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Chitosan and grape secondary metabolites: A proteomics and metabolomics approach

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Abstract. Chitosan is a polysaccharide obtained by deacetylation of chitin, and it is involved in defence mechanisms of plants toward diseases. In the present work, *V. vinifera* L. cv. Ortrugo, grafted on 420A rootstock was grown in pot and treated, at veraison, by 0.03% chitosan solution at cluster level. Just before the treatment (T0) and 24 hours (T1), 48 hours (T2), 72 hours (T3) and 10 days (T4) later, the concentration of stilbenic compounds was detected, and at T1 proteomics and metabolomics analyses were done. Proteomics relies on the analysis of the complete set of proteins existing in a given substrate, while metabolomics relies on the analyses of the complete set of metabolites in a given substrate. The treatment improved the stilbene concentration over the control at T1. Proteomic analysis showed that superoxide dismutase (SOD) and phenylalanine ammonia-lyase (PAL) were overexpressed in the treated grapes. SOD is known to be an enzyme active against reactive oxygen species (ROS) while PAL is a key enzyme in the phenylpropanoids pathway. Metabolomics analysis highlighted the positive role of the treatment in improving the triperpenoid concentration (betulin, erythrodiol, uvaol, oleanolate); these compounds are known to be effective against microbes, insects and fungi.

1. Introduction

The importance of chitosaccharides, as plant growth promoters and disease control agents has been recently emphasized [1,2]. Chitosan, a linear D-(1,4)-glucosamine polymer produced by deacetylation of chitin and an important structural component of several plant fungi cell walls, can be applied to plants as antimicrobial agent (against fungi, bacteria and viruses) and as elicitor of plant defence mechanisms [3,4]. As concerning the latter, evidences have been obtained on the stimulation of phenylalanine ammonia lyase (PAL), peroxidase and lopoxygenase activities as well as the accumulation of phytoalexins and PR proteins [5–7]. Their biological activity is most likely resulting from the binding to membrane receptors and it depends on the molecular weight and degree of N-acelylation of the molecule [8,9]; chitosan oligomers of low molecular weight were shown to be more effective in inducing defence responses than those of higher molecular weight. Moreover chitosan has been reported to enhance the systemic resistance [1].

V. vinifera plantlets and leaves treated *in vitro* by chitosan and infected by *Botrytis cinerea* and *Plasmopara viticola*, did not show any disease symptom [10,11]. Chitosan showed to be effective during table grape post harvest storage, by replacing the SO_2 treatments, resulting in the control of fungal diseases [12,13].

The aim of the research was to investigate the role of cluster treatments with chitosan on the plant (grapes) response in terms of enzymes regulation and secondary metabolites production, by applying new high performing technologies such as proteomics and metabolomics.

2. Materials and methods

Plant material. *V. vinifera* L. cv. Ortrugo clone PC ORT 81 (white berry color) grafted on *V. berlandieri* x *V. riparia* 420A rootstock was grown on pot (70 L volume) containing sandy soil. The vines were cane pruned and trained vertical shoot positioning (VSP) with 10 buds/vine. The pots were placed outdoor on a platform covered by a hail protection net, basic fertilizer supply was provided at bud burst and the soil was kept near field capacity by drip irrigation.

Treatments. Chitosan from shrimp shell, low viscosity, purchased from Sigma-Aldrich (50494-100G-F), was utilized for the spray treatments. After the preparation of the mother solution with acetic acid, which was kept overnight at −20° C, a 0.03% chitosan solution was sprayed at cluster level at veraison, by treating all the clusters of 8 vines; untreated vines (8) were kept as control, by spraying the clusters only with 0.1% acetic acid.

Tests. The following tests were performed, on both control and treated clusters, at time 0 (corresponding to veraison, just before the treatment), time 1 (24 h after the treatment), time 2 (48 h after the treatment), time 3 (72 h after the treatment), time 4 (10 days after the treatment): total soluble solids (° Brix), pH, stilbenic compounds; only at T1 proteomics and metabolomics analyses were done.

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Stilbenes were extracted from frozen berries by the method of Bavaresco et al. [14] by using methanol 95% and phase partitioning; a 1200 series liquid chromatograph system, equipped with a quaternary pump and an electrospray ionization system, and coupled to a G6410A triple quadrupole mass spectrometer detector (Agilent, Santa Clara, CA, USA) was used. Chromatographic separation was performed by a Synergy Fusion C 18 column from Phenomenex. The LC mobile phase A consisted of 0.05% ammonium formate in water, while mobile phase B was 0.05% ammonium formate in methanol. The gradient started with 50% B, to be increased till 75% at 5 minutes, 80% B at 7.5 minutes, 85% B till 20 minutes; the flow was 0.2 mL/min, at a temperature of 45° C. The electrospray source was set using N at 340° C, flowing at 10 L/min, and operating in the negative ionization mode (Vcap = 4000 V).

Proteomics. After extraction of the proteins from the berry skins using TCA/acetone, followed by reduction and alkylation, the analysis of tryptic peptides was performed by using a shotgun MS/MS approach, by a hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer coupled to a nano LC Chip Cube source. MS/MS spectra analysis of peptide identification allowed protein inference, against the proteome of *V. vinifera*, using Spectrum Mill MS Proteomics Workbench.

Metabolomics. An untargeted screening was performed via high-resolution mass spectrometry by using a hybrid Q-TOF spectrometer coupled to an UHPLC chromatographic system. MS acquisition was performed in positive mode, in the range 100–1200 m/z and compounds identification carried out according to isotopic patterns (accurate mass, isotopic spacing, isotopic ratio).

Partial Least Square-discriminant analysis (PLS-DA) was the statistical approach used for both proteomics and metabolomics, and differential metabolites/proteins were then exported from covariance structures.

3. Results and discussion

TSS increased from 11.4° Brix at T0 till 16.2° Brix at T4, while pH changed from 2.78 (T0) to 3.08 (T4), as expected (data not shown).

As concerning the stilbenes, resveratrol, resveratroloside, piceid and pterostilbene were detected, and only at T1 the treatment induced a significantly higher concentration of resveratroloside, while no statistical differences were calculated at the other sampling times and for the other compounds (Table 1a, Table 1b).

Proteomics results are reported in Table 2, where proteins expressed at a score (WC0) above 0.2 and below −0.2 are listed since they are considered the most prominent for class prediction. The proteins with WC0 above 0.2 were more expressed in the control (untreated) samples, whilst the proteins with WC0 below −0.2 were more expressed in the treated samples. According to Table 2 the following proteins were more expressed under chitosan spray treatment: superoxide dismutase, proteasome subunit beta type, nascent polypeptide-associated complex subunit beta, ATP-dependent Clp protease proteolytic subunit, phenylalanine ammonia-lyase (PAL). Each of those is related to biochemical function (pathways) which can be identified by screening the Uniprot database. For instance the treatment was involved in oxidative stresses (superoxide dismutase increasing, providing an action against ROS) and in the phenylpropanoid pathway through PAL enzyme. Phenylpropanoid pathway is involved in the biosynthesis of flavonoids and stilbenes.

Table 1a. Resveratrol and resveratroloside in grapes, depending on the sampling time and the treatment.

Sampling Time	Tr.	Resveratrol (μg/ kg f.w.)	Resveratroloside (μg/ kg f.w.)
T0	C.	25 a	344 a
	T.	12 a	224 a
T1	C.	16 a	223 b
	T.	18 a	452 a
T2	C.	17 a	360 a
	T.	15 a	370 a
T3	C.	24 a	397 a
	T.	13 a	426 a
T4	C.	16 a	352 a
	T.	13 a	371 a

Tr.: Treatment; C.: Control; T.: Treated. Letters represent comparison between control and treated grapes per each sampling time. Values followed by a different letter are significantly different at $p < 0.05$.

Table 1b. Piceid and Pterostilbene in grapes, depending on the sampling time and the treatment.

Sampling Time	Tr.	Piceid (μg/ kg f.w.)	Pterostilbene (μg/ kg f.w.)
T0	C.	1417 a	83 a
	T.	1405 a	90 a
T1	C.	1430 a	92 a
	T.	1480 a	74 a
T2	C.	1332 a	68 a
	C.	1238 a	102 a
T3	T.	1482 a	73 a
	C.	1259 a	67 a
T4	T.	1298 a	65 a
	C.	738 a	64 a

Tr.: Treatment; C.: Control; T.: Treated. Letters represent comparison between control and treated grapes per each sampling time. Values followed by a different letter are significantly different at $p < 0.05$.

The main metabolomics results are reported in Table 3. where it is shown that some triterpenoids (botulin, erythrodiol, uvaol, oleanolate) increased very much under the chitosan treatment (up to 65 fold change). Triterpenoids including steroids are a highly diverse group of natural products widely distributed in plants. including grapevine [15]. Plants often accumulate these compounds in their glycosylated form – saponin, which are considered defensive compounds against pathogenic microbes, insects or fungi [16]. Triterpenes are synthesized via mevalonate pathway [17] and their accumulation is known to be triggered by biotic and abiotic stresses [18].

4. Conclusions

Chitosan spray treatment at cluster level was able to enhance phenylpropanoid and mevalonate pathways, while further investigations are needed to check the role of the metabolites produced from those pathways on the defence reactions of the vine toward diseases.

Table 2. List of differential proteins.

PLS-DA important proteins		
Compound	**WC0**	**Mass**
Peroxidase 4	0.217184	34516.2
Superoxide dismutase	−0.22816	31569.7
Pathogenesis-related protein 10	0.266685	17356.5
Cytochrome b-cl 7 complex subunit 7	0.211336	14706.9
Chalcone-flavonone isomerase 1	0.214391	25310.5
Aspartate aminotransferase	0.271999	45286.1
Proteasome subunit beta type	−0.23107	23205.3
Nascent polypeptide-associated complex subunit beta	−0.2325	17173.2
ATP-dependent Clp protease proteolytic subunit	−0.24473	31202.3
Sucrose synthase	0.269306	92937.3
Phenylalanine ammonia-lyase	−0.22497	78591.9
Carotenoid cleavage dioxygenase 1	0.262366	61965.1
Chalcone-flavonone isomerase family protein	0.244305	27747.5
Ribulose bisphosphate carboxylase small chain	0.244345	20713.5
Serine/threonine-protein kinase	0.24413	98336.8

Table 3. Enhanced accumulation of triterpenoids shows fold change in treatment samples as compared to control.

Fold-change	
Compound	**FC (abs) ([treated] vs [control]): Raw**
Betulin	64.67152
Erythrodiol	64.67152
Uvaol	64.67152
Oleanolate	11.47518

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