Industrial Crops & Products 117 (2018) 235–244

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Hairy root cultures of *Salvia viridis* L. for production of polyphenolic compounds

Izabela Grzegorczyk-Karolak[a,*], Łukasz Kuźma[a], Ewa Skała[a], Anna K. Kiss[b]

[a] Department of Biology and Pharmaceutical Botany, Medical University of Lodz, Muszynskiego 1, 90-151 Lodz, Poland
[b] Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland

ARTICLE INFO

Keywords:
Rol genes
Rosmarinic acid
S. viridis
Salvianolic acids
Transformed roots

ABSTRACT

Salvia viridis is a containing bioactive phenols plant, which has been used in Turkish medicine. The aim of the study was to establish hairy root culture of the species in order to increase the production of the compounds with pharmacological activity. Transformed roots are promising biotechnological systems that produce great amounts of secondary metabolites; this is attributed to their fast growth and their genetic and biosynthetic stability. Stable hairy root lines of *S. viridis* were established from shoot explants using *Agrobacterium rhizogenes* strain A4. The highest frequency of transformation (45%) was achieved when the shoot was inoculated at the node or internode by bacteria cultivated on Yeast/Mannitol/Broth (YMB) medium supplemented with acetosyringone. The transformation of the root clones was confirmed by polymerase chain reaction using *aux*1, *aux*2, *rol*B and *rol*C primers. Of the five obtained transformed root clones, the highest biomass was achieved for clone K3 (13.63 ± 0.5 g/L after 5 weeks) grown in Woody Plant (WP) medium in darkness. The UPLC-PDA-ESI–MS analysis of hydromethanolic extracts of transformed roots of *S. viridis* revealed the presence of 10 compounds identified as caffeic acid derivatives. Quantitative analysis showed that rosmarinic acid (RA) was the predominant compound in all clones. The highest RA (35.8 mg/g dry weight) and total polyphenol (41.24 mg/g dry weight) content were evaluated in clone K3 cultured in WP medium. These values were 8-fold higher than those of the non-transformed roots, indicating that hairy root cultivation could be a promising technique for the production of valuable compounds from *S. viridis*.

1. Introduction

Salvia viridis L. (syn. *S. horminum* L.), of the Lamiaceae family, is an annual herb native to the Mediterranean area. It has been used in traditional medicine as a mouthwash for treating inflammation and sore gums (Dweck, 2000). Phytochemical reports have revealed the presence of essential oils (Kokkalou et al., 1982), triterpenoids (Ulubelen and Brieskorn, 1975; Ulubelen et al., 1977), phenolic acids and flavonoids (Rungsimakan and Rowan, 2014) in the aerial and underground parts of *S. viridis*. In addition, diterpenoids displaying antibacterial and antihyperitive activity, such as ferruginol, 1-oxoferruginol, aethiopinone, salviviridinol, viridinol, viridone, sugiol, and microstegiol, have been isolated from the roots (Ulubelen et al., 2000).

The biotechnological production of high-quality compounds represents a promising alternative to classical cultivation; *in vitro* plant culture is commonly used to enhance the production of bioactive metabolites. One such method is hairy root culture obtained by *Agrobacterium rhizogenes* transformation. The roots of higher plants transformed with *A. rhizogenes* grow fast in media without growth regulators and produce great amounts of bioactive compounds without being affected by seasonal variation (Mehrotra et al., 2008; Shinde et al., 2010; Weremczuk-Jeżyna et al., 2013); in contrast, *in vitro* cultures of conventional roots are slow growing and require the addition of growth regulators to the medium. Another advantage of using transformed roots is that they can be successfully cultured on the target scale in bioreactors.

Despite the importance of *Salvia* plants as a source of high-value metabolites, only a few studies describe the establishment of hairy root cultures from this genus. So far, among *Salvia* species, hairy roots have been obtained for *S. miltiorrhiza* Bunge (Zhi and Alfermann, 1993), *S. officinalis* L. (Grzegorczyk et al., 2006), *S. sclarea* L. (Kuźma et al., 2006), *S. castanea* Diels. (Li et al., 2016) and *S. austriaca* Jacq (Kuźma et al., 2012). The cultures have been studied for the *in vitro* production of diterpenes, triterpenoids and polyphenols (Zhi and Alfermann, 1993; Grzegorczyk et al., 2006; Kuźma et al., 2012; Li et al., 2016). Such polyphenol compounds include caffeic acid derivatives such as rosmarinic acid (RA) or salvianolic acids, and these have been found to have antioxidant and anti-inflammatory properties (Won et al., 2003;

* Corresponding author.

https://doi.org/10.1016/j.indcrop.2018.03.014
Received 17 October 2017; Received in revised form 2 March 2018; Accepted 5 March 2018

Petersen and Simmonds, 2003). The compounds have also demonstrated antibacterial and antiviral activity (Tewtrakul et al., 2003). The biological properties of rosmarinic acid have been widely examined for recent years. The compound proved to be promising drug for cancer prevention and treatment of various types of human cancers (Moon et al., 2010). Rosmarinic acid exhibited potent anti-inflammatory effects in in vivo models of acute lung injury induced by lipopolysaccharide (Chu et al., 2012). Sánchez-Campillo et al. (2009) demonstrated the capacity of RA to inhibit cutaneous alternations caused by UV exposure, and Lee et al. (2008) found that RA could be used as a therapeutically agent for atopic dermatitis. RA improved also cognitive performance and showed neuroprotective effect. According Hamaguchi et al. (2009) the compound inhibited amyloid-β aggregation and prevents the development of Alzheimer's disease. Additionally, RA is considered particularly valuable objects of research as its application does not result in adverse side −effects.

The present study describes the first successful establishment of hairy roots of *S. viridis* and evaluates their potential to produce bioactive compounds. Transformed root cultures were obtained, and then root clones were selected on the basis of their growth and bioactive metabolite production during five-week culture in WP medium under various photoperiod conditions. Rosmarinic acid and its derivative show similar biological activity; however, compounds with higher numbers of caffeoyl groups were found to possess greater activity (Ito et al., 1998). Therefore, rosmarinic acid, the main extract compound, and the total amount of polyphenols in hairy roots were chosen to select the most productive root clone.

2. Materials and methods

2.1. Induction and establishment of hairy roots of S. viridis

Five-week-old aseptic shoots of *S. viridis* cultured on MS (Murashige and Skoog, 1962) agar (0.7%) solidified medium supplemented with 0.5 mg/L BAP (6 benzylaminopurine) and 0.1 mg/L IAA (indole-3-acetic acid) were used as a source of explants for hairy root induction. Two types of explants were used: leaves and shoots. The explants were wounded with a needle dipped into *Agrobacterium rhizogenes* strain A4 (plasmid pRiA4) culture. Before transformation, the bacterial culture was incubated on fresh YMB (Hooykaas et al., 1977) solid medium with or without acetosyringone (AcS) (200 μM) for 48 h at 26 °C in the dark. Control explants were wounded with sterile needle without bacteria. Infected and control explants were placed on hormone-free MS agar (0.7%) medium with or without acetosyringone (200 μM) and incubated in the dark for five weeks. The experiment was repeated three times; 8–10 explants were used for each treatment: explant and site of infection/medium with or without AcS for bacteria and explant after infection cultivation. Five weeks after infection, the transformation frequency (the percentage of explants forming roots after infection with *A. rhizogenes*), the number of roots per responding explants and the root length were determined (Table 1).

The obtained roots, longer than 1 cm, were excised from explants and transferred individually into 100 mL Erlenmeyer flasks containing 20 mL Woody Plant (WP) (Lloyd and McCown, 1980) liquid medium without growth regulators and supplemented with 500 mg/L ampicillin to prevent bacterial spread. The cultures were maintained in the dark, on a rotary shaker at 80 rpm. After eight subcultures, when the antibiotic was eliminated from the medium, five fast growth root lines were obtained (K1–K5).

2.2. Confirmation of transformation

The genomic DNA was extracted from the fresh plant materials (200 mg) of five potential transformed root clones (K1-K5) of *S. viridis* using Nucleospin Plant II Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The roots of field-grown *S. viridis* were used as negative controls; the plant was obtained from seeds and identified in the Department of Biology and Pharmaceutical Botany, Medical University of Lodz, where a voucher specimen was deposited. The fresh plant materials were powdered in liquid nitrogen. Polymerase chain reaction (PCR) using *rol*B, *rol*C, *aux*1 and *aux*2 specific primers were used to confirm successful transformation. PCR amplification was performed according to Skała et al. (2015). The reaction products (a fragment size of 386 bp for *rol*B, 500 bp for *aux*1, 582 bp for *rol*C and 774 bp for *aux*2) were run on 1.2% agarose gel stained with ethidium bromide, along with a standard DNA marker. The plasmid DNA isolated from 24-h cultures of *A. rhizogenes*, strain A4 (OD_{600} = 0.4) was used as a positive control; it was isolated using the Plasmid Mini AX Kit (A&A Biotechnology, Poland) according to the protocol given by the manufacturer.

2.3. Hairy root growth rate

To determine growth, five root clones of about 0.55–0.7 g total fresh weight (cir. 0.045–0.06 g of dry wt) were cultured in 300 mL Erlenmeyer flasks containing 80 mL WP medium. The cultures were cultivated on a rotary shaker (80 rpm) in a growth chamber at 26 °C in the dark or under light from cool white fluorescent lamps (16-h light/8-h dark photoperiod; PPFD of 40 $\mu M\, m^{-2}\, s^{-1}$). After five weeks of culture, root biomass accumulation, calculated as dry weight (DW) per liter, was evaluated. The experiment was repeated three times (15–17 passages) for each clone and culture condition (dark/photoperiod).

2.4. Phytochemical analysis of phenolic compounds

2.4.1. Extraction procedure

Lyophilized and powdered samples of hairy roots (K1–K5) and untransformed roots of *S. viridis* (100 mg) were extracted with 30 mL of

Table 1
Hairy root induction frequency from *S. viridis* explants within five weeks following infection with *A. rhizogenes* strain A4.

Explant type	Medium		% of explant producing roots	Mean number of roots per explant
	Per bacteria	Per explant		
Shoot infected in node	YMB	MS	26.7	3.6
Shoot infected in internode	YMB	MS	25	3.2
Midrib of the leaf	YMB	MS	15.4	1.5
Shoot infected in node	YMB	MSAcS	0	0.0
Shoot infected in internode	YMB	MSAcS	3.3	1.0
Midrib of the leaf	YMB	MSAcS	0	0.0
Shoot infected in node	YMBAcS	MS	45	4.4
Shoot infected in internode	YMBAcS	MS	45	5.2
Midrib of the leaf	YMBAcS	MS	3.1	1.0

The bacteria were cultured on Yeast/Mannitol/Broth (YMB) medium without acetosyringone or containing acetosyringone – YMBAcS.
The explant were cultured on Murashige and Skoog (MS) medium without acetosyringone or containing acetosyringone – MSAcS.