Industrial Crops and Products 60 (2014) 52–59

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Production of guaianolides in *Agrobacterium rhizogenes* - transformed chicory regenerants flowering *in vitro*

Milica D. Bogdanović[a,*], Slađana I. Todorović[a], Tijana Banjanac[a], Milan B. Dragićević[a], Francel W.A. Verstappen[b], Harro J. Bouwmeester[b], Ana D. Simonović[a]

[a] *Institute for Biological Research "Siniša Stanković", Bul. Despota Stefana 142, University of Belgrade, 11060 Belgrade, Serbia*
[b] *Laboratory of Plant Physiology, Wageningen University and Research Centre, 6708PB Wageningen, The Netherlands*

ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form 8 May 2014
Accepted 31 May 2014
Available online 24 June 2014

Keywords:
Sesquiterpene Lactones
Guaianolides
Hairy root
Cichorium intybus
Rol genes
Flowering

ABSTRACT

Chicory (*Cichorium intybus* L.) is rich in bitter sesquiterpene lactones, mainly guaianolides: lactucin, 8-deoxylactucin, lactupicrin and their 11(*S*),13-dihydroderivatives—compounds recognized for their antimicrobial and anti-cancer effects. *In vitro* plant tissue culture, and particularly *Agrobacterium rhizogenes*—generated hairy root (HR) cultures, have many advantages as systems for production of valuable secondary metabolites. Although chicory HRs grow better than control culture, having nearly 60 times greater fresh weight gain, they do not contain a higher content of guaianolides than wild type (wt) roots. Thus we have established *in vitro* system comprised of wt root and HR cultures, and wt and transformed regenerated plants of the same age, in rosette and flowering stage, in order to study the effects of transformation, organogenesis and flowering on guaianolides production. Both regeneration and flowering *in vitro* were spontaneous, so the results were not influenced by exogenous growth regulators. Some of the transformed clones grew better, but all flowered earlier in comparison to wt plants. Floral transition increased guaianolides content in both roots and leaves of transformed, but not of wt plants. Expression of *RolC* oncogene correlated with floral transition and with guaianolides accumulation. We propose *A. rhizogenes*—transformed plants at the flowering stage as an alternative source of free guaianolides, where, in contrast to HRs, entire plants can be used for the extraction.

1. Introduction

Chicory or Belgian endive (*Cichorium intybus* L., *Asteraceae*) is a perennial herbaceous plant, known to grow as a weed on roadsides. Various chicory varieties are cultivated for salad leaves and chicons (var. *foliosum*), roots used as a coffee substitute (var. *sativum*), and as forage. Wild and cultivated chicory varieties are used in traditional medicine to promote appetite and digestion (Malarz et al., 2002), for the treatment of intestinal worms, malaria, gallstones, gastroenteritis, cuts, bruises, and even cancer. Chicory extracts, especially root extracts, have anti-bacterial (Nandagopal and Kumari, 2007; Petrovic et al., 2004), anti-inflammatory, hepatoprotective (Bais and Ravishankar, 2001; Zafar and Mujahid Ali, 1998), antimicrobial, nematicidal (Nishimura and Satoh, 2006), herbicidal, allelopathic (Wang et al., 2011), and anti-cancer effects (Conforti et al., 2008; Csupor-Löffler et al., 2009).

Chicory plants contain a number of medicinally important secondary metabolites including fructose polymer inulin, used as a sweetener and prebiotic (Baert and Van Bockstaele, 1992), esculin, coumarins, flavonoids and vitamins (Nandagopal and Kumari, 2007). However, most of chicory pharmaceutical properties, as well as its bitter taste, are attributed to germacrene-derived sesquiterpene lactones (SLs)—guaianolides, eudesmanolides and germacranolides (de Kraker et al., 2002; Kisiel and Zielinska, 2001; Rees and Harborne, 1985). Chicory leaves, and especially roots, are rich in lactucin-like guaianolides: lactucin, its *p*-hydroxyphenylacetic acid ester lactupicrin (or lactucopicrin), 8-deoxylactucin, as well as their 11(*S*),13-dihydroderivatives and glycosides, while the levels of eudesmanolides and germacranolides are lower (Kisiel and Zielinska, 2001; Malarz et al., 2002; Van Beek et al., 1990). Guaianolides are shown to have antifeedant (Rees and Harborne, 1985), antifungal (Barrero et al., 2000), antiprotozoal and cytotoxic activity, as well as potent antimalarial effect (Bischoff et al., 2004).

Plant cell and tissue *in vitro* culture, as a source of secondary metabolites, has many advantages over field agriculture, including controlled and readily manipulated conditions that are independent of geographical and climate factors, pests and pathogens

Abbreviations: SL, sesquiterpene lactone; GUS, β-glucuronidase; MS, Murashige and Skoog basal medium; HR, hairy roots; FW, fresh weight; Wt, wild type.

* Corresponding author. Tel.: +381 11 2078 431; fax: +381 11 2078 404.
E-mail address: milica84bog@gmail.com (M.D. Bogdanović).

http://dx.doi.org/10.1016/j.indcrop.2014.05.054
0926-6690/

(Georgiev et al., 2012). Growing plants and tissues *in vitro* allows for better control of target metabolites levels and quality, resulting in improved products consistency (Talano et al., 2012). Moreover, genetically modified plants grown in a contained system can be readily used without regulatory constrains. Hairy root (HR) cultures obtained by *Agrobacterium rhizogenes* infection are often a technology of choice for production of valuable secondary metabolites, because HRs have biosynthetic capabilities of differentiated tissues, high growth rate on hormone-free media, genetic and biochemical stability and provide possibility for scaling-up in bioreactors (Georgiev et al., 2012). Transformation with *A. rhizogenes*, as well as separate or combined introduction of its *Rol* genes, driven by their native or constitutive promoters, boosts secondary metabolism from 2- to 300-fold depending on the group of secondary metabolites and the plant species (Bulgakov, 2008) by yet unresolved mechanisms. Several recent reviews list a number of secondary metabolites successfully produced by wild type HR cultures (*e.g.* where no foreign genes other than Ri-plasmid oncogenes, including *Rol* genes, are introduced) of various plant species (Georgiev et al., 2012; Giri and Narasu, 2000; Srivastava and Srivastava, 2007; Talano et al., 2012; Zhou et al., 2011). Even though transgenic plants are readily regenerated from HR cultures, often spontaneously (*e.g.* Momčilović et al., 1997; Vinterhalter et al., 2006; Sretenović-Rajičić et al., 2006; Subotić et al., 2009), reports on the level of secondary metabolites produced by the regenerants and how do they compare to the HR cultures are scarce (Chaudhuri et al., 2006). There is virtually no information on how flowering *in vitro* affects secondary metabolite production.

Chicory is readily transformed with *A. rhizogenes* (Bais et al., 2000, 2001; Kamada et al., 1992; Limami et al., 1998; Malarz et al., 2002, 2013; Sun et al., 1991). However, the obtained HR cultures contained very little free (unglycosylated) SLs—less than intact untransformed plants (Malarz et al., 2013). The aim of this study is to improve the existing system by exploring whether whole chicory plants regenerated from HR cultures produce more guaianolides than HRs or wt plants, and to assess the influence of flowering *in vitro* on guaianolides production.

2. Materials and methods

2.1. Plant material

Cichorium intybus L. Blue seeds (Samen Mauser, Switzerland) were surface sterilized with 20% bleach (0.8% active chlorine) for ten minutes and rinsed five times in sterile distilled water. Sterilized seeds were set to germinate in Petri dishes on MS medium (Murashige and Skoog, 1962) without sucrose, solidified with 0.6% agar. The seedlings were transferred to MS supplemented with 2% sucrose until maturation. All cultures were maintained under long-day (16/8 light/dark) at 25 ± 2 °C and 60–70% relative humidity, under photon flux rate of 42 μmol m^{-2} s^{-1} at the cultures level.

2.2. Hairy root induction and culture

Hairy roots were induced using *A. rhizogenes* A4M70GUS strain harboring pRiA4 plasmid with β-glucuronidase (GUS) cassette integrated into the T_L region, between the *RolC* and *RolD* genes (Tepfer and Casse-Delbart, 1987). The GUS construct contains *UidA* gene, driven by doubled 35SCaMV promoter and followed by *nos* (nopaline synthase) polyadenylation signal. Bacterial culture was maintained at 28 °C in yeast extract broth medium supplemented with 100 mg l^{-1} neomycin. Overnight culture was used for plant inoculation. Fully grown leaves from 10 weeks old plants were inoculated with liquid bacterial culture along the leaf veins using a sterile needle dipped in the culture. Four weeks after the transformation, roots that emerged on leaves were excised and grown separately on MS supplemented with 2% sucrose, 0.6% agar and 500 mg l^{-1} cefotaxime to stop bacterial growth. Cefotaxime concentration was gradually reduced over a six-month period. The roots were then subcultured on MS without antibiotic.

HR cultures were started from 1 cm long root tip explants in liquid MS supplemented with 2% sucrose, and were propagated with continuous shaking. Roots excised from untransformed plants were used as controls. Fresh weight (FW) of roots blotted dry was recorded after one month of cultivation; samples were frozen in liquid nitrogen and kept at −80 °C until use.

2.3. Regeneration of transgenic plants

Regeneration of shoots from root cultures was spontaneous, on MS supplemented with 2% sucrose, without growth regulators. Regenerated plants were excised from roots and grown separately for 10 weeks under the conditions described in Section 2.1. After this time, fresh weight of rosettes and flowering plants was measured, and the material was frozen and stored at −80 °C until further use.

2.4. PCR, RT-PCR and QRT-PCR

Genomic DNA was extracted from leaves of regenerated plants by CTAB method (Stewart and Via, 1993). RNA was isolated from leaves and roots of regenerated plants using Trizol reagent (Invitrogen, USA) following manufacturer's protocol. All isolations were performed in biological triplicates. Total RNA was treated with DNase I (Fermentas, USA) according to manufacturer's protocol and reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA) at 42 °C, with poly$(dT)_{18}$ primers.

Genomic PCR and RT-PCR were performed using Taq recombinant polymerase (Fermentas, USA). The PCR mixtures contained 100 ng of genomic DNA or cDNA corresponding to 100 ng RNA, 1 μM specific primers (listed in Table 1) and standard components according to Fermentas protocol, in a 25-μl volume. The PCR program included initial denaturation (95 °C/5 min), 40 cycles of denaturation (95 °C/30 s), annealing (61 °C for *virD1* or 56 °C for other genes, for 30 s), and extension (72 °C/30 s), followed by final extension (72 °C/10 min).

Quantification of *RolA*, *B* and *C* expression was performed by QRT-PCR. The qPCR reactions were set with Maxima SYBR Green mix (Fermentas, USA), with cDNA corresponding to 100 ng RNA and 0.3 μM primers (Table 1). Specificity of primers was verified by Primer-BLAST, electrophoretic sizing, and melting curve analysis. The amplification was carried out on ABI PRISM 7000 SDS thermal cycler (Applied Biosystems, USA) using the described cycling program for PCR, followed by melting curve analysis. Standards for absolute quantification were prepared from PCR amplicons of *Rol* and 18S rRNA genes that were electrophoretically purified, extracted from gel with GeneJET Gel Extraction Kit (Fermentas, USA) and serially diluted in a 10^9–10^2 copies range. Constitutive expression of 18s rRNA gene was confirmed in parallel using the same poly$(dT)_{18}$–primed RT reactions as templates and universal plant 18S rRNA primers (Bogdanović et al., 2013).

2.5. GUS staining

To determine GUS expression, root tip explants were incubated in a reaction mixture containing 50 mM NaH_2PO_4, 10 mM Na_2EDTA, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$ and 1 mg ml^{-1} X-Gluc (Sigma, Switzerland) at 37 °C for 12 h.