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Chitosan elicits mono-glucosylated stilbene production and release in fed-batch bioreactor cultures of grape cells

Maura Ferri [a], Samuele C.F. Dipalo [b], Nello Bagni [a], Annalisa Tassoni [a,*]

[a] Department of Experimental Evolutionary Biology, University of Bologna, Via Irnerio 42, 40126 Bologna, Italy
[b] Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

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This work is dedicated to the memory of Prof. Nello Bagni, pioneer in plant biology research, who inspired this research and to whom we will always be indebted for the invaluable support as teacher and mentor.

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ABSTRACT

The present paper reports the study of the optimal conditions for grape (*Vitis vinifera* cv. Barbera) cell suspensions in batch and fed-batch bioreactor cultures, in order to specifically improve the production of mono-glucosylated stilbenes, which are resveratrol derivatives. These compounds are physiologically as active as free resveratrol in cardio- and chemoprotection, but are more stable and bioavailable after ingestion through diet. In fed-batch conditions the production of mono-glucosides was considerably increased together with that of free resveratrol. For the first time, an elicitor (chitosan) was tested in a bioreactor system, demonstrating its efficacy in inducing the production of stilbenes. The bioreactor culture conditions also allowed the accumulation of other polyphenols, such as catechins. The vast majority of the produced compounds was released into the culture media, which represents a relevant advantage for the recovery of specific molecules or of polyphenol-enriched mixtures. These results represent a further step toward the employment of grape cells in fed-batch cultures, as a promising alternative to whole plant extraction for the industrial production of plant polyphenols, considering the necessity for developing sustainable processes.

1. Introduction

Plants are the most abundant source for numerous compounds, including pharmaceuticals, nutraceuticals and food additives. In particular, polyphenols, which are one of the most widespread groups of plant secondary metabolites, are present in many crop plants and widely proven to be beneficial to health, because of their known antioxidant properties.

Polyphenols are synthesised through the very complex phenylpropanoid pathway, via phenylalanine ammonia lyase, that leads to the production of flavonoids, stilbenes and other phytochemicals (Iriti & Faoro, 2004; Winkel-Shirley, 2001). The production of *p*-coumaroyl-CoA represents an important branching point, supplying the biosynthesis of both flavonoids and stilbenes (Jeandet et al., 2002).

Flavonoids are ubiquitous in plants and occur both as glucosides and aglycones (Winkel-Shirley, 2001). Glucosylation increases their water solubility, allowing flavonoid storage in the plant cell vacuole and reduces their reactivity towards free radicals (Iriti & Faoro, 2004). Humans ingest significant quantities of flavonoids through the diet due to their widespread distribution and variety and heat stability. After ingestion, both glucosides and aglycones are absorbed in the small intestine, providing health benefits such as an increase in the antioxidant capacity of blood and potential prevention of cancer and cardiovascular diseases (Iriti & Faoro, 2004).

Flavonoid biosynthesis leads to several major subgroups, among which are catechins (Winkel-Shirley, 2001). Catechins are polyphenolic antioxidant metabolites particularly abundant in tea, wine, cocoa and chocolate. They are the building blocks for other compounds, such as condensed tannins and anthocyanidins, but after intestinal absorption monomers are the prevalent forms identified in the blood. Their effects on the cardiovascular system have been extensively studied in human and animal models and include, among others, an increase of plasma antioxidant activity, a decrease of LDL-cholesterol fraction and oxidative stress-derived substances and a decrease of blood pressure (Espín, García-Conesa, & Tomás-Barberán, 2007).

Over the last few years, extensive studies have been performed on resveratrol (RESV), a low molecular weight phytoalexin

* Corresponding author. Tel.: +39 051 2091278; fax: +39 051 242576.
E-mail address: annalisa.tassoni2@unibo.it (A. Tassoni).

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belonging to the stilbene family that occurs in both *trans*- and *cis*-isomeric forms in several plants including grape (*Vitis vinifera* L.). In plants, RESV is present at a basal level, but its synthesis can be induced by biotic and abiotic stress (Jeandet et al., 2002). Besides the antioxidant properties that provide protection against cardiovascular diseases such as arteriosclerosis (Das et al., 1999), it has been demonstrated that RESV acts as chemopreventive agent against several types of cancer by modulating tumour initiation, promotion and progression phases (Delmas, Lancon, Colin, Jannin, & Latruffe, 2006; Jannin et al., 2004). Moreover, RESV seems to extend the lifespan of diverse species including *Saccharomyces cerevisiae*, *Drosophila melanogaster* and mouse (Baur et al., 2006; Howitz et al., 2003; Valenzano et al., 2006). At present, RESV is under phase-II clinical trials to test its effect on the prevention of colon cancer (www.cancer.gov).

RESV is the most bioactive natural stilbene, although great attention has been given to RESV mono-glucosides such as piceid and resveratroloside (Waffo-Teguo et al., 1998). The mono-glucosylated stilbenes are physiologically as important as free RESV, possessing a relevant antioxidant activity that depends on the location of the derivatized hydroxyl groups, the type of sugar residue and the *cis*-/*trans*- configuration of the double bond (Orsini, Pelizzoni, Verotta, Aburjai, & Rogers, 1997). Moreover, glucosylation protects the aglycone from enzymic oxidation, extending the RESV half-life and bioavailability (Regev-Shoshani, Shoseyov, Bilkis, & Kerem, 2003). The first absorption step of the glucosylated form is the deglucosylation by intestinal β-glucosidases, to release free RESV (Aumont et al., 2001; Henry-Vitrac, Desmouliere, Girard, Merillon, & Krisa, 2006; Walle, Hsieh, DeLegge, Oatis, & Walle, 2004).

The levels of stilbenes and flavonoids in grapes and their derived products, especially red wine, follow from several factors such as grape cultivar, agronomic conditions, geographic region and oenological procedures. However, there is no standardised procedure to obtain RESV-enriched grapes that may be subsequently used to prepare nutraceuticals. Currently, increasing demand for natural nutraceutical, food, cosmetic and pharmaceutical compounds, such as resveratrol, makes their production from sustainable sources a necessity (Donnez, Jeandet, Clément, & Courot, 2009). In this context, the use of biotechnology is viewed as a particularly promising alternative to whole plant extraction to obtain valuable secondary metabolites under controlled conditions through plant cell cultures. *In vitro* cultures provide a source of homogeneous highly active cells that allow some plant limits such as slow growth, seasonal and environmental variations and diseases to be overcome. At present, only few plant cell culture processes for the production of bioactive compounds are undertaken commercially. The major problems hindering the development of large-scale culture of plant cells include low productivity, cell line instability and difficulty in the process of scale-up. Media, elicitors, culture conditions, bioreactor design and other critical parameters influence the behaviour of plant cell cultures, allowing the improvement of the production of one or few compounds that would not normally accumulate in the intact plant.

Grape cell cultures synthesise all the major polyphenols found in wine and whose production can be induced as a response to biotic and abiotic stimuli (Decendit et al., 2002; Ferri et al., 2007, 2009; Krisa et al., 1999; Vitrac et al., 2002; Waffo-Teguo et al., 1998). The further elicitation of plant-cell metabolism might represent a powerful strategy for increasing stilbene bioproduction (Donnez et al., 2009). One of the commonly known biotic elicitors is chitosan, a linear d-(1,4)-glucosamine polymer and an important structural component of the cell walls of several plant pathogen fungi (Hadwiger, 1999). The ability of chitosan to induce defense responses has been widely demonstrated in several plants (Iriti et al., 2006; Kim, Chen, Wang, & Rajapakse, 2005; Rabea, Badway, Stevens, Smagghe, & Steurbaunt, 2003), including grape (Aziz et al., 2006). Recently, Ferri et al. (2009) demonstrated that chitosan increases the production of stilbenes and anthocyanins in grape cell suspensions.

Currently, only a few reports have been published on grape cell bioreactor cultures (Aumont et al., 2004; Decendit et al., 1996; Honda et al., 2002; Tapia et al., 2009), probably due to specific issues such as low growth rate (Ferri et al., 2009), cell aggregation and adhesion to the vessel wall (Kieran, MacLoughlin, & Malone, 1997). Moreover, no elicitor has been tested in grape batch bioreactor.

In the present paper, a fed-batch process was set up, to optimise stilbene and catechin production in *V. vinifera* cv. Barbera cell cultures and, the effect of an elicitor (chitosan) in a bioreactor system was studied for the first time.

2. Materials and methods

2.1. Cell cultures and fermentative conditions

Liquid cultures of *V. vinifera* cv. Barbera petiole cells were obtained and propagated using Murashige and Skoog medium (MS) (Murashige & Skoog, 1962), with 1 mg l^{-1} benzylaminopurine (BAP), 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-d) and 10 g l^{-1} sucrose as the carbon source. Suspensions were subcultured every 2 weeks with an inoculum at 50% (v/v inoculum/fresh medium) (Ferri et al., 2009; Tassoni et al., 2005).

The cultures were carried out in a 1 l stirred-tank bioreactor (ADI 1025 Bio Console and ADI 1010 Bio Controller; Applikon Biotechnology, Schiedam, The Netherlands) and all the parameters were controlled by BioXpert Lite software (Applikon Biotechnology). Several trial bioreactor processes (not described in the present paper) were carried out to set up the best operational conditions such as impeller type, configuration, rotational speed, air flow rate, time and amount of fresh medium feeding and length of culture. Finally the following culture conditions were used: marine impeller (3 blades, 45° angle, 4 cm diameter; Applikon Biotechnology); temperature, air flow and stirring rate kept constant at 24 °C, 0.2 l min^{-1} and 100 rpm, respectively; pH and dO_2% were monitored but not adjusted.

Batch processes were performed in 800 ml of the MS medium previously described with the addition of 50 mg l^{-1} of rifampicin which not only reduces the risk of microbial contamination, but also elicits the synthesis of polyphenols (Ferri et al., 2007). Sucrose was supplied at concentrations of 10 or 30 g l^{-1}. Fourteen-day old grape cell suspensions were used to inoculate the bioreactor at 50% (v/v inoculum/fresh medium). Fed-batch fermentations were performed by feeding MS medium enriched with 30 g l^{-1} sucrose. After 14 days of batch process, approximately 400 ml of exhausted broth were removed and 400 ml of fresh medium plus an appropriate volume of water, were fed to restore a working volume of 800 ml. The new culture cycle was carried out for further 14 days with and without the addition of chitosan at the final concentration of 50 and 100 mg l^{-1} (Ferri et al., 2009).

2.2. Cell growth analysis

The biomass yield was estimated by measuring different growth parameters such as cell number, fresh weight, dry weight and viability. The cell number was evaluated using a Nageotte chamber. Fresh weight and dry weight were determined by filtration of suspension culture, using a nylon mesh filter (50 μm) and by drying fresh cells at 80 °C to a constant dry weight, respectively. Cell viability was measured by the selective labelling of viable cells with 75 μg ml^{-1} fluorescein diacetate and expressed as percentage of living/total cells (Darzynkiewicz, Li, & Gong, 1994).