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Short communication

Identification of potential protein markers of noble rot infected grapes

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ABSTRACT

The evaluation of *Botrytis cinerea* as noble rot on withered grapes is of great importance to predict the wine sensory/organoleptic properties and to manage the winemaking process of Amarone, a passito dry red wine. This report describes the first proteomic analysis of grapes infected by noble rot under withering conditions to identify possible markers of fungal infection. 2-D gel electrophoresis revealed that protein profiles of infected and not infected grape samples are significantly different in terms of number of spots and relative abundance. Protein identification by MS analysis allowed to identify only in infected berries proteins of *B. cinerea* that represent potential markers of the presence of the fungus in the withered grapes.

1. Introduction

Grape withering, a technological process used to produce the so-called "passito" wines, allows the partial removal of water from the grape berries, with a consequent weight loss up to 40%. During this process, *Botrytis cinerea* can colonize grape berries. This fungus, under favorable conditions, can develop as noble rot, inducing chemical modifications that lead to a grape composition change (Costantini, Bellincontro, De Santis, Botondi, & Mencarelli, 2006; Guarrera, Campisi, & Asmundo, 2005; Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud-Funel, 2006; Tosi et al., 2012).

The presence of the fungus affects specifically the aroma of some wines such as Tokaji Aszu, Sauternes, and Trockenbeerenauslese, called "botrytized wines" (Ribéreau-Gayon et al., 2006), that are intentionally produced from grapes infected by *B. cinerea*. On the other hand, the oxidase activity of the *B. cinerea* enzymes can affect the organoleptic properties of other wines, inducing a loss of color, alterations of flavor and aroma, not appreciated by many consumers (Li, Guo, & Wang, 2008)

Amarone is the most important "passito" red wine mainly produced from non-botrytized withered Corvina grapes. The presence of noble rot however, cannot be excluded for sure, since *B. cinerea* can grow in the thermo-hygrometric conditions occurring in the withering process. Although the noble rot impact on aroma and sensory quality of Amarone has been investigated (Fedrizzi et al., 2011; Tosi et al., 2012), so far only few studies have been carried out on the effects of *Botrytis* infection on withered grape proteins (Vincenzi et al., 2012)

Since the noble rot infection used for the Amarone production cannot be easily assessed (being the mold not visible on red berries), the possibility to detect *B. cinerea* on grapes before the winemaking could be of great importance for the producers in order to predict the wine sensory/organoleptic properties and to manage the winemaking process.

In this study, a proteomic analysis using two-dimensional electrophoresis (2-DE) combined with mass spectrometry (MS) was carried out to compare healthy *vs* noble rot infected grapes. The aim of this study was to investigate the effects of noble rot infection on the protein pattern of Corvina withered grapes and to identify possible markers of infection. These could be used to assess the presence of noble rot infected berries before the process of winemaking. Such information could be very valuable for the producers to correctly manage the winemaking process.

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2. Materials and methods

2.1. Grape sample preparation and infection by B. cinerea

Grapes (cv. Corvina) were collected from vineyards of Valpolicella area (Italy). The infection of grape berries was performed using a suspension of *B. cinerea* strain B2 as described by Lorenzini, Azzolini, Tosi, and Zapparoli (2013). A total of 100 infected and 100 healthy berries were incubated under withering conditions until they reached 35% of weight loss.

2.2. Chemical and enzymatic analysis of grapes

The analyses were carried out on juices obtained from the berries. Organic acids, glycerol, glucose, and fructose were measured enzymatically using a commercial kit (La Roche, Basel, Switzerland). Laccase activity was determined as previously described (Tosi et al., 2012).

2.3. Protein extraction

Frozen healthy and botrytized grapes berries were separately powdered by grinding in a mortar with liquid nitrogen. Proteins were extracted from 2 g of the resulting powder as described by Di Carli et al. (2011). The final dried protein pellet was dissolved at room temperature in IPG buffer (7 M urea, 2 M thiourea, 2% triton 100×, 65 mM dithiothreitol). The supernatant obtained after centrifugation at 20,000*g* for 15 min was used for 2-DE analysis.

2.4. Protein quantification

The protein content was quantified with the BCA™ Protein Assay Kit (Pierce) and bovine serum albumin (BSA) as a standard. A microplate reader (Bio-Tek Instruments) was used. Quantification was performed in triplicate.

2.5. Two-dimensional electrophoresis (2-DE)

IPG strips (11 cm, 3–10 linear pH gradient, Bio-Rad) were passively rehydrated with 100 μg of protein in 200 μL of IPG buffer, for 10 h. IEF was carried out at 20 °C using a Protean IEF Cell (Bio-Rad) and the following program: 5 h linear gradient at 500 V, 4 h rapid gradient at 1000, 8000 V rapid gradient until 32,000 V/h was reached. After the first-dimension, the IPG strips were equilibrated in a buffer containing urea 6 M, glycerol 30%, SDS 2%, Tris–HCl 50 mM pH 8.8 and DTT 1%, and then in the same buffer containing iodoacetamide 2.5%, instead of DTT. The second dimension was carried out using 16% polyacrylamide precast Criterion Strain Free Gels (Bio-Rad) and a Criterion Cell system (Bio-Rad). Separation was performed at 30 mA constant current. Gels were stained with Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO). Five technical replicates for each condition (berries infected and not infected with *B. cinerea*) were performed.

2.6. Gel image analyses

The images were recorded using an Epson Expression 1680 Pro scanner (Seiko-EPSON Corp., Japan) with 16 bit dynamic range and 300 dpi resolution.

Gel image analysis was performed using the Proteomweaver® software (Bio-Rad, Hercules, CA).

The quantitative comparison of the spots was carried out by the scanner-generated spot volume that was expressed as a numeric value of optic density after subtraction of background. Spot intensities were normalized by the software to compare the different gels. Normalization was performed by an algorithm and did not require any internal standard: the software computes an intensity factor for every gel, which makes all the normalization factors as close to one as possible. For every gel match, the ratio between the pair matched spots was calculated. The normalization factor is the median of these ratios. After completion of spot matching in 2-DE gels, the normalized intensity values of individual protein spots were used to compare the protein quantitative levels between the two groups (treated *vs* untreated).

2.7. Statistical analysis

A paired *t*-test analysis was performed and significance was accepted at $p < 0.05$. To provide high statistical confidence in the subsequent identification of proteins affected by the treatment, MS analysis was conducted only on spots whose difference in intensity between groups reached both statistical significance and at least 1.5-fold-change. Multivariate analysis was also performed to investigate patterns in protein profiles. Principal component analysis (PCA) was performed using the statistical software Statgraphics (Statpoint Technologies, Inc., USA).

2.8. In-gel digestion and LC–MS/MS analysis

Spots were excised from 2D gels and protein digested with sequencing grade-modified trypsin (Promega) as described in (Tibaldi, Arrigoni, Brunati, James, & Pinna, 2006). Samples were then dried under vacuum and suspended in 10 μL of water/0.1% formic acid (FA) for MS analysis.

LC–MS/MS analyses were performed as previously described (Tolin, Pasini, Simonato, Mainente, & Arrigoni, 2012) using a 6520 ESI-Q-TOF mass spectrometer (Agilent Technologies) coupled to a nano-HPLC 1200 series through a Chip-Cube interface (Agilent Technologies).

A large Capacity Chip was used and 2 μL of samples were injected into the column at a flow rate of 4 μL/min. Peptides were separated in the C18 nano-column (40 mm × 75 μm) at a flow rate of 0.3 μL/min using Water/FA 0.1% (eluent A) and ACN/FA 0.1% (eluent B). Chromatographic separation was achieved by a linear gradient of eluent B from 5% to 50% in 15 min. The instrument operated in a data dependent mode: MS/MS spectra of the 3 most intense ions were acquired for each MS scan. Data files were analyzed using Proteome Discoverer Software (version 1.4, Thermo Fisher Scientific) connected to a Mascot Search Engine server version 2.2.4 (Matrix Science, London, UK). Spectra were searched against an in-house database obtained by a *Vitis vinifera* database (version November 2013, 80431 entries) concatenated with the protein databases of *B. cinerea*, and a database of the most common contaminant proteins usually found in proteomics experiments. Data were filtered to exclude MS/MS spectra containing less than 5 peaks and with a total ion count lower than 50. Enzyme specificity was set to trypsin with 1 missed cleavage, while precursor and fragment ions tolerances were set to 10 ppm and 0.05 Da, respectively. Carbamidomethylation of cysteine residues was set as fixed modification and oxidation of methionine as variable modification. A False Discovery Rate (FDR) of 5% and 1% was calculated by the software based on the search against the corresponding randomized database. Peptides were classified as high (99%) and medium (95%) confidence, according to the corresponding FDR. Only proteins identified with at least 2 independent, unique peptides were considered as positive hits. Proteins were grouped into protein families according to the principle of maximum parsimony.