Enzyme and Microbial Technology 46 (2010) 14–20

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Significant enhancement of oleanolic acid accumulation by biotic elicitors in cell suspension cultures of *Calendula officinalis* L.

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ARTICLE INFO

Article history:
Received 2 June 2009
Received in revised form 27 August 2009
Accepted 3 September 2009

Keywords:
Marigold
Cell suspension culture
Biotic elicitors
Oleanolic acid

ABSTRACT

The effects of elicitors on cell growth and oleanolic acid (OA) accumulation in shaken cell suspension cultures of *Calendula officinalis* were investigated. Elicitors were added individually at various concentrations to 5-day-old cell cultures and their effects monitored at 24 h intervals for 4 days. Different effects on OA accumulation were observed depending on the day of treatment. Jasmonic acid was the most efficient elicitor. After 72 h of treatment with 100 μM JA, the intracellular content of OA reached its maximum value (0.84 mg g^{-1} DW), which was 9.4-fold greater than that recorded in an untreated control cultures. The addition of chitosan at 50 mg l^{-1} produced a 5-fold enhancement of OA accumulation (0.37 mg g^{-1} DW) after 48 h of treatment. Treatment with yeast extract at 200 mg l^{-1} for 96 h or with pectin at 2 mg l^{-1} for 48 h produced identical cellular levels of OA (0.22 mg g^{-1} DW). Lastly, 48 h elicitation with homogenate of the fungus *Trichoderma viride* produced a 1.8-fold increase in oleanolic acid content (0.12 mg g^{-1} DW). In addition to significantly stimulating OA accumulation and its secretion into the culture medium, the elicitors also caused slight inhibition of cell growth.

1. Introduction

The marigold, *Calendula officinalis* L., from the family Asteraceae, is an annual plant that has been used for centuries in traditional folk medicine for the treatment of various ailments. Marigold tissues contain oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) (Fig. 1) in the form of the aglycone of triterpenoid saponins, namely glucosides (derivatives of 3-O monoglucoside) [1] and glucuronides (derivatives of 3-O-monoglucuronide) [2]. Oleanolic acid glucuronides extracted from the flowers are relatively non-toxic and has been used in cosmetics and also represent an important source of pharmaceuticals. Moreover, our previous studies have shown that oleanolic acid glycosides isolated from marigold can play a role in chemical defense against pathogens and environmental interactions. These compounds exhibit hemolytic, allelopathic, fungistatic [3], antibacterial and antiparasitic activities [4]. Free oleanolic acid has numerous important medicinal activities including anti-inflammatory, anti-viral, anti-fungal, anti-nociceptive, anti-allergic, anti-ulcer, anti-hyperlipidemic, anti-tumor, anti-cariogenic, hepatoprotective, hypoglycemic [5] and anti-HIV properties [6].

Commercial importance of *C. officinalis* and its bioactive compounds has in recent years resulted in a great interest. We have chosen the model plant marigold as a suitable species for the study of regulation of biosynthetic pathway of pentacyclic triterpenoids in view of the interesting saponin profile of this species.

Although oleanolic acid as well as its glycosides are common phytochemicals which naturally occur in many medicinal plants, little is known about the production of these compounds in *in vitro* cultures.

Because of the important biological activities of OA and its glycosides from *C. officinalis*, biotechnological production of these compounds using suspension cultures is of great practical value. The advantage of this method is that it may provide continuous and reliable source of natural products of industrial importance. Moreover, plant cell cultures of marigold seem to be an attractive alternative to intact plant tissues for the production of high-valuable secondary metabolites.

We have previously shown that cell suspension cultures of *C. officinalis* synthesize oleanolic acid glycosides, but the amount of these compounds produced is less than that obtained from the intact plant. Numerous strategies have been investigated to try and enhance the production of OA in marigold suspension cultures, including the optimization of medium composition, inoculum size, and physical parameters of the culture environment [7], plus the selection of highly productive cell lines, and the influence of various sugars and osmotic stress (unpublished). However, the potential utilization of these compounds by the pharmaceutical industry has been limited by the low yields from *in vitro* cultures. Elicitation has been one of the most effective strategies to improve the production of useful secondary metabolites in plant cell cultures [8]. Elicitors act as signalling molecules for the cells and generate a multicompo-

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doi:10.1016/j.enzmictec.2009.09.002

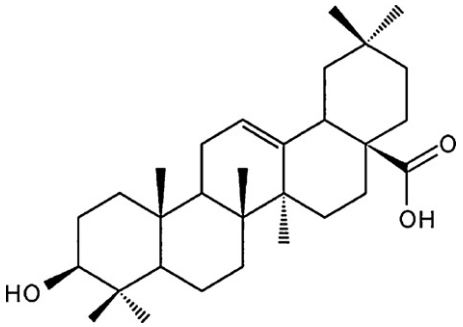

Fig. 1. Structure of oleanolic acid.

nent response by triggering various signal-transduction pathways [9]. Both biotic and abiotic elicitors are known to stimulate or induce *de novo* synthesis of a wide range of bioactive compounds in plant cells as a result of their defensive reaction against pathogen attack. Chitosan is the deacetylated form of chitin, which is the main component of the cell walls of some fungal species and mimics the effects of pathogenic microorganisms to stimulate plants to biosynthesize secondary metabolites [10]. Pectin is a family of complex polysaccharides present in all plant primary cell walls. Pectic oligosaccharides (oligogalacturonides – OGAs) are released when enzymes secreted from the pathogen degrade the homogalacturonan in the cell wall. OGAs can be detected by plants as signals to initiate defense responses [11]. Gundlach et al. [12] demonstrated that jasmonic acid acts as a second messenger transmitting elicitor signals to stimulate the biosynthesis of many secondary metabolites in plants cells, and it plays a significant role in the induction and regulation of defense genes. Jasmonic acid, pectin and yeast extract have been reported to enhance the production of triterpenoids in suspension cultures of various plant, e.g. *Perilla frutescens* [13], *Uncaria tomentosa* [14] and *Scutellaria baicalensis* [15]. This suggests that also production of oleanolic acid and its glycosides in suspension cultures of *C. officinalis* can be enhanced if these elicitors are used.

In this study, we examined the effects of elicitors, added at a range of concentrations to the culture medium for different periods, on oleanolic acid accumulation and cell viability in *C. officinalis* suspension cultures.

2. Materials and methods

2.1. Cell suspension cultures

Cell cultures of *C. officinalis* were established from 3-month-old callus tissue as previously described by Grzelak and Janiszowska [7] and were maintained in Murashige and Skoog medium [16] supplemented with 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 mg l^{-1} 6-(γ,γ-dimethylallylamino)purine and 30 g l^{-1} sucrose. The pH of the medium was adjusted to 5.8 with 0.1 M NaOH before autoclaving for 15 min at 121 °C. The cell suspensions were grown in flasks with shaking (110 rpm) at 22 °C in the dark. Subculturing was performed every 3 weeks, by transferring approximately 2.5 g of cells into 100 ml of fresh medium.

2.2. Elicitation

After 16 months in culture, cells (approx. 5 g) were transferred to flasks containing 100 ml fresh medium and grown under conditions as described above. After 5 days, different elicitors were individually added to these suspension cultures at various concentrations.

2.3. Preparation of elicitors

A stock solution of jasmonic acid (JA, Sigma) was prepared by dissolving 250 mg in 70% (v/v) ethanol. A further dilution was made in distilled water. This solution was filter sterilized using a 0.22-μm syringe filter (Millipore) and added to the culture medium to give concentrations of 50, 100 and 150 μM.

Chitosan (Ch, Sigma) from crab shells was prepared by the method of Popp et al. [17]. It was dissolved in 5% (v/v) 1 M HCl by gentle heating and continuous stirring. The pH of the solution was adjusted to 5.0 with 1 M NaOH. The final solution was autoclaved at 121 °C for 15 min and added to the culture medium to give chitosan concentrations of 50, 100 and 150 mg l^{-1}.

Yeast extract (YE, Sigma) was prepared according to the method of Peltonen et al. [18]. It was diluted in distilled water, autoclaved and added to the culture medium to give concentrations of 100, 200 and 300 mg l^{-1}.

Pectin (P, Sigma) from citrus peel was dissolved in distilled water and autoclaved. It was then added to the culture medium to give concentrations of 2, 4 and 6 mg l^{-1}.

The fungal strain *Trichoderma viride* was cultured as described by Namdeo et al. [19]. Mycelia were harvested by filtration, dried at 65 °C for 24 h and then ground with a mortar and pestle. The powder was resuspended in distilled water and autoclaved. This *T. viride* homogenate was added to the culture medium to give concentration of 0.5 mg l^{-1}.

2.4. Determination of dry cell weight (DW)

C. officinalis cells were separated from the medium by vacuum filtration. The cells were dried at 50 °C to constant weight and the dry cell weight calculated.

2.5. Extraction of intracellular and extracellular oleanolic acid

The dried cells were ground with methanol and boiled for 60 min. The methanol extract was then evaporated to dryness. The culture medium was extracted three times with an equal volume of 1-butanol and this extract was evaporated to dryness under vacuum. Dried samples containing a mixture of oleanolic acid glycosides from the cells or culture medium were hydrolyzed in Kiliani mixture (acetic acid:hydrochloric acid:water, 35:10:55, v/v/v) for 2 h. Five volumes of water were added to the acidic hydrolysate and the OA was extracted four times with diethyl ether. The ether extract was then evaporated to dryness, dissolved in ether and separated by thin layer chromatography (TLC) on silica-gel plates with chloroform:methanol (95:5, v/v) as the mobile phase. The OA was visualized by spraying the plates with 0.1% (w/v) rhodamine in acetone and located by comparison with a standard. The purified OA was methylated twice with an ethereal solution of diazomethane.

2.6. Quantification of oleanolic acid

Quantitative measurements of oleanolic acid methyl ester were made using gas chromatography (GC). GC was performed at 260 °C using a Shimadzu GC-2014 instrument equipped with flame ionization detection (FID) and fitted with a 2 m × 3 mm 3% SE-30 Chromosorb WHP column (Supelco). The temperature of the injector and detector was 275 °C and nitrogen was used as the carrier gas at a flow rate of 120 ml min^{-1}. Peak identification and quantification of oleanolic acid were carried out by referring to a calibration curve produced using an authenticated sample of methylated oleanolic acid as the standard.

2.7. Cell viability test

The viability of cells treated with elicitors was assessed by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF) by the action of mitochondrial dehydrogenases in mitochondria, as described by Towill and Mazur [20]. Briefly, 200 mg fresh weight of cells was rinsed with 5 ml of distilled water and incubated with 4 ml of TTC solution (0.08% (w/v) TTC in 50 mM potassium phosphate buffer, pH 7.5) for 24 h at 23 °C in the dark. The cells were then rinsed three times with distilled water. The TPF was extracted with 5 ml of 95% ethanol for 25 min at 60 °C. Absorbance of the extract was measured spectrophotometrically at 485 nm using 95% ethanol as the blank. The TPF content in elicited cells was expressed as a percentage of the content in untreated control cells (100%) and is referred to as the cell viability.

3. Results

3.1. Effect of different elicitors on cell growth

The effects of different concentrations of elicitors following their application to 5-day-old cultures of *C. officinalis*, corresponding to the early-exponential phase of cell growth, were investigated. Cell growth was monitored by measuring the dry cell weight (DW). The addition of jasmonic acid, chitosan or yeast extract (Fig. 2a–c) had almost no effect on cell growth for the first 72 h. At this time, slight increases in cell growth were recorded for cultures treated with JA at 50 μM and YE at 200 mg l^{-1}, but these were not statistically significant (Fig. 2a and c). However, after 96 h of treatment with elicitors cell growth was almost uniformly decreased. The largest decreases in cell growth of 18 and 22% compared with the control (9.3 mg l^{-1}) were observed after 96 h in cultures treated with JA