ANTIOXIDANT COMPOUNDS IN *SALVIA OFFICINALIS* L. SHOOT AND HAIRY ROOT CULTURES IN THE NUTRIENT SPRINKLE BIOREACTOR

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ABSTRACT

The study focused on the production of compounds with antioxidant activity in hairy root and shoot cultures of *Salvia officinalis* grown in laboratory-scale sprinkle nutrient bioreactors. HPLC analysis showed that production of rosmarinic acid in transformed roots (34.65±1.07 mg l^{-1}) was higher that in shoot culture (26.24±0.48 mg l^{-1}). In the latter diterpenoids: carnosic acid (1.74±0.02 mg l^{-1}) and carnosol (1.34±0.01 mg l^{-1}) were also found. Biomass accumulation after a growth period in the bioreactor was also studied. An 18-fold increase in hairy root biomass was recorded after 40 days of culture. In sage shoot culture, biomass increased 43 times after 21 days of bioreactor run. The current operating conditions of the bioreactor were not suitable for the propagation of *Salvia officinalis* mainly due to the hyperhydricity problem of leaves and stems.

KEY WORDS: antioxidant compounds, bioreactor, *Salvia officinalis*.

INTRODUCTION

Salvia officinialis L. (sage) is an important and well-known medicinal plant. Sage (beside rosemary) is among the species of herbs considered to have the strongest antioxidant activities (Cuvelier et al. 1994; Miura et al. 2002). This is attributed to the presence of abietane diterpenoids (first of all carnosic acid – CA and carnosol – Car) in the aerial parts and rosmarinic acid (RA) in the leaves and roots of *S. officinalis*. We have previously studied the production of these compounds in various sage culture systems at small scale level i.e. in callus and cell suspension, shoots grown in solid and liquid medium as well as in hairy roots in shake flask culture (Grzegorczyk et al. 2005; Grzegorczyk et al. 2006). We have also shown that organ cultures (hairy roots for RA and shoots for CA and Car) had a higher potential for the production of antioxidant compounds than undifferentiated cultures of sage (Grzegorczyk et al. 2005; Grzegorczyk et al. 2006) and the extracts of the cultures have the same or even higher antioxidant activity than extracts from leaves of intact plants of *S. officinalis* (Grzegorczyk et al. 2007).

The present study was undertaken to investigate of the accumulation of antioxidants in sage shoots and hairy roots grown in a 5L sprinkle nutrient bioreactor. Up to now there have been no reports on the cultivation of sage in bioreactors.

MATERIALS AND METHODS

The shoot and hairy root cultures

The shoot cultures were established from shoot tips of 3--week-old *S. officinalis* seedlings (Grzegorczyk et al. 2005). A stock of shoots was maintained on MS (Murashige and Skoog 1962) agar medium supplemented with IAA (0.1 mg l^{-1}) and BAP (0.45 mg l^{-1}). The cultures were routinely subcultured at 3-week intervals.

The hairy root culture was obtained upon infection with *Agrobacterium rhizogenes* ATCC 15834 and the hairy root line HR-1 was chosen for study in the bioreactor. The roots were characterized by fast growth and high rosmarinic acid production when they were grown in shake flasks (Grzegorczyk et al. 2006). The culture was maintained by regular subcultures every 40 days in 300 ml shake flasks containing 80 ml hormone-free WP liquid medium (Lloyd and McCown 1980). The root culture was incubated at 100 rpm on a rotary shaker. The transformed nature of the roots was confirmed by PCR analysis (Grzegorczyk et al. 2006).

The shoot and root cultures were maintained in growth chamber at temperature 26±2°C under light from cool white fluorescent lamps (16 h light/8 h dark period; 40 µM m^{-2} s^{-1}). At the time of experiments described in this work, the shoot culture and hairy root culture were about two and two and a half years old (23-25th passages), respectively.

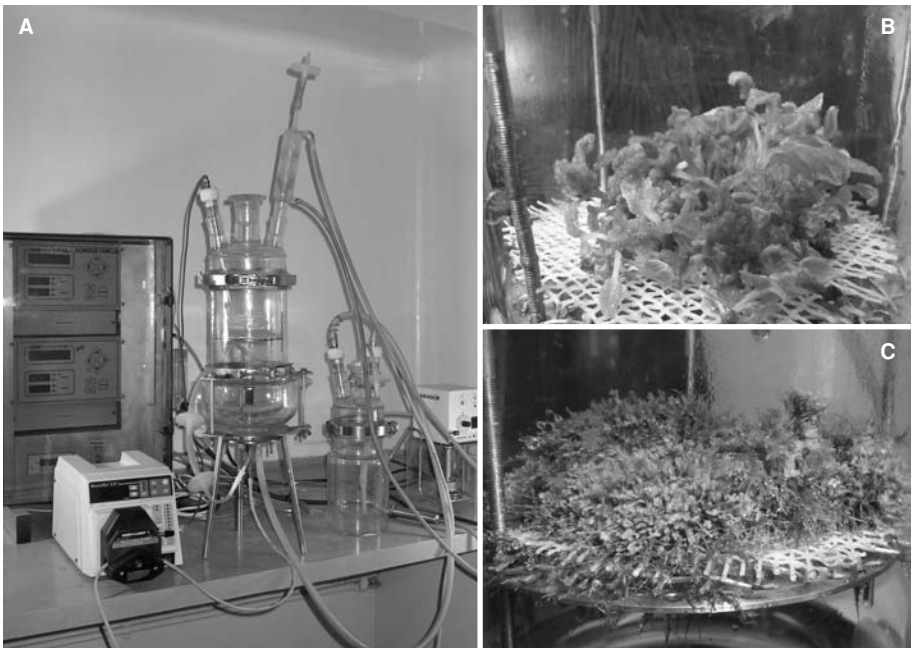

Fig. 1. Nutrient sprinkle bioreactor tested in the experiments: set-up for the design (A); biomass of *Salvia officinalis* shoots (B) and hairy roots (C) harvested after 21 and 40 days of inoculation, respectively.

Bioreactor cultures

The nutrient sprinkle bioreactor (Fig. 1A) previously described by Chmiel et al. (2001) was used in this study. It was made of glass (Büchi) and consisted of two vessels: one (internal volume: 5L) for growth of plant material and another as a nutrient medium reservoir (volume: 1.5L). Before starting the cultivation, the bioreactor was sterilized by autoclaving at 121˚C for 17 min. The liquid MS or WP medium (of the same composition as for small scale cultures) was taken from the reservoir by peristaltic pump (type CMF 10 Chemap) and flowed through a spraying nozzle to the growth chamber, where it sprinkled the plant material and then it returned to the medium reservoir. The nozzle was situated at the bottom of the growth chamber. Roots or shoots were placed in the growth chamber on stainless steel mesh (with 5 mm pore size) which was situated at 18 cm from the bottom. For shoots, pump operating time was 40 s (about 75 ml of the medium) and breaks in medium supply were 50 s. The parameters for root cultures were: 45 s (about 85 ml of the medium) and 40 s, respectively. The parameters were controlled by an MC 5B clock. The medium temperature was kept at 26±2˚C by a Therma mix thermostat (Braun Biotech International).

Inoculation of the bioreactor was carried out by aseptically transferring the shoots or roots from the inoculum vessels with sterile forceps in a laminar flow cabinet. The shoot culture inoculum was 15 shoot tips (0.5 cm in length), which weighed 0.6±0.01 g fresh weight (FW) and 0.052±0.001 g dry wt (DW). Inocula were taken from three-week-old cultures. For hairy roots, roots grown in shake flasks for 40 day were used as bioreactor inoculum. The roots were cut into four pieces weighing 8.7±0.29 g FW and 0.76±0.006 g DW. The culturing periods were the same as described for small scale cultures: 21 days for shoots and 40 days for hairy roots. The bioreactor cultures were incubated in the light (40 µM m^{-2} s^{-1}; 16 h/8 h light/dark regime).

Analytical procedures

The growth of roots and shoots in the bioreactor was measured in terms of fresh and dry weights. Additionally for shoot culture, the total number of shoots produced in the bioreactor over a three week period (multiplication rate) was counted.

At the end of each bioreactor run, the content and production of diterpenoids (CA and Car) and RA were measured. For RA analysis, plant material (dried shoot and hairy roots) was extracted with acetone and diterpenoids were extracted with methanol. Quantitative analysis was carried out by HPLC, using a Waters Symetry® C 18 column (4.6×150 mm, 3.5 µm) with 2 cm guard column. The Waters LC system, consisting of a Water 600 pump and a Water 996 Photodiode detector, a Water 717 plus Autosampler was used. The solvent systems were acetonitrile/0.1% o-phosphoric acid 65:35 (v/v) and acetonitrile/0.1% o-phosphoric acid 30:40 (v/v) for diterpenoids and RA analysis, respectively. The details of the procedure were as reported earlier (Grzegorczyk et al. 2005). The compound concentrations were achieved using calibration curves prepared with standard compounds. The content was expressed as milligrams per gram of dry weight. The production of analyzed compounds, expressed in mg per culture volume, was calculated based on the initial volume of the medium (1L), which was also taken as the working volume of the bioreactor. The bioreactor experiments consisted of three replications per each type of plant material.

RESULTS AND DISCUSSION

The shoot and hairy root cultures of *S. officinalis* were scaled up using a 5L-sprinkle bioreactor, in which liquid nutrient medium (1L) was dispersed into droplets and sprayed onto the root and shoot biomass. The efficiency of growth (on the basis of fresh and dry weight) and antioxidant phenolic compound accumulation (RA, CA, Car) were determined at the end of each bioreactor run and the results are presented in Table I.

The cultivation of sage shoots in the bioreactor resulted in biomass accumulation of 25.2±2.5 g FW l^{-1} and 2.25±0.18 g DW l^{-1} on the 21st day (Fig. 1B). There was roughly a 42-45 fold increase over the initial inoculum. For

TABLE 1. Growth and production of antioxidant compounds in shoots and hairy roots of *Salvia officinalis* cultivated in the nutrient sprinkle bioreactor.

Parameters	Shoots*	Hairy roots**
Total biomass (g/bioreactor)		
FW	25.2±2.5	154.6±5.97
DW	2.25±0.18	13.77±0.54
Biomass increase***		
FW	42	18
DW	45	18
CA		not detected
mg g^{-1} DW	1.74±0.02	
mg l^{-1}	3.92±0.03	
CAR		not detected
mg g^{-1} DW	1.34±0.01	
mg l^{-1}	3.02±0.02	
RA		
mg g^{-1} DW	26.24±0.48	34.65±1.07
mg l^{-1}	59.04±1.09	477.13±14.73

* Data recorded after 21 days of culture in MS medium supplemented with IAA and BAP

** Data recorded after 40 days of culture in WP medium

*** x^t/x^0 –the ratio of final to initial biomass

Results are the mean of three independent experiments (three bioreactor runs) ± standard error.

comparison, sage shoots simultaneously grown under similar conditions (the same subculture, medium and harvesting time) in Magenta vessels exhibited a 27-29 fold increase in DW and FW (unpublished data). As shown in our previous studies, static liquid culture in Magenta vessels was better for sage shoot multiplication and production of antioxidant compounds than the other shoot culture systems (on agar solidified medium and liquid agitated culture) (Grzegorczyk and Wysokińska 2004; Grzegorczyk and Wysokińska 2008). Also, differences in multiplication rate (estimated by the number of shoots produced after 3 weeks) were observed between cultures in the bioreactor (4.4±0.31 shoots per explant) and Magenta vessels (3±0.19 shoots/explant). However, all shoots formed in bioreactor had hyperhydrated leaves (translucent, curled and thickened) and stems. The reason was probably the high water potential of sage leaves. Earlier, the sprinkle bioreactor was used in our laboratory for *Centurium erythraea* shoot proliferation and no hyperhydricity symptoms were observed in shoots (Piątczak et al. 2005).

HPLC analysis revealed that the content of antioxidant diterpenoids in sage shoots cultured in the sprinkle bioreactor can reach 3.08±0.02 mg g^{-1} DW (sum of CA and Car), which was about half that in shoots from Magenta vessels (6.2±0.09 mg g^{-1} DW). The reason for the loss may be attributed to the hyperhydricity of sage shoots in bioreactors. Sales et al. (2002) have reported that hyperhydricity reduced the cardenolide content in *Digitalis minor* shoot culture. The decrease of total diterpenoid content of sage shoots grown in the sprinkle bioreactor was due to a lower concentration of CA (1.74±0.02 mg g^{-1} DW), while the level of Car (1.34±0.01 mg g^{-1} DW) was slightly higher than that obtained in static liquid culture shoots in Magenta vessels at the same age (0.96±0.01 mg g^{-1} DW) and shoots of naturally grown plants (1.1±0.01 mg g^{-1}) (Grzegorczyk and Wysokińska 2008). Car is an oxidized derivative of CA and oxygen is involved in its biosynthesis. It is possible that greater oxygen availability under sprayed conditions in the bioreactor may induce degradation of CA to higher oxidized derivatives, including Car. Instability of CA in contact with oxygen has been reported by many researchers (Cuvelier et al. 1994; Schwarz and Ternes 1992).

Another compound with antioxidant activity produced by sage shoots cultured in the bioreactor was RA. Its content was 26.2±0.48 mg g^{-1} DW after three weeks of culture (Table 1). The value was similar to that detected in sage shoots grown in Magenta vessels (24.89±0.59 mg g^{-1} DW) and over two times higher than RA level in the leaves of *S. officinalis* plants growing in the field (12.2±0.17 mg g^{-1} DW) (Grzegorczyk and Wysokińska 2008).

In another set of experiments, the sprinkle bioreactor was used for sage hairy root cultivation. The roots with high growth potential and RA production (HR-1 line) (Grzegorczyk et al. 2006) were transferred from shake flasks to bioreactor supported on a steel net and sprinkled with WP liquid medium incorporating 30 g l^{-1} sucrose. The roots displaying plagiotropic growth were short and branched with a large number of characteristic white root hairs. After a 40 day culture period, the roots formed a dense mat and entire available surface of the steel net was occupied with the tissue (Fig. 1C). The final biomass concentration was 154.6±5.97 g FW and 13.8±0.54 g DW. It represents an 18-fold increase over the inoculum biomass. We can also compare the results to our data obtained earlier with sage hairy roots grown in shake flasks after 40 days of cultivation (Grzegorczyk et al. 2006). In the latter, biomass accumulation was 107.3±4.38 g FW l^{-1} and 8.41±0.16 g DW l^{-1}. Thus, biomass of the roots grown in bioreactor was 1.4-1.6 times higher than that achieved for roots cultured in shake flasks.

The hairy roots of *S. officinalis* cultivated in the bioreactor did not produce diterpenoids (CA and Car). This is not surprising because the secondary metabolites are accumulated in aerial parts and only trace amounts of the compounds were found in roots of plants (Grzegorczyk et al. 2005). Neither of these diterpenoids were previously detected in transformed root lines of sage grown in Erlenmeyer flask cultures (Grzegorczyk et al. 2006). In the sprinkle bioreactor culture, transformed roots of sage exhibited a high concentration of rosmarinic acid, on average 34.7±1.07 mg g^{-1} DW. This concentration was higher (4-5 times) than those in the roots of naturally grown plants (Grzegorczyk et al. 2005) and the commercially available samples of *S. officinalis* dried leaves (Grzegorczyk and Wysokińska 2008). On the other hand, the level of RA in hairy roots in bioreactor culture was a little lower than that found in the HR-1 line grown in shake flasks (41.7±0.53 mg g^{-1} DW) (Grzegorczyk et al. 2006). Many studies have reported a decrease of secondary metabolite level after transferring culture from shake flasks to a bioreactor (Chattopadhyay et al. 2002; Scragg et al. 1987). The reason for the reduction has been attributed to the fact that bioreactors and shake flasks are completely different systems. The difference is most evident in the area of nutrient availability and gas environment (oxygen, carbon dioxide, ethylene as well as other unknown gaseous compounds) (ten Hoopen et al. 1994; Scragg et al. 1995). However, because of an in-

crease in biomass, RA production in sage hairy roots grown in the sprinkle bioreactor (477.1±14.73 mg l^{-1}) was enhanced by 35% compared to that of shake flask culture (350.8±9.08 mg l^{-1}).

Our results demonstrate that the bioreactor configuration is suitable for scaling up cultivation of sage hairy roots, and the cultures can be an appropriate source of superior RA production. On the other hand, the bioreactor is not suitable for sage shoot multiplication because of hyperhydricity problems. Further studies are needed to optimize culture conditions such as regulating the time that the shoots are in contact with the liquid medium to overcome the hyperhydricity problem and enhance production of diterpenoids in sage shoots grown in the bioreactor.

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LITERATURE CITED

CHATTOPADHYAY S., SRIVASTAVA A.K., BHOJWANI S.S., BISARIA V.S. 2002. Production of Podophyllotoxin by plant cell cultures of *Podophyllum hexandrum* in bioreactor. J. Biosci. Bioeng. 93: 215-220.

CHMIEL A., LEDAKOWICZ S, PAWŁOWSKA B. 2001. The mist bioreactor for cultures of transformed roots. In: 28th Conference SSCHE, Proceedings on CD ROM, May 21-25, Tatranske Matliare (SK).

CUVELIER M.E., BERSET C., RICHARD H. 1994. Antioxidant constituents in sage (*S. officinalis*). J. Agric. Food Chem. 42: 665-669.

GRZEGORCZYK I., BILICHOWSKI I., MIKICIUK-OLASIK E., WYSOKIŃSKA H. 2005. *In vitro* cultures of *Salvia officinalis* L. as a source of antioxidant compounds. Acta Soc. Bot. Pol. 74: 17-21.

GRZEGORCZYK I., KRÓLICKA A., WYSOKIŃSKA H. 2006. Establishment of *Salvia officinalis* L. hairy root cultures for the production of rosmarinic acid. Z. Naturforsch. 61c: 351-356.

GRZEGORCZYK I., MATKOWSKI A.,WYSOKIŃSKA H. 2007. Antioxidant activity of extracts from *in vitro* cultures of *Salvia officinalis* L. Food Chem. 104: 536-541.

GRZEGORCZYK I., WYSOKIŃSKA H. 2004. Mikrorozmnażanie *Salvia officinalis* L. z wierzchołków pędów. Biotechnologia. 65: 212-218.

GRZEGORCZYK I., WYSOKIŃSKA H. 2008. Liquid shoot cultures of *Salvia officinalis* L. for micropopagation and production of antioxidant compounds; effect of triacontanol. Acta Soc. Bot. Pol. 77: 99-104.

TEN HOOPEN H.J.G., VAN GULIK W.M., SCHLATMANN J.E., MORENO P.R.H., VINKE J.L., HEIJNEN J.K.J., VERPOORTE R. 1994. Ajmalicine production by cell cultures of *Catharanthus roseus*: from shake flask to bioreactor. Plant Cell Tiss. Organ Cult. 38: 85-91.

LLOYD G.B., MCCOWN B.H. 1980. Commercially-feasible micropropagation of mountain laurel – *Kalmia latifolia* by use of shoot-tip culture. Proc. Int. Plant Prop. Soc. 30: 421-427.

MIURA K., KIKUZAKI H., NAKATANI N. 2002. Antioxidant activity of chemical components from sage and thyme measured by the oil stability index method. J. Agric. Food Chem. 50: 1845-1851.

MURASHIGE T., SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

PIĄTCZAK E., WIELANEK M., WYSOKIŃSKA H. 2005. Liquid culture system for shoot multiplication and secoiridoid production in micropropagated plants of *Centaurium erythraea* Rafn. Plant Sci. 168: 431-437.

SALES E., NEBAUER S.G., ARRILAGA I., SEGURA J. 2002. Plant hormones and *Agrobacterium tumefaciens* strain 82139 induce efficient plant regeneration in the cardenolide – producing plant *Digitalis minor*. J. Plant Physiol. 159: 9-16.

SCHWARZ K. TERNES W. 1992. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis* II: Isolation of carnosic acid and formation of other phenolic diterpenes. Z. Lebens. Unters. Forsch. 195: 99-103.

SCRAGG A.H. 1995. The problems associated with high biomass level in plant cell suspensions. Plant Cell Tiss. Organ Cult. 43: 163-170.

SCRAGG A.H., MORRIS P., ALLAN E.J., BOND P., FOWLER M.W. 1987. Effect of scale-up on serpentine formation by *Catharanthus roseus* suspension culture. Enz. Microbial Technol. 9: 619-624.