Proteins and enzymatic activities in Erbaluce grape berries with different response to the withering process.

Proteins and enzymatic activities in Erbaluce grape berries with different response to the withering process

Simone Vincenzi[a*], Serena Tolin[b], Luca Cocolin[c], Kalliopi Rantsiou[c], Andrea Curioni[a], Luca Rolle[c]

[a] University of Padova, Centro Interdipartimentale per la Ricerca in Viticoltura ed Enologia (CIRVE), Legnaro (PD), Italy

[b] University of Padova, Biotecnologie Agrarie, Legnaro (PD), Italy

[c] Department of Exploitation and Protection of the Agricultural and Forestry Resources (Di.Va.P.R.A.), Microbiology and Food Technology sector, Turin University, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy

*Author to whom correspondence should be addressed. Telephone: +39 0438453052; fax: +39 0438453736; email: simone.vincenzi@unipd.it.

ABSTRACT

During the off-vine natural withering process of Erbaluce (white) grapes to obtain “Erbaluce Caluso” Passito wine, some berries change in color from green-yellow to blue. This phenomenon appears to a different extent in different years and it might be related to several parameters, such as temperature and humidity during withering, grape composition and *Botrytis* loading. To better understand the mechanism involved in this phenomenon, the metabolic changes corresponding to this color variation were studied.

At the end of the withering process berries with different colors were separated using a reflectance spectrophotometer, obtaining three color classes identified as “green” (L*= 40.3, a*= -0.56, b*= 15.20), “gold”(L*= 37.7, a* = 5.01, b*= 14.12) and “blue” (L*= 28.6, a* = 0.89, b*= -0.67).

The three groups of berries had a different water content, the blue berries containing about 30% less water than the green ones. Samples were crushed and the juices were analyzed. The yield in juice for blue berries was less than 50% of that of the other two classes, confirming their higher dehydration level.

Protein extraction from de-seeded berries was carried out using two different protocols, the first involving a treatment with phenol (to remove polyphenolic substances) and the second based on an extraction with a mild detergent (to recover the proteins to be used for enzyme analyses).

No trace of laccase activity was found in any of the samples, although DNA analysis, by quantitative PCR, suggested the presence of *Botrytis cinerea* infection in the blue grapes. Chitinase activity of the blue berries was only 30% of that of the other two samples, as confirmed also by zymographic analysis on electrophoretic gels. The same was found also for esterase activity, which was decreased (of about 85%) in the blue berries. In contrast, the highest beta-glucosidase activity was found the in the blue berries.

The electrophoretic analysis of the protein extracts revealed strong differences between the profile of the blue berries and those of the other two samples. Compared to the green and gold ones, the blue berries showed a complete absence of bands corresponding to chitinases, and the appearance of unusual protein bands. Mass spectrometry (MS) analysis showed that these latter proteins were of grape origin, the main of them corresponding to the PR-protein β-1,3-glucanase, although no significant increase in glucanase activity was measured in the corresponding protein extract.

Keywords: Dehydrated grapes; Grape color; Grape Proteins; Botrytis; Enzymatic activities

1. Introduction

The withering process is particularly important in winemaking because many types of dry and sweet wines (*dessert* wine) are produced by using over-ripe and/or dehydrated grapes. The grape dehydration process essentially results in the increase of sugar content and volatile compounds, but other significant metabolic changes have been observed in grapes during post harvest drying, including metabolization of the organic acids, formation of new aroma compounds and polymerization of skin tannins [1], with a significant effect on the organoleptic characteristics of the final wine. In addition the drying process induces several changes in the physicochemical characteristics of the berries, including modifications of the visual attributes of the berry skins.

Only a few studies have investigated the effect of the withering process on the protein content and composition in grapes, despite the fact that proteins and peptides significantly contribute to the quality of wine because they affect taste, clarity and stability. A biphasic pattern of total protein accumulation in the berries was observed during the gradual weight loss, this being attributed essentially to a synthesis of new proteins involved in stress response [1]. *De novo* protein synthesis was confirmed at a molecular level by a transcriptional profiling of grapes during post harvest drying. In particular a high level of expression of genes involved in stress protection, in hexose metabolism and transport, in cell wall composition and in secondary metabolisms was found [2]. More recently a proteomic analysis of grape berries during withering confirmed that the majority of proteins differentially expressed during post harvest drying are related to stress and defense activity, energy and primary metabolism, cytoskeleton remodeling and secondary metabolism [3].

In addition to the modifications induced in response to the withering process, mainly related to the osmotic stress, some fungal pathogen can infect grapes during the drying period thus modifying the protein pattern of the grapes. The fungal decay is the major factor affecting the success of the grape dehydration process and *Botrytis cinerea* infection is one of the most important post harvest diseases because of its ability to develop even at low temperatures. The effects on grape proteins of *Botrytis cinerea* in the form of grey mould have been extensively studied, and generally a complete [4] or partial degradation of the grape proteins [5] by the fungus was found, depending on the infection rate. In addition, induction of some grape proteins (different isoforms of invertase and chitinase) has been observed in berries [5, 6] in response to the fungal attack, and also some fungal proteins, as endopolygalacturonase 4 and pectin methylesterase, were found in grape juice and wine.

However *B. cinerea*, when developing as noble rot, has some positive effects on high quality white wines (Sauternes, Tokay), favoring the release of aroma compounds from the berry skin [7], thus changing the varietal aroma through oxidation of monoterpenes [8], producing glycerol and enhancing the wine mouthfeel. It is then possible that the different quality of wines produced with grapes infected by noble rot or gray mould can be also related to a different protein expression, both in fungus and in grape.

In this paper we describe the protein composition and some enzymatic activities in grape berries in samples of Erbaluce grapes showing different colors, which depend on the dehydration level obtained during the withering process and on the *Botrytis cinerea* development. The presence of the fungus on grapes during the production of “Erbaluce Caluso” passito is not as important as for other sweet wine productions (Sauterne wine, for example), but the development of noble rot to different extent may lead to a quality improvement of the final wine.

2. Experimental

2.1 Grapes and drying system

Grapes clusters of *Vitis vinifera L*. cv Erbaluce from a vineyard located at Caluso (Piedmont, North West Italy) were carefully harvested when a soluble solids content (SSC) of 18±1 °Brix was reached during 2009 season. Three batches of 30 kg of grapes were placed in perforated boxes (60 × 40 × 15 cm, 6 kg grapes for each box) in a single layer. For the natural off-vine dehydration process, the boxes were introduced inside a typical room without temperature, relative humidity (RH) and speed air control. The dehydration process was carried out for 141 days, from 12 September 2009 to 31 January 2010 (autumn-winter thermo hygrometric drying conditions) in accordance with Denomination Origin (D.O.) Erbaluce di Caluso DOCG wines production rules.

2.2 Samples classification

At the end of the withering process, berries with different colors were separated by CIELab parameters[9], using a reflectance spectrophotometer (Minolta colorimeter CR 410), obtaining three classes identified as: “green” (L*= 40.34 ± 1.91, a*= -0.56 ± 1.51, b*= 15.20 ± 2.85), “gold”(L* = 37.65 ± 3.63, a*= 5.01 ± 2.23, b*= 14.12 ± 3.23) and “blue” (L*= 28.64 ± 2.74, a*= 0.89 ± 1.13, b*= -0.67 ± 2.48). About 400 berries for both color group and batch were used for the chemical analysis.

2.3 Technological parameters

Reducing sugars and pH were determined according to International Organization of Vine and Wine methods [10].

2.4 Water content determination

Samples of 20 berries were manually de-seeded, weighed and freeze dried. The difference in weight before and after lyophilization was used to calculate the water content in the different samples.

2.5 Total Protein extraction (Protocol 1)

Deseeded berries were ground to a fine powder in a mortar with liquid nitrogen. Two grams of powder were extracted as reported by Deytieux et al. [11] with slight modifications. Briefly the sample was extracted with 5 volumes of extraction buffer (0.1 M Tris-HCl pH 7.5, 5 mM EDTA, 1 mM PMSF (phenylmethanesulfonyl fluoride), 0.8% β-mercaptoethanol, 0.1 M KCl, 0.7 M sucrose, 1% PVPP), stirred for 1 h at 4°C, then an equal volume of Tris-HCl pH 7.5 buffer- saturated phenol was added. After 1 h of agitation at 4°C, the sample was centrifuged (9000 g, 30 minutes, 4°C) and the phenol phase was collected. The aqueous phase was re-extracted with 5 mL of buffer and 5 mL of phenol, stirred for 30 minutes and centrifuged. The phenol phases were combined and washed three times with an equal volume of extraction buffer, then the proteins were precipitated by adding 5 volumes 0.1 M ammonium acetate in methanol and incubating overnight at -20°C. The proteins were recovered by centrifugation (9000 g, 30 minutes, 4°C) and the pellet was washed once with 0.1 M ammonium acetate in methanol, twice with ice-cold methanol and twice with ice-cold 80% acetone.

The resulting pellet was resuspended in 500 µL of 7 M urea, 2 M thiourea, 4% CHAPS. Protein concentration was measured with Bradford assay (BioRad Protein Assay) using bovine serum albumin as standard.

2.6 Protein extraction for enzymatic activities (Protocol 2)

Three grams of powder obtained by grounding grape samples with liquid nitrogen were extracted with 2 volumes of extraction buffer (0.5 M Tris-HCl pH 8.5, 1% Triton X-100, 20% glycerol, 5% PVPP, 2 M NaCl and 14 mM β-mercaptoethanol) by stirring for 3 h at 0°C, as reported by Zocca et al. [12]. The solution was centrifuged (20000 g, 30 minutes, 4°C) and the aqueous phase was collected. The pellet was re-extracted with the same procedure, then the supernatants were combined, filtered at 0.45 µm, and dialysed at 4°C against water (3500 Da cutoff). Finally the extracts were lyophilized and resuspended in water at 10 mg ml^{-1}.

2.7 SDS-PAGE

SDS-PAGE analysis in polyacrylamide gels (14% T, 3.3% C) was performed according to Laemmli [13]. Ten µL of the total protein obtained with Protocol 1 were mixed with 15 µL of loading buffer (containing 4% β-mercaptoethanol), heated at 100°C for 5 min and loaded onto the gels. SDS-PAGE was carried out in a Mini-Protean III apparatus (Bio-Rad, Milano, Italy) at 50 mA constant current until the tracking dye bromophenol blue ran off the gel. Gels were stained for protein with Coomassie Brilliant Blue G-250, destained with distilled water and analyzed with a ChemiDoc XRS Imaging System (Bio-Rad, Milano, Italy).

2.8 Measurement of enzymatic activities

Esterase activity was determined as reported by Lomolino et al. [14]. Fifty µL of substrate (2% p-nitrophenyl acetate, 10% Triton X-100) were mixed with 1.9 mL of phosphate buffer 10 mM, pH 7.5 and 50 µL of the protein extract obtained with Protocol 2.

The mix was incubated at 37°C and the absorbance was read at 400 nm for 1 h taking a measure every 5 minutes. One Unit was defined as the quantity of protein able to produce an increase of 1 AU per minute.

Chitinase activity was estimated as described by Derckel et al. [15]. Basically, 10 µL of the extract obtained with Protocol 2 were mixed with 190 µL of water, 400 µL of the substrate solution (1% CM-chitin-RBV) and 800 µL of 100 mM sodium acetate buffer, pH 4.0. The reaction was run at 30°C and stopped at 30 min with 400 µL of 0.1 M HCl before being left on ice for 10 min. The undigested substrate was removed by centrifugation (5000 g for 10 min) and the absorbance of the supernatant measured at 550 nm. One unit will produce an increase at 550 nm of 1 AU per minute at pH 4.0 at 30°C.

The laccase activity was determined using syringaldazine as substrate [16]. Five hundred µL of protein extract obtained with Protocol 2 were mixed with 2.2 mL of potassium phosphate buffer 100 mM, pH 6.5 and 300 µl of syringaldazine 0.216 mM in methanol. The mix was incubated at 30 °C and the increase in A530nm was recorded for approximately 10 minutes. One unit will produce an increase at 530 nm of 0.001 AU per minute at pH 6.5 at 30°C.

The beta-glucosidase activity was measured using p-nitrophenyl-glucopyranoside (Sigma, Milano, Italia). 50 µL of protein extract were mixed with 250 µL of sodium acetate buffer 0.1 M, pH

5.1 and 300 µL of substrate 1 mM in sodium acetate buffer. The reaction was performed at 37°C for 30 min and blocked with 400 µl of sodium carbonate 1 M. The absorbance was read at 400 nm. One unit will produce an increase at 400 nm of 1 AU per minute at pH 5.1 at 37°C.

Pectin methyl esterase activity was measured as reported by Hagerman and Austin [17] with some modifications. The reaction mix was composed of 2 mL of pectin solution 0.5% in phosphate buffered saline, pH 7.5, 0.8 mL of water, 0.15 mL of bromothymol blue 0.04% and 50 µL of protein extract obtained with Protocol 2. The enzymatic activity was followed at 25°C for 40 min at 620 nm. One unit will produce a decrease at 620 nm of 1 AU per minute at pH 7.5 at 25°C.

Glucanase activity was measured using the protocol from Silveira Celestino et al. [18] with some modifications. Fifty µL of laminarin solution 1% in 100mM sodium acetate buffer, pH 5.0, were incubated at 50°C for 30 minutes with 50 µL of protein extract obtained with Protocol 2. The reaction was stopped by adding 900 µL of water and 650 µL of dinitrosalicylic acid solution [19]. After boiling for 5 minutes, absorbance was read at 540 nm. The amount of reducing sugar produced was determined using a curve prepared with glucose as standard. One unit will produce 1 µmole of reducing sugar per minute.

For each enzymatic activity, all measurements were done in triplicate.

2.9 In gel chitinase activity

Staining for chitinolytic activity was performed according to Trudel and Asselin [20], by incorporating into SDS-PAGE gels 0.01 % (w/v) glycol chitin, which was prepared as reported by Molano et al. [21]. Five µL of protein extract obtained with Protocol 2 were mixed with 15 µL of loading buffer (without β-mercaptoethanol), boiled for 5 min and loaded onto the gel. After the

electrophoretic migration the gel was soaked in sodium acetate buffer, 50 mM, pH 5, containing 1% Triton X100 and was incubated overnight at room temperature under agitation. The following day the gel was rinsed for 10 min with distilled water and incubated for 10 min with Calcofluor white M2R (Sigma) 0.01% in Tris-HCl 500 mM, pH 8.9. The chitinolytic bands were visualized under UV light.

2.10 Quantification of Botrytis cinerea by quantitative PCR

Sample of grapes were crushed and stored at -20°C as must. One ml of the must was subjected to DNA extraction according to Mills et al. [22]. The obtained DNA was subjected to quantitative PCR (qPCR) analysis by using a primer set and probe specific for *B. cinerea* , as described by Cadle-Davidson [23]. Quantification of *B. cinerea* was performed based on calibration curves prepared from serial decimal dilutions of a culture of *B. cinerea* in ringer. Initial load of *B. cinerea* used for the construction of the calibration curves was determined microscopically counting the spores in a Burker chamber.

2.11 LC-MS/MS analysis

After electrophoresis, bands of interest were excised from the gel, dehydrated with acetonitrile for 10 min and dried in SpeedVac. Cysteines were reduced with 10 mM dithiotreitol in 50 mM NH_4HCO_3 for 1 h at 56°C, and alkylated with 55 mM iodoacetamide for 45 min at room temperature in the dark. Gel bands were repeatedly washed with 50 mM NH_4HCO_3 and acetonitrile, and dried under vacuum. In gel protein digestion was performed using 10 µL of sequencing grade modified trypsin (Promega, Madison, WI) (12.5 ng μL^{-1} in 50 mM NH_4HCO_3). Digestion was carried out

at 37°C overnight. The peptides were extracted 3 times with 50 µL of 50% acetonitrile/1% formic acid, dried under vacuum and dissolved in 10 µL of 0.1% formic acid. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed with a 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled to a chip-based chromatographic interface. 4 µL of samples were injected into the chip and loaded into the enrichment column (C18, 160 nL volume) at a flow rate of 4 µL min^{-1}. Peptides were separated in the C18 nano-column (150 mm × 75 µm) at a flow rate of 0.5 µL min^{-1}. Water/formic acid 0.1% and acetonitrile/formic acid 0.1% were used as eluents A and B, respectively. The chromatographic separation was achieved using a gradient of eluent B from 3% to 50% in 30 min. Mass spectra were acquired in a data dependent mode: MS/MS spectra of the 3 most intense ions were acquired for each MS scan in the range of 350-2400 m/z. Scan speed was set to 4 MS spectra/sec and 3 MS/MS spectra/sec. Capillary voltage was set to 1750 V and drying gas to 5 L sec^{-1}. Raw data files were converted into Mascot Generic Format (MGF) files with MassHunter Qualitative Analysis Software version B.03.01 (Agilent Technologies) and searched using Mascot Search Engine (version 2.2.4 Matrix Science, London, UK) against the UniRef100 database (version 2011x (12162651 sequences, 4264415143 residues). Enzyme specificity was set to trypsin with one missed cleavage using a mass tolerance window of 10 ppm for the precursor ion and 0.05 Da for the fragment ions. Carbamidomethylcysteine and oxidation of methionine were selected as fixed and variable modification, respectively. Proteins with at least 2 peptides with individual significant score ($p < 0.05$) were considered as positively identified.

2.12 Statistical analysis

Statistical analyses were performed using the statistical software package SPSS (version 17.0; SPSS Inc., Chicago, IL, USA). Tukey-b test for $p<0.05$ was used in order to establish statistical differences by one-way analysis of variance (ANOVA).

3. Results and Discussion

3.1 Berry characteristics at the end of the withering process

In this study, during the off-vine natural withering process of Erbaluce grapes to obtain "Erbaluce Caluso" Passito wine, some berries on the bunch changed in color from green-yellow to blue. In the samples here studied, at the end of the withering process, about the 56-58% of the berry belonged to the gold group, 43-46% to the green group while only 1-3% were classified as blue.

The three groups of berries had a different water content, the blue berries containing about 30% less water than the green ones. Samples were crushed and the juices were analyzed. The yield in juice for blue berries was less than 50% of that of the other two classes, confirming their greater dehydration level. Consequently, the must obtained from each group of different colored berries showed significant differences in sugars concentration and acidity (Table 1).

In order to obtain a protein extract suitable for SDS-PAGE analysis, a phenol-based protocol that provided good results in the extraction of the skin from red berries [11] was used (Protocol 1). Phenol is a protein denaturant and therefore some proteins, like enzymes, can lose their functionality during the extraction procedure. In order to avoid protein denaturation and to compare some enzymatic activities among the three samples of berries, a second extraction protocol based on the mild detergent Triton X100 was used (Protocol 2).

In order to correlate the color of the berries with *B. cinerea* infection, a qPCR approach was carried out to determine the fungus loads in the different samples (blue, green and gold). The calibration curve obtained by the serial dilutions of a *B. cinerea* culture was characterized by a

correlation coefficient (R^2) equal to 0.9624. Detection and quantification was possible only for the blue grapes sample and the *B. cinerea* load was determined to be 7.94 x 10^5 spores mL^{-1} of must.

3.2 Enzymatic acivities

Unexpectedly, considering the dark color in blue grapes that indicated the occurrence of high levels of enzymatic browning, no trace of laccase activity was found in any of the samples. Therefore, the presence of laccase activity deriving from *B. cinerea* in the original grapes could not be completely excluded, since the enzyme may have been denatured during the prolonged drying stage or during the freezing of the samples, as reported for the laccase extracted from other fungi [24].

In grapes, chitinases and Thaumatin-like proteins (TLP) accumulate in berries in a constitutive manner from veraison to maturity [25]. Accumulation of different isoforms of chitinases and TLPs even during post harvest has been demonstrated, probably as a result of both concentration (water loss) and synthesis of new proteins [3].

Analysis of chitinase activity showed that the blue berries contained less activity than the other two samples (about 70% of reduction on a dry weight basis) (Table 2).

This finding was confirmed by activity detection on electrophoretic gels (Figure 1). In those experiments the green and gold samples were very similar, showing three different isoforms, named A, B and C. The presence of multiple isoforms of chitinases in grape berries has already been reported [15, 26]. In contrast, in the sample obtained from the blue berries the two bands showing less intensity (A and C) were completely lacking, but a new band with higher electrophoretic mobility (D) appeared. This band could correspond to a chitinase fragment retaining its activity or to a chitinase isoform synthesized *ex novo* in response to the fungal attack.

The overall reduction in chitinases found in the blue berries is in agreement with previous studies, in which it was reported that, among the grape Pathogenesis related (PR)-proteins, chitinases are the less resistant, showing both protein [5] and activity [27] reduction after *B. cinerea* infection.

Esterases are enzymes widespread in the plant kingdom, and are frequently present as large families of many isoforms. In *V. vinifera* the esterase isoenzymatic pattern has been used for varietal or clone differentiation[28]. Esterase activity has been found also in grape berries [28, 29], and there is some evidence that this activity can reduce the accumulation of esters produced by the yeasts during fermentation [30]. Also *B. cinerea* can produce some esterases able to degrade the esters and to reduce the fruity aroma of the final wine [31]. In our case esterase activity, was very similar in green and gold berries but decreased of about 85% in the blue ones (Table 2). This should indicate a major effect of the withering process and the very low contribution of the esterase enzymes secreted by *B. cinerea* in the blue berries. As a consequence of the low esterase activity, the wine obtained from the blue berries might contain a higher content of fruity aromas.

Beta-Glucosidase activity, that contributes to the release of aroma compounds in wine starting from glycosilated precursors, showed a different behavior compared to that of the previous enzymes, the blue berries containing the highest activity (increase of 44-67%) (Table 2). This is probably due to the *B. cinerea* infection of the blue berries. As a matter of fact, the presence of at least one intracellular beta-Glucosidase enzyme in *B. cinerea* has been reported [32]. However, the synthesis of a new protein in the blue berries in response to stress cannot be excluded. Despite glucosidase enzymes are not included in the PR-protein family, beta-Glucosidases can enhance resistance to plant diseases by cleaving hydroxycinnamic acid glucosides [33], salicylic acid glucosides [34] and phytohormone conjugates [35]. It is important to consider also the possible effect of the pH on the glucosidase activity. In the blue berries not only the glucosidase activity was the highest at pH 5 (pH of the assay), but also the pH of the resulting juice (5.38) was the closest to the enzyme pH

optimum. Therefore the differences in glucosidase activities in the three juices would be most probably even more pronounced in winemaking conditions. The increased glucosidase activity in the blue samples may then result in an intensification of the wine bouquet deriving from the higher rate of hydrolysis of the aroma precursors present in the grape.

Pectin methylesterase (PME), an enzyme involved in pectin degradation, showed the lowest activity in gold berries (Table 2), whereas the highest value was obtained in the green ones, the blue berries showing an intermediate value. Therefore, such differences are unlikely to be related to the different dehydration level of the samples.

3.3 Protein analysis

The proteins extracted with phenol were analyzed by SDS-PAGE, loading the same volume of extract in each well (Figure 2).

Though the conditions of phenol extraction (Protocol 1) were studied specifically to reduce the protein-polyphenol interactions, some Coomassie-stained material remained at the top of the stacking gel, suggesting the presence of high molecular weight aggregates. This could be due to irreversible proteins-polyphenols aggregation occurring during the withering. Nevertheless, some well defined protein bands were detectable on the gel, even in the *Botrytis* infected grapes (blue berries).

The protein profiles of green and gold berries were very similar and characterized mainly by two intense bands at 25-28 kDa, that should correspond to chitinase isoforms [36] and by several faint bands between 35-42 kDa and 50-65 kDa. In the protein extract obtained from blue berries the two bands at 25-28 kDa were missing and a new band at a lower molecular weight appeared. These

data confirm that a drastic chitinase degradation is caused by *Botrytis* infection, as previously observed by chitinolitic activity detection on gel. In addition, some new intense bands were evident in the range 37-40 kDa, probably corresponding to beta-glucanases [37].

The changes in the protein profile of berries infected by *B. cinerea* generally shows a complete [4] or partial degradation of the grape proteins [5] depending on the infection level. In addition, it has been found that several new protein bands are synthesized, either by the fungus during infection, and by the plant, as a response triggered by the pathogen. Grape proteins induction has been observed in both leaves [38] and berries [6]. In this latter case a group of proteins synthesized in response to the fungal attack was recorded between 46 and 50 kDa. Later, nano-LC-MS/MS analysis of wine proteins, showed that some grape invertase and chitinase isoforms were induced by the infection, whereas two pectinolytic enzymes from *B. cinerea*, endopolygalacturonase 4 (gi|3282222) and pectin methylesterase (gi|12964194), were found in the botrytized wine at about 45 kDa [5]. On the other hand, the grape β-glucanase was identified only in one spot in the healthy wine and decreased significantly in botrytized wine.

In order to better understand the effect of withering in Erbaluce berries, the new bands in the blue berries extract were excised from the gel (Figure 3 shows a detail of the region between 31 and 45 kDa with bands B1-B4) and analyzed by LC-MS/MS..

The proteins identified in bands B1-B4 (Table 3) are mostly of grape origin, but some of them correspond to glyceraldehyde-3-phosphate dehydrogenase of *B. cinerea* . This enzyme was identified, along with malate dehydrogenase, as the main protein expressed in the mycelia of *B. cinerea* when cultured in synthetic medium [39]. The four spots identified as glyceraldehyde-3-phosphate dehydrogenase were present only in the more virulent *B. cinerea* strain. This is not surprising because, apart from its role in the glycolytic cycle, glyceraldehyde-3-phosphate dehydrogenase has been reported to influence many other cellular processes and act as a virulence factor in different organisms [40, 41].

Significantly, the grape protein UPI00015CB0BD, identified with the highest score in the most intense band B4, has about 80% of sequence identity with the protein beta 1-3 glucanase (Q9M3U4_VITVI) and could be therefore produced by the berries in response to the infection.

In contrast with data obtained with MS analysis, the glucanase activity in blue berries was not significantly different from the other two samples when expressed on a dry weight. As reported for laccase activity, probably some enzymatic activities were lost during the prolonged drying period or during the storage of samples before analysis.

4. Conclusions

The changes in color of Erbaluce grapes during the withering process can be related to a different kinetic of water loss, which seems affected by *B. cinerea* infection occurring only in the blue-colored berries in the form of “noble rot”. The activity of some enzymes, some of them having a potential effect on wine quality, is also related to the development of the blue color in the berries, being probably affected by the presence of the fungus. The protein profile of berries infected by *B. cinerea* has already been reported, and generally shows a complete or partial degradation of grape proteins by the fungus depending on the infection level. In our case a severe degradation of chitinases was observed in infected berries, but several new bands were newly synthesized. These proteins are mostly of grape origin, in particular β-1,3-glucanase, in contrast with previous reports, that showed the release of pectinolytic enzymes from *B. cinerea* and the induction of some grape invertase and chitinase [5]. To our knowledge this is the first report of protein analysis in grapes infected by noble rot.

Although the mechanism responsible for the different colors of the Erbaluce berries has not been determined with precision, it seems that the berry color is associated to metabolic changes

leading to different characteristics of the grapes, which are, at least in part, related to the potential quality of the wine.

References

[1] V. Costantini, A. Bellincontro, D. De Santis, R. Botondi, F. Mencarelli, J. Agric. Food Chem. 54 (2006) 3334-3340.

[2] A. Zamboni, L. Minoia, A. Ferrarini, G. B. Tornielli, E. Zago, M. Delledonne, M. Pezzotti, J. Exp. Botany 59 (2008) 4145-4159.

[3] M. Di Carli, A. Zamboni, M. E. Pè, M. Pezzotti, K. S. Lilley, E. Benvenuto, A. Desiderio, J. Proteome Research 10 (2011) 429-446.

[4] R. Marchal, L. Berthier, L. Legendre, L. Marchal-Delahaut, P. Jeandet, A. Maujean, J. Agric. Food Chem. 46 (1998) 4945-4949.

[5] C. Cilindre, S. Jégou, A. Hovasse, C. Schaeffer, A. J. Castro, C. Clément, A. Van Dorsselaer, P. Jeandet, R. Marchal, J. Proteome Res. 7 (2008) 1199-1208.

[6] C. Cilindre, A. J. Castro, C. Clément, P. Jeandet, R.Marchal, Food Chem. 103 (2007) 139-149.

[7] A. Genovese, A. Gambuti, P. Piombino, L. Moio, Food Chem. 103 (2007) 1228-1236.

[8] J.S. Câmara, P. Herbert, J.C. Marquez, M.A. Alves, Anal. Chim. Acta 513 (2004) 203-207.

[9] L. Rolle, S. Guidoni, J. Int. Sci. Vigne Vin. 41 (2007) 193-201.

[10] OIV, 2008. Recueil international des méthodes d'analyse des vins et des moûts. Paris, France.

[11] C. Deytieux, L. Geny, D. Lapaillerie, S. Claverol, M. Bonneau, B. Donèche, J. Experimental Botany 58 (2007) 1851-1862.

[12] F. Zocca, G. Lomolino, A. Curioni, P. Spettoli, A. Lante, Food Chem. 102 (2007) 59-65.

[13] U. K. Laemmli, Nature 227 (1970) 680–685.

[14] G. Lomolino, C. Rizzi, P. Spettoli, A. Curioni, A. Lante, Agro Food Industry Hi-Tech (2003) 32–35.

[15] J.P. Derckel, L. Legendre, J. C. Audran, B. Haye, B. Lambert, Plant Science 119 (1996) 31-37.

[16] J.P. Ride, Physiol. Plant Pathol. 16 (1980) 187-196.

[17] A. E. Hagerman, P. J. Austin , J. Agric. Food Chem. 34 (1986) 440–444.

[18] K. R. Silveira Celestino, R. B. Cunha, C. R. Felix, BMC Biochem. 7 (2006) 23-31.

[19] G. L. Miller, Anal. Biochem. 31 (1959) 426-428.

[20] J. Trudel, P. Audy, A. Asselin, Molecular plant-Microbe Interactions 2 (1989) 315-324.

[21] J. Molano, I. Polacheck, A. Duran, E. Cabib, J Biol Chem. 254 (1979) 4901–4907.

[22] D. A. Mills, E. A. Johannsen, L. Cocolin, Appl. Env. Microbiol. 68 (2002) 4884-4893.

[23] L. Cadle-Davidson, Am. J. Enol. Vitic. 59 (2008) 387-395.

[24] R. S. S. Teixeira, P. M. Pereira, V. S. Ferreira-Leitão, Enzyme Res. 2010 (2010) 905896.

[25] D. B. Tattersall, R. van Heeswijck, P. B. Høj, Plant Physiol. 114 (1997) 759-769.

[26] S. Vincenzi, A. Curioni, Electrophoresis 26 (2005) 60-63.

[27] T. Girbau, B.E. Stummer, K.F. Pocock, G.A. Baldock, E. Scott, E.J. Waters, Australian J. Grape Wine Res. 10 (2004) 125–1335.

[28] S. A. Oliveira-Collet, M. A. Collet, M. F. P. S. Machado, Biochem. Syst. Ecol. 33 (2005) 691-703.

[29] F. Zocca, PhD Thesis, Padua University (2009).

[30] R.B. Boulton, V.L. Singleton, L.F. Bisson, R.E. Kunkee, Principles and practices of winemaking, Chapman & Hall, New York, 1996.

[31] D. Doubourdieu, PhD Thesis, Bordeaux II University (1983).

[32] I. Sasaki, H.P Nagayama, Biosci. Biotech. Biochem. 58 (1994) 616-620.

[33] M. Frey, R. Kliem, H. Saedler, A. Gierl, Mol. Gen. Genet. 246 (1995) 100-109.

[34] Z. Chen, J. Malamy, J. Henning, U. Conrath, P. Sanchez-Casas, H. Silva, J. Ricigliano, D.F. Klessig, Proc. Natl. Acad. Sci. USA 92 (1995) 4134-4137.

[35] B. Brzobohaty, I. Moore, P. Kristoffersen, N. Campos, J. Schell, K. Palme, Science 262 (1993) 1051-1054.

[36] M. Marangon, S. Van Sluyter, K.A. Neilson, C. Chan, P.A. Haynes, E.J. Waters, R.J. Falconer, J. Agric. Food Chem. 59 (2011) 733-740.

[37] M. Esteruelas, P. Poinsaut, N. Sieczkowski, S. Manteau, M.F. Fort, J.M. Canals, F. Zamora, Food Chem. 113 (2009) 28-35.

[38] A.S. Renault, A. Deloire, J. Bierne, Vitis 35 (1996) 49-52.

[39] F. J. Fernandez-Acero, I. Jorge, E. Calvo, I. Vallejo, M. Carbù, E. Camafeita, C. Garrido, J. A. Lopez, J. Jorrin, J. M. Cantoral, Arch. Microbiol. 187 (2007) 207-215.

[40] M. Deveze-Alvarez, J. Garcia-Soto, G. Martinez-Cadena, Microbiol. 147 (2001) 2579-2584.

[41] V. Pancholi, G.S. Chhatwal, Int. J. Med. Microbiol. 293 (2003) 391-401.

Figure 1. Chitinolytic activity detection after SDS-PAGE under non-reducing conditions of grape berries extracts (obtained with Protocol 2). In each lane 50 µg of extract were loaded.

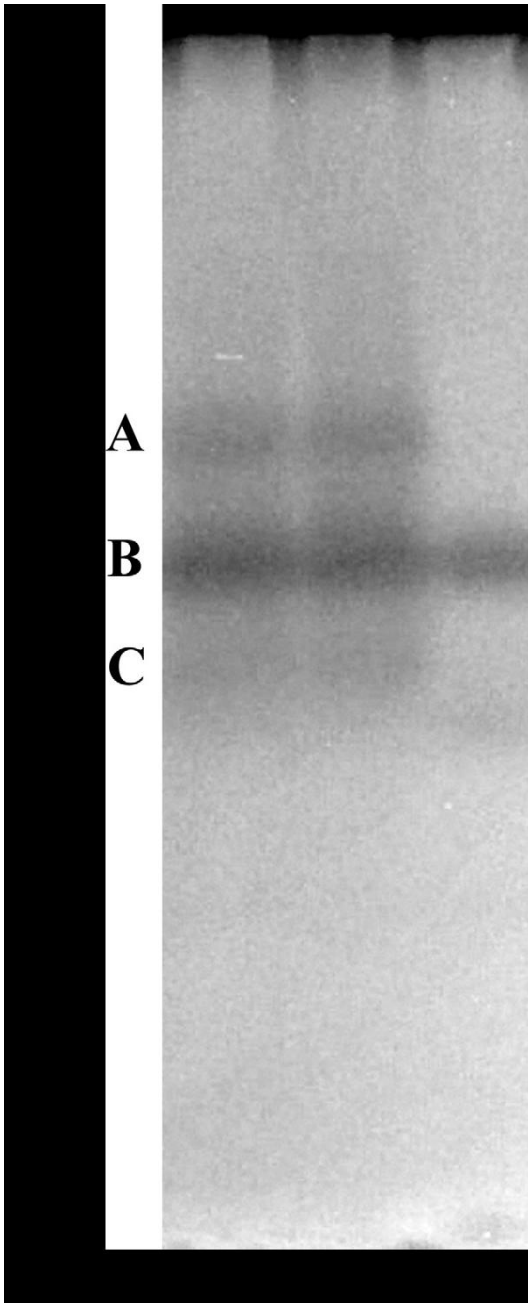

Figure 2. SDS-PAGE analysis under reducing conditions of the extracts of the grape berries of different color (as indicated on the top). In each lane 20 µL of extract were loaded.

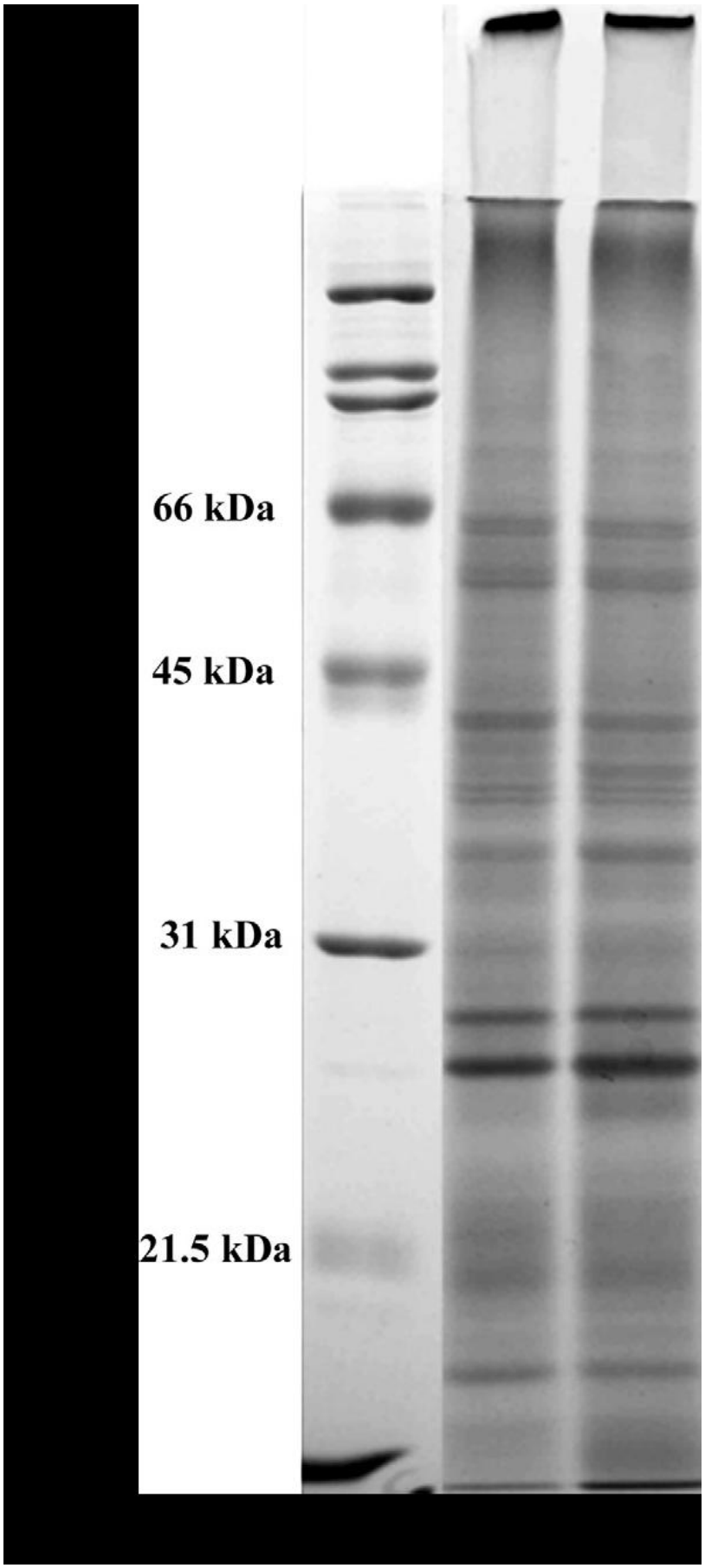
66 kDa
45 kDa
31 kDa
21.5 kDa

Figure 3. Detail of the SDS-PAGE gel showing the four major bands appearing in the blue berries. These bands were excised from the gel and analyzed by LC-MS/MS.

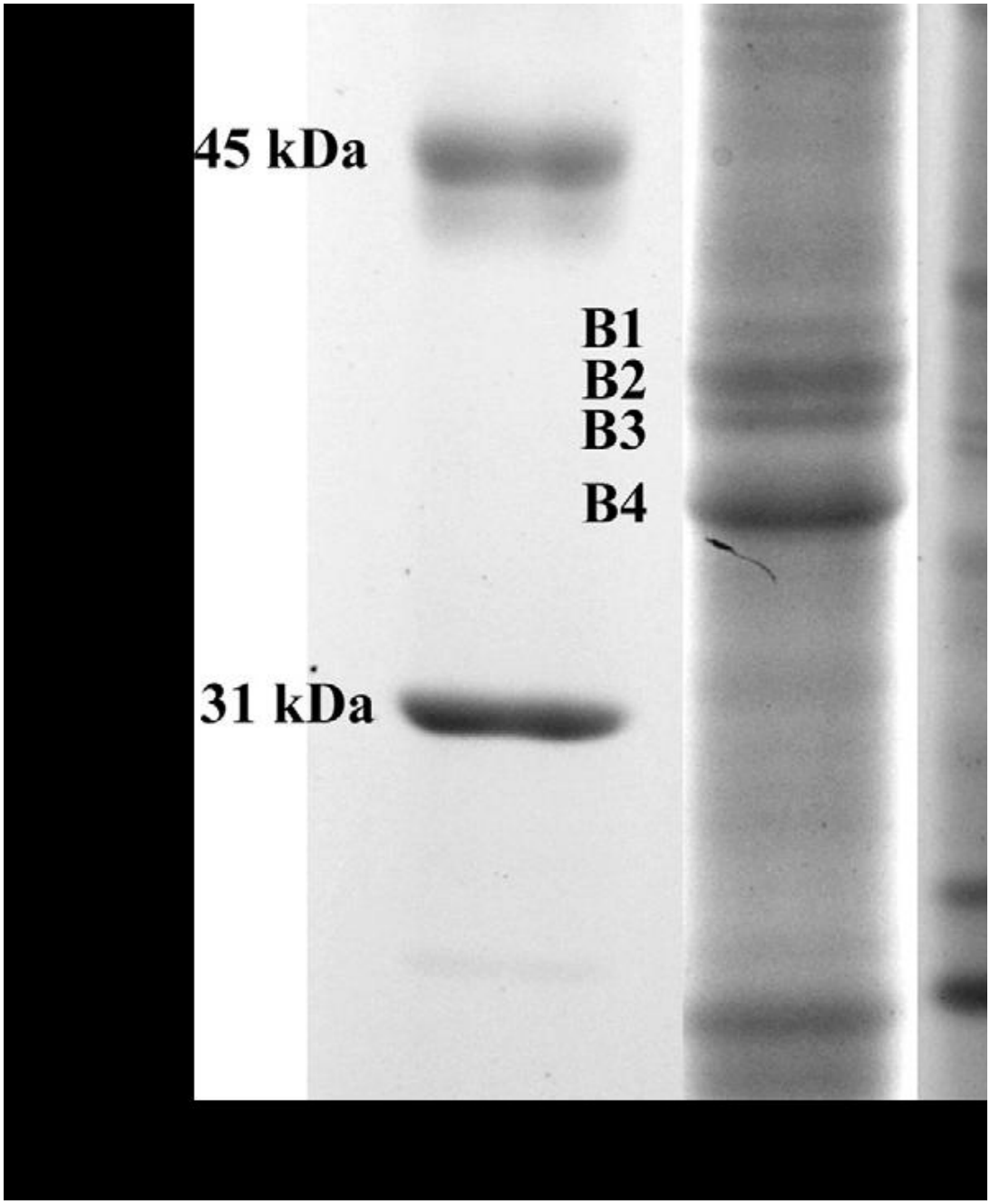

Table 1. Main technological parameters of musts obtained from Erbaluce dried berries separated on the basis of the colour.

Grape	Water content (%)	Reducing sugars (g L^{-1})	pH
Green	58.8 ± 3.7^{a}	407 ± 4^{b}	3.46 ± 0.08^{b}
Gold	67.0 ± 6.1^{a}	480 ± 6^{b}	3.62 ± 0.07^{b}
Blue	38.9 ± 3.1^{b}	620 ± 80^{a}	5.38 ± 0.02^{a}

All data are expressed as average value ± standard deviation (n=3). Means followed by different letters are significantly different ($P < 0.01$).

Table 2. Enzymatic activities (expressed as U g^{-1} of dry weight) measured on the protein extracts of different classes of berries.

	Chitinase	Esterase	Glucosidase	Pectin methyl esterase	Glucanase
GREEN	0.80 ± 0.06^{a}	0.78 ± 0.25^{a}	0.197 ± 0.021^{b}	0.483 ± 0.08^{a}	1.14± 0.06
GOLD	0.79 ± 0.04^{a}	0.65 ± 0.01^{a}	0.228 ± 0.007^{b}	0.209 ± 0.034^{c}	0.90± 0.12
BLUE	0.25 ± 0.05^{b}	0.11 ± 0.06^{b}	0.329 ± 0.008^{a}	0.365 ± 0.011^{a}	1.22± 0.01

All data are expressed as average value ± standard deviation (n=3). Means followed by different letters are significantly different ($P < 0.01$).

Table 3: Proteins identified in bands excised from gel electrophoresis of the blue berries extract.

Gel band	Accession number	Protein	Taxonomy	Mascot score	#Peptides
B1	D7U304	Whole genome shotgun sequence of line PN40024, scaffold_5.assembly12x	*Vitis vinifera*	486	6
	D7U303	Whole genome shotgun sequence of line PN40024, scaffold_5.assembly12x	*Vitis vinifera*	345	5
	Q7XAU7	Thaumatin-like protein	*Vitis vinifera*	248	3
	D8WSH4	Glyceraldehyde-3-phosphate dehydrogenase	*Botryosphaeria dothidea*	233	5
	Q9M6B3	Malate dehydrogenase	*Vitis vinifera*	214	5
	D7TDZ8	Whole genome shotgun sequence of line PN40024, scaffold_151.assembly12x	*Vitis vinifera*	185	5
	A5C7L5	Putative uncharacterized protein	*Vitis vinifera*	176	4
	A6SGS7	Glyceraldehyde-3-phosphate dehydrogenase	*Botryotinia fuckeliana*	122	3
	D7SH97	Whole genome shotgun sequence of line PN40024, scaffold_0.assembly12x (Fragment)	*Vitis vinifera*	113	5
	O04708	VVTL1	*Vitis vinifera*	79	2
	Q7XAU6	Class IV chitinase	*Vitis vinifera*	78	2
	A6RJ45	ATP-dependent RNA helicase eIF4A	*Botryotinia fuckeliana*	38	2
B2	D8WSH4	Glyceraldehyde-3-phosphate dehydrogenase	*Botryosphaeria dothidea*	446	5
	UPI00019839ED	PREDICTED: hypothetical protein isoform 1	*Vitis vinifera*	437	9
	UPI00019839EA	PREDICTED: hypothetical protein isoform 2	*Vitis vinifera*	374	8
	Q7XAU7	Thaumatin-like protein	*Vitis vinifera*	294	4
	A5C7L5	Putative uncharacterized protein	*Vitis vinifera*	172	4
	Q9M6B3	Malate dehydrogenase	*Vitis vinifera*	165	5
	D7TDZ8	Whole genome shotgun sequence of line PN40024, scaffold_151.assembly12x	*Vitis vinifera*	160	5
	A6SGS7	Glyceraldehyde-3-phosphate dehydrogenase	*Botryotinia fuckeliana*	86	3
	UPI00015C9152	PREDICTED: hypothetical protein	*Vitis vinifera*	59	3
B3	UPI00019839ED	PREDICTED: hypothetical protein isoform 1	*Vitis vinifera*	672	11
	UPI00019839EA	PREDICTED: hypothetical protein isoform 2	*Vitis vinifera*	595	9
	Q9M6B3	Malate dehydrogenase	*Vitis vinifera*	292	6
	D8WSH4	Glyceraldehyde-3-phosphate dehydrogenase	*Botryosphaeria dothidea*	203	4
	D7TDZ8	Whole genome shotgun sequence of line PN40024, scaffold_151.assembly12x	*Vitis vinifera*	203	5
	Q7XAU7	Thaumatin-like protein	*Vitis vinifera*	198	3
	A5C7L5	Putative uncharacterized protein	*Vitis vinifera*	133	2
	O04708	VVTL1	*Vitis vinifera*	125	2
	UPI00015C9152	PREDICTED: hypothetical protein	*Vitis vinifera*	112	3
	D7TJ27	Fructose-bisphosphate aldolase	*Vitis vinifera*	86	2
	A5B7S5	Putative uncharacterized protein	*Vitis vinifera*	30	2
	Q7XAU6	Class IV chitinase	*Vitis vinifera*	21	2

B4	UPI00015CB0BD	PREDICTED: hypothetical protein	*Vitis vinifera*	572	4
	UPI00019839EE	PREDICTED: hypothetical protein isoform 2	*Vitis vinifera*	561	7
	O04708	VVTL1	*Vitis vinifera*	136	2
	Q7XAU7	Thaumatin-like protein	*Vitis vinifera*	245	3
	D7U304	Whole genome shotgun sequence of line PN40024, scaffold_5.assembly12x	*Vitis vinifera*	307	4
	A6S494	Malate dehydrogenase	*Botryotinia fuckeliana*	294	4
	D7TDZ8	Whole genome shotgun sequence of line PN40024, scaffold_151.assembly12x	*Vitis vinifera*	164	4
	UPI00015CA2F6	PREDICTED: hypothetical protein	*Vitis vinifera*	70	2