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Chitosan treatment elicited defence mechanisms, pentacyclic triterpenoids and stilbene accumulation in grape (*Vitis vinifera* L.) bunches

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ABSTRACT

The stimulation of the plant response to pathogen attack by the application of resistance inducers, called elicitors, could represent an environmentally and commercially viable alternative or complement to existing pathogen control methods. In this work, the elicitor chitosan was sprayed on grape (*Vitus vinifera* L.) berries growing on the vine to shed light into the elicitation mechanisms underlying its application, with untreated bunches as controls. To gain a more comprehensive picture of the complex molecular processes elicited by chitosan, a proteomic approach was complemented by target and untargeted mass spectrometric analyses. The treatment altered the regulation of reactive oxygen species, with Cu/Zn superoxide dismutase and glyoxal oxidase showing up-accumulation. This might lead to an increased lignification via hypersensitive response mechanisms. Furthermore, enzymes involved in anthocyanin rather than stilbene phytoalexins accumulated in treated bunches. Stilbenes increased from 1.6 times (resveratrol) up to 3.8 times (piceid) over untreated bunches. The up accumulation of hydroperoxide lyase might lead to accumulation of oxylipins. Furthermore, the pentacyclic triterpenoids ursolate, oleanoate and betulinate increased by 1.25, 1.47 and 3.68 times in treated grape bunches ($p < 0.01$). Hence, the main processes underlying the response of grape fruits to chitosan treatment involved the accumulation of phenylpropanoid and triterpenoids phytoalexins, as well as the modulation of oxidative stress–related enzymes.

1. Introduction

In recent years, advances have been made in understanding the sequential events taking place during the induction of resistance strategies in plants as a response to pathogen attack, ultimately leading to the accumulation of defence gene products (Benhamou, 1996). The speed and extent of the plant response to intracellular signaling determines the outcome of a plant-pathogen interaction. Given this, the stimulation of a plant response by the application of resistance inducers, termed elicitors, could provide a biologically, environmentally and commercially viable alternative to existing pathogen control methods (Benhamou, 1996).

Elicitors are biological, chemical and physical external factors able to switch on enzymatic responses to biotic/abiotic stresses as well as the signaling pathway leading to the accumulation of secondary metabolites (Trivellini et al., 2016). Among the known elicitors, chitosan is included among the best prospects as a biocontrol agent (El Ghaouth et al., 1994). This compound is a polycationic β-1,4-linked D-glucosamine biopolymer that occurs naturally within the cell wall of several fungi, insect exoskeletons and crustaceous shells (Pichyangkura and Chadchawan, 2015), Indeed, chitosan has been widely studied as an efficient tool to inhibit/decrease microbial growth by inducing several defensive genes in plants (Pichyangkura and Chadchawan, 2015), including the agricultural crop grape (*Vitis vinifera* L.).

For example, the efficacy of chitosan to control grapevine diseases such as gray mold and powdery mildew has been previously demonstrated (Iriti et al., 2011; Romanazzi et al., 2002). Indeed, it has been shown that chitosan improves development and protects *V. vinifera* against *Botrys cinerea* (Barka et al., 2004). In addition to its antimicrobial properties, chitosan treatment was able to induce changes in protein expression profile and stilbene production (resveratrol and its mono-glucosylated piceid and resveratroloside) in grape cell

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suspensions (Ferri et al., 2009, 2011). Although changes in the grape proteome have been reported from cell culture in response to chitosan application (Ferri et al., 2014), very little and fragmentary information is available regarding the effect of chitosan as an elicitor in terms of the protein expression profile. Monitoring of the complete set of proteins could offer the opportunity to unravel the specific physiological and molecular mechanisms underlying stilbene and other phytoalexin increases caused by chitosan.

In addition, over the past twenty years, health-promoting metabolites in fruits have generated significant interest among scientists, food nutritionists and consumers, due to their pivotal role in supporting human health and longevity (Slavin and Lloyd, 2012). Grapes and their derivatives such as wine, juice and dried fruit, constitute a large source of healthy polyphenols for human diets (Ferri et al., 2014). In grapes, polyphenols are divided into two main classes: flavonoids (anthocyanins, dihydroflavonols, flavonols and falvanols) and non-flavonoids (hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols and stilbenes) (Fanzone et al., 2012; Murcia et al., 2017).

Among these secondary metabolites, stilbenes are of particular interest, since they are responsible for various beneficial effects (Gil-Muñoz et al., 2017). They have significant antifungal, antibacterial and antioxidant properties as well as strong neuroprotective and cardio-protective effects (Guerrero et al., 2009; Gil-Muñoz et al., 2017; Murcia et al., 2017). For these reasons, high amounts of stilbenes, in particular 3,4',5-trihydroxy-*trans*-stilbene (resveratrol) in commercial wines have been claimed to decrease risk of coronary heart diseases and other degenerative disorders (Fauconneau et al., 1997; Jang et al., 1997; Renaud and de Lorgeril, 1992).

Biosynthesis of secondary metabolites, such as stilbenes in grapes, depend upon many factors such as genetic materials, environmental conditions as well as cultural practices (Bavaresco et al., 2012) and can be stimulated by elicitors (Gil-Muñoz et al., 2017) thus contributing to basic nutritional characteristics of fruits (Lairon, 2010). Therefore, elicitors can be considered a meaningful strategy for a sustainable and high quality grapevine production. For this reason, several attempts have been made regarding stilbene elicitation by biological elicitors such as fungi (Dercks and Creasy, 1989; Schmidlin et al., 2008), chemical elicitors such as jasmonic acid and its ester methyl jasmonate, salicylic acid, abscisic acid (Bavaresco et al., 2009, 2016; Gil-Muñoz et al., 2017; Portu et al., 2016; Tassoni et al., 2005) and physical elicitors such as UV-C light radiation (González-Barrio et al., 2005; Cantos et al., 2003). The interest in the use of chitosan has also been growing as a means to providing more protection against plant pathogens since it has been shown to have a wide range of anti-fungal activity (Barka et al., 2004) while potentially increasing health-promoting compounds.

Taking this background into consideration, a spray treatment of chitosan at the beginning of the veraison stage of grape production was used with the aim to evaluate its role as elicitor by analysis of concentration of phytoalexins as well as the proteomic changes induced by treatment in fruit.

2. Results and discussion

Chitosan is known to form a semi-permeable film around plant tissues and to induce an inhibitory effect towards a number of pathogenic fungi through stimulation of host defense responses. The interaction of elicitor molecules such as chitosan with membrane receptors involves a complex response in which some events are triggered, thus resulting in altered gene transcription (Benhamou, 1996).

The technological parameters of grape berries, i.e. the ranges for titratable acidity (6.6–6.8 $g\,kg^{-1}$), pH (3.21–3.25) and soluble solids (22.7–23.3 °Brix), were not significantly affected ($p < 0.01$) by treatments. These results were in line with previous findings (Portu et al., 2016).

Proteomics has been used to investigate the response in grape skins under chitosan treatment, as a result of gene reprogramming occurring when plants are sprayed with this biopolymer. Supervised PLS-DA class prediction analysis allowed to clearly discriminating chitosan-treated from control proteins (Fig. 1), as confirmed by N-fold validation (overall accuracy = 100%). A loading plot generated from the PLS-DA analysis allowed determination of those proteins having the highest scores in the discriminant analysis. Discriminant proteins, determined from nanoscale liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry on grape bunch skin following chitosan treatment, are shown in Table 1.

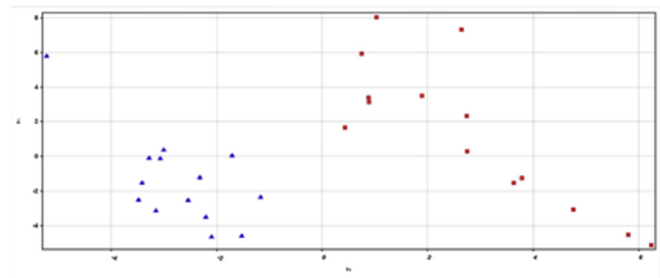

Fig. 1. Partial Least Squares Discriminant Analysis (PLS-DA) supervised score plot, carried out on the proteomic profile gained from chitosan treated bunches (triangle-shaped), as compared to control (square-shaped).

As shown, in the treated grapes, 59 proteins were identified as the most discriminant and differentially expressed proteins in PLS-DA, some up- and some down accumulated, compared to the control. From a general point of view, our findings highlighted a modulation of proteins related to oxidative stress response, protein turnover and energy metabolisms, photosynthesis, phenylpropanoids synthesis, as well as proteins generally related to plant stress response. Interestingly, a down-accumulation of enzymes involved in protein metabolism and energy processes has been reported also by Ferri et al. (2009, 2014). These authors postulated that the chitosan-related induction of defence mechanisms might lead to the consumption of cellular resources, including the diversion of substrates and energy from normal metabolic processes (Ferri et al., 2014).

Analyzing grape proteins after chitosan treatment, an increased accumulation of enzymes involved in secondary metabolism pathways, related to defense mechanisms, was observed. Although the simple identification and quantification of proteins from a tissue is not always sufficient for the understanding of the complex mechanisms occurring in biological systems, it is interesting to point out that most of changes were related to basic cellular or plant stress responses.

2.1. Oxidative stress

Seven differentially accumulated enzymes, involved in the response to oxidative stress, could be identified in treated grapes. Pathogen-derived elicitors initiate a very rapid production of reactive oxygen species (ROS), which act as signals to activate the stress response. Plant cells contain an array of protective and repair systems that, under normal circumstances, minimize the occurrence of oxidative damage. After the treatment with chitosan, an increase of two hydrogen peroxide (H_2O_2) related enzymes, namely copper/zinc superoxide dismutase (SOD) and glyoxal oxidase (GLOX), was observed.

SOD, the first enzyme in the detoxifying process, reacts with superoxide radicals to produce H_2O_2. Three different types of SOD, classified according to their metal cofactor, are known: copper/zinc (Cu/Zn SOD), manganese (Mn SOD) and iron (Fe SOD). These SODs are located in different compartments of the plant cells: Fe SODs are situated in the chloroplasts, Mn SODs in the mitochondria and peroxisomes, and Cu/Zn SODs in the chloroplast, the cytosol and possibly the extracellular space (Alscher et al., 2002). An up-accumulation of copper/zinc SOD and a down-accumulation of superoxide dismutase (SOD) could be observed in treated grapes.