Plant Physiology and Biochemistry 99 (2016) 73–78

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

New strategies for the use of *Linum usitatissimum* cell factories for the production of bioactive compounds

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ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form
9 December 2015
Accepted 11 December 2015
Available online 18 December 2015

Keywords:
β-glucan
Cyclodextrins
Flax
Phytosterols
Suspension-cultured cell
Tocopherols
(Z)-3-hexenol

ABSTRACT

In this work, suspension-cultured cells of *Linum usitatissimum* L. were used to evaluate the effect of two types of cyclodextrins, β-glucan and (Z)-3-hexenol separately or in combination on phytosterol and tocopherol production. Suspension-cultured cells of *L. usitatissimum* were able to produce high levels of phytosterols in the presence of 50 mM methylated-β-cyclodextrins (1325.96 ± 107.06 μg g dry weight^{-1}) separately or in combination with β-glucan (1278.57 ± 190.10 μg g dry weight^{-1}) or (Z)-3-hexenol (1507.88 ± 173.02 μg g dry weight^{-1}), being cyclodextrins able to increase both the secretion and accumulation of phytosterols in the spent medium, whereas β-glucan and (Z)-3-hexenol themselves only increased its intracellular accumulation. Moreover, the phytosterol values found in the presence of hydroxypropylated-β-cyclodextrins were lower than those found in the presence of methylated-β-cyclodextrins in all cases studied. However, the results showed that the presence of methylated-β-cyclodextrins did not increase the tocopherols production and only an increase in tocopherol levels was observed when cells were elicited with 50 mM hydroxypropylated-β-cyclodextrins in combination with β-glucan (174 μg g dry weight^{-1}) or (Z)-3-hexenol (257 μg g dry weight^{-1}). Since the levels of tocopherol produced in the combined treatment were higher than the sum of the individual treatments, a synergistic effect between both elicitors was assumed.

To sum up, flax cell cultures elicited with cyclodextrins alone or in combination with β-glucan or (Z)-3-hexenol were able produce phytosterols and tocopherols, and therefore, these elicited suspension-cultured cells of *L. usitatissimum* can provide an alternative system, which is at the same time more sustainable, economical and ecological for their production.

1. Introduction

Linum usitatissimum L. (flax) is a native plant from the Mediterranean and Southwest Asian regions and it is one of the oldest crops being widely grown for its oil, fiber content, and food nutrients (Oomah, 2001). Among these food nutrients, it is important to note for the beneficial properties on human health, the abundant content of tocopherols and phytosterols.

In this sense, tocopherols are lipophilic antioxidants and together with tocotrienols belong to the vitamin-E family. In plants, tocopherols act as agent antioxidants and their levels increase when they are exposed to different stresses. In addition, tocopherols have been associated with the prevention of several human diseases like atherosclerosis, diabetes, Parkinson's disease, Alzheimer's disease, and coronary heart disease (Salinthone et al., 2013; Giordano et al., 2014; Jain and Jain, 2012; Yilmaz et al., 2013).

On the other hand, phytosterols are essential components of the membranes of all eukaryotic organisms, and are responsible for their permeability and fluidity (Posé and Botella, 2009). The most important natural sources of plant sterols in human diets are oils, margarines, legumes, vegetables and seeds (Dutta and Appelqvist, 1996). Phytosterols have beneficial effects on human health because of their capability to lower serum cholesterol levels (Hicks and Moreau, 2001). Phytosterols also have beneficial effects against colon cancer (Awad and Fink, 2000), and they have anti-inflammatory, antibacterial, antioxidant, and anti-ulcerative properties (Beveridge et al., 2002).

Due to beneficial properties of these bioactive natural compounds, new sustainable and more environment-friendly methods

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http://dx.doi.org/10.1016/j.plaphy.2015.12.009
0981-9428/

have emerged, especially those based on plant biomass generation using in vitro culture techniques. One of these alternative methods for the production of these bioactive natural compounds is the use of elicited suspension-cultured cells (SCC) (Chavan et al., 2011). In this sense, we have developed a method for the production of these metabolites based on the elicitation of SCC with β-cyclodextrins (CD), which are cyclic oligosaccharides consisting of seven glucopyranose residues linked by a (1 → 4) glucosidic bonds. In fact, their elicitor activity is due to their chemical similarity with the alkyl-derived oligosaccharides that are released from plant cell walls during fungal attacks (Bru et al., 2006). CD can also form inclusion complexes with non-hydrophilic compounds, allowing their secretion from the producer cells to the culture medium. Therefore, not only are CD able to act as inducers of metabolite biosynthesis but also as promoters of the adsorption of these compounds from the culture medium, enabling their accumulation, and recovery directly from the spent medium with no biomass destruction (Belchí-Navarro et al., 2012).

On the other hand, green leaf C6-volatiles play important roles in mediating the behavior of herbivores and their natural enemies, as well as triggering plant–plant communication to prevent further attacks. The most important green leaf C6-volatiles are (Z)-3-hexenal, (Z)-3-hexenyl acetate and (Z)-3-hexenol (Hex) (Engelberth et al., 2013). The exposition of plants to these volatile compounds usually induces phytoalexin biosynthesis as well as the expression of defense-related genes in several plant species (Matsui, 2006).

β-Glucans (Glu) are oligosaccharides derived from fungi and are generated from the fungal cell walls at the site of infection through the action of plant β-glucanases (Okinaka et al., 1995). These Glu have been used as elicitors on a broad range of plant species. In fact, Glu obtained from *Phytophthora megasperma* and *Pyricularia oryzae* induced phytoalexin biosynthesis in soybean and rice cells, respectively (Cheong and Hahn, 1991; Yamaguchi et al., 2000). All these findings suggest that both Hex and Glu act as a signal compounds to initiate defense responses, and for this reason, it will be useful to examine whether the treatment of flax SCC with Hex or Glu increases bioactive compound production or it does not.

The aim of this study was to characterize the total production (intra and extracellular) of phytosterols and tocopherols in flax SCC treated with CD separately or in combination with Glu or Hex.

2. Materials and methods

2.1. Plant materials

L. usitatissimum L. calli were established in our laboratory in 2013 from stem explants obtained from flax vitroplants, and they were maintained under a 16 h light/8 h dark photoperiod with a light intensity of 18.4 w m^{-2} at 25 °C. These calli were subcultured in 100 ml of MS medium supplemented with thiamin (0.4 mg L^{-1}) and myo-inositol (100 mg L^{-1}), and 30 g L^{-1} sucrose. In addition, an hormonal dose was added in the culture medium to allow the growth of friable calluses of flax (0.22 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 0.04 mg L^{-1} benzyladenine and 0.186 mg L^{-1} naphthaleneacetic acid). This culture medium was adjusted at pH 6.0 before autoclaving.

Flax calli were subcultured in this solid culture medium every 21 days. Flax SCC were initiated by inoculating friable callus pieces into 250 ml flasks containing 100 ml of the same medium without agar, and maintained at 25 °C, under a 16 h light/8 h dark photoperiod with a light intensity of 18.4 w m^{-2} in a rotary shaker at 110 rpm. These SCC were routinely maintained by periodical subcultures every 13 days by diluting with one volume of culture medium, and then distributing it into two flasks.

2.2. Determination of cell viability

Cell viability was assessed by incubating a small aliquot of SCC for 1–2 min culture medium containing 0.01% (p/v) fluorescein diacetate (Sigma–Aldrich, Spain) for the selective labeling of live cells. Fluorescence was observed with a fluorescence microscope (DMRB, Leica Microsystems Inc. Wetzlar, Germany) using specific filters (λexc = 490 nm, λemi = 520 nm).

2.3. Elicitation of flax suspension cultured cells

Elicitation experiments were performed in triplicate using 13 day-old flax SCC. For this, 4 g fresh weight (FW) of washed cells were transferred into 100 ml flasks and suspended in 20 ml of culture medium supplemented with CD (Wacker Chemie, Spain), during 144 h of incubation at 25 °C under a 16 h light/8 h dark photoperiod with a light intensity of 18.4 w m^{-2} in a rotary shaker at 110 rpm.

The effect of hydroxypropylated-β-cyclodextrins (CDH) or methylated-β-cyclodextrins (CDM) on phytosterol and tocopherol production was assessed by transferring cells into the culture medium supplemented with 25 and 50 mM CD at the beginning of elicitation experiments. Control treatments without elicitors were always run in parallel. Glu (Sigma, Spain) or Hex (Sigma, Spain) was added at 1 mg L^{-1} and 40 μM, respectively when it was required.

After elicitation treatments, cells were separated from the culture medium under a gentle vacuum and the phytosterol and tocopherol content was quantified in both the spent medium and the cells.

2.4. Extraction of phytosterols

Phytosterols were extracted from elicited culture media by phase partitioning with ethyl acetate. The organic phase was collected and evaporated at 40 °C in vacuum. Later, the residue was dissolved in methanol and considered as phytosterol enriched extracts. These extracts were filtered through 0.22 μm filters before injecting them into a gas chromatography-mass spectrometry (GC/MS) system.

The intracellular phytosterol content was extracted from 100 mg freeze-dried cells with 4 ml of methanol: water (9:1 v/v); this was warmed up during 8 min in the microwave at 80 W, and filtered through nylon filter. The process was repeated twice, and the methanol extracts were mixed and evaporated with N_2 gas. The aqueous phase was recovered, mixed with 6 ml ethyl acetate: water (2:1, v/v) and vortex until an emulsion of both phases was obtained. After recovering the organic phase, the aqueous phase was vortexed again with 6 ml ethyl acetate: water (2:1 v/v). Finally, the organic extracts were mixed and evaporated at 40 °C in vacuum. All samples were suspended in 1 ml methanol and filtered (0.22 μm) prior to GC/MS analysis.

2.5. Extraction of tocopherols

The intracellular tocopherol content was extracted from 1 g FW cells or 5 ml elicited culture medium with methanol: pyrogallol (6%) sonicating for 10 min. KOH to a final concentration of 6% was added and then, the samples were saponified at 70 °C for 30 min. After that, the extracts were mixed with 1.5 ml of NaCl (10%) and tocopherols were extracted by phase partitioning with 15 ml of n-hexane. The organic phase was evaporated at 40 °C in vacuum, and all samples were suspended in 500 μL methanol and filtered (0.22 μm) prior to GC/MS analysis.