Industrial Crops & Products 122 (2018) 732–739

Contents lists available at ScienceDirect

Industrial Crops & Products

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

# Growth of *Leonurus sibiricus* L. roots with over-expression of AtPAP1 transcriptional factor in closed bioreactor, production of bioactive phenolic compounds and evaluation of their biological activity

Przemysław Sitarek[a,*], Tomasz Kowalczyk[b], Laurent Picot[c], Dorota Michalska-Hejduk[d], Michał Bijak[e], Adam J. Białas[f], Marzena Wielanek[g], Tomasz Śliwiński[h], Ewa Skała[a]

[a] Department of Biology and Pharmaceutical Botany, Medical University of Lodz, Muszyńskiego 1, 90-151, Łódź, Poland
[b] Department of Genetics and Plant Molecular Biology and Biotechnology, The University of Lodz, Banacha 12/13, Łódź, Poland
[c] UMRi CNRS 7266 LIENSs, Université de la Rochelle, La Rochelle, France
[d] Department of Geobotany and Plant Ecology, Faculty of Biology and Environmental Protection, University of Lodz, Banacha 1/3, 90-237, Lodz, Poland
[e] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236, Lodz, Poland
[f] Department of Pneumology and Allergy, 1st Chair of Internal Medicine, Medical University of Lodz, Łódź, Poland
[g] Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Łódź, Poland
[h] Laboratory of Molecular Genetics, University of Lodz, Pomorska 141/143, Łódź, 90-236, Poland

ARTICLE INFO

*Keywords:*
Bioreactor
AtPAP1transgenic roots of *Leonurus sibiricus*
*PAL*
*C4H* and *4CL* genes expression
Antioxidant activity
Antiproliferative effect

ABSTRACT

Fast-growing transgenic root cultures of *Leonurus sibiricus* with the AtPAP1 transcriptional factor may be used as a base for potential large-scale phenolic acids production. The present study investigates the effect of different volumes of medium (300 mL, 1 L, 3 L, 5 L flasks) and a 5 L bioreactor on biomass increase and phenolic acids production by transgenic roots of *L. sibiricus*. Of these cultures, those from the 5 L bioreactor demonstrated the greatest increase in dry weight (20.83 g/L) and highest yields of phenolic acids (chlorogenic acid 448 mg/L and caffeic acid 302 mg/L). Additionally, they also displayed a cytotoxic effect on melanoma cells across the range of tested concentrations, as well as antioxidant activity on human blood plasma. This approach may serve as an alternative to conventional field crops for enabling large-scale production of the active constituents of AtPAP1 root extract, which would be of great value for pharmaceutical production.

## 1. Introduction

*Leonurus sibiricus* L. belongs to the Lamiaceae family. It is an aromatic, herbaceous plant, which can be annual, bisannual or perennial. Most significantly from a pharmacological perspective, the species has been reported to have a wide range of anti-inflammatory, antioxidant, anti-diabetes, anti-bronchitis and anti-cancer effects (Sayed et al., 2016; Oliveira et al.,2017; Sitarek et al., 2017a,b, 2016a). The present study describes a procedure to increase the production of secondary metabolites from the *Leonurus sibiricus* based on genetic manipulation. Previous studies describe the successful transformation of *L. sibiricus* by *Agrobacterium rhizogenes* infection (Sitarek et al., 2016b) and note that the hairy root cultures obtained have a different secondary metabolite content to untransformed roots (Sitarek et al., 2016b). Given the improved pharmacological content of hairy roots and the rich variety of beneficial compounds in *Leonurus sibiricus*, the species represents a good model for improving the production by refining biotechnology techniques (Sitarek et al., 2016b).

In the last two decades, genetic transformation and metabolic engineering have become powerful tools for transferring new genes into plants. This approach offers an attractive alternative to conventional breeding, because specific traits can be transferred into selected genotypes without adverse effects on desirable genetic backgrounds (Raghavendrarao et al., 2017; Rivera et al., 2012). Furthermore, they offer tremendous potential to modify, improve and enhance the production of various compounds. One such factor which can enhance the production of phenolic acids is AtPAP1 (*Arabidopsis thaliana* transcription factor) (Qiu et al., 2014; Zhang et al., 2010). One effective approach to engineering high levels of bioactive compounds is by simulating the overexpression of the factors that regulate the transcription of the genes involved in the phenylpropanoid pathway (*PAL, 4CL, C4H*) (Anh Tuan et al., 2014). The use of transgenic root cultures provides novel opportunities for the production of valuable phytochemicals synthesized in roots. They are genetically stable, they can accumulate

* Corresponding author.
*E-mail address:* przemyslaw.sitarek@umed.lodz.pl (P. Sitarek).

https://doi.org/10.1016/j.indcrop.2018.06.059
Received 10 April 2018; Received in revised form 10 June 2018; Accepted 14 June 2018

biomass in a culture system and they are able to grow in hormone-free culture media (Srivastava and Srivastava, 2007). Moreover, they demonstrate rapid growth and promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types (Banerjee et al., 2012). Our earlier studies revealed that transgenic roots of *L. sibiricus* with AtPAP1 transcriptional factor produce higher levels of phenolic acids than roots without any such construct; in addition, they showed better biological properties Sitarek et al., 2018). However, despite these successes, no commercial process currently exists for the production of phenolic acids by transgenic roots. There remains a need to increase the rates of phenolic acid production for commercial exploitation.

One of the methods for increasing production is by the use of a bioreactor (Sivakumar, 2006). In a biochemical context, bioreactors are defined as self-contained, sterile environments which capitalize on liquid nutrient or liquid/air inflow and outflow systems. The use of bioreactors may be an effective way for increasing the production of transformed roots and their secondary metabolites to an industrial scale: many studies have discussed such upscaling (Wu et al., 2007; Kusakari et al., 2012). However, their use can be complicated by the continuous growth of the transformed roots. The bioreactor must have a special configuration to compensate for the heterogeneous, structured and entangled nature of fibrous roots (Sivakumar, 2006).

The current study presents the first analysis of the phenolic acid content of the transgenic roots of *Leonurus sibiricus*, and their productivity, following transformation with a AtPAP1 transcription factor, while examining the effects of using various media volumes and bioreactor size that may be suitable for commercial-scale production. It also evaluates the expression of some of the genes involved in the phenylpropanoid pathway and determines the antiproliferative activity of the transgenic root 80% aqueous methanolic extract in human melanoma cells and its antioxidant effect on human plasma.

## 2. Materials and methods

### 2.1. Establishment and confirmation of L. sibiricus transgenic root culture with transcriptional factor AtPAP1

The establishment and confirmation of the AtPAP1 transgenic root were described previously (Sitarek et al., 2018).

### 2.2. Plant material and extract preparation

Lyophilized and powdered AtPAP1 root was used for analysis (10 g d.w.). The material was subjected to extraction in an ultrasonic bath for 15 min with 80% (v/v) aqueous methanol (500 mL) at 35 °C, followed by two further 15-min extractions with 300 mL 80% (v/v) aqueous methanol. The root extract was filtered, combined and evaporated under reduced pressure and then lyophilized. It was kept dry and stored in the dark until further use. The yield (w/w) was 50.25% for the AtPAP1 extract (Sitarek et al., 2018).

### 2.3. Determination of phenolic acids by HPLC analysis

LC–MS/MS was used to identify the phenolic acids. The content of the phenolic acids in the AtPAP1 root extract was determined by HPLC according to our earlier studies (Sitarek et al., 2018).

### 2.4. Growth of AtPAP1 transgenic roots of L. sibiricus culture in various volumes of medium

To determine the effect of growth volume, 6 g/L f.w. of *L. sibiricus* AtPAP1 transgenic roots were cultured in full strength SH medium in four Erlenmeyer flasks of different volume: a 300 mL flask containing 80 ml of medium, a 1 L flask with 500 mL medium, a 3 L flask with 1.5 L medium and a 5 L flask with 2.5 L medium. No phytohormone was added to any of the cultures. The AtPAP1 transgenic root cultures were kept on a rotary shaker at 75 rpm and incubated at 25 ± 2 °C. The fresh and dry weights of AtPAP1 transgenic roots were measured after 30 days of culture. The data was recorded as mean mass of three transgenic root cultures after each incubation period.

### 2.5. Scaling up the AtPAP1 transgenic root culture in the bioreactor

An attempt was made to scale up the transformed *L. sibiricus*roots to grow in a glass and stainless steel 5 L bioreactor consisting of two containers: a main container (volume 5 L) in which AtPAP1 root growth took place, and an auxiliary container (volume 1.5 L) serving as a reservoir for the nutrient medium [liquid SH medium supplemented with 3% (w/v) sucrose)]. The medium (1 L of SH medium) was supplied through with a polypropylene spray nozzle using a peristaltic pump which operated in bursts of 40 s, supplying 75 ml of medium, separated by 1.5 min breaks. The nozzle was situated at the bottom of the growth chamber; from here it provided the nutrient medium to the AtPAP1 roots, which were supported on a stainless steel wire mesh (with 10 mm pore size), situated 18 cm above the bottom. A more detailed description of the bioreactor configuration and operation is given by Chmiel et al. (2001). During the experiment, an autoclavable nylon mesh was tightened just beneath the medium surface to prevent the roots from sinking to the bottom or becoming submerged in the medium during the growth phase. The entire vessel was divided into lower and upper compartments by a mesh septum. The sterile culture vessel was inoculated under aseptic conditions. The vessel was inoculated with 0.6 g/L of transgenic AtPAP1 root. The experiment duration was 30 days and cultures were incubated at 25 ± 2 °C. Growth was measured as fresh and dry weight.

### 2.6. RNA extraction, cDNA synthesis and real-time PCR

Total RNA from the transgenic roots was isolated with Syngen Plant RNA MINI Kit reagent First strand cDNA synthesis was performed using a TranScriba kit (A&A Biotechnology, Poland) in a 20 µL reaction mix according to the manufacturer's protocol. Quantitative real-time PCR analysis was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) using a RT-PCR Mix SYBR® (A&A Biotechnology, Poland). The following Primer sequences were used: *PAL* F-5′CATGGACAACACTCGTTTGG-3′ and R-5'TTACGAGCTCGGAG AATTGG-3′; *C4H* F-5'GCCCATACGGAGAAGCAATA-3′ and R-5'CAAGC TTCCACCTGGACCTA-3′; *4CL* F-5' CTTAACGATCCGGAAGCAAC3′ and R-ATCAATGAGCCCGATGTCTC3′. Briefly, each reaction was performed in a 25 µL mix containing RT-PCR SYBR® Master Mix (10 µL), 0.5 µL of each primer (10 µM), 1 µL of cDNA and water. The elongation factor 1α (*EF-1α*) gene was chosen as an internal control for normalization (F5'-TGAGATGCACCACGAAGCTC-3′ and R- 5′CCAACATTGTCACCAGGAA GTG -3′). The PCR procedure was performed under the following conditions: five minutes at 95 °C, followed by 35 cycles of 95 °C at 30 s, 60–65 °C for each gene at 30 s and 72 °C at 60 s. Each sample was analysed in triplicate. In the melting curve analysis, the levels of the genes were normalized to that of the elongation factor 1α (*EF-1α*) gene used to test the specificity of amplification. The expression of the genes was calculated by the comparative $C_t$ method (Schmittgen and Livak, 2008).

### 2.7. Antiproliferative activity of AtPAP1 roots extracts in human melanoma cells

The antiproliferative activity of AtPAP1 root extract on melanoma cells was studied in the A2058 (ATCC® CRL-11147) cell line. A2058 are highly invasive human epithelial adherent melanoma cells, derived from lymph node metastatic cells obtained from a 43-year-old male patient. They are tumorigenic at 100% frequency in nude mice, and considered as very resistant to anticancer drugs. All cell culture experiments were performed at 37 °C. Cells were grown to confluence in