Establishment of *Salvia officinalis* L. Hairy Root Cultures for the Production of Rosmarinic Acid

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Shoots of *Salvia officinalis*, a medicinally important plant, were infected with *Agrobacterium rhizogenes* strains ATCC 15834 and A4 which led to the induction of hairy roots in 57% and 37% of the explants, respectively. Seven lines of hairy roots were established in WP liquid medium under light and dark conditions. The transformed nature of the root lines was confirmed by polymerase chain reaction using *rol*B and *rol*C specific primers. Transformed root cultures of *Salvia officinalis* showed variations in biomass and rosmarinic acid production depending on the bacterial strain used for transformation and the root line analyzed. Both parameters (growth and rosmarinic acid content) of ATCC 15834-induced lines were significantly higher than the A4-induced lines. The maximum accumulation of rosmarinic acid (about 45 mg g^{-1} of dry weight) was achieved by hairy root line 1 (HR-1) at the end of the culture period (45–50 days). The level was significantly higher than that found in untransformed root culture (19 mg g^{-1} of dry wt).

Key words: Agrobacterium rhizogenes, Hairy Root Cultures, Rosmarinic Acid

Introduction

Salvia officinalis is probably the most known species of the *Salvia* genus and since ancient times it has been used for culinary and medicinal purposes. This plant has been reported to contain various types of secondary metabolites (compounds of essential oil, polyphenols, di- and triterpenoids) with antibacterial, antiviral, anti-inflammatory and antioxidant activities (Baricevic *et al*., 2001; Cuvelier *et al*., 1994; Farag *et al*., 1989; Tada *et al*., 1994). Recently, antioxidant plant metabolites have attracted a great deal of attention due to their role in the protection against diseases caused by oxidative stress, such as heart disease and cancer (Haraguchi *et al*., 1995). Antioxidant activity of *Salvia officinalis* plant extracts is mainly attributed to abietane-type diterpenoids (carnosic acid and carnosol) as well as rosmarinic acid (*α-O*-caffeoyl-3,4-dihydroxyphenyllactic acid, RA). The strong antioxidant activity connected with the low toxicity makes RA also interesting for food industry (Parnham and Kesselring, 1985). In our laboratory various *in vitro* systems (callus and cell suspension cultures as well as shoot cultures and whole regenerated plantlets) of *S. officinalis* were used for the production of the compounds with antioxidant activity but their level was found to be usually lower than that from intact plants (Grzegorczyk *et al*., 2005). It is well known that root cultures of higher plants transformed with the soil-borne pathogen *Agrobacterium rhizogenes* are able to grow intensively in media without growth regulators and produce amounts of secondary metabolites equivalent or higher than in the roots of intact plants.

In this work we report for the first time the successful establishment of hairy root cultures of *S. officinalis* through infection with *A. rhizogenes* strains ATCC 15834 and A4. The hairy roots were studied for their ability to synthesize RA under light and dark conditions. So far, out of other members of the *Salvia* genus transformation procedure was used only for *S. miltiorrhiza* (Chen *et al*., 1999) and *S. sclarea* (Kuźma *et al*., 2005).

Materials and Methods

Establishment of hairy root cultures

Hairy roots were initiated on sterile 5-week-old shoots of *Salvia officinalis*. Seeds of *S. officinalis*

provided by the Garden of Medicinal Plants of Łódź were chosen as the initial plant material. The specimen was identified by Prof. J. Siciński (Department of Plant Ecology and Phytosociology, University of Łódź). A voucher specimen was deposited at the Department of Biology and Pharmaceutical Botany, Medical University of Łódź. The shoots were grown on Murashige and Skoog (MS) agar (0.7%) medium (Murashige and Skoog, 1962) supplemented with 0.57 μM IAA (indole-3-acetic acid) and 2.22 μM BAP (6-benzylaminopurine) (Grzegorczyk and Wysokińska, 2004). Inoculation was carried out at the second node of stems as well as at the midrib of the leaves by wounding with a sterile needle dipped into bacterial culture. Two agropine strains of *A. rhizogenes*: ATCC 15834 and A4 were used in the experiments. The strain A4 was obtained from Dr. Simpson (USA), and the strain ATCC 15834 was a gift from Dr. Furmanowa collection (Poland). Before infection, the *A. rhizogenes* strains were incubated on YMB agar medium (Vervliet *et al*., 1975) with or without 200 μM acetosyringone (AcS) at 26 °C for 48 h. AcS dissolved in 1 drop of ethanol was filter sterilized before adding to the autoclaved medium. Control explants were wounded with a sterile needle without bacteria. After inoculation, all explants were incubated on MS agar medium supplemented with AcS (200 μM) or without this compound in the dark at 26 °C. The transformation frequency (expressed as the percentage of inoculated explants producing roots) and number of roots per explant were studied after a 6-week period.

Roots (about 1 cm long) that formed at the wound sites of the infected explants were excised and transferred into WP liquid medium (Lloyd and McCown, 1980) containing ampicillin (500 mg l^{-1}) and incubated in the dark. After four passages, 7 each, the concentration of ampicillin was reduced to 300 mg l^{-1}. After next four weeks ampicillin was omitted from the medium and axenic root lines (HR-1, HR-4 and HR-5 by ATCC 15834 and HR-2, HR-3, HR-6 and HR-7 by A4) which showed good growth were selected and used for this study. The cultures were incubated on a rotary shaker (100 rpm) in a growth chamber at 26 °C in the dark or under light from cool white fluorescent lamps (16 h light/8h dark period; 40 μM m^{-2} s^{-1}).

Growth studies

For the determination of the growth of seven root lines transformed by *A. rhizogenes* ATCC 15834 and A4, about 0.7 g of fresh weight (0.06 g of dry weight) of roots was cultured in 80 ml of WP liquid medium in 300-ml Erlenmeyer flasks under the above mentioned conditions. The cultures were grown for 28 d and fresh and dry weights were determined. For each line three flasks were used and the experiment was repeated three times.

The results were presented as the growth index (GI) based on dry weight after 28 d of culture: GI = (final dry wt – initial dry wt)/initial dry wt.

For the determination of the time course of changes in the growth and RA production we chose a high productivity root line (HR-1). The roots (about 0.55 g fresh weight and 0.045 g dry weight) were transferred into WP liquid medium (80 ml) and cultured in the light (see above) for 50 d. Triplicate flasks were harvested every 5 d for the analysis of fresh and dry weight as well as RA content. The experiments were repeated three times.

Establishment of untransformed root culture

An untransformed root culture of *S. officinalis* was established by excising roots (about 1 cm) from 5-week-old *in vitro* regenerated plantlets and placing them in MS (macronutrients were reduced to half of normal concentration) agar-solidified medium supplemented with IAA (0.57 μM). After 4 weeks, the roots were transferred into WP liquid medium containing IAA (0.57 μM). The cultures were maintained for eight passages (28 d each) under the same conditions as hairy roots.

Confirmation of transformation

DNA from seven putative transformed root lines and untransformed roots (negative control) was extracted from 50 mg of plant tissue following the procedures described by Pirttilä *et al.* (2001). The DNA concentration was estimated using a Beckman Du-640 spectrophotometer. PCR analysis was performed by using specific primers (Interactiva Biotechnologie GmbH, Germany) recognizing *rol*B (5′ GCT CTT GCA GTG CTA GAT TT 3; 5′ GAA GGT GCA AGC TAC CTC TC 3′) and *rol*C (5′ CTC CTG ACA TCA AAC TCG TC 3′; 5′ TGC TTC GAG TTA TGG GTA CA 3′) genes in T_L fragment of R_i plasmid. Each PCR

reaction contained: standard PCR buffer with $(NH_4)_2SO_4$ (Fermentas, Germany), 1.0 unit *Taq* DNA polymerase (Fermentas), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Fermentas), 50 pM of each primer and 10 ng of the target DNA. PCR conditions were: 94 °C for 3 min (initial denaturation) followed by 35 cycles of 1 min at 94 °C (denaturation), 53.5 °C for 1 min (annealing) and 72 °C for 1 min (extension) with final extension at 72 °C for 6 min. Amplification products were analyzed by electrophoresis on 1.0% agarose gel and detected by straining with ethidium bromide. For all samples, apart from control (untransformed roots), the predicted products of about 383 bp for *rol*B and about 585 bp for *rol*C were obtained (Fig. 1).

Extraction and quantitative analysis of RA

RA was extracted from transformed and untransformed roots cultures. Dried and powdered roots (250 mg) were extracted with methanol. After filtration and evaporation to dryness, the residue was dissolved in 5 ml methanol and analyzed by HPLC as described earlier (Grzegorczyk *et al.*, 2005). The identification of RA was done by comparing its retention time and UV spectrum with the standard compound. The standard of RA was purchased from Roth. The RA concentration was estimated by interpolation of the peak area with a calibration curve constructed for standard RA. RA content of the analyzed samples was expressed as mg g^{-1} of dry weight. The presented results are the means of three replicates. The recovery rate of RA in the crude extract was 98.6%.

Statistical analysis

The data were calculated as means ± standard error. Significant differences were determined using U Mann-Whitney test (at a 5% probability level).

Results and Discussion

The appearance of hairy roots on the wound sites of *S. officinalis* explants was observed, usually with no callus formation, within 6 weeks after inoculation with *A. rhizogenes* strains (ATCC 15834 or A4). The frequency of positively responding explants (*i. e.* percentage of explants forming roots at the inoculation site after 6 weeks) was dependent on the explant type, bacterial strain and the presence of acetosyringone (Table I). The best response was obtained on the shoots infected with strain ATCC 15834 at the second node. In this case, the root induction frequency was 57% and the mean number of roots from inoculation sites was 2. The roots appeared also on 37% of the shoot explants infected with strain A4. The differences in the virulence could be explained by plasmids harboured by the bacterial strains (pR_iA4 and pR_i15834). In order to improve the frequency of root induction, acetosyringone (an activator of *vir* genes) (200 μM) was added to the medium on which bacteria or infected explants were cultivated. We found that the frequency of root formation from *S. officinalis* shoots can be increased from 37% to 67% following infection with *A. rhizogenes* strain A4 cultivated in YMB medium containing AcS. Also, the presence of the *vir*-inducing compound in MS inoculation medium slightly increased the percentage of shoot forming roots (from 37% to 42%) after infection with strain A4. This is in contrast to the results for strain ATCC 15834, which was the most effective without the use of AcS (Table I). It has been earlier demonstrated that the addition of AcS in case of some

Table I. Hairy root induction frequency *Salvia officinalis* shoots within 6 weeks after infection with two *A. rhizogenes* strains.

Bacterial strain	Number of tested explants	Medium [a]	Medium [b]	% of explant producing roots	Mean number of roots per explant
A4	35	YMB	MS	37.1	2.15
	31	YMB	MSAcS	41.9	3.14
	30	YMBAcS	MS	66.7	3.30
ATCC 15834	30	YMB	MS	56.7	2.00
	30	YMB	MSAcS	46.7	3.43
	30	YMBAcS	MS	33.3	3.60

[a] The bacteria were cultured for 48 h without (YMB medium) or with 200 μM acetosyringone (YMBAcS).
[b] The explants were cultured for 6 weeks without (MS medium) or with 200 μM acetosyringone (MSAcS).

Agrobacterium strains may have an inhibitory effect on transformation (Godwin *et al*., 1991). It is possible that wounded tissues of the plant species produce sufficient amounts of phenolic compounds for induction of transcription of *vir* region of the *A. rhizogenes* strain, as has been suggested for members of the Solanaceae family (Spencer and Towers, 1991). Another possibility may be the toxic effect of AcS to the bacterial cells of strain ATCC 15834, although Stachel *et al*. (1985) found that the compound at the concentration of 200 μM was not significantly toxic to *Agrobacterium* cells.

No hairy roots were initiated from *S. officinalis* leaf explants irrespective of the bacterial strain (ATCC 15834 and A4) and the presence or absence of AcS.

Seven axenic *S. officinalis* hairy root lines (HR-1 to HR-7), three obtained through inoculation with ATCC 15834 strain (HR-1, HR-4, HR-5) and four induced with A4 strain (HR-2, HR-3, HR-6, HR-7), were selected after 10 passages on the basis of the growth rate. The root lines were grown in WP liquid medium under light and dark conditions. Their growth (measured as growth index in terms of dry weight) and the content of RA were examined after 28 days and compared with untransformed roots grown in WP medium supplemented with IAA (0.57 μM). All seven hairy root lines used in this study were confirmed to have T_L-DNA (*rol*B and *rol*C genes) in the genome (Fig. 1). However, the response of the root cultures in terms of morphology, growth and RA accumulation varied as shown in Figs. 2–4. Roots induced with strain A4 were short with thick lateral branches and formed dense spherical structures, while roots originating from strain ATCC 15834 were longer, thin with evident plagiotropic growth (Fig. 2). Also, differences in biomass production between the hairy root lines of different origins were observed. The hairy roots transformed with ATCC 15834 showed a higher growth rate as compared to those induced by A4. The differences were statistically significant at $p \leq 0.05$. On the other hand, the growth of roots was not significantly different in the light or the dark (Fig. 3). RA was identified in hairy roots of all lines by

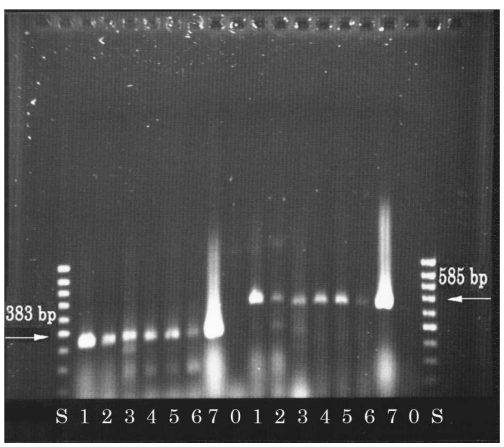

Fig. 1. PCR analysis of *S. officinalis* roots transformed by *A. rhizogenes* ATCC 15834 (lanes 1, 4, 5) and A4 (lanes 2, 3, 6, 7) and untransformed roots (lane 0), GeneRuler™ 100 bp DNA ladder (lane S). Arrows show amplified fragments of *rol*B (383 bp) and *rol*C (585 bp) genes.

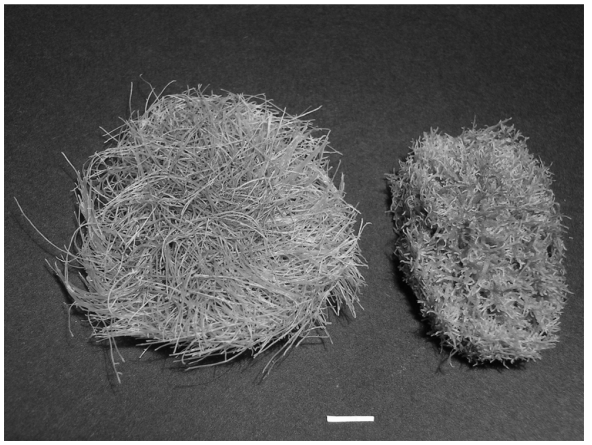

Fig. 2. The hairy roots of *S. officinalis* obtained through inoculation with *A. rhizogenes* strain ATCC 15834 (left) and A4 (right). The hairy roots were grown for 28 days in WP liquid medium (bar 1 cm).

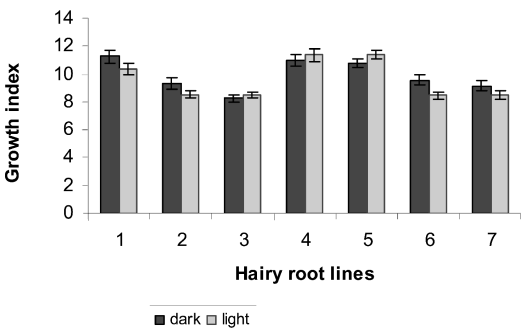

Fig. 3. Growth of hairy root lines of *S. officinalis* after 28 days of culture in WP liquid medium under light and dark conditions [initial inoculum (0.06 ± 0.003) g dry weight/flask]. Growth indexes were calculated on the basis of dry weight of the cultures. Vertical bars represent the standard error of the means of three replicates.

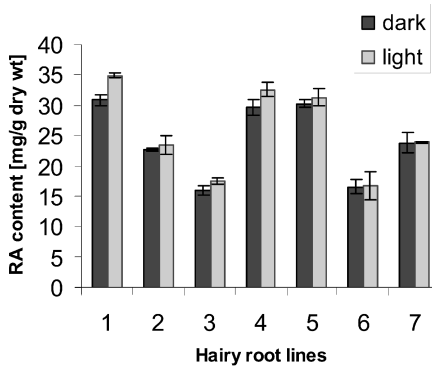

Fig. 4. RA content (mg g^{-1} dry weight) of different hairy root lines of *S. officinalis*. The hairy roots were grown for 28 days in WP liquid medium under light and dark conditions. The results show the means ± standard error of three replicates. RA content in untransformed root culture was 19 mg g^{-1} of dry weight.

HPLC. Only small amount of the metabolite was detected in the medium after 28 days of culture, suggesting that RA was retained within the tissues. We also demonstrated that the root cultures of *S. officinalis* did not produce diterpenoids with antioxidant activity, such as carnosol and carnosic acid. This is not surprising because the secondary metabolites are accumulated in aerial chlorophyllous tissues and were found only in trace amounts in roots of intact plants of *S. officinalis* (Grzegorczyk *et al.*, 2005). In relation to RA production, the lowest RA level was achieved in line HR-3 which showed also the lowest biomass accumulation. The highest content of RA was found in line HR-1 which had a high growth rate (Figs. 3 and 4). It suggests that in *S. officinalis* transformed roots the capacity to synthesize RA is associated with biomass production. It is also possible that the differences in RA production between hairy root lines can be originated from dissimilarities of the bacteria used for infection because RA accumulation was significantly higher in root cultures induced by strain ATCC 15834 than in those transformed with A4. This is consistent with data on hairy roots of *Hyoscyamus albus*, where the relationship between the bacterial strain and alkaloid production was also observed (Zehra *et al.*, 1999). We found the hairy roots differed in their content of RA even when the same *Agrobacterium* strain A4 was used, suggesting that it might be a consequence of the different sites of T-DNA insertion into the plant genome (Zehra *et al.*, 1999). We also demonstrated that RA levels were always slightly higher in sage root cultures grown in the light than in the dark, but the differences were statistically significant only for line HR-1 (Fig. 4).

In order to get more detailed information about the growth and RA accumulation the time course during a 50-day culture period was made. The highest productivity line HR-1 grown in the light was chosen for this purpose. After an initial lag phase, exponential growth (between 5^{th}–15^{th} day) was observed, with a dry weight doubling time of 7.7 days. The maximum biomass [(9.01 ± 0.27) g fresh wt/flask and (0.72 ± 0.01) g dry wt/flask] was achieved at the 35^{th} day of culture. The values represent a 16-fold increment of the inoculum biomass [(0.55 ± 0.02) g fresh wt/flask and (0.045 ± 0.002) g dry wt/flask]. The RA content appeared to be related to growth. The maximum content of this compound in line HR-1 [(44.98 ± 0.26) mg g^{-1} dry wt] was recorded at the end of the growth phase (at 45–50 days). Similar behavior has also been observed in the production of RA by a cell suspension culture of *Coleus blumei* (Gertlowski and Petersen, 1993). The RA level in HR-1 root line was over twice as high as in untransformed roots of *S. officinalis* cultured *in vitro* (19 mg g^{-1} dry wt). The untransformed roots showed only little growth in WP liquid medium supplemented with IAA (0.57 μM), did not branch and died after eight passages. The RA content achieved by hairy root line HR-1 was also over 6 times higher than in roots of 10-week-old *in vitro* regenerated plants of *S. officinalis* (Grzegorczyk *et al.*, 2005) and 90-times higher than that of a commercial sage sample (482 μg g^{-1} dry wt) reported earlier by Santos-Gomes *et al.* (2002).

It is also important that RA production in line HR-1 remained stable for over at least 23 passages, during almost 2 years of cultivation. In passages 14 and 23 the roots produced, respectively, 35.3 mg g^{-1} dry wt (303 mg l^{-1}) and 37.3 mg g^{-1} dry wt (320 mg l^{-1}) of RA, after 28 days.

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