

The effect of yeast extract and methyl jasmonate on rosmarinic acid accumulation in *Coleus blumei* hairy roots

N. BAUER*, D. KISELJAK and S. JELASKA

Department of Molecular Biology, Faculty of Science, University of Zagreb,
Horvatovac 102a, 10000 Zagreb, Croatia

Abstract

The leaves of axenically grown *Coleus blumei* were inoculated with *Agrobacterium rhizogenes* strain A4 and hairy root were established. PCR and Southern hybridization analysis confirmed transgenic nature of hairy root clones. Cultures of normal roots, induced by α -naphthaleneacetic acid on leaf explants, and hairy roots were evaluated for growth and rosmarinic acid content. Significantly better growth and up to 2.8 higher amount of rosmarinic acid was detected by HPLC analysis in hairy root clones. Methyl jasmonate stimulated rosmarinic acid accumulation in 6 out of 11 tested clones, while yeast extract induced RA accumulation in two and diminished it in 5 out of 11 tested hairy root clones.

Additional key words: *Agrobacterium rhizogenes*, elicitors, phenolic compounds, phenylalanine ammonia lyase, transformation.

Introduction

Rosmarinic acid (α -*O*-caffeoyl-3,4-dihydroxyphenyllactic acid, RA) is a hydroxycinnamoyl ester, most commonly found in plant species of the family *Boraginaceae* and *Lamiaceae*, and also in lower plants such as hornworts (Petersen and Simmonds 2003, Vogelsang *et al.* 2006). RA is readily accumulated in undifferentiated plant cell cultures, callus and hairy roots, sometimes in much higher concentrations than in intact plants (Pavlov *et al.* 2000, Tada *et al.* 1996, Chen *et al.* 1999a,b, Kochan *et al.* 1999). Rosmarinic acid has various biological activities, which makes it a very interesting product for pharmaceutical, cosmetic and food industry (Petersen and Simmonds 2003).

The first *Coleus blumei* cell cultures accumulating RA were described by Razzaque and Ellis (1977), Zenk *et al.* (1977) and Ulbrich *et al.* (1985). A rapid and highly-effective method for micropropagation of *Coleus blumei* was established (Rani *et al.* 2006). The RA accumulation has been shown to be dependent on a medium composition, especially sucrose content (Ulbrich *et al.* 1985, Hippolyte *et al.* 1992, Gartlowski and Petersen 1993, Martinez and Park 1993). In addition, several authors discovered that rosmarinic acid accumulation can be enhanced by fungal elicitors (Bais *et al.* 2002, Yan

et al. 2006) or methyl jasmonate (Szabo *et al.* 1999, Matsuno *et al.* 2002, Kim *et al.* 2006). Fungal (or yeast) elicitors and methyl jasmonate increased phenylalanine ammonia lyase (PAL) activity which is a key enzyme of RA biosynthetic pathway (Petersen *et al.* 1993). Positive correlation between PAL activity and RA biosynthesis was confirmed in cell cultures of *Boraginaceae* and *Lamiaceae* species (Mizukami *et al.* 1992, 1993, Szabo *et al.* 1999).

Coleus blumei is susceptible on *Agrobacterium* infection (Bauer *et al.* 2002). Transformed callus cultures of *Coleus blumei* have been established and show faster growth and higher RA accumulation than normal calli and intact plants (Bauer *et al.* 2004). The *Agrobacterium rhizogenes* caused hairy roots characterized by fast growth on hormone-free medium, lack of geotropism, abundant lateral branching and genetic stability. Recent progress in scaling-up of hairy root cultures makes this system an attractive tool for industrial production of plant secondary metabolites (Guillon *et al.* 2006a,b).

The aim of the investigation reported here was to obtain hairy root cultures of *Coleus blumei* suitable for biotechnological application.

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Abbreviations: CB - medium proposed for *Coleus blumei* (Petersen and Alfermann 1988); FM - fresh mass; MJ - methyl jasmonate; MS - Murashige and Skoog medium (1962); NAA - α -naphthaleneacetic acid; NR - normal, non-transformed roots; PAL - phenylalanine ammonia lyase; RA - rosmarinic acid; YE - yeast extract.

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* Corresponding author; fax: (+358) 1 4606286, e-mail: nbauer@zg.biol.pmf.hr

Materials and methods

Plants and hairy root induction: For the experiments, *Coleus blumei* Benth. plantlets cultivated *in vitro* were used. Hairy root cultures were established after transformation of leaf explants with *Agrobacterium rhizogenes* A4 (pHRI) according to Bauer *et al.* (2002) and Karami *et al.* (2009). Briefly, leaf segments were incubated for 15 min in 2-d-old bacteria suspension. Explants were dried on a sterile filter paper and inoculated upside-down on solid, hormone-free Murashige and Skoog (1962; MS) medium. After 2 d, explants were sub-cultured on hormone-free MS medium with 500 mg dm⁻³ carbenicillin. Normal (non-transformed) roots (NR) were induced on leaf explants cultured on MS medium supplemented with 1.0 mg dm⁻³ α -naphthaleneacetic acid (NAA). The stocks of hairy root clones were maintained on solid (8 g dm⁻³ agar) hormone-free MS medium and normal root cultures on MS with 1.0 mg dm⁻³ NAA. For experiments, roots were cultured in 100 cm³ Erlenmeyer flasks filled with 30 cm³ medium and incubated on an orbital shaker at 100 rpm. Approximately 0.3 g of root tissue (2 - 15 mm long tips) from 4-week-old stock culture was inoculated in each flask. All cultures were incubated at 24 °C in the dark. The effect of MS and CB (Petersen and Alfermann 1988) media on the growth and RA accumulation was analyzed.

Roots were harvested from the culture medium, washed with distilled water and blotted dry by paper towels. Fresh mass (FM) was determined. Growth index (GI) was calculated according to formula: $GI = (\text{final FM} - \text{initial FM}) / \text{initial FM}$. Tissue was lyophilized and the yield of dry mass was measured.

Polymerase chain reaction: DNA was extracted from the tips of roots using *Qiagen* (Hilden, Germany) DNeasy plant mini-kit. The presence of T-DNA in hairy roots was detected by PCR. PCR amplification of the target sequences was performed in 50 μ m³ of the following reaction mixture: 0.2 mM of each dNTP, 10 \times buffer for *DyNAzyme* DNA polymerase, 3 mM MgCl₂, 10 pmol of each primer (forward: 5'-CAGCTTCTAAATGTG GTGGAGGCC-3', reverse: 5'-CCTTGCCGATTGCCAG TATGGC-3'), 1.0 U *DyNAzyme I* DNA polymerase (*Finnzymes*, Espoo, Finland) and 50 ng of plant DNA. In the transformed roots, *orf13* gene was amplified. No amplification of *virB10* gene confirmed absence of agrobacteria in the root tissue. The cycling conditions were: one cycle at 94 °C for 5 min; 30 cycles at 94 °C for 45 s, at 60 °C for 45 s and at 72 °C for 1 min; one cycle at 72 °C for 5 min. PCR products were analyzed on 1 % (m/v) agarose gel.

Southern hybridization: To confirm T-DNA insertions in plant genome, Southern blot analysis was performed. Genomic DNA (15 μ g) from hairy root lines was digested with *Bam*HI. Fragments were fractionated on 0.7 % (m/v) agarose gel and transferred to a positively charged nylon

membrane by vacuum (*Roche Diagnostics*, Mannheim, Germany). DNA fragment 30a (*Bam*HI 1.52 kb) of cosmid pLJ1 (Jouanin 1984) was eluted from the gel, labeled by random primer method with fluorescein-11-dUTP and used as a probe. The cosmid pLJ1 contains TL-DNA of pHRI (identical to pRiA4). The blot was hybridized at 65 °C overnight, followed by two washes at room temperature with 1 \times SSC, 0.1 % SDS and then two washes at 65 °C with 0.1 \times SSC, 0.1 % SDS. For the detection, Gene images CDP-Star detection module (*Amersham Biosciences*, Buckinghamshire, UK) was used. Probe bound anti-fluorescein alkaline phosphatase conjugate catalyses light production by enzymatic decomposition of a stabilized dioxetane substrate. Finally, the blot was exposed to *Hyperfilm-MP* for 3 h.

Elicitor treatment: Methyl jasmonate (MJ; *TCI Europe* nv, Zwijndrecht, Belgium) was dissolved in dimethylsulfoxide, filter sterilized (*Nalgene*, 0.2 μ m) and added (100 μ M final concentration) in freshly prepared and autoclaved MS medium (Matsuno *et al.* 2002). Yeast extract (YE; *Sigma-Aldrich*, St. Louis, USA) was dissolved in distilled water, autoclaved and added (5 g dm⁻³ final concentration) in freshly prepared MS medium (Mizukami *et al.* 1992). Hairy roots were cultured for 33 d (end of exponential growth phase), and then the old medium was substituted with 30 cm³ of fresh MS medium supplemented with MJ or YE. MS medium without elicitors was added to control cultures. PAL activity was analyzed 24 h after treatment and RA content 24 h, 2, 4 or 8 d after media substitution. Each treatment was performed in 3 replicates.

Extraction and determination of rosmarinic acid content: RA was extracted from lyophilized roots. Roots were homogenized in mortar and 40 mg of tissue was extracted with 2 cm³ of 70 % ethanol in ultrasonic bath at 70 °C for 20 min. This extraction protocol was the most suitable among others that were evaluated (extraction with 70 % ethanol at 4, 23 or 70 °C, or 80 % methanol at 4, 23 or 70 °C; results not shown). The extract was centrifuged at 10 000 g for 15 min. Supernatant was filtered through a membrane filter (*Nalgene*, 0.45 μ m) to eliminate undissolved particles. The filtered sample was diluted (1:5) with 50 % methanol in water (v/v) acidified with 0.01 % H₃PO₄ (v/v). RA concentration was determined by HPLC *PerkinElmer Series 200* chromatograph system consisting of a vacuum degasser, quaternary pump, autosampler and diode array detector. *Hypersil* ODS column, 250 \times 46 mm, particle size 5 μ m was used. The solvent for separation was 50 % methanol in water (v/v) acidified with 0.01 % H₃PO₄ (v/v). The flow rate was 0.85 cm³ min⁻¹ and detection wavelength was at 330 nm. Authentic RA (*Fluka*, Buchs, Switzerland) was used as reference substance.

Determination of soluble phenols, proteins and PAL activity: The total phenolic compounds of ethanol extracts were determined by the Folin-Ciocalteu reagent. The reaction was performed by mixing 20 mm³ of extract, 1.58 cm³ water and 0.1 cm³ Folin-Ciocalteu reagent and completed to 2 cm³ with 200 g dm⁻³ Na₂CO₃. After 2 h at 37 °C, the intensity of the colour was measured at 765 nm and compared to a gallic acid calibration curve. Soluble phenols were estimated as gallic acid equivalents.

Soluble proteins were extracted from lyophilized hairy roots with 2 cm³ of 200 mM borate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 20 000 g at 4 °C for 30 min. Supernatant was desalted on PD-10 column (Amersham Biosciences, Buckinghamshire, UK). Protein content of

the filtrate was quantified with Bradford reagent using bovine serum albumin as standard (Bradford 1976).

PAL activity was measured in protein extracts. The reaction mixture containing 0.8 cm³ 0.5 M borate buffer (pH 8.8), 0.05 cm³ of 20 mM L-phenylalanine and 0.2 cm³ enzyme extract was incubated at 40 °C for 3 h. During that time, a change in absorbance at 300 nm was monitored (*t*-cinnamic acid production).

Statistical analysis: To analyze significance of the difference between control and treatment, Duncan New Multiple Range Test (DNMRT) was used (Duncan 1955). The results were analyzed using computer program *Statistica 7.0* (StatSoft Inc., USA). Each experiment measurements were replicated 3 times. The data reported here are the means of 3 or 6 replicates.

Results and discussion

Establishment of hairy root culture: 14 d after inoculation, roots started to grow from leaf explants. Roots developed on all inoculated explants cultured on hormone-free MS medium and no roots developed on uninfected explants. After three weeks, more than 200 roots were excised from infected leaf explants and grown separately for three months. Sixteen hairy root lines that grew vigorously on MS medium without growth regulators and had typical hairy root phenotype were selected. The presence of *orf13* gene in the hairy root lines was tested by PCR amplification of DNA using *orf13* forward and reverse primers. DNA from

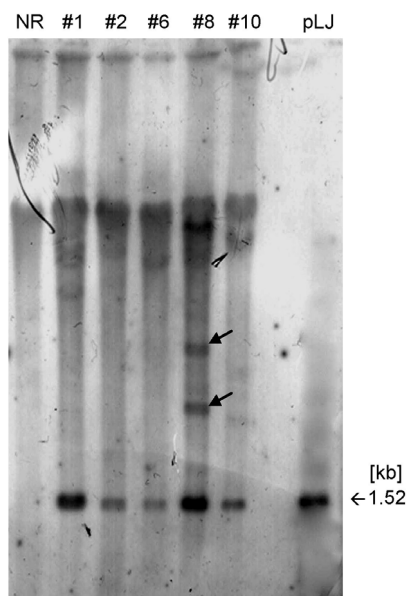


Fig. 1. Southern blot of *Bam*HI-digested genomic DNA of selected hairy root clones of *C. blumei* using fluorescein labeled 1.52 kb fragment of pLJ1. Lanes: NR - non-transformed root; #1, #2, #6, #8 and #10 - hairy root clones; pLJ - cosmid pLJ1 digested with *Bam*HI. Additional fragments detected in clone #8 (arrows) may indicate its possible multiple transformation.

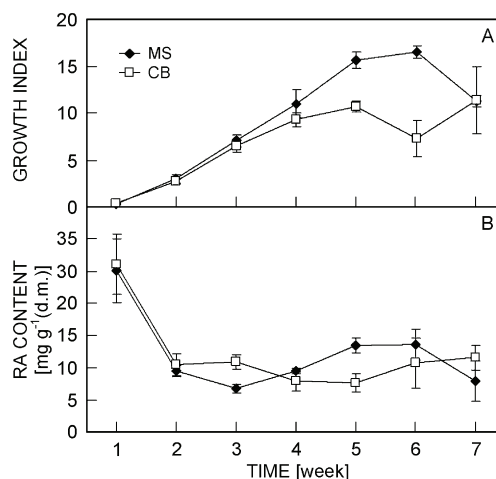


Fig. 2. Growth (A) and rosmarinic acid content (B) in *C. blumei* hairy root line #12 cultivated in liquid MS and CB medium. Data are means \pm SD of six replicates.

A. rhizogenes A4 (pHRI) served as positive control and DNA from non-transformed roots served as negative control. All transformants showed presence of 498-bp fragment of *orf13*. The presence of transgenes was confirmed by Southern hybridization of hairy roots genomic DNA (#1, #2, #6, #8, #10) using fluorescein labeled 30a fragment from cosmid pLJ1 as a probe. A fragment of the expected size (1.52 kb) in positive control (cosmid pLJ1) and hairy root lines hybridized with the probe, confirming the transgenic nature of hairy root lines (Fig. 1). Additional fragments were detected in clone #8, indicating its possible multiple transformation. For further experiments, 11 lines (#1, #2, #3, #4, #6, #8, #9, #10, #12, #15, #16) were used.

CB medium is proposed for *C. blumei* callus culture (Petersen and Alfermann 1988) and we confirmed its efficiency on growth and RA production in *Coleus blumei* transformed callus cultures (Bauer *et al.* 2004).

However, according to Li *et al.* (2005) MS medium was the best for growth of *Coleus forskohlii* hairy roots. Therefore, we compared growth and RA production of *C. blumei* hairy roots cultured in hormone-free CB and MS medium. Roots grew better in MS medium and maximum growth index was 16.5 after 42 d in culture. In CB medium, growth was slower and maximum growth index was 11.37 after 49 d (Fig. 2A). In addition, RA yield was significantly higher in MS medium than in CB medium (Fig. 2B). Therefore, for further experiments, roots were cultured in MS medium.

Growth and RA accumulation: Considerable variation between non-transformed and hairy roots was observed in morphology and growth. Non-transformed roots were thinner with rare branches. They have strikingly lower growth rate than hairy roots and they started to necrotize after 28 d. Hairy roots displayed a typical phenotype marked by plagiotropic growth and had higher incidence of lateral branching. Several clones of hairy roots, with stable phenotype, were subcultured in liquid hormone-free medium for three years. To evaluate growth and RA accumulation of different clones, hairy roots were grown in liquid hormone-free MS medium, and normal roots in liquid MS medium supplemented with 1 mg dm⁻³ NAA. Six hairy root clones accumulated significantly higher amount of RA than non-transformed roots. The best producing clone #8 accumulated 50 mg(RA) g⁻¹(d.m.), while RA content of non-transformed roots was only 17 mg g⁻¹(d.m.) (Fig. 3A). In addition, growth of hairy root lines was stable and 3 - 5 times higher than growth of normal roots (Fig. 3B). If we take into account better growth and RA production in clone #8, the production of RA per flask was 13 times higher than in non-transformed roots. Hairy root cultures of *Coleus forskohlii* produced roughly 4.8 % RA (Li *et al.* 2005), *Salvia*

multiorrhiza 4.6 or 3 % RA (Yan *et al.* 2006, Chen *et al.* 2001), and *Salvia officinalis* 4.5 % RA (Grzegorzczuk *et al.* 2006). Recently, we introduced hairy root clone #8 in 1-dm³ bioreactor. Cultures demonstrate higher growth and permanent RA production (data not shown). These preliminary results make the base for biotechnological application of *Coleus blumei* hairy roots in RA production.

Effects of elicitors: It has been reported that RA biosynthesis was affected by elicitors, such as YE and MJ. Particularly, MJ induces RA biosynthesis in *Ocimum basilicum* plants (Kim *et al.* 2006), *Coleus blumei* suspension culture (Szabo *et al.* 1999), *Lithospermum erythrorhizon* suspension culture (Tsuruga *et al.* 2006) and *Coleus forskohlii* hairy root culture (Li *et al.* 2005). YE induced RA biosynthesis in *Agastache rugosa* (Kim *et al.* 2001), *Lithospermum erythrorhizon* (Tsuruga *et al.* 2006) and *Orthosiphon aristatus* (Sumaryono *et al.* 1991) cell suspension cultures, and *Salvia multiorrhiza* (Yan *et al.* 2006) and *Coleus forskohlii* (Li *et al.* 2005) hairy root cultures.

We examined the effects of YE and MJ on PAL activity and RA accumulation in hairy root clones of *Coleus blumei* (Fig. 4A,B). One day after treatment hairy root clones showed different responses to elicitors. The addition of YE reduced RA accumulation in five clones (#1, #2, #9, #10, #12), did not significantly affect RA accumulation in four clones (#3, #6, #15, #8) and enhanced RA accumulation in two clones (#4, #16). Clones #4 and #16 showed 44.0 and 20.2 % increase in RA accumulation, respectively. MJ enhanced RA accumulation in six clones (#1, #2, #3, #4, #8, #10), did not influence on RA accumulation in four clones (#6, #12, #15, #16) and diminished it in clone #9. MJ increased RA accumulation in clone #8 for 21 % so this

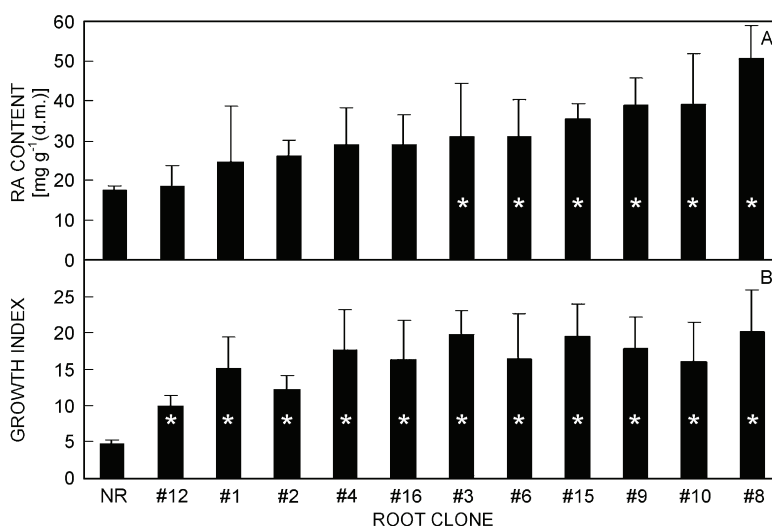


Fig. 3. Rosmarinic acid content (A) and growth index (B) of hairy root clones (#1, #2, #3, #4, #6, #8, #9, #10, #12, #15, #16) and non-transformed roots (NR) of *C. blumei*. Hairy roots were cultured in liquid hormone-free MS medium and NR in MS supplemented with 1 mg dm⁻³ NAA for 28 d. Data are means \pm SD of six replicates. * - statistically significant differences ($P \leq 0.05$) from non-transformed roots.

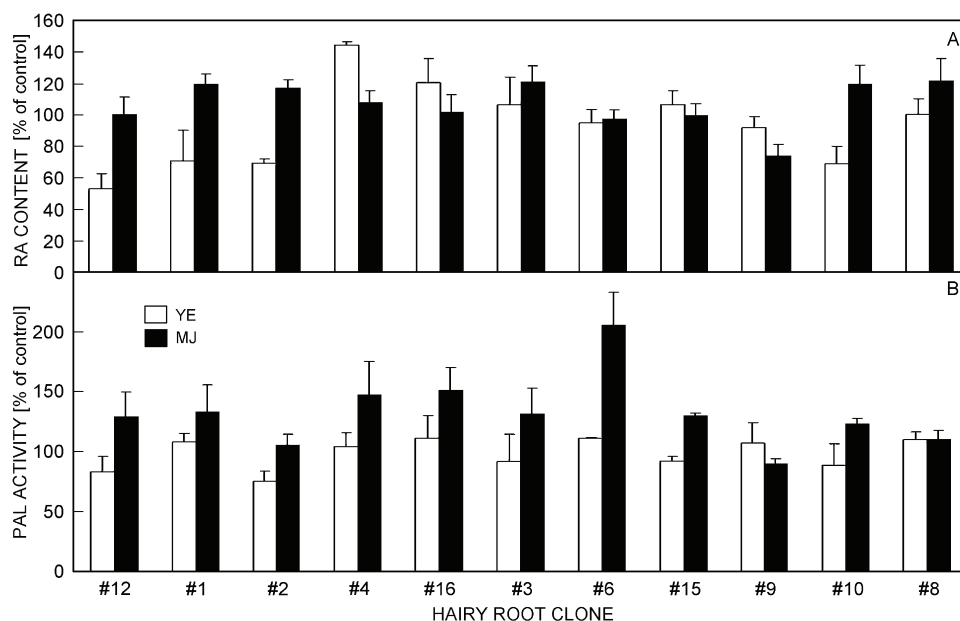


Fig. 4. Rosmarinic acid content (A) and PAL activity (B) in *Coleus blumei* hairy roots (#1, #2, #3, #4, #6, #8, #9, #10, #12, #15, #16) treated with 5 g dm⁻³ yeast extract (YE) and 100 µM methyl jasmonate (MJ). YE and MJ were added to 33-d-old hairy root cultures. Roots were harvested 24 h post inoculation. Results are expressed as percentages of increase of RA content or PAL activity with respect to the mean of control hairy roots. Data are mean ± SD of three replicates.

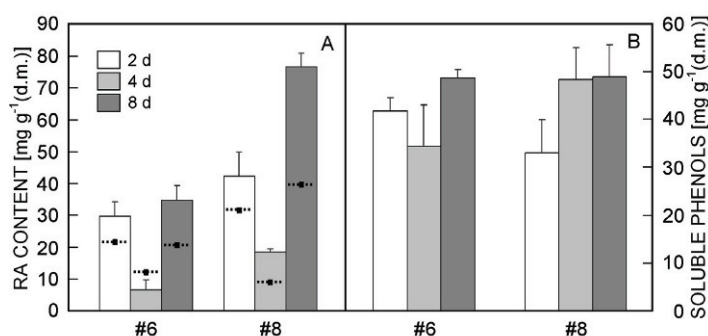


Fig. 5. Rosmarinic acid content (A) and soluble phenolic content (B) in *Coleus blumei* hairy root clone #6 and #8 treated with 100 µM methyl jasmonate (MJ). MJ was added to 33-d-old hairy root cultures. Roots were harvested 2, 4 and 8 d post treatment. Values for control are denoted with interrupted line with dot. Soluble phenols were estimated as gallic acid equivalents. Data are mean ± SD of three replicates.

clone on average accumulated 14.5 mg RA per flask (Fig. 4A). Increase in the PAL activity preceded the RA accumulation in *Lithospermum erythrorhizon* (Mizukami *et al.* 1992, 1993, Ogata *et al.* 2004) and *Coleus blumei* cell suspension cultures (Szabo *et al.* 1999). Mostly, maximum PAL activity was reached 24 h after elicitor treatment, and then it decreased. However, *Salvia miltiorrhiza* hairy roots showed reduction of PAL activity after elicitor treatment (Yan *et al.* 2006). PAL activity of *C. blumei* hairy roots was differentially affected with YE and MJ. YE did not significantly affect PAL activity or diminished it. MJ enhanced PAL activity in all clones except clone #9 (Fig. 4B). Significant increase in PAL activity implied on possible higher RA accumulation few days after MJ treatment. Clones #6 and #8 were analyzed for RA production 2, 4 and 8 d after MJ treatment

(Fig. 5A). Surprisingly, moderate increase of PAL activity 24 h after MJ treatment (Fig. 4B) led to progressively higher RA accumulation in clone #8. However, extremely increased PAL activity (Fig. 4B) of clone #6 stimulated RA accumulation after 2 d, decreased it after 4 d and increased again after 8 d when RA content was 34.6 mg g⁻¹(d.m.) that was 170 % higher than in control tissue. These high variations in RA production were even more obvious in clone #6 treated with double (200 mM) MJ concentrations (data not shown). It is also reported that RA biosynthesis was reduced after yeast elicitor treatment in Ti transformed *Salvia miltiorrhiza* cell suspension culture (Chen and Chen 2000) and after jasmonic acid treatment in hairy roots of *Ocimum basilicum* (Bais *et al.* 2002). Similar results are noticed after *Pseudomonas* treatment of *Thymus vulgaris* (Kwok

and Shetty 1996), *Origanum vulgare* (Eguchi *et al.* 1996) and *Rosmarinus officinalis* (Yang *et al.* 1997). Clones, tolerant to *Pseudomonas*, show reduction in RA accumulation, probably because of redirection of metabolism toward lignin synthesis (Yang *et al.* 1997).

We analyzed soluble phenolics in hairy roots after MJ treatment. The content of soluble phenolics decreased or increased like RA accumulation. In clone #6 phenolics first dropped down (after 4 d), and then increased (after 8 d). Clone #8 progressively increased phenolic content after MJ (Fig. 5B). In elicited *Echinacea purpurea* cell cultures different phenolics are accumulated in medium and in cultured cells (Li and Barz 2006). Whether phenolic biosynthesis in clone #6 was re-channeled, and/or clone #6 drained phenolics into the surrounding medium after MJ treatment left to be investigated. Mano (1989) underlined that there are variation in the

characteristics of hairy root lines and, moreover, that the characteristics of each line are very stable. If we select a superior clone that has desirable characteristics, we can maintain it with minimal alterations for a long period of time.

To our knowledge, this is the first report of hairy root establishment in *Coleus blumei*. The established hairy roots demonstrate high RA content and vigorous growth, significantly better than normal roots. In comparison to other hairy roots, *Coleus blumei* clones show the best RA production recorded till now. Different response of hairy roots to elicitation with YE and MJ was found. In some clones YE and MJ did not influence RA content or even diminished it. On the other hand, high RA content was found after elicitation in some hairy root clones and this make them suitable for biotechnological application.

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