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Genetic transformation of *Salvia austriaca* by *Agrobacterium rhizogenes* and diterpenoid isolation

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Hairy roots of *Salvia austriaca* Jacq. transformed with *Agrobacterium rhizogenes* strain A4 were obtained and transgenic status of the roots was confirmed by polymerase chain reaction (PCR) using *rol*B and *rol*C specific primers. The root cultures growing in half-strength Gamborg (1/2 B5) liquid medium supplemented with sucrose (30 g L^{-1}) under light conditions (photoperiod: 16 h light/8 h dark) were examined for their ability to produce diterpenoids. From *n*-hexane extract the abietane-type diterpenoids royleanone, 15-deoxyfuerstione and taxodione were isolated and identified. This is the first report on the genetic transformation of *S. austriaca*.

1. Introduction

Salvia is the largest genus in the family Lamiaceae with over 900 species in the world. Many sage species have been cultivated and used as medicinal plants for the treatment of common colds, acute and chronic bronchitis, various stomach disorders and problems associated with heart and circulatory system (Akin et al. 2010; Dweck 2000; Ulubelen 2003). Chemical constituents of sage species include essential oils, diterpenoids, triterpenoids, flavonoids and caffeic acid oligomers (Güllüce et al. 2006; Lu and Foo 2002). Flavonoids, triterpenoids and essential oils are mainly localized in aerial parts of these plants, while various types of diterpenoids are major constituents of their roots.

In our previous work, hairy roots of *Salvia sclarea* were investigated chemically and several abietane-type diterpenoids with different biological activities were isolated (Kuźma et al. 2006, 2007; Różalski et al. 2006). The root cultures transformed with *Agrobacterium rhizogenes* represent an attractive experimental system for secondary metabolite production since they exhibit genetic and biochemical stability and accumulate considerable amounts of valuable metabolites (Giri and Laxmi 2000), in some cases even higher than in roots of intact plants (Allan et al. 2002; Tiwari et al. 2008).

In continuation of our studies on diterpenoids from *Salvia* sp., we have now investigated hairy root culture of *Salvia austriaca* Jacq. It is a herbaceous plant native to Russia and Eastern Europe (Clebsch and Barner 2003). Roots of this species have been reported to accumulate abietane-type diterpenoids, derivatives of royleanone (Nagy et al. 1999). In the present study, we describe for the first time genetic transformation of *S. austriaca* by *Agrobacterium rhizogenes* (strain A4), biomass production by fast growing hairy root clones in half-strength B5 (½ B5) Gamborg (Gamborg et al. 1968) liquid medium and characterisation of diterpenoids accumulated in the culture.

2. Investigations, results and discussion

Hairy roots of *S. austriaca* were induced by infection of leaves (leaf laminas and petioles) and shoot tips from *in vitro* grown shoots with *Agrobacterium rhizogenes* strain A4. After 2 weeks of culture on hormone-free LS agar medium containing 30 g L^{-1} sucrose, roots started to emerge from infected sites of explants and the process was continued up to 4 weeks. At that time, approximately 50% of each kind of explants responded. Five weeks after infection the roots were excised and transferred individually into ½ B5 liquid medium containing ampicillin 400 mg L^{-1} without addition of phytohormone. The concentration of ampicillin was gradually lowered and finally omitted. After 6 passages (14 days, each) axenic root cultures were obtained and seven clones (C1-C7), which were able to grow in ½ B5 liquid medium, were chosen for further investigations. All the clones exhibited the visible factures typical of hairy roots i.e. increased lateral branching and absence of geotropism (Fig. 1). Transformation was confirmed by PCR using *rol*B and *rol*C specific primers. The results showed that two 423 bp and 626 bp fragments corresponding to *rol*B and *rol*C genes, respectively, were amplified only from hairy root cultures, but not from untransformed tissue (Fig. 2). PCR with *vir*G primers revealed the absence of - *A. rhizogenes* contamination (Fig. 2).

Our previous study on *S. sclarea* hairy roots showed that light had a positive effect on diterpenoid levels (Kuźma et al. 2006). Therefore, axenic hairy root clones of *S. austriaca* starting from passage 7 were cultured under the light conditions (8 h dark/16 h light; 40 μmol m^{-2} s^{-1}). We found that under these conditions after 30 days of culture in ½ B5 liquid medium, the fresh weight of the transformed root clones increased 5–11-fold i.e., from 0.6 − 0.7 g to 3.2 − 6.3 g per flask (80 mL medium) (Fig. 3a). At the time the dry weight increased 7–9 times (0.38−0.57 g per flask) over initial inoculum (0.056 − 0.06 g per flask) (Fig. 3b). Clones

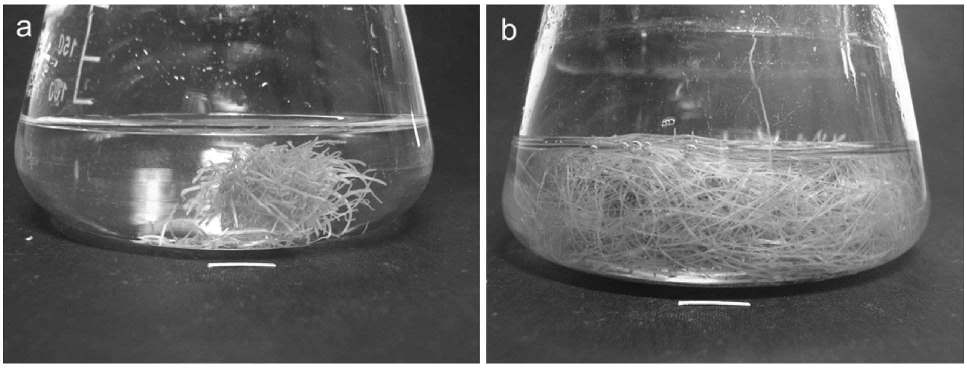


Fig. 1: Hairy roots of *Salvia austriaca* obtained after transformation with *Agrobacterium rhizogenes* (strain A4) cultured in ½ B5 liquid medium: (a) begining of the culture, (b) culture day 30. Bar 1 cm

C1 and C7 showed the highest biomass accumulation (over 6 g FW per flask and 0.57 g DW per flask). Clone C5 showed the smallest growth; its biomass was 3.2 g FW and 0.38 g DW per flask (Fig. 3a and 3b). As shown in Fig. 3c, the root clones differed also in their dry matter content (% DW). Clone C3, C5 and C6 showed high % DW (11 − 12%). Clone C4 showed the lowest percentage of DW (8%) (Fig. 3c), which indicated the highest water content. Differences in growth between different hairy root lines have been observed in other plant species (Baíza et al. 1998; Tiwari et al. 2008).

Our study showed that repeated preparative thin layer chromatography (TLC) (normal phase and reversed phase) was an effective technique for isolation and purification of diterpenoids. Three known abietane-type diterpenoids were isolated from *n*-hexane extract of hairy roots of *S. austriaca* and were identified as royleanone (**1**), 15-deoxyfuerstione (**2**) and taxodione (**3**) (Fig. 4). These compounds have been reported as root constituents of several *Salvia* species, such as *S. hypargeia* (Ulubelen et al. 1999), *S. mountbretii* (taxodione) (Topcu and Ulubelen 1996), *S. moorcroftiana* (taxodione and 15-deoxyfuerstione) (Simöes et al. 1986), *S. officinalis* (royleanone) (Slameňová et al. 2004) and *S. barrelieri* (royleanone and taxodione) (Kolak et al. 2009). Interest in these compounds has been stimulated by the fact that they exhibit various biological properties. Both taxodione and royleanone have antibacterial (Gaspar-Marques et al. 2006; Moujir and Gutiérrez-Navarro 1996; Yang et al. 2001) and cytotoxic activities (Moujir and Gutiérrez-Navarro 1996; Kupchan et al. 1969; Slameňová et al. 2004; Ulubelen et al. 1999). Reports on antioxidant properties of taxodione (Kolak et al. 2009) and 15-deoxyfuerstione (Hannedouche et al. 2002) have also been available. To date, royleanone (**1**), 15-deoxyfuerstione (**2**) and taxodione (**3**) have not been found in *Salvia austriaca*. From roots of the plant only royleanone derivatives, i.e. 7-α-acetoxyroyleanone and 7-α-hydroxyroyleanone have been isolated (Nagy et al. 1999). This data and our results suggest that the isolated constituents of hairy roots differ from those reported from roots of the intact plant. The occurrence of new compounds in transformed root cultures, which are absent from untransformed tissues of intact plants, has already been reported (Asada and Yoshikawa 1998; Fukui et al. 1998; Sidwa-Gorycka et al. 2009). For example, two coumarins that have never been found in *Ruta graveolens* were identified in hairy roots of this species (Sidwa-Gorycka et al. 2009). However, our preliminary analysis using ultra performance liquid chromatography (UPLC) of *n*-hexane root extract of field-grown *S. austriaca* suggests the presence of taxodione, which was based on the comparison of UV spectra (DAD detector) and retention time values with those for a standard sample.

3. Experimental

3.1. Establishment of hairy roots

Shoot tips and leaves of *in vitro* grown shoots of *S. austriaca* were used for inoculation with *Agrobacterium rhizogenes* strain A4. The shoots were derived from *S. austriaca* seeds (Jena Botanical Garden, Friedrich Schiller University, Germany) and grew on LS (Linshmaier and Skoog 1965) agar (0.7%) medium supplemented with 0.1 mg L^{-1} indole 3-acetic acid (IAA) and 0.9 mg L^{-1} 6-benzylaminopurine (BAP). *A. rhizogenes* agropine strain A4 (obtained from University of Łódź, Poland) was grown on YMB agar medium (Vervliet et al. 1975) for 48 h at 26° C. The explants were inoculated with the culture of *A. rhizogenes* by direct wounding using sterile needle immersed in the bacterial culture. The shoot tips (*ca.* 2 cm in length with one pair of leaves) were infected below the apical bud. The leaves were wounded in leaf lamina along of the leaf-mid-vein or petiole end. The infected explants were incubated in the dark on LS agar (0.7%) medium supplemented with 30 g L^{-1} sucrose. Under these conditions roots appeared at the inoculated sites after 14–28 days. After 5 weeks the roots achieved 1–1.5 cm in length. They were excised from explants and transferred individually (each root was treated as a separate clone) into half-strength Gamborg (½ B5) liquid medium containing ampicillin (400 mg L^{-1}). The medium was supplemented with 30 g L^{-1} sucrose. After two subcultures (14 days each) the content of ampicillin was decreased to 300 mg L^{-1} and then (after two successive subcultures) to 100 mg L^{-1}. The cultures were maintained at 26 °C in the dark on a rotary shaker (100 rpm). After six subcultures axenic

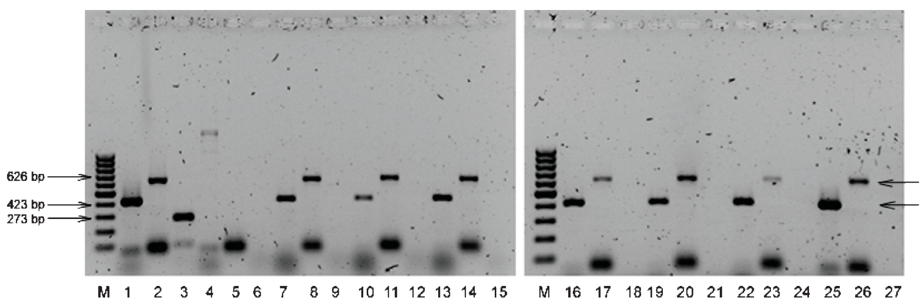


Fig. 2: PCR analysis of genomic DNA isolated from *S. austriaca* hairy root clones transformed by *A. rhizogenes* strain A4. Lanes M molecular weight marker: GeneRuler™ 100 bp DNA ladder; lanes 1–3 positive control of *A. rhizogenes* strain A4 showing *rol*B (423 bp), *rol*C (626 bp) and *vir*G (273 bp) genes; lanes 4–6 DNA from untransformed tissue as a negative control; lanes 7–9 DNA isolated from clone C1; lanes 10–12 from clone C2; lanes 13–15 from clone C3, lanes 16–18 from clone C4; lanes 19–21 from clone C5; lanes 22–24 from clone C6; lanes 25–27 DNA isolated from clone C7

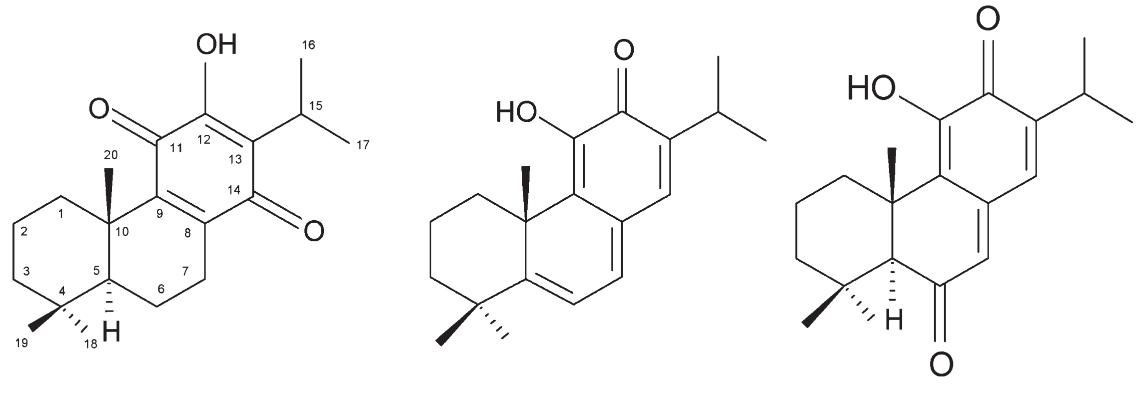
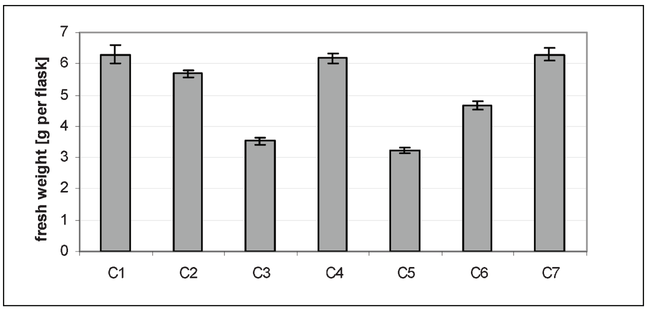
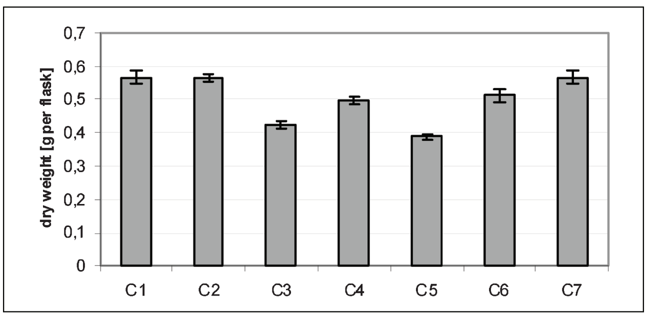


Fig. 4: Chemical structures of royleanone (**1**), 15-deoxyfuerstione (**2**), and taxodione (**3**)

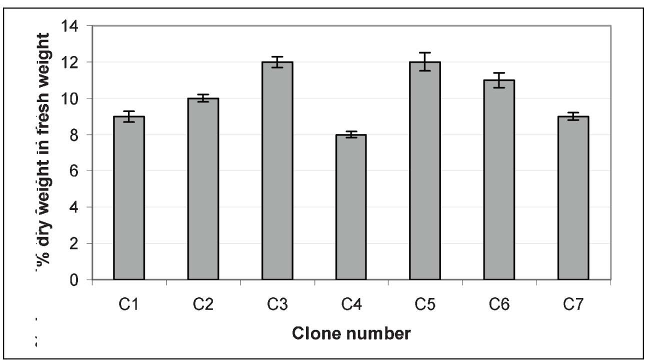
hairy roots were obtained and then cultivated in 300 mL Erlenmeyer flasks containing 80 mL ½ B5 liquid medium at 26 °C on a rotary shaker, at 16/8 h (light/dark) photoperiod with light provided by cool-white fluorescent lamps at an intensity of 40 μmol m^{-2} s^{-1}. Subcultures were carried out every 30 days. Seven fast growing clones (called C1 - C7) were chosen for further studies on biomass production and diterpenoid isolation. The clones were derived from leaf laminas (clones C1, C3), petioles (clones C5, C7) and shoot tip explants (clones C2, C4, C6). To measure growth of the cultures (fresh and dry weights as well as the percentage of dry weight), hairy roots from 3 flasks of each root clone were harvested after 30 days of culture. The experiments were repeated in five subsequent passages (12–16). The fresh weight of inoculum was 0.58–0.66 g (0.056–0.064 g dry weight) per flask.



(a)



(b)



(c)

Fig. 3: Comparative growth of *S. austriaca* transformed root clones after 30 days in ½ B5 liquid medium in the light (photoperiod: 16 h of light: 40 μmol m^{-2} s^{-1}). (a) Fresh weight [g] of harvested biomass per flask (80 mL of medium); (b) Dry weight [g] of harvested biomass per flask (80 mL of medium); (c) Amount (%) of dry matter from fresh root biomass. The values are the mean ± standard error of five experiments (12–16 passages)

3.2. PCR analysis

Due to the presence of secondary metabolites in plant tissue DNA from seven hairy root clones (C1 - C7) and untransformed roots (from 5-week-old plantlets) of *S. austriaca* was isolated using a method with CTAB with some modifications (Bekesiova et al. 1999). In order to confirm the transformation of *S. austriaca* on the molecular level, the presence of the T-DNA fragment (*rol*B and *rol*C genes) in hairy roots was determined by the use of polymerase chain reaction (PCR). As a positive control plasmid DNA isolated from *A. rhizogenes* cells (strain A4) was used. In this case DNA from bacteria was extracted from 24 h cultures (OD_{600} 4.0) using alkaline lysis (Maniatis et al. 1982). Oligonucleotide primers and procedure described by Królicka et al. (2001) were used for PCR detection of the sequence homologous to bacterial *rol*B and *rol*C genes (present in T-DNA) in *S. austriaca* genome. In order to confirm that hairy roots are free of *A. rhizogenes* cells the PCR with primers homologous to the sequence of *vir*G gene (gene beyond the transferred T-DNA) was performed according the procedure described by Sidwa-Gorycka et al. (2009).

3.3. Extraction and isolation of diterpenoids

TLC chromatograms showed no chemical differences among hairy root clones of *S. austriaca,* therefore roots of all lines (C1–C7) were combined and used for isolation of diterpenoids. Lyophylised (Freeze Dryer 1–2/LD Christ) and powdered plant material (130 g) was extracted with *n*-hexane (3 x 100 mL) for 15 min at room temperature using ultrasonic bath. After filtration, the extracts were combined and evaporated to dryness *in vacuo* providing a residue (6 g), which was dissolved in acetone (50 mL) and purified on a Sephadex LH-20 column (20 g, Pharmacia). Further separation was conducted by preparative TLC on silica gel (Merck, Art. 1.05553, *n*-hexane:ethyl acetate, 9:1 v/v, UV detection at 366 nm). Six fractions were obtained and compounds **1**, **2** and **3** (Fig. 4) present in fractions I, II and III, respectively, were isolated by repetitive preparative TLC. Fractions I (69.4 mg) and II (31 mg) were separated on silica gel plates in dichloromethane and then on RP-18 (Merck, Art. 13724) plates in methanol, finally yielding **1** (2.8 mg) and **2** (1.2 mg), respectively. Fraction III (87.6 mg) was subjected to repeated preparative TLC on silica gel (*n*-hexane:ethyl acetate, 9:1 v/v) to afford **3** (42 mg). The isolated compounds were identified by comparison of their spectroscopic data with those reported for royleanone (**1**) (Carreno et al. 2000; Rodriguez 2003), 15-deoxyfuerstione (**2**) (Tada et al. 2010) and taxodione (**3**) (Rodriguez 2003; Tada et al. 2010).

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