Rosmarinic Acid and Other Phenolic Acids in Hairy Roots of *Hyssopus officinalis*

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Hairy roots of *Hyssopus officinalis* L. were induced by infection of petioles with *Agrobacterium rhizogenes* LBA 9402 and studied for production of phenolic acids, especially rosmarinic acid (RA). The highest content of rosmarinic acid (about 6% of dry weight) was obtained in hairy roots grown in Gamborg's B5 liquid medium containing 10% (w/v) sucrose. The level was at least 60% higher than those found in callus, cell suspension culture and roots of one-year-old field grown plants. Apart from RA, nine other phenolic acids were detected in transformed roots and quantified by gas chromatography.

Introduction

Hyssopus officinalis L. is a herbaceous perennial plant which belongs to the Lamiaceae family. The plant is well known in medicine for its spasmolitic, antiinflammatory and antiseptic properties against bacteria, fungi and viruses (Kreis *et al.*, 1990; Ghfir *et al.*, 1994). The therapeutic activity of the herb of *H. officinalis* has usually been attributed to the components of its essential oil. The species contains *ca* 1% of essential oil (Tsankova and Konaktchiev, 1993). Lamaison *et al.* (1990) detected rosmarinic acid in inflorescences of *H. officinalis* in about 0.5% yield on the dry weight of plant material.

Rosmarinic acid (RA, Fig. 1) is one of the most common caffeic acid esters occurring particularly in Lamiaceae and Boraginaceae families. In plants RA is thought to be a part of the defense system against bacterial infections and predators (Ravn *et al.*, 1989). The pharmacological properties RA are described to be antioxidant, antibacterial, antiviral, and antiinflammatory (Parnham and Kesselring, 1985; Holzmannova, 1995). The antioxidative activity and the low toxicity make RA also interesting for food industry.

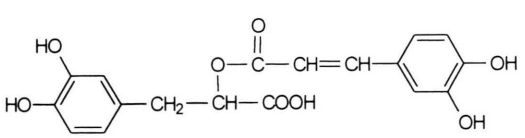

Fig. 1. Rosmarinic acid.

RA has also been reported to be accumulated in cultured cells of a number of species, including *Coleus blumei* (Zenk *et al.*, 1977), *Anchusa officinalis* (De Eknamkul and Ellis, 1984), *Lithospermum erythrorhizon* (Fukui *et al.*, 1984). *Orthosiphon aristatus* (Sumaryono *et al.*, 1991) and *Salvia officinalis* (Hippolyte *et al.*, 1992). However, only few reports on the use of transformed roots for the production of the compound have been reported in the literature (Tada *et al.*, 1996). Transformed root cultures provide useful substances as well as constitute an appropriate experimental system to study root physiology and biochemistry (Wysokińska and Chmiel, 1997).

In the present study hairy root cultures of *H. officinalis* induced by *Agrobacterium rhizogenes* (LBA 9402 strain) were established and the contents of RA as well as other phenolic acids (derivatives of hydroxycinnamic and benzoic acids) in the cultures were determined. This paper also described the results of comparative analysis on RA produced *in vitro* by various tissue cultures (organized and unorganized) and *in vivo* by plant organs (shoots and roots) of *H. officinalis*. To our

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knowledge there are no reports on hyssop tissue cultures.

Experimental

Plant material

Seeds of *Hyssopus officinalis* L. were obtained from Prof. Kozłowski (Institute of Medicinal Plants, Poznań, Poland). The seeds were surface-sterilized with 0.1% solution of $HgCl_2$ for 10 min and then rinsed three times in sterile distilled water (15 min each). The desinfected seeds were germinated in the dark, at 26 °C on Murashige and Skoog (1962) medium (MS) supplemented with 3% sucrose and 0.8% agar.

Transformed root cultures

Leaves of four-week old plantlets from the seedlings were used as explants for the induction of hairy roots. *Agrobacterium rhizogenes* strain LBA 9402 harbouring the Ri plasmid 1855 was used for transformation after 48 hours growth in YMB medium (Vervliet *et al.*, 1975), at 25 °C, in the dark. Leaves were inoculated at the cut end of the petioles with syringe needle which had been dipped into the bacterial broth. The inoculated leaves were incubated in hormone-free B5 Gamborg's *et al.* (1968) agar medium, at 26 °C, in the dark. Three to four week after infection the adventitious roots appeared at the infection sites. Putative transformed roots (1–2 cm long) were excised from explants and transferred into hormone-free B5 liquid medium containing 3% (w/v) sucrose and ampicillin initially at a concentration of 500 mg/l but after three passages reduced to 300 mg/l. After four passages (7–10 days each) the antibiotic was omitted and axenic hairy root cultures were obtained. One clone that showed sufficient growth in B5 medium was selected and used for the experiments. To prove transformation of this clone, opines (agropine and mannopine) were extracted and detected by paper electrophoresis (Petit *et al.*, 1983).

The cultures were incubated on a rotary shaker (100 rpm), at 26 °C, in the dark and subcultured every 4 weeks by transferring about 0.3 g fresh weight into 80 ml fresh medium in 300 ml Erlenmeyer flasks. The roots were maintained for about 12 months prior to the start of experiments. The effect of initial sucrose concentration of 1, 3, 5, 7, 10 and 12% (w/v) was examined using B5 basal medium. After 28 days culture, five flasks of each treatment were harvested and analyzed for biomass (fresh and dry weights) and RA production. Kinetics growth and RA production were studied in B5 medium supplemented with 10% (w/v) sucrose. For this purpose, three flasks were harvested over a time period of 35 days, at 5 day intervals. B5 medium supplemented with 10% sucrose was also used to investigate effect of light (continuous cool-white fluorescent light, 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on growth and RA content in roots. All experiments were repeated three times.

Callus, cell and shoot cultures

In order to determine RA contents in various *in vitro* tissues, callus, cell and shoot cultures of *H. officinalis* were obtained. Hypocotyls (*ca.* 1 cm segments) from two-week old aseptically grown seedlings were used for callus initiation. MS basal medium supplemented with 0.2 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l benzylaminopurine (BAP) was used for this purpose. After 4 weeks the calli were subcultured on the same medium. After three passages (35–40 days, each) calli were transferred to Schenk and Hildebrandt (1972) agar medium (SH) containing 3% sucrose and 1 mg/l NAA, 0.1 mg/l indole-3-butyric acid (IBA) and 0.1 mg/l BAP. The calli maintained over almost one year on the medium were used as source of material for RA quantity determination.

Suspension cultures were obtained from friable callus in liquid MS medium containing 3% sucrose, 1 mg/l NAA and 0.2 mg/l BAP. Cultures were maintained on rotary shaker (100 rpm) and subcultured every 2–3 weeks into the same medium by adding 5 ml of suspension (about 0.2 g fresh weight) to 80 ml fresh medium in 300 ml Erlenmeyer flasks.

Shoot-tips (*ca.* 0.5 cm, with one apical and two axillary buds) were excised from 4-week-old plantlets, placed on MS agar medium containing 3% sucrose, 0.1 mg/l indole-3-acetic acid (IAA) and 0.5 mg/l BAP and multiple shoots were obtained. The cultures used in this study had been maintained for one year by subculturing into the same medium, every 35 days.

All cultures (callus, cell suspension and shoots) were incubated at 26 °C under continuous cool white fluorescent light (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Isolation of phenolic acid fractions

Dried and powdered roots (10 g) were extracted twice (3h) with boiling 80% ethanol. The fraction of free phenolic acids (called F-1) was obtained using ethyl ether and 5% $NaHCO_3$, according to known methods (Świątek and Dombrowicz, 1984). Water solution after extraction of F-1 fraction was

divided into two equal parts; one was subjected to acid hydrolysis (2 M HCl, 100 °C, 1 h) to give 70 mg F-2 fraction and second to alkaline hydrolysis ($NaBH_4$ + $Ba(OH)_2$, 100 °C, 15 min) to yield F-3 fraction (165 mg) (Schmidtlein and Hermann, 1975).

Chromatographic analysis of phenolic acid fractions

The obtained fractions (F-1, F-2 and F-3) were subjected to silylic reaction with N-bis (trimethylsilyl)-trifluoroacetamide (BSTFA, REGIS Chem. Comp., Morton Grove, USA). The analysis of silylic derivatives of phenolic acids was performed with a gas chromatograph HP-5890 (Hewlett-Packard) with a mass detector HP-5970 MSD. A glass capillary HP-5 (50 m × 0.2 mm) was used, diameter of filling was 0.5 μm, injector temp. 100–250 °C, increase 5 °C/min up to 150 °C and 8 °C/min up to 250 °C; gas carrier flow speed (hel) 24 ml/s; internal standard: pseudocumen.

High performance liquid chromatography (HPLC)

The gas chromatography (GC) method did not allow for detection and quantity determination of depsid compounds. Therefore, for separation and quantification of RA, HPLC method was used. The dried plant materials (0.5 g) were extracted twice with boiling 70% ethanol (for 2 h). The combined extracts were evaporated to dryness and washed with hot water. The impurities were filtered off and the filtrate was evaporated to dryness, resolved in methanol and subjected to HPLC. HPLC analysis was performed on Lichrosorb column RP18 (4 × 250 mm, 5 μm) using a linear gradient acetonitrate: water: *o*-phosphoric acid 10:90:1, changed within 20 min; flow rate of 1 ml/min. RA was detected by measuring UV absorption at 332 nm. The retention time of RA was 3.4 min, which corresponded to that of the RA standard. The RA content was determined with reference to a standard curve constructed for known amounts of RA. Each determination was repeated in triplicate and the mean together with the standard error was calculated. The results were expressed as % dry weight or mg/l of culture.

Results

Growth of hairy roots and RA production; effect of sucrose

Hairy roots of *H. officinalis* were induced on the leaves of axenic plants by infection with *A. rhizogenes* LBA 9402 strain harbouring Ri 1855 plasmid. The roots which appeared at the infected sites were cut off and transferred into hormone-free B5 liquid medium containing 3% sucrose and antibiotic (300–500 mg/l ampicillin). After several passages in order to eliminate bacteria, hairy roots were cultured on the same medium without antibiotic. They exhibited the transformed phenotype with regular continuous lateral root formation. Transformation was proved by opine (agropine and mannopine) assay. The growth of hairy roots (fresh and dry biomass) tended to enhance when sucrose concentration was increased from 3 to 12% w/v in B5 basal medium. We found that both roots growth (1.1 g dry wt. of biomass per flask) and RA content (5.7% of biomass dry wt.) were the highest in the medium containing high sucrose concentration (10%); relative to 3% sucrose the biomass dry wt. was higher by over 5 times. The hairy roots in 10% sucrose medium contained almost twice as much RA as roots grown in 3% sucrose. When both biomass production and RA accumulation were taken into account the highest RA production (0.8 g/l culture) was achieved in the media containing 10% sucrose. The productivity was remained stable during at least 2 years of continuous subculturing of hairy roots into the medium of the same composition.

Hairy roots were cultured under optimum conditions (B5 medium containing 10% sucrose) for time course study (Fig. 2). From the initial inoculum of 0.3 g (fresh wt.), fresh and dry weights increased approximately 30 times over 35 days of culture and reached 8.7 fresh wt./flask (0.15 g dry wt./flask). The specific growth rate based on dry weight was 0.13 day $^{-1}$ at the exponential growth phase (between 5 and 25 days of culture). This indicates that the doubling time was 5.5 days during the fastest growth phase. It was similar to the doubling times reported in the literature for a variety of hairy root cultures (Wilson *et al.*, 1987). RA production of *H. officinalis* root cultures increased over the period of subculture reaching a maximum (0.9 g/l; equivalent to 4.9% of dry weight) after 30–35 days of culture during the stationary and deceleration of growth phases (Fig. 2).

It is known that light is a factor that may increase biomass growth and production of useful secondary metabolites in transformed roots from some plant species. In the case of hairy roots of *H.*

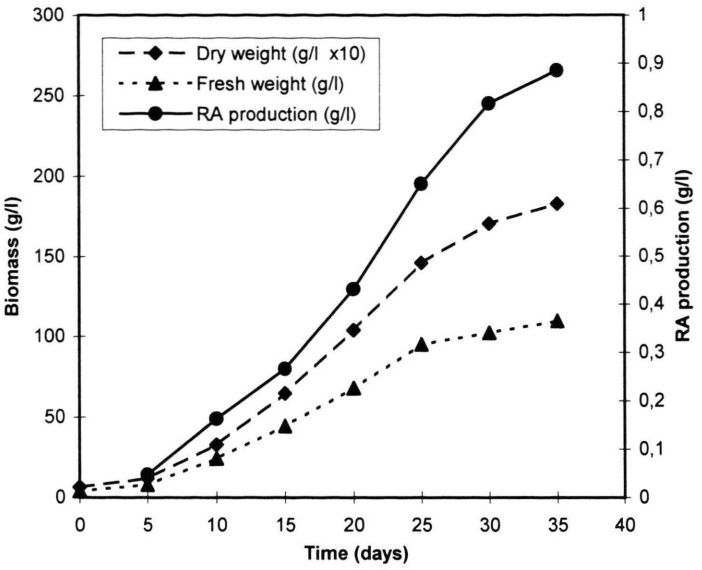

Fig. 2. Time course of biomass (fresh and dry weights) and rosmarinic acid (RA) production in hairy root culture of *Hyssopus officinalis*. Values are expressed as means of there replicates.

officinalis in this study, however, the light did not affect the growth and RA production.

RA production in vitro and in vivo

Production of RA in various cultured tissues (callus, cells and shoots) was also investigated and compared to those in the various organs (roots and shoots) of the original plants of *H. officinalis* (Table I). Callus tissue was induced from hypocotyl explants (see Material and Methods) on MS agar medium supplemented with 0.2 mg/l NAA and 0.1 mg/l BAP, but this culture resulted in poor growth of the calli. Therefore, after three subcultures they were transferred into SH medium containing 1 mg/l NAA, 0.1 mg/l IBA and 0.1 mg/l BAP. Such calli were subcultured for one year and proliferated mostly into an unorganized light-brownish mass of cells. On the other hand, MS medium was selected for the initiation and the growth of cell culture since it provided a finer and more homogenous cell suspension than the SH medium. The best concentration of growth regulators was 1 mg/l NAA and 0.2 mg/l BAP. Under these conditions *H. officinalis* cell suspension gave about 10 g fresh weight of biomass per flask (an increase of 50 times from the inoculum), within 21 days.

RA contents in callus and suspension cultures were equal. It was noteworthy that production of the compound in these unorganized cultures was as high as that of the roots of one year-old field grown plants (Table I). The value, however, was at least 60% lower than that of in transformed roots cultured under optimum conditions (B5 medium containing 10% sucrose). A comparison of RA accumulation in roots collected from one- and two-years-old plants showed that it was 3 times lower in older roots (Table I).

For shoot cultures, the shoot-tips from aseptically grown plantlets were subcultured on MS medium with 0.1 mg/l IAA and 0.5 mg/l BAP. Proliferation showed to be rather efficient yielding about 8 axillary shoots (0.5 cm in length) per explant after 35 days. The *in vitro* cultured shoots were found to contain an average 1.5% RA. This

Table I. Rosmarinic acid (RA) content in plants and tissue cultures of *Hyssopus officinalis*. Values are the means of the three replicates ± standard error (n = 3).

Analysed material	RA content (% dry wt.)
***In vitro* cultures**	
Callus	3.33±0.13
Cell suspension	3.32±0.11
Multiple shoots	1.66±0.14
Intact plants	
Shoots	0.45±0.01 [a]
	0.45±0.03 [b]
Roots	3.32±0.22 [a]
	1.26±0.06 [b]

[a] One-year-old plants grown in field.
[b] Two-years-old plants grown in field.

was 3 times higher than the amount detected in the shoots of the field grown plants (0.5% dry wt.).

Other phenolic acids

Further investigations were carried out to detect other phenolic acids in transformed roots maintained in B5 liquid medium supplemented with 10% sucrose. They were examined by GC. The phenolic acids are presented in Table II. They include 3 derivatives of cinnamic acid and 6 derivatives of benzoic acid. The majority of phenolic acids was present both unbound and bound since they were also present in fractions after hydrolysis. The amounts of the phenolic acids ranged between 0.05 mg (*p*-coumaric acid) and 21 mg/100 g dry roots (caffeic acid).

Discussion

Comparative studies of unorganized and organ cultures of *H. officinalis* showed that RA production not be improved by the increase of the degree of differentiation. A prerequisite for this purpose is high concentration of sucrose in the nutrient medium. It is also known from *Coleus blumei* (Zenk *et al.*, 1977) and *Salvia officinalis* (Hippolyte *et al.*, 1992) cell suspension cultures that high concentration of sucrose stimulated growth rate and RA production. The osmotic pressure of the medium may be involved in this phenomena. On the basis of our results it can also be suggested that the accumulation of RA is determined by the age of plants or several environmental factors which were not controlled; the roots obtained from one-year-old plants of *H. officinalis* were superior in terms of RA production as compared to roots from older (two-years-old) plants.

The presented results indicate that RA production in hairy roots *of H. officinalis* can be greater than in an intact plant. However, it is smaller than has been reported previously for cell cultures of *Coleus blumei* (Zenk *et al.*,1977) or *Salvia officinalis* (Hippolyte *et al.*, 1992). Thus, biotechnological value of hairy roots of *H. officinalis* does not lie in their impressive productivity, but rather in their potential to provide a simple experimental system for studies on regulation of biosynthesis of RA and other phenolic compounds. For example, the explanation why this pathway is so well expressed in both unorganized and organized cultures may be important.

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Table II. Phenolic acids detected by GC in hairy root culture of *Hyssopus officinalis*. The roots were cultured in the dark in B5 liquid medium containing 10% (w/v) sucrose; culture period of 28 days. Values of single measurements.

	Retention time	Phenolic acid content (mg/100 g dry wt.)			
Acid	[min]	F-1	F-2	F-3	total
Salicylic	20.47	n. d.	0.10	n. d.	0.10
p-Hydroxybenzoic	21.25	0.09	1.45	0.11	1.65
Wanilic	24.70	0.03	3.59	0.08	3.70
Gentisic	24.74	0.03	2.26	n. d.	2.29
Protocatechuic	25.69	0.08	4.54	0.10	4.72
Syringic	27.23	n. d.	1.62	0.13	1.75
p-Coumaric	28.20	n. d.	0.05	n. d.	0.05
Ferulic	32.13	0.53	0.56	0.39	1.48
Caffeic	33.44	12.00	4.97	4.20	21.17

n. d. – not detected.
F_1- fraction of free phenolic acids.
F_2, F_3 – fractions of phenolic acids after acid and alkaline hydrolysis, respectively.

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