extraction

0.5 g of the lyophilized tissue was extracted with 25 ml of ethanol at room temperature. The ethanol extract was concentrated in vacuo. The residue was dissolved in 1 ml of methanol and filtered through the membrane filter (0.45 µm).

Thin-layer chromatography (TLC)

TLC was carried out using precoated plates (Merck, Art. 5554). Solvent systems employed: hexane – ethanol, 1:1 (v/v) and chloroform – methanol, 85:15 (v/v). Spots were visualized by UV light (254 nm) and after spraying with 20% solution of sulphuric acid with subsequent heating (100°C).

High-performance liquid chromatography (HPLC)

The extracts were analysed by RP-HPLC, under conditions as follows: column – Nova-Pak C8 cartridge 8x100 mm (Waters, Art. 35884), eluent: methanol – water, 35:65 (v/v) isocratic mode, flow rate: 2 ml/min, photodiode array detector: 200-300 nm.

RESULTS AND DISCUSSION

The individual roots, of those emerged in the sites of inoculation (Fig. 1), were used to establish finally 6 clones of hairy roots, which appeared to contain compounds showing the same mobilities in paper electrophoresis as authentic samples of agropine and mannopine, and reducing silver nitrate reagent (Fig. 2). However, the compounds were no more detected in some of the root clones, after one year of the cultivation. The same phenomenon was observed by Kamada et al. (1986) in hairy root cultures of *Atropa belladonna* and was also reported by Tepfer (1984) who established that opines undergo spontaneous disappearance and reappearance in prolonged cultures and that the presence of opines in the tissue is a poor marker for Ri T-DNA. Ambros et al. (1986) proved by *in situ* hybridization, the presence of Ri T-DNA in the chromosomes of *Crepis capillaris* transformed roots, al-

Fig. 1. Hairy roots originating from *Lactuca virosa* L. leaf explants.

Fig. 2. Detection of agropine (A) and mannopine (M) in two LBA 9402-transformed root clones of *Lactuca virosa* L. (lane 1-2). Lane 3 – agropine and mannopine standards, lane 4 – extract from untransformed roots of *L. virosa*, N – neutral sugars.

though not all of them were positive, when tested for mannopine content.

Other features of hairy roots such as the rapid growth on the hormone-free media and the enhanced production of laterals were observed in our cultures during the all culture period (Fig. 3).

On the rifampicine containing medium the growth of the untransformed calli was completely arrested (Fig. 4), whereas the calli obtained from the hairy roots continued growing (Fig. 5).

Although we did not perform molecular analyses to demonstrate transgenic nature of the roots, their persistent hairy root phenotype together with the other mentioned features suggests that some genetic information encoded in *A. rhizogenes* T-DNA is integrated and expressed in the plant genome.

The effect of the medium composition on the fresh and dry weights of the one, selected hairy root clone is shown in Fig. 6. The most favourable for the biomass accumulation was the MS medium with reduced concentration of macronutrients (B). The dry weight of the tissue reached almost the same value in MS medium (A), although the fresh weight of the roots was significantly lower. The relatively low biomass increments in B5 medium (I) contrast with the reported data as the B5 medium is preferably used for root cultivation (Hashimoto and Yamada 1991).

Composition of the medium affected morphology of the cultures i.e. lateral root formation, thickness and colour (creamy to brown) of the roots. The latter feature of the cultures is at least partly connected with the presence of phenolics.

The sucrose concentration in the culture medium had no any marked influence on the fresh weight of the tissue (Fig. 7). The dry weight increment was proportional to the sucrose concentration employed, and was the best in the medium containing 5% of the sugar (Fig. 7).

The plant material for phytochemical analysis was harvested after three weeks of culture in the MS medium with reduced macronutrients concentration (B), containing 3% sucrose. During the prolonged culture in this medium, regeneration of shoots occurred spontaneously.

Fig. 3. Hairy roots of *Lactuca virosa* L. cultivated in the MS medium with reduced concentration of macronutrients.

Fig. 4. Untransformed callus of *Lactuca virosa* L., three weeks after inoculation on rifampicine containing medium.

Fig. 5. Callus derived from the transformed root of *Lactuca virosa* L., three weeks after inoculation on rifampicine containing medium.

Fig. 6. Growth indexes (GI) of *Lactuca virosa* L. hairy roots, cultivated in different nutrient media (A-I), calculated on the basis of the fresh (FW) and the dry (DW) weights of the cultures. Vertical bars denote standard error.

Fig. 7. Growth indexes (GI) of *Lactuca virosa* L. hairy roots, cultivated in the MS medium with reduced concentration of macronutrients, supplied with 2-5% sucrose, calculated on the basis of the fresh (FW) and the dry (DW) weights of the cultures. Vertical bars denote standard error.

Fig. 8. HPLC chromatograms of the ethanol extracts prepared from different tissues and organs of *Lactuca virosa* L.: a – callus, b – roots of the intact plant, c – hairy roots, 1 – lactuside A, 2 – jacquinelin glucoside, 3 – jacquinelin.

A preliminary phytochemical examination of the hairy root extract revealed the presence of compounds showing the same Rt values in HPLC, and UV spectra as the authentic samples of some sesquiterpene lactones characteristic of the roots of the intact plant (jacquinelin, jacquinelin glucoside, lactuside A). The compounds were not detected in callus and suspension cultures of the plant which were examined previously (Stojakowska et al., 1994). The extracts of the hairy and normal roots showed some quantitative differences as well as changes of the aglycone/glycoside ratio, whereas qualitative composition of sesquiterpene lactones remained the same (Fig. 8).

The hairy roots, in contrast with the callus and cell suspension, are capable to synthesize biologically active sesquiterpene lactones and can provide a suitable material to investigate the biosynthetic pathway of the compounds.

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HODOWLA KORZENI WŁOŚNIKOWATYCH *LACTUCA VIROSA* L.

STRESZCZENIE

Hodowlę korzeni włosnikowatych *Lactuca virosa* L. (*Asteraceae*) uzyskano infekując eksplantaty liściowe aseptycznych roślin za pomocą *Agrobacterium rhizogenes* (linia LBA 9402). Do dalszych badań użyto klonu odznaczającego się najszybszym wzrostem. Testowano wpływ dziewięciu pożywek hodowlanych oraz różnych stężeń sacharozy (2-5%) na przyrost biomasy. Najkorzystniejsze dla wzrostu hodowli były pożywki: MS i MS z obniżoną o połowę zawartością makroelementów, zawierające 5% sacharozy. Wstępna analiza chromatograficzna wyciągów z liofilizowanych korzeni włosnikowatych potwierdziła obecność laktonów seskwiterpenowych charakterystycznych dla korzeni rośliny macierzystej.

SŁOWA KLUCZOWE: *Lactuca virosa* L., *Agrobacterium rhizogenes*, korzenie włosnikowate, laktony seskwiterpenowe.