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ABSTRACT

Viral infections are known to have a detrimental effect on grapevine yield and performance, but there is still a lack of knowledge about their effect on the quality and safety of end products.

Vines of *Vitis vinifera* cv. Nebbiolo clone 308, affected simultaneously by Grapevine leafroll-associated virus 1 (GLRaV-1), Grapevine virus A (GVA), and Rupestris stem pitting associated virus (RSPaV), were subjected to integrated analyses of agronomical performance, grape berry characteristics, instrumental texture profile, and proteome profiling.

The comparison of performance and grape quality of healthy and infected vines cultivated in a commercial vineyard revealed similar shoot fertility, number of clusters, total yield, with significant differences in titratable acidity, and resveratrol content, and in texture parameters such as cohesiveness and resilience.

The proteomic analysis of skin and pulp visualized about 400 spots. The ANOVA analysis on 2D gels revealed significant differences among healthy and virus-infected grape berries for 12 pulp spots and 7 skin spots. Virus infection mainly influenced proteins involved in the response to oxidative stress in the berry skin, and proteins involved in cell structure metabolism in the pulp.

INTRODUCTION

Due to its economic importance, the wine-making industry occupies a leading position in Italian agriculture. Some of the world's most appreciated and valued red wines, such as Barolo and Barbaresco, are produced in Piedmont from Nebbiolo grapes. Grapevines in this region are threatened by several viruses and virus-like pathogens [1]. Among these, *Grapevine leafroll associated virus-1* (GLRaV-1; family *Closteroviridae*, genus Ampelovirus) and *Grapevine virus A* (GVA; family *Betaflexiviridae*, genus *Vitivirus*), are the causal agents of two important diseases, leafroll (LR) and rugose wood (RW) diseases, respectively. *Rupestris stem pitting associated virus* (RSPaV; family *Betaflexiviridae*, genus *Foveavirus*), also present in north-western Italian vineyards, induces few, if any, symptoms in singly-infected *Vitis* spp., but, as it replicates in the plant, it can contribute to the development of RW in the presence of other viruses, such as GVA [2]. Since no natural resistance to viruses has been identified in *V. vinifera*, the management of viral diseases relies on preventive cultural practices and the mandatory use of certified virus-free propagation material [3]. Programs to eradicate the infections are ongoing; meanwhile, however, berries from infected plants are routinely used for winemaking.

The effects of viral infections on grapevine agronomic and oenological performance are still under investigation, since the plants' response to infection may be strongly influenced by the virus strain, the plant genotype, and the environment. Leafroll-infected grapevines show a reduction in the overall rate of photosynthesis [4], and a marked reduction in yield in susceptible cultivars [5]. Moreover, a significant decrease of the overall quality of both grapes and musts is reported for LR- and RW-infected vines [6-10].

A detailed knowledge of the molecular effects of viral infection in grapevines is still lacking, despite the recent completion of *Vitis* genome sequencing programmes [11,12]. In general, evolutionary studies suggest that viruses manipulate host cellular processes for their life cycle [13,14]. In the grapevine, GLRaV-3 infection induces an increased expression of several genes involved in a wide spectrum of biological functions, including cell defence [15]. In compatible virus-infected grapevines, an increase in the expression of senescence-associated genes, and the accumulation of some proteins with known allergenic properties [16] are known to occur [17].

During the last decade, the number of proteomic studies devoted to the investigation of grape physiology and wine characteristics has boosted. The main issues investigated to date are grape berry ripening, grapevine response to biotic stresses, mainly drought and salinity, and the formation of haze in white wine following thermal abuses. Comprehensive reviews on these issues have been published in 2002 [18,19], 2007 [20], 2010 [21]. Despite its potential, proteomics has rarely been applied to study plant-virus interactions [22,23], and the questions surrounding fruits have not been addressed through this method.

This study reports the first investigation on agronomic performance, fruit texture and composition, and proteomic changes occurring in berries of virus-infected Nebbiolo grapes grown in field conditions.

MATERIALS AND METHODS

Vineyard.

The experimental vineyard was located in the Langhe area (Neive, Cuneo, Italy), a hilly viticultural area characterized by a loamy-clay soil. The vineyard, north-west oriented, was planted in 1992 with infected Nebbiolo vines, clone 308, derived from one originally infected mother plant carrying GLRaV-1, GVA and RSPaV in mixed infection. In the same vineyard, the healthy progeny of clone 308, free of viruses by heat treatment [6], was planted in the adjacent row (2.5 m distance). All vines were grafted onto healthy 420 A rootstock, Guyot pruned and vertically trained. Plant density was about 5000 vines/hectare. The vineyard was subjected to ordinary agronomical and disease-control practices.

Plant selection.

In winter 2007, dormant canes were collected from 30 plants of both the Nebbiolo clone 308 infected progeny and the healthy progeny. Total RNA was extracted from 0.1 g of cortical scraping using the Concert™ Plant RNA Isolation Reagent (Invitrogen) following the manufacturer's instructions. The phytosanitary status of each plant was checked by Reverse transcription (RT)-PCR of several grapevine-infecting viruses (ArMV, GFLV, GFkV, GLRaV-1, GLRaV-2, GLRaV-3, GVA, GVB and RSPaV) according to Gambino et al., 2006 [24]. Twenty healthy and twenty infected plants, carrying GLRaV-1, GVA and RSPaV in mixed infection, were selected and labelled for observations throughout the 2008 growing season.

Sampling.

Observations were carried out throughout the 2008 growing season on the 20 healthy and 20 infected plants chosen.

Virus detection and quantification. In July 2008, three basal and three fully expanded apical leaves were harvested from the first three shoots of infected and healthy plants. At harvest, leaf samples for viral quantification were collected only from infected vines.

Berry chemical composition, physical characteristics and proteomics. At harvest, berries were sampled from the chosen vines according to the different targets of the experiment. The berries were randomly picked with attached pedicels from both sides of each cluster to avoid the effect of shadowing. Berry juice chemical composition was measured on 30 berry samples for each

individual vine. Three groups of three adjacent plants among those chosen for each sanitary status were selected for berry phenol composition (500 berry pools) and instrumental texture analysis (500 berry pools). For each instrumental texture test, 30 berries were randomly selected from the 500 berry pools, after visual confirmation that they were intact. For proteomic analysis, 400 berries (20 berries from each individual plant) were immediately frozen in the vineyard using dry ice, after washing with tap water, and stored at -80°C until analysis.

Virus diagnosis and quantification.

Total RNA was extracted from 0.1 g of midribs using the Concert™ Plant RNA Isolation Reagent (Invitrogen) following the manufacturer's instructions. The health status of each selected plant sampled in July was checked by Reverse Transcription (RT)-PCR, with primers specific for several grapevine-infecting viruses (ArMV, GFLV, GFkV, GLRaV1, GLRaV-2, GLRaV-3, GVA, GVB and RSPaV), as indicated in [24]. GLRaV-1 and GVA titres in the infected plants were measured by reverse transcription TaqMan Real Time PCR (qRT-PCR) on the RNA-dependent RNA polymerase gene of each virus, and expressed as viral genome units per *V. vinifera* glyceraldehyde-3P-dehydrogenase (GAPDH) transcript copy, following indications given in [25]. For the quantitation of the GVA isolate infecting the Nebbiolo 308 clone, specific primers and a specific probe were designed, namely GVAPrep347F (5'-CCTACACTCAGCCCGCAAA-3'), GVAPrep385R (5'-GCGAGTCCTCGGTTTTCGA-3'), and the probe GVAPrep367P (5'-CCTTGGCTGCTGAGAT-3).

Agronomical performances and berry chemical composition.

The agronomical performances (bud burst index, shoot fertility, yield, cluster number and weight, and berry weight) of healthy and infected vines were assessed individually on the 20 selected vines throughout the growth season. Bud burst index was analysed in spring as indicated in [26]. Bud burst index indicates the degree of bud development after winter dormancy on a scale from 1 to 5, the shoot fertility is the number of inflorescences on each shoot at spring, yield is the weight of grapes per vine at harvest.

Soluble solids were measured by a portable refractometer, titratable acidity and pH were measured using the International Organization of Vine and Wine methods [27].

Phenolic compounds in berry skin extracts were evaluated using an UV-1601PC spectrophotometer (Shimazdu Scientific Instruments) as proposed in [28]. Total anthocyanins were expressed as malvidin-3-glucoside chloride and total flavonoids were expressed as (+)-catechin. Resveratrol was determined by HPLC applying the chromatographic condition proposed in [29]. Total anthocyanidins, flavonoids and resveratrol were quantified using external standards supplied by

Extrasynthèse (Genay, France).

Briefly, the berry skins were manually removed and dried with adsorbent paper. Afterwards, they were quickly immersed in 25 mL of a pH 3.2 buffer solution containing 12% ethanol and 600 mg/L sodium metabisulphite. After homogenization at 6000 *g* for 1 min with an Ultraturrax T25 high-speed homogenizer (IKA Labortechnik), the extracts were centrifuged at 3000 *g* for 10 min at 20ºC. The supernatants were then used for the determination of phenolic compounds.

Instrumental texture analysis.

A Universal Testing Machine TAxT2i Texture Analyzer (Stable Micro System, Godalming, UK) equipped with a HDP/90 platform and a 5 kg load cell was used for mechanical testing of skin and whole berries. Data were acquired at 400 Hz and evaluated using the Texture Expert Exceed software version 2.54.

The skin hardness, evaluated as resistance to probe penetration, was assessed by a puncture test using an SMS P2/N needle probe as described in [30]. The skin hardness was expressed in terms of skin break force (N), skin break energy (mJ) and resistance to axial deformation (Young's modulus, N mm^{-1}). The skin thickness (µm) was assessed using a P2 flat probe and a speed test of 0.2 mm s^{-1}, as in [31], on a skin fragment (ca. 0.25 cm^2) manually excised from the side of each berry.

For the Texture Profile Analysis (TPA) test, each whole berry was compressed in the equatorial position with an SMS P/35 flat probe under 25% deformation, with a waiting time between the two bites of two seconds, using 1 mm s^{-1} as speed test [30].

Typical texture parameters, *i.e.* hardness (N), cohesiveness (adimensional), gumminess (N), springiness (mm), chewiness (mJ) and resilience (adimensional) were provided by the software as described in [31].

Protein extraction and 2-D electrophoresis.

100 berries (5 berries per plant) were cut, deseeded and peeled while frozen, the mesocarp (pulp) and pericarp (skin) were separated and finely ground in a mortar with a pestle, working in liquid nitrogen to ensure sample conservation. The resulting powder was lyophilized and stored at -20°C until analysis.

Proteins were extracted from the berry skin powder (0.3 g) following Saravanan and Rose's phenol based protocol [32], while for the berry pulp powder (2 g) Sarry's protocol was used [33]. Final pellets were re-suspended in IEF rehydration solution (urea 7M, thiourea 2M, 4% CHAPS, DTT 130mM, 0.2% IPG buffer 4-7, 0.5% IPG buffer 3-10). Total protein concentration was assessed using Plus One 2DQuant kit (GE Life Sciences).

IEF was carried out with 60 µg of proteins per strip using 7 cm long ReadyStrips IPG strips, pH 3-

10 Non Linear (Biorad) in an Agilent 3100 OFFGEL Fractionator up to 13 kVh, after passive rehydratation for 12h. Running conditions per strip were: max voltage 5 kV; max current 50 µA. Strips were then equilibrated under gentle agitation, on a stirrer for 15 minutes, twice, in standard equilibration buffer containing 2% DTT the first time, and 2.5% iodoacetamide the second time.

SDS-PAGE [34] was performed on 8-16% gradient polyacrylamide mini gels using a Mini PROTEAN Tetra cell apparatus (Biorad) at RT. Running conditions per gel were: 10 mA for 10 minutes, 15 mA for 10 min, 20 mA until bottom of the gel. LMW-SDS marker kit (GE Bioscience) was used as molecular mass standard. Gels were stained with colloidal CBB G-250 [35]. Gel images were acquired using a ProXpress 2D (PerkinElmer) cooled CCD camera equipped with ProScan software package. The acquisition was performed by bottom illumination with the use of one UV-to-visible light converter plate. Flat field correction was used to minimize variance in gel acquisition. The chosen emission filter was ND/2, with exposure time of 2 seconds, 33 µm resolution and 16-bits.

The PDQuest software package (Biorad) was used for image analysis. Three replicate gels were run from each sample. Gel images were filtered, and spots were detected using the spot detection tools. The first matching among gels was run automatically, and then about 20 landmark spots were added to refine the match, which was finally checked manually and eventually corrected. The normalized volume of each spot was calculated dividing spot volume value by the sum of total spot volume values. Total spot volume was calculated by the software, and this referred to the sum volume of all the spots on the gel. Statistical analysis on the resulting normalized spot values was performed as described below.

Mass spectrometry analysis.

Significantly different spots were excised from the gel and prepared for mass spectrometry as described in [36]. Spectra of protein digests were obtained using a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, USA) in a positive reflecton mode in the range 680-4200 Da. The spectra were analyzed using the Flex-Analysis 3.3 software package (Bruker Daltonics) and calibrated internally with the autoproteolysis peptides of trypsin. Before database search was performed, the spectra were depleted of contaminating peaks deriving from both trypsin autodigestion and the digestion of a blank piece of the gel.

The MS-Fit (http://prospector.ucsf.edu) software package was used to interpret the MS spectra, through the PMF method [37]. Data were searched against the nrNCBI2010.09.24 database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), including 11894394 entries, on *Vitis vinifera*, *Saccharomyces cerevisiae*, a type of yeast which could be normally found in vineyard, and on GLRaV-1, GVA and RSPaV viruses. The parameters used for the search were: S-carbamidomethyl

derivate on cysteine, as fixed modification, oxidation on methionine as variable modification, and up to two missed cleavage sites for trypsin digestion. The peptide mass tolerance was 20 ppm. After identification, theoretical molecular mass, pI of proteins and GO terms were calculated by processing sequence entries at http://www.expasy.org/tools/pi_tool.html and proteins were assigned to a functional category (FunCat) by the Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de/projects/funcat) according to their role described in the literature.

Statistical analyses.

Agronomical performance, berry composition, and instrumental texture analysis parameters. Data obtained by measuring parameters were analyzed by t-test of infected versus uninfected samples. Data expressed as percentage (P%) were subjected to angular transformation [arcsin (√P%)] before t-testing. Sprouting index, shoot fertility and number of clusters per vine were compared by the Mann-Whitney non-parametric U test.

2-D Electrophoresis. The normalized spot intensity data were exported from the software of origin and analyzed using SAS statistic software. Normalized spot volumes were analyzed by ANOVA, and significantly different spots ($p \leq 0.05$) were selected if detectable in at least two out of three replicates, having a minimum volume of at least 500 ppm, and showing at least a three-fold variation.

RESULTS

Plant health status and virus quantification.

RT-PCR results confirmed the presence of GLRaV-1, GVA and RSPaV in the infected plants, and the healthy status of control plants. Mean loads of GLRaV-1 and GVA in the infected plants were 2.5 (SD=0.8) and 0.4 (SD=0.2) viral genomes/100 GAPDH transcript copies, respectively. The distribution of the viral loads was uniform among the infected plants (Supplemental Table S1).

Vine agronomical performances and berry chemical composition.

The infected plants showed a significant decrease in the bud burst index and lower (though not significantly so) shoot fertility and total yield, compared to healthy controls. The berry weight of infected vines was significantly higher than that of healthy plants, due to the slightly smaller bunches (Table 1). The health status did not affect berry composition in terms of soluble solids and phenol contents; however, titratable acidity and resveratrol were higher in the infected vines (Table 1).

Table 1.

Instrumental texture analysis.

The mechanical properties of berry skin and whole berries are reported in Table 2. No significant difference was noticed between the mechanical characteristics of infected and healthy grape skin, due to high data dispersion. Significant differences were detected in whole berry cohesiveness and resilience, which are useful parameters to evaluate pulp texture characteristics [38]. Cohesiveness is a measure of the strength of internal bonds making up the berry body, while resilience indicates how well the berry regains its original position after deformation.

Table 2.

Protein expression and identification.

The protein yield per g of lyophilized tissue powder was 2.5 mg for the healthy skin and 2.4 for the infected skin, and 0.1 mg for both pulp samples. The dissection of the berries into skin and pulp, and their subsequent separate proteomic analysis, allowed for the visualization of a mean of 460 spot in the skin and 380 in the pulp. No significant difference was detected for protein yield and total spots between healthy and infected status.

ANOVA analysis on 2D gels revealed significant differences between healthy and virus-infected grape berries for 7 skin and 11 pulp spots (Figure 1). Only in the pulp was complete disappearance of 3 out of 11spots observed. Among the selected spots, numbers 4811 and 5708 could not be identified.

The characteristics of the spots selected as differentially expressed, and the list of proteins identified, are reported in Table 3. The mass of most identified proteins fell within a 25% tolerance limit of published masses. The only exception was the C term fragment of cytochrome P450 protein (spot 8102). RSPaV coat protein was identified, together with *V. vinifera* proteins, in pulp spots 1604 and 8309.

Data regarding the PMF identifications of each spot are reported in supplemental table S2.

Fig. 1: 2D gels from V. vinifera cv Nebbiolo: healthy (A) and infected (B) berry skins and healthy (C) and infected (D) berry pulps.

Table 3.

DISCUSSION

Grape berry proteomic analysis of healthy and infected plants was integrated with agronomical analyses to provide a complete picture of the effects of viral infection on both the field behaviour and berry quality of Nebbiolo grapevines, an important and characteristic Italian cultivar. Differences found can be attributed only to the different sanitary status of the grapes since healthy and infected plants had the same genetic background, the same age, and were planted in adjacent rows of the same vineyard, so they were exposed to the same environmental factors and agronomical practices.

Viral infection in vineyards is endemic worldwide [39], and little is known on the effect of viruses on the final product. Some differences in fruit composition have been reported between healthy and virus-infected grapevines of different cultivars, such as Nebbiolo [6,7], Albariño [8] and Pinot noir [9-10]. In our study, healthy plants showed a better general growth performance compared to infected vines, as indicated by the higher bud burst index, and the higher, although not significant, shoot fertility and yield. Virus infection, however, did not affect berry composition in terms of either soluble solids or phenol content. The higher titratable acidity and resveratrol content of infected berries may reflect a response to biotic stress [40].

This research comprises the first proteomic study applied to the comparison of healthy and virus-infected Nebbiolo grape berries. Proteomic analysis was conducted on berries harvested at commercial maturity, as indicated by the content in soluble solids, pH, titratable acidity and total anthocyanins. No difference in the ripening parameters was detected between healthy and virus-infected plants. This is consistent with the fact that also virus infected vineyards are routinely used for winemaking.

The dissection of berries into skin and pulp, and their subsequent separate proteomic analysis, visualized about 400 spot. Despite the different efficiencies of the two protein extraction protocols, the total number of detected spots was constant in all gels, since an equal amount of proteins was subjected to 2DE. The similarity between the proteome of Nebbiolo grape pulps and skins is consistent with the data reported in [41], that only 8.6% of spots doubled or more between the two tissues in Cabernet Sauvignon grapes, although cross-contamination could not be ruled out.

As a general trend, in the present study virus infection mainly affected proteins involved in the response to oxidative stress in the berry skin, and proteins involved in cell structure metabolism in the pulp (Tab. 3). Proteomic analysis showed that GLRaV-1, GVA and RSPaV mixed infection does not dramatically change the protein pattern of ripe Nebbiolo berries, therefore indicating that these viruses are well tolerated by Nebbiolo in our experimental conditions.

The coat protein of RSPaV was the only viral protein detected in berry mesocarp, in spots 8309 and 3403. The two spots were characterized by different pI and molecular mass values, possibly due to the presence of virus genetic variants [2], or to differential PTMs, as reported for *Cucumber mosaic*

virus coat protein in infected tomato plants [42]. There may be several reasons for the detection of only RSPaV coat protein in extracts from grapes infected also with GLRaV-1 and GVA. The RSPaV coat protein may be more stable than those of GLRaV-1 and GVA, and therefore it may accumulate in infected cells, while the others are present in lower concentrations perhaps due to degradation. Moreover, RSPaV coat protein is a good target for serological diagnosis of the virus in infected grapes [43], in line with a high concentration of the antigen in infected tissues. Alternatively, GLRaV-1 and GVA could be present at much lower concentration than RSPaV in the infected plants, as it has been reported for *Grapevine fanleaf virus* and *Grapevine fleck virus* in Nebbiolo grapes [25].

All the other proteins modified by the infection belonged to the grape and were involved in energy metabolism, stress response, aminoacid and protein turnover, cell structuring and signal transduction.

In the energy metabolism, the ATP synthase and the phosphoglycerate kinase (PGK) were altered by the viral infection. The ATP synthase beta subunit, upregulated in the pericarp of the infected Nebbiolo berries, is part of an enzyme complex responsible for ATP synthesis. Although it is generally found in the inner membrane of mitochondria [44], recent reports describe the localization of ATP synthase components on the plasma membrane of several human, mouse, rat and insect cell types, where they function as receptors for multiple ligands in diverse processes, as reviewed in [44,45]. Upregulation of the ATP synthase beta subunit has been reported in soybean leaves infected with *Soybean mosaic Potyvirus* [46] and in the lymphoid organ of the freshwater shrimp *Paeneus monodon* following infection with yellow head virus [47]. In infected Nebbiolo grape skins, where phloematic viruses are absent, upregulation of the ATP synthase beta subunit is probably associated with an increased plant metabolism.

Phosphoglycerate kinase expression in virus-infected Nebbiolo grapes was altered depending on the tissue: decreased in pericarp and increased in mesocarp. PGK is a transferase enzyme of the cell glycolytic pathway. In some prokaryotes, beside its role in glycolysis, PGK is also able to bind host cytoskeletal proteins and it plays a role in internalizing bacteria in the insect vector cell [48]. In infected *Nicotiana benthamiana*, a specific binding of PGK to the RNA of *Bamboo mosaic potexvirus* has been demonstrated, and silencing of host PGK has a detrimental effect on synthesis of the viral coat protein in the same system [49], although no information is available about PGK expression in infected cells. RSPaV coat protein was detected in pulp extracts only, where PGK was over-expressed.

The proteins owing to the stress response machinery are normally affected by pathogens [17]. This metabolic pathway is the most altered in our experiment in berry skin tissue. Both ferritin and aldo/keto reductase decreased in the pericarp of infected grapes. In plants, ferritins are involved in

iron storage and metabolism in different organs [50], and play a role in the defence against iron-induced oxidative stress [51]. Virus infections are known to alter the oxidative stress response in several plants [42,52,53]. This alteration is necessary for the induction of specific plant responses to biotic stress, such as the synthesis of phytoalexins, including resveratrol [54]. Also aldo/keto reductases are a class of enzymes involved in the scavenge of oxygen radicals. Different grapevine cultivars react with increased levels of ROS [55] and of scavenger enzymes [56] upon infection with phloematic bacteria. In this context, the resveratrol increase observed in virus-infected berry skins appears consistent (Tab. 1). No information is available to date about the antioxidant capacity of musts and wines from infected and healthy vines. In infected Nebbiolo mesocarp, an increased amount of polyphenol oxidase was found. Polyphenol oxidase is involved in senescence and oxidative browning of plant tissues, and its increase after infection has been reported for several plants and pathogens [57]. One markedly down-regulated pulp spot in infected plants represented a cyt-P450 C-term fragment. The cytochrome P450 monooxygenase is an enzyme superfamily involved in plant response to abiotic and biotic stresses. Differential expression of cyt-P450 is involved in the grapevine response to *Xylella fastidiosa* infection [58]. The modification of proteins involved in the oxidative stress response indicates that the evaluation of oxidative status in wines derived from infected grapes needs further investigation.

Proteomic analysis of GLRaV-1, GVA, and RSPaV infected Nebbiolo berries revealed also the modification of two proteins involved in aminoacid and protein turnover. The increased expression of N-acetyl-gamma-glutamyl-phosphate reductase (AGPR) was found in the mesocarp. AGPR is involved in the synthesis of ornithine and arginine, precursors of polyamines [59], which are small basic molecules involved in plant growth and stress response [60]. Alteration of grape polyamines has also been reported as a consequence of infection with several phloematic viruses, including GLRaV-1 and RSPaV [61,62]. However, co-migration of AGPR and RSPaV coat protein means that the individual contributions of each protein to the over-expression of the spot cannot be assessed. The over-expression of the alpha subunit of the 20S proteasome detected in the infected pericarp is in line with the decrease of proteins responsible for the degradation of ROS (ferritin, aldo/keto reductase) in the same tissues, as 20S represents the proteolytic core of the 26 proteasome, and is involved in the degradation of proteins modified by oxidation [63]. The complex is implicated in the degradation of pathogen proteins (including viral movement proteins), but it can also be exploited by pathogens to enhance the infection process [64]. In Nebbiolo vine leaves, induction of two components of the ubiquitin/26S proteasome complex is associated with *Flavescence dorée* phytoplasma infection [56]. Due to their role described in literature, as well as in our study, both AGPR and proteasome 20S subunit are involved in the response machinery to biotic and abiotic stresses.

Viral infection caused marked modifications in the expression of cell structure related proteins in the mesocarp: complete induction of one pectin methylesterase (PME), over-expression of plastid movement impaired 1 and alpha tubulin, and under-expression of a putative fimbrin.

The PME spot, appearing only in infected pulp, showed a molecular mass lower than predicted, as it represents the active form of the enzyme, derived from cleavage of the N-term inhibitor [65]. The softening of the grape during maturation is a result of significant changes in the cell-wall constituent composition, particularly at the cellular level of the pulp [66]. The activation of PME causes an increase in the degradation of the cell wall pectins in the pulp, resulting in increased softening [67]. The significant increase in cohesiveness and resilience in the infected berry (Tab. 2), suggests cell wall degradation, as cohesiveness is inversely correlated with elasticity, resistance to touch, and firmness, which are known to decrease with softening [38]. PME is also involved in the systemic movement of the tobacco mosaic virus in tobacco plants [68], and one PME gene is induced in leaves of Carménère grapevines infected with GLRaV-3 [15]. Plastid movement impaired 1 is involved in anomalous chloroplast movements in Arabidopsis [69], and shows some similarities to one rice protein involved in elicitor binding in stress responses, and probably cooperating in Ca^{2+}-mediated signal transduction [70]. Alpha tubulin, a globular protein of the microtubules, is involved in positioning and intra-cell transport of organelles, as well as in the movement of pathogens [71]. The increase in alpha tubulin indicates an alteration of the cytoskeleton. Several viruses are known to interact with cell microtubules [72], but nothing has been reported to date on the interactions of grapevine viruses and alpha tubulin. Alteration of the cytoskeleton is also suggested by under-expression of a putative fimbrin, an actin-binding protein, in the pulp of infected berries.. Taken together, these results suggest a reorganization of the cytoskeleton in grape mesocarp in the presence of virus infection, possibly in relation to the intracellular and intercellular movement of viral particles [73]. The changes in berry structure due to viral infection may have some consequences, though unexplored, in the winemaking process.

The last pathway affected by viral infection is signal transduction. Two spots representing heterotrimeric G-proteins with a WD40 motif were modified in the mesocarp of infected Nebbiolo grapes. They probably contain the same protein bearing different PTMs, the more acidic one being induced by the presence of the virus. G-binding proteins (G-proteins) are found in all organisms, are activated by exchange of GDP to GTP, and are important switches that cycle between an active and an inactive state. G-proteins can be grouped into heterotrimeric and monomeric family members. Heterotrimeric G-proteins contain a conserved structural motif of approximately 40 aminoacids (WD40), and are involved in signal transduction, RNA processing, gene regulation, vesicular traffic, regulation of cytoskeleton and cell cycle [74]. In plants, G-binding proteins are implicated in several mechanisms, including defence against pathogens [74], reaction to abiotic stresses [75,76]

and interaction with microorganisms of the rhizosphere [77]. Also in this case, these spots may have implications in the plant defence machinery as described for aminoacid and protein turnover related proteins. One Rab11 containing spot was strongly under-expressed in infected grape skin. The Rab proteins belong to the superfamily of monomeric small GTPases, play several roles in plants, and are involved in membrane trafficking and signalling. Rab11 isoforms are known to be preferentially expressed in fruits or in fruit-deriving tissues, where they might be responsible for the secretion of enzymes involved in fruit softening [78]. The decrease in Rab11 after viral infection could thus be correlated to changes in skin hardness. The results of the puncture test show that both skin break force and skin break energy were slightly higher in infected plants, although not significantly so. Nevertheless, the Rab11 protein may play a role in structuring the berry skin, one parameter known to be modified by viral infection [79]. Changes in skin structure have some technological impact in winemaking. During maceration in a model hydroalcoholic solution, the Nebbiolo grapes with higher values of skin break force produced extracts with a higher total anthocyanin content [80] and with slower release kinetics of cyanidin 3-*o*-glucoside and peonidin 3-*o*-glucoside, both easily oxidised pigments [81].

CONCLUSIONS

Grape berry proteomic analysis was integrated with the evaluation of agronomical performance, to provide a more complete picture of the effects of viral infection on both field behaviour and berry composition of Nebbiolo grapes. Minor differences were observed between healthy and infected plants, in terms of agronomical performance and fruit quality. Proteomic analysis showed that virus infection mainly affected proteins involved in the response to oxidative stress in the berry skin, and proteins involved in cell structure metabolism in the pulp. These results indicate that infection increases pulp cell wall degradation, as confirmed by instrumental texture analysis data, and that it apparently causes an alteration in the skin structure. Further investigations will be required to clarify whether the changes in berry metabolism induced by the viruses can affect winemaking and the quality of the resulting wine.

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671

672 **Figure captions.**

673

674 Figure 1: 2D gels from *V. vinifera* cv Nebbiolo: healthy (A) and infected (B) berry skins and healthy
675 (C) and infected (D) berry pulps.

676

Table 1 - Plant agronomical and juice qualitative parameters of healthy and virus infected Nebbiolo vines.

	Healthy	Infected	Sample sizes (H, I) or DF	Significance
Bud burst index	4.5[a] ± 0.5	3.9 ± 0.3	20,20	** [b]
N° clusters/vine	8.8 ± 2.3	8.0 ± 3.4	20,20	ns
Shoot fertility (n° inflor./shoot)	1.4 ± 0.4	1.2 ± 0.4	20,20	ns
Berry weight (g)	1.95 ± 0.09	2.05 ± 0.10	38	**
Cluster weight (g)	352 ± 66	339 ± 48	38	ns
Yield (kg/vine)	3.1 ± 1.0	2.6 ± 1.0	38	ns
Soluble solids (°Brix)	23.8 ± 0.6	23.8 ± 0.7	38	ns
pH	3.02 ± 0.04	3.03 ± 0.04	38	ns
Titratable acidity (g/L)	7.6 ± 0.6	8.2 ± 0.5	38	**
Total anthocyanins (mg/kg grape)	556 ± 73	551 ± 40	4	ns
Total flavonoids (mg/kg grape)	2687 ± 275	2348 ± 314	4	ns
Resveratrol (mg/kg grape)	0.25 ± 0.10	0.52 ± 0.10	4	*

[a] Values are means ± SD.

[b] ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$.

Table 2 – Skin and whole berry mechanical properties of healthy and virus infected Nebbiolo vines.

	Parameter	Healthy	Infected	Significance
Skin	Break force (N)	0.52 ± 0.13[a]	0.55 ± 0.13	ns[b]
	Break energy (mJ)	0.36 ± 0.15	0.40 ± 0.14	ns
	Young's modulus (N/mm)	0.35 ± 0.05	0.36 ± 0.06	ns
	Thickness (μm)	203 ± 25	200 ± 29	ns
Berry	Hardness (N)	4.91 ± 0.77	4.96 ± 0.98	ns
	Cohesiveness (-)	0.71 ± 0.03	0.73 ± 0.04	*
	Gumminess (N)	3.45 ± 0.45	3.57 ± 0.56	ns
	Springiness (mm)	2.20 ± 0.17	2.25 ± 0.13	ns
	Chewiness (mJ)	7.62 ± 1.36	8.06 ± 1.67	ns
	Resilience (-)	0.37 ± 0.02	0.39 ± 0.03	*

[a]Values are means ± SD, n=30.

[b] ns = $P > 0.05$; * = $P \leq 0.05$.

Table 3

Table 3 - Identified proteins changing in abundance in virus-infected *V. vinifera cv* Nebbiolo berry pericarp (skin) and mesocarp (pulp) .

Skin

Spot code	*Fold variation*	*Identified proteins*	*GenBank ID*	*Exp. Mw (kDa)/pI*[a]	*Hyp. Mw (kDa)/pI*	*MIPS/GO*
2710	+4.0	ATP synthase subunit beta	XP_002280824	52/5.23	59.9/5.8	20.03 transport facilitation/0042777
1407	+3.9	Proteasome subunit alpha type	XP_002281948	32.5/5.11	30.8/5.0	14.07 protein modification/0006511
5509	-3.2	Phosphoglycerate kinase	CBI24183	37.6/6.15	36.6/5.7	02.01 glycolysis and gluconeogenesis/0006096
4306	-3.3	Ferritin	CAN59741	26.7/5.96	25.4/5.7	32.01 stress response/0055114
7501	-3.5	Aldo/keto reductase	CAN68994	35.6/6.63	37.5/6.5	32.01 stress response/0055114
6302	-23.4	GTPase Rab11	CBI31604	28.0/6.19	26.9/6.3	30.01 intracellular signalling/0007264

Pulp

Spot code	*Fold variation*	*Identified proteins*	*GenBank ID*	*Exp. Mw (kDa)/pI*	*Hyp. Mw (kDa)/pI*	*MIPS/GO*
8507	Induction	Pectin methylesterase (C term fragment)	CBI36883	45.3/7.38	60.5/9.3	42.01 cell wall/0042545
8309	Induction	G protein-WD40 + coat protein RSPaV	XP_002281279 + ABD98736	33.2/7.61	36.0/7.6 28.2/6.48	-
3403	+17.2	N-acetyl-gamma-glutamyl-phosphate reductase + coat protein RSPaV	CAN73785 + ABD98736	36.4/5.77	36.0/6.0 28.2/6.48	01.01 amino acid metabolism/0003942
1908	+9.2	Plastid movement impaired 1	XP_002273127	93.2/4.95	94.4/5.5	42.26 plastid/0009902
2406	+4.1	Phosphoglycerate kinase	XP_002263950	36.5/5.48	36.6/5.7	02.01 glycolysis and gluconeogenesis/

						0006096
7702	+3.5	Polyphenol oxidase	AAB41022	58.8/6.94	67.4/6.3	32.01 stress response/0055114
1604	+3.4	Alpha-tubulin	XP_002285563	50.4/5.11	49.6/4.9	20.09 transport routes/0007018
8305	-3.2	G protein-WD40	XP_002281279	33.5/7.77	36.0/7.6	-
2705	-15.0	Putative fimbrin	XP_002276851	67.6/5.27	78.8/5.3	16.06 motor protein/0003779
8102	-21.3	Cyt P450 (C-terminal fragment)	CBI20810	25.4/7.84	47.4/8.2	32.01 stress response/0055114

[a]Exp. Mw/pI: molecular mass and pI on gel; Hyp. Mw/pI: predicted molecular mass and pI; MIPS/GO: metabolic pathway code and Gene Ontology code.

Figure 1

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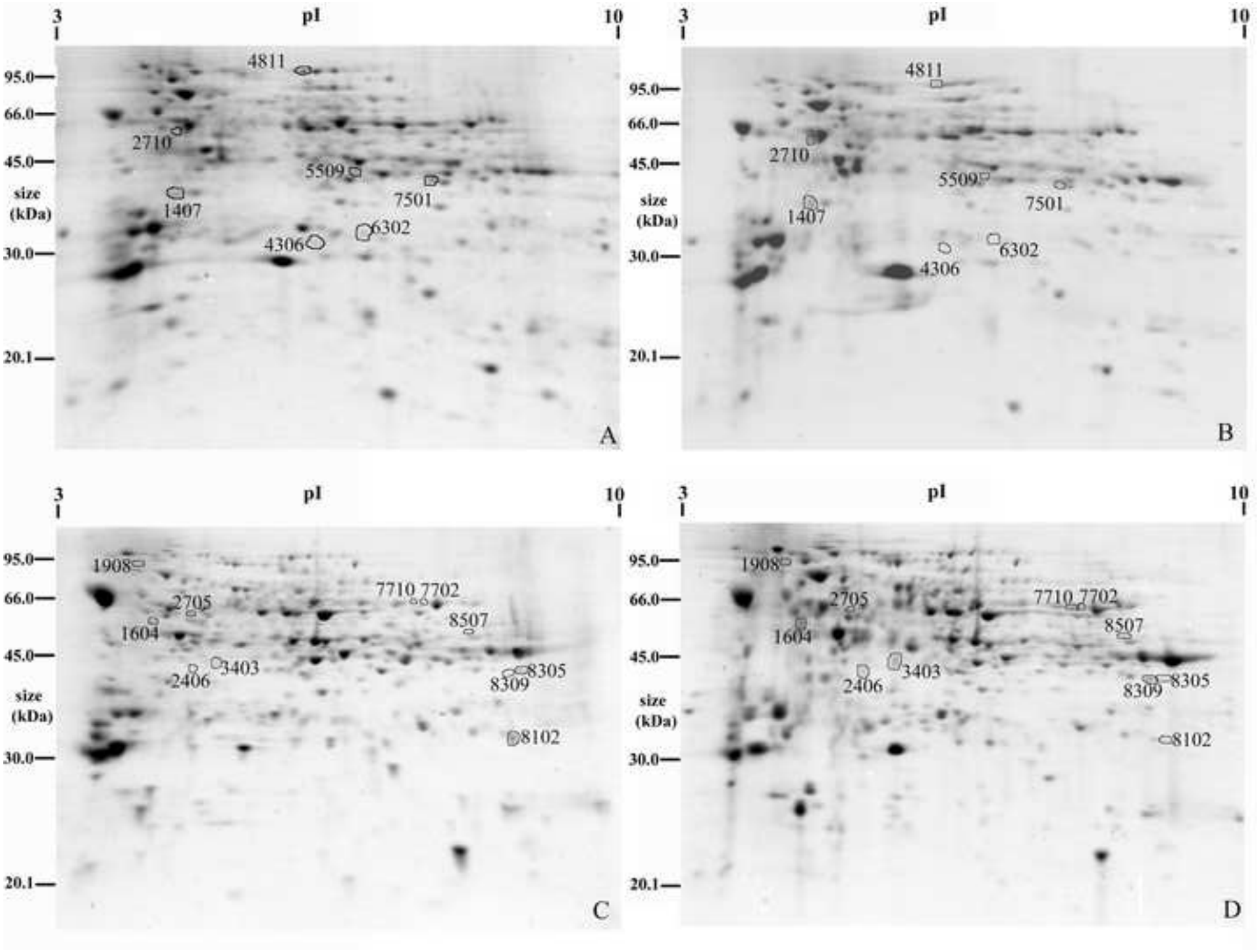

Supplementary table 1

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Supplementary table 2

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