Diterpenoid Production in Hairy Root Culture of *Salvia sclarea* L.

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Growth and diterpenoid accumulation (salvipisone, ferruginol, aethiopinone and 1-oxoaethiopinone) during the growth cycle of a *Salvia sclarea* hairy root culture are described. The roots transformed by *Agrobacterium rhizogenes* (LBA 9402) were cultured in half-strength B5 liquid medium supplemented with 30 g L^{-1} sucrose under light (16 h/8 h light/dark). A culture period of 30 days was optimal for both biomass and diterpenoid production. The total content of four diterpenoids in the hairy roots [(27.3 ± 0.6) mg g^{-1} dry weight] was higher than that of roots of field-grown *S. sclarea* plants [(3.15 ± 0.15) mg g^{-1} dry weight]. In transformed roots, aethiopinone was the main diterpenoid, whereas the principal diterpenoid of natural roots was salvipisone.

Key words: Diterpenoids, Hairy Roots, *Salvia sclarea*

Introduction

Recently, *Agrobacterium rhizogenes*-mediated hairy roots of *Salvia sclarea* were established (Kuźma *et al.*, 2006). The genetically modified root cultures have several advantages. They usually demonstrate genetic and biochemical stability, fast growth in hormone-free media and the ability to synthesize root-derived secondary metabolites (Giri and Narasu, 2000). From a *S. sclarea* hairy root culture, four diterpenoids, namely salvipisone, ferruginol, aethiopinone and 1-oxoaethiopinone, were isolated (Kuźma *et al.*, 2006) (Fig. 1). In this study, we present data on the growth and diterpenoid production of *S. sclarea* hairy roots during a 50-day culture period in liquid $^1/_2$ B5 medium supplemented with 30 g L^{-1} sucrose. The diterpenoid content in the hairy root culture is compared with that in roots of *S. sclarea* intact plants.

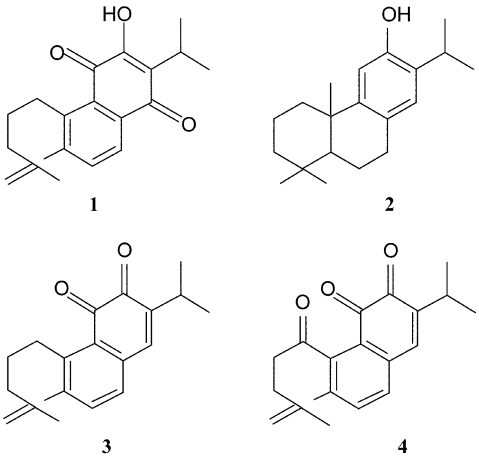

Fig. 1. Chemical structure of salvipisone (**1**), ferruginol (**2**), aethiopinone (**3**) and 1-oxoaethiopinone (**4**).

Material and Methods

Hairy root culture

The transformed root culture of *S. sclarea* was obtained through infection of sterile shoots with *Agrobacterium rhizogenes* (LBA 9402) (see Kuźma *et al.*, 2006). For the time course, the hairy roots were cultured for 50 d, and three flasks were collected every five d during each of three successive subcultures. Fresh and dry weights, as well as the diterpenoid content in the roots were examined. All results are means of three independent replications from three successive passages ± standard error (SE). Each replication was done in three Erlenmeyer flasks.

Diterpenoid determination

Diterpenoid extraction from the plant material was performed after lyophilization according to the procedure described before (Kuźma *et al.*, 2006). Quantitative analysis of diterpenoids was performed by high performance liquid chromatography (HPLC). For HPLC analysis, a Varian 9050 chromatograph with a Varian 9012 pump and a UV detector and a Nucleosil C18 column (250 × 4.6 mm, 5 μm Supelco) were used with a solvent system of water acidified with 85% H_3PO_4 (pH 2.6) (A) and acetonitrile/methanol (2:1 v/v) (B). The gradient elution was as follows: 0–12 min 75–95% B; 12–15 min 95% B; 15–16 min

95–70% B; 16–20 min 70% B. The flow rate was 1.5 mL min^{-1}. Detection was at 270 nm. The identification of diterpenoids was done by comparison of their retention times and UV spectra with those of standard compounds, which had been earlier isolated from the hairy roots of clary sage, chromatographically purified and identified by spectral methods (^{1}H NMR, ^{13}C NMR). Further details had been described previously (Kuźma *et al.*, 2006). The total diterpenoid content and contents of individual diterpenoids of the analyzed samples were expressed as mg per g of root dry weight.

Results

Root growth and diterpenoid accumulation

Our earlier studies had indicated that light significantly promotes the growth and diterpenoid biosynthesis in hairy roots of *Salvia sclarea*. The diterpenoid level (25.4 mg g^{-1} dry weight) in the light-incubated culture was 2.5-times higher than that in the dark-incubated culture (10.9 mg g^{-1} dry weight) (Kuźma *et al.*, 2006). Based on these results, the time course of changes in growth and diterpenoid accumulation (salvipisone, ferruginol, aethiopinone, 1-oxoaethiopinone and the sum of these four compounds) of *S. sclarea* hairy roots grown in $^1/_2$ B5 liquid medium (Gamborg *et al.*, 1968) supplemented with 30 g L^{-1} sucrose under light conditions (16 h/8 h light/dark photoperiod; light intensity: 40 μmol m^{-2} s^{-1}) during a 50-days culture period was studied. The culture reached a maximum biomass on the 30th day (Fig. 2a). At that time the fresh and dry weights increased about 14-times [(76.25 ± 3) g and (7.25 ± 0.4) g L^{-1} of culture, respectively] (Fig. 2a). After 30 days, fresh weight as well as dry weight decreased and roots turned brown. It may be related to diminishing of nutritive compounds in the medium, ageing and/or cell death. Simultaneously, with biomass measurement, the hairy roots of *S. sclarea* were assessed for diterpenoid accumulation over the same period of 50 days. The total diterpenoid content (calculated as the sum of salvipisone, ferruginol, aethiopinone and 1-oxoaethiopinone) in hairy roots of *S. sclarea* decreased during the earlier exponential phase, when the roots grew actively and started to increase from 12 mg g^{-1} on day 15 to reach a maximum of 27.3 mg g^{-1} on day 30 (Fig. 2b). At that time total diterpenoid production (per liter of culture) was 198 mg. Diterpenoids on the 30th day of culture were retained within the root cells and were not detected in the culture medium. However, after 30 days of culture, in accordance with our preliminary results obtained by TLC, diterpenoid secretion into the culture medium was observed. It can explain the drastically reduced diterpenoid content in roots (by about 30%) during the period between day 30 and day 35 (Fig. 2b). Generally, the content of individual diterpenoids followed the same trend as that of the total diterpenoid content. The most evident changes over the growth cycle were observed for the content of aethiopinone which was the major constituent of the diterpenoid mixture synthesized by *S. sclarea* hairy roots. Its level increased 2-fold between the 25th and 30th day of culture, when the diterpenoid content reached the value of 12 mg g^{-1} dry weight (87.3 mg L^{-1}). Also salvipisone and 1-oxoaethiopinone contents increased rapidly between the 25th and 30th day of culture, although their levels remained much lower than that of aethiopinone. Only the amount of ferruginol was rather stable during the 50-days culture cycle except for a slight decrease on day 35 (Fig. 2b). The above results indicate that in a hairy root culture the maximum of the biomass production coincides with the maximum of the diterpenoid accumulation. This behaviour has been detected for many other metabolites produced in *in vitro* cultures, for expample taxanes in a *Taxus baccata* cell culture (Navia-Osorio *et al.*, 2002) or tanshinones in hairy roots of *Salvia miltiorrhiza* (Yan *et al.*, 2005). In the case of *S. sclarea* transformed roots, both parameters, biomass and diterpenoid content, achieved the highest values on 30 day of the culture. Our study also showed that the culture of hairy roots of *S. sclarea* is stable and only a small variation in growth and diterpenoid contents during 6 years of continuous subculturing of 30-day intervals was observed. For example, the total diterpenoid content in hairy roots from the 40th subculture (for over three years) was (25.4 ± 0.4) mg g^{-1} dry weight (Kuźma *et al.*, 2006), whereas after the 70th passage (almost six years in culture) the value was (22.4 ± 0.8) mg g^{-1} dry weight. The stability can be a great advantage in the use of *S. sclarea* hairy roots for the diterpenoid production.

Comparison of diterpenoid accumulation in transformed root culture and plant roots

The diterpenoid content in roots collected from 2-year-old *Salvia sclarea* plants grown in the field

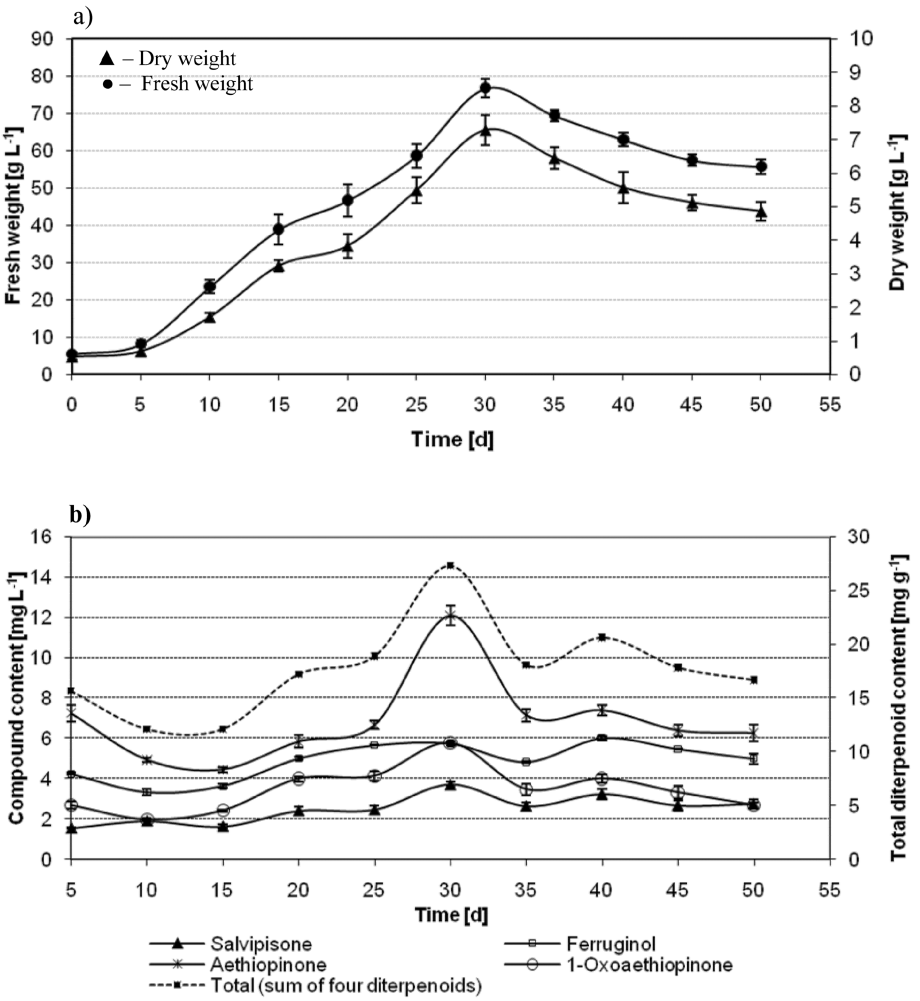

Fig. 2. Time course of (a) growth (fresh and dry weight) and (b) diterpenoid accumulation in transformed roots of *Salvia sclarea* cultured in $^1/_2$ B5 liquid medium supplemented with 30 g L^{-1} sucrose over a period of 50 days in the light (16 h/8 h light/dark photoperiod; light intensity: 40 μmol m^{-2} s^{-1}). Each data represent means ± SE of three replications.

[(3.15 ± 0.15) mg g^{-1} dry weight] was almost 9-times lower than that found in genetically modified roots. In both types of roots the same diterpenoids *i.e.* salvipisone, ferruginol, aethiopinone and 1-oxoaethiopinone were identified by HPLC. However, relative proportions of diterpenoids in roots of field-grown plants were different from those in hairy roots. The most important difference was high accumulation of aethiopinone in hairy roots: up to 48% of the total diterpenoid content, whereas in the normal roots aethiopinone represented only 8% of the sum of the detected diterpenoids. In the latter, the main compound was salvipisone (60% of the total diterpenoids). A modification of secondary product biosynthesis following transformation by *A. rhizogenes* is also known for some other plant species. For example, Babaoglu *et al.* (2004) have demonstrated that main secondary metabolites in hairy roots of *Lupinus mutabilis* are isoflavones: genistein, 2′-hydroxygenistein and wighteone, whereas genistin was more abundant in roots of intact plants. Fu *et al.* (2006) suggested that the genetic modification of roots can lead to a differential regulation mechanism of gene expression for even closely related compounds.

In conclusion, it is evident that a stable and high-productivity root culture can be obtained by genetic transformation of *S. sclarea* with *A. rhizogenes*. The aethiopinone content in the genetically modified roots grown in liquid $^1/_2$ B5 medium supplemented with sucrose was even 50-times the amount detected in roots of 2-year-old plants collected from the field. Also, the amount of another bioactive diterpenoid, salvipisone, from the transformed root extract was almost 3-times higher than that from the extract of roots of clary sage intact plants. Similar higher levels of secondary metabolites produced by transformed roots in comparison with either a non-transformed root culture or roots of original plants have been reported for many other plant species, *e.g. Linum flavum* (Lin *et al.*, 2003), *Tylophora indica* (Chaudhuri *et al.*, 2005) and *Physalis minima* (Putalun *et al.*, 2004). According to Chaudhuri *et al.* (2006) changes in the secondary metabolite accumulation brought about by RiT-DNA could result in changes in the polyamine metabolism and/or changes in the sensitivity of plant cells to auxins. The high and stable production of aethiopinone and salvipisone makes hairy roots of *Salvia sclarea* a potential source of diterpenoids and opens the way for further investigations focused on the production of useful metabolites in bioreactors.

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