Industrial Crops and Products 75 (2015) 24–28

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Extraction and characterization of brassinosteroids from residues of the biodiesel chain

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ARTICLE INFO

Article history:
Received 8 July 2014
Received in revised form 5 March 2015
Accepted 7 March 2015
Available online 18 March 2015

Keywords:
Brassinosteroids
Phytostimulation
Biodiesel
Biorefinery

ABSTRACT

Brassinosteroids are plant hormones whose stimulating activity on plant growth and development has been fully characterised and already investigated for agricultural applications. Straw and deoiled cakes, that are main residues from crops and processing for biodiesel production, of *Brassica carinata* and *Brassica napus* var. *oleifera* were analyzed for their content of brassinosteroids and compared to the concentrations detected in seeds. Brassinosteroids in these extracts were quantified by both HPLC analysis and bioactivity test, and a very high residual content was found in these by-products. *B. carinata* showed a higher concentration of brassinosteroids than *B. napus* var. *oleifera*. A new extraction method was optimized to accelerate the process. As brassinosteroids are hydrophobic compounds, then characterised by low water-solubility, the addition of HP-β cyclodextrin as coadjuvant was tested in order to improve their bioavailability in a commercial formulation for agricultural uses. These extracts were tested for their phytostimulating activity in pot-grown annual plants (lettuce and chard) under controlled conditions in a plant growth chamber. A significant increase of biomass yield was reached with both foliar and root treatments; no significant yield increased was observed when HP-β cyclodextrin was added. Then the formulation of a commercial bio-based product to be proposed as phytostimulant for agricultural uses is possible by extraction of brassinosteroids from residues of the biodiesel chain; this co-product can improve the addition value and reduce the environmental impact of a biodiesel biorefinery converting a byproduct, coproduct or residue in a source.

1. Introduction

Natural compounds characterised for their phytostimulating activity can be used in agriculture to increase crops yields and quality of products and to optimize the resource use efficiency. Brassinosteroids (BSs) are plant hormones whose positive effect on plant growth and development has been detected and characterised: they stimulate cell division and elongation, increase the efficiency of the photosynthetic activity, improve the reproductive development and regulate the flowering time, the seed development, viability and germination, modulate the plant response to biotic and abiotic stresses such as salt and drought tolerance, thermotolerance, oxidative stress tolerance, pathogen resistance, herbicide and pesticide tolerance (Vriet et al., 2012; Teixeira Zullo and Adam, 2002; Gruszka, 2013; Clouse and Sasse, 1998; Brosa, 1999; Khripach et al., 1999; Arora et al., 2008; Bajguz and Hayat, 2009; Friebe, 2006; Gill and Tuteja, 2010; Krishna, 2003; Nakashita et al., 2003). When tested for agricultural uses, a positive role of brassinosteroids has been reported for several crops, such as rice, wheat, soybean, bean, coffea, potato, tomato, orange, grapevine, spruce and also in fungi (Teixeira Zullo and Adam, 2002; Divi and Krishna, 2009; Morinaka et al., 2006; Kamuro and Takatsuto, 1999); they are active at very low concentrations and they are non toxic and environmental friendly (Khripach et al., 1999).

Brassinosteroids are present in all plant tissues at greatly different concentrations, with the highest levels in pollen, followed by seeds; the lowest levels are found in leaves and shoots (Khripach et al., 1999; Bajguz and Tretyn, 2003). In plant tissues they are usually present as glycosides or conjugated to fatty acids. Chemical synthesis of brassinosteroids is possible, but it is very expensive and time-consuming; moreover the biological activity of synthetic compound is lower than natural compounds (Ishiguro et al.,

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http://dx.doi.org/10.1016/j.indcrop.2015.03.019
0926-6690/

1980; Back et al., 1997; Brosa et al., 1998). Biological methods to produce brassinosteroids are known through crown gall cells cultures (USPatent 5,084,388).

Residues from the biodiesel chain processes are potentially a good, cheap and easily available source of natural brassinosteroids, as these lipophylic compounds are usually concentrated in high lipid content fractions such as oils; then the extraction of brassinosteroids can become an alternative use for these wastes, whose amounts produced in the world is increasing following the increasing production of biofuels.

The aim of the present work was to evaluate the extraction of brassinosteroids from the residues of the biodiesel chain (straw and deoiled cakes) to reach simultaneously a co-product of these processes and a reduced cost and environmental impact due to waste disposal; then these co-products, to be used as bio-based components of a commercial phytostimulant formulation for agriculture, could improve the value added of a biodiesel biorefinery. Isolation of brassinosteroids is a time-consuming and tedious task because of their very low concentrations in plant tissues (Gamoh and Takatsuto, 1994). In the present work a fast and efficient method to extract brassinosteroids from the residues of *Brassica napus* var *oleifera* and *Brassica carinata* was optimised and compared to current methods available in literature. Moreover, as brassinosteroids are hydrophobic compounds, then characterised by low water-solubility, a commercial formulation for agricultural uses has been tested in which HP-β cyclodextrin as coadjuvant was added in order to improve their water solubility and bioavailability (De Azevedo et al., 2002). The phytostimulating effect of these extracts and formulations was tested on pot-grown annual plants (lettuce and chard) under controlled conditions in a plant growth chamber.

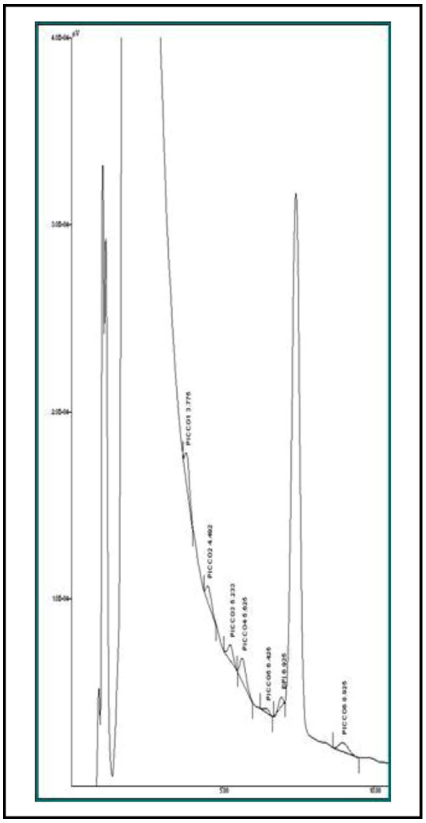
2. Materials and methods

2.1. Extraction of brassinosteroids

Brassinosteroids were extracted and analysed according to Gamoh et al., 1989: 70 g of ground plant material (seeds, cake or straw) from *B. napus* var *oleifera* (rapeseed) and *B. carinata* were extracted by shaking at 190 rpm with 200 ml methanol for one week, then with 200 ml 1:1ethylacetate:methanol for another week; the two extracts were combined.

The extracts were filtered on paper filters, then mixed to 400 ml 1:1 ethylacetate:water saturated with NaCl; the organic layer was then transferred to a clean bottle and the methanolic residue furtherly extracted with 100 ml ethylacetate, then added to the first organic layer. This extract was dried at 33 °C *in vacuo* and dissolved in 100 ml hexane, then extracted in 2 × 100 ml 9:1 methanol:water. The aqueous methanolic layer was separated and added with 200 ml ethylacetate and 150 ml saturated sodium hydrogencarbonate solution. The organic layer was transferred to a clean bottle and the residual aqueous methanolic phase furtherly extracted with 50 ml ethylacetate, then mixed to the first organic layer fraction. The organic phase was then concentrated at 33 °C *in vacuo*.

To this method, two new protocols, aimed to reduce the time necessary to reach a highly-efficient extraction of brassinosteroids, were compared. Then three extraction methods were compared:. Method 1 (EL): according to Gamoh et al., 1989, as described above; Method 2 (EB): as method 1, but both extractions lasted one day instead of one week; Method 3 (EB + S): as method 2, but 3 × 10 min sonication cycles (S100 Elmasonic, Elma Hans Schmidbauer GmbH & Co., KG), separated by 30 min intervals, preceded each extraction phase.



Peaks	tR (min)
1	4.1
2	5.0
3	5.8
4	6.3
5	7.4
6	7.8
7	10.4

Fig. 1. HPLC chromatograms and Retention time (tR) of brassinosteroids detected in *B. napus* var. *oleifera* seeds extracts.