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Seasonal Variation of Defense-Related Gene Expression in Leaves from Bois noir Affected and Recovered Grapevines

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Supporting Information

ABSTRACT: Although Bois noir is one of the main phytoplasma diseases of grapevine, the gene expression and enzyme activities that underlie physiological changes occurring in symptomatic and recovered (with spontaneous or induced symptom remission) plants are mostly unknown. Bois noir symptomatic leaves (September 2006, 2007) and symptomless leaves from infected symptomatic plants (September 2007) of Sangiovese (moderately susceptible) and Chardonnay (highly susceptible) cultivars were collected. Moreover, leaves from infected symptomless plants of both cultivars were harvested in June 2007. Leaves from recovered plants were also collected in the same periods. In recovered plants of both cultivars, class III chitinase and almost every time phenylalanine ammonia-lyase and chalcone synthase expression were increased for all collection periods. In symptomatic leaves of both cultivars, the expressions of the same genes were up-regulated and also those of β-1,3-glucanase and flavanone 3-hydroxylase. The activities of chitinase, phenylalanine ammonia-lyase, β-1,3-glucanase, and superoxide dismutase generally correlated with gene expression. For the moderately susceptible Sangiovese, the defense genes were generally up-regulated in both symptomatic and symptomless leaves (for all collection periods). This behavior was not observed in the highly susceptible Chardonnay, in which changes in gene expression were linked to evident symptom display. Therefore, the physiological response of the plants to this pathogen infection appear to be the reason for the resistance of the cultivar to the disease.

KEYWORDS: enzyme activity, grapevine yellows, phenylpropanoid genes, plant−phytoplasma interaction, quantitative real-time PCR, recovery, *Vitis vinifera*

INTRODUCTION

Bois noir (BN) is a severe phytoplasma disease of grapevine (*Vitis vinifera*) that is associated with stolbur (16SrXII-A subgroup), and it poses a serious phytosanitary problem in Europe and the Mediterranean area.[1] Phytoplasma are nonculturable degenerate Gram-positive prokaryotes that are obligate symbionts of plants and insects and that need both hosts for their dispersal in nature.[2] Grapevines infected by BN have an array of symptoms on leaves, shoots, and grapes that suggest profound disturbances in the balance of plant hormones and disorders in phloem functions via localized phytoplasma accumulation or multiplication in symptomatic leaves.[3,4] The symptomatology associated with the expression of phytoplasma disease has been correlated with plant phenology and the location and abundance of phytoplasma within individual grapevines.[5] However, reduced translocation of phloem has also been observed in plants with a low titer of phytoplasma, suggesting a more indirect influence of the parasite on host metabolism and phloem function.[6] Grapevine sensitivities to BN change according to cultivar, and symptoms range from very severe, as in highly susceptible cultivars (e.g., Chardonnay), to mild, as in tolerant cultivars, whereas no completely resistant cultivars have been described to date.[7] The typical BN symptoms on the leaves can appear at different periods, according to the cultivar and latitude. In central Italy, BN symptoms are usually visible from July to October.[8] The symptomatic plants infected by phytoplasma can undergo recovery, which is seen as a spontaneous remission of disease symptoms, and which may or may not involve the elimination of the causal pathogen from the host.[9,10]

This recovery phenomenon has been observed for different grapevine cultivars affected by both BN and Flavescence dorée in different viticultural regions,[11] and it can be spontaneous or induced.[11−13] In recovered grapevines, the disease symptoms and the phytoplasma causal agent disappear from the canopy.[12]

To date, little is known of the mechanisms involved in the phytoplasma−plant interactions. The information available after sequencing of two '*Candidatus* Phytoplasma asteris' strains,[14] '*Ca.* P. australiense'[15] and '*Ca.* P. mali',[16] have provided an initial understanding of the physiology and requirements of these phytoplasma and, in particular, of the genetic bases of their pathogenicity.[17] Initial investigations into phytoplasma−plant interactions have shown changes in polyphenol production and sugar and amino acid transportation as well as comprehensive differences in gene expression, as mainly reported for the experimental host plant, the periwinkle (*Catharanthus roseus*).[3,4,18] Recently, changes in gene expression related to carbohydrate metabolism in grapevine infected with BN were reported by Hren et al.[19,20]

At the same time, some studies have investigated the mechanisms involved in recovery from phytoplasma diseases in different plant species, including apple, apricot, and grapevine.[21−23] These studies have shown that the reactions in these plants appear to be induced systemic resistance or systemic acquired resistance (SAR),[24,25] which represent states of heightened defense to secondary infections. This suggests that the metabolic

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pathways involved in this recovery are the same as those involved in the mechanisms of defense responses in compatible and incompatible pathogen interactions.[26]

A way to monitor physiological changes in plant tissues is to determine the relative expression levels of the genes that appear to be involved. Recently, studies performed on a genomic scale in BN-infected plants using GeneChip microarrays have underscored an alteration, which can be cultivar specific, in the expression of many genes involved in different pathways, for example, related to the cell wall, defense mechanisms, and responses to stress.[20,27] To date, however, the physiological changes in BN-infected symptomatic and recovered plants according to phenological plant development and the presence or absence of symptoms remain unknown.

The aim of this study was to investigate the expression of genes related to the plant defense mechanisms on symptomatic and recovered BN affected plants. These were determined in highly sensitive (Chardonnay) and moderately sensitive (Sangiovese) grapevine cultivars in two different phenological stages, according to the presence/absence of disease symptoms, to assess if gene expression and enzyme activities that underlie physiological changes are linked to the disease and/or to symptom display. The study analyzed the relative expression levels of several marker genes that covered a large set of defense classes of proteins involved in grapevine−pathogen interactions.[28,29] Some of these genes are known as pathogenesis-related proteins (β-1,3-glucanase and class III chitinase), others belong to secondary metabolism (phenylalanine ammonia-lyase, chalcone synthase, and flavanone 3-hydroxylase), and some were related to oxidative stress (superoxide dismutase, catalase, class III peroxidase) and electron transport (NADPH). Furthermore, the β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase, and superoxide dismutase activities were examined for samples harvested in September 2007.

MATERIALS AND METHODS

Plant Materials. The two cultivars used, Sangiovese and Chardonnay, were grown in the ASSAM Experimental Farm of Petritoli (AP), in the Marche region of central eastern Italy. From each cultivar, five each of symptomatic (infected) and recovered from BN plants and control (healthy) plants were randomly selected. The plants recovered from BN that were analyzed in this study obtained their recovery after partial uprooting.[12] These plants lost the disease symptoms in 2006 and were found to be free of the pathogen after molecular diagnosis. Three different time points were chosen for the study: September 2006 and 2007, when BN symptoms are clearly visible, and June 2007, when plants that were symptomatic the previous September did not show any leaf symptoms. The leaf samples from the same plants at each sampling point were collected and analyzed separately. The leaves from each symptomatic plant included only symptomatic leaves in September 2006 and only symptomless leaves in July 2007, but both symptomatic and symptomless leaves in September 2007. At least two leaf samples, corresponding to 10−12 leaves (about 30 g), were collected from each leaf typology, and they were immediately frozen in dry ice. These samples were maintained for 1−2 months at −80 °C, until RNA extraction. To determine the presence of phytoplasma and to assess the enzyme activities, additional leaf samples were collected from both of the cultivars at each time point. Moreover, all of the plants in this study had been tested in November 2005 for the presence of the main grapevine viruses (by ELISA: GFLV, GLRaV-1, GLRaV-2, GLRaV-3, GVA, and GFkV).

Phytoplasma Identification. Phytoplasma identification was performed in all of the samples, and DNA was extracted from the grapevine leaves using DNeasy Plant Mini kits (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The universal primers P1[30] and P7[31] for ribosomal phytoplasma sequences were used in direct PCR assays. Irrespective of the results, the reaction products were diluted 1:30, and 1 μL was used as template in nested reactions driven by the primer pair R16(I)F1/R1.[32] Each reaction was performed in a total volume of 25 βL, containing 20−30 ng μL^{-1} DNA, 12.5 mL of 2× Ready Mix Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO), and 1 μM of each forward and reverse primer. All of the PCR amplifications were carried out according to the following parameters: 1 denaturation cycle of 5 min at 94 °C, then 36 cycles with a denaturation step of 1 min at 94 °C, annealing for 2 min at 50 °C, and elongation for 3 min at 72 °C, with a final cycle of 10 min at 72 °C. Ten microliter aliquots of the R16(I)F1/R1 amplicons were digested for 3 h at 37 °C with 3 U of *Mse*I restriction endonuclease (New England BioLabs).

The PCR-RFLP products were analyzed by electrophoresis in 1.5% agarose (Sigma-Aldrich) gels, along with a 1 kb DNA size marker (New England BioLabs), and the DNA bands were visualized under UV light after staining with ethidium bromide.

Gene Expression Analysis. Gene expression analysis on symptomatic, symptomless, symptomless recovered, and control leaves was performed according to reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), using a SYBR Green dye system, according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[33] The comparative $\Delta\Delta C_t$ method was used to evaluated the relative quantities of each of the amplified products in the samples.[34]

RNA Extraction. High-quality total RNA was obtained from the leaves according to the method of Costantini et al.[35] Two independent RNA extractions were performed on each sample. Briefly, 15 g of leaf tissue was ground in liquid nitrogen, and 200 mg of the resulting leaf powder was randomly collected for RNA extraction. Extraction buffer was added [1 mL: 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2% (w/v) CTAB (Sigma), 2% (v/v) β-mercaptoethanol, 2.5 M NaCl, 2% (w/v) soluble PVP-40], and the samples were incubated at 65 °C for 30 min. Following centrifugation at 10000*g* for 10 min at 4 °C, the lysates were transferred to QIAShredder spin columns (RNeasy Plant mini-kit, Qiagen) and centrifuged at maximum speed for 2 min. The supernatants were transferred to new tubes with an equal volume of chloroform/isoamyl alcohol (24:1), mixed, and centrifuged at 10000*g* for 5 min at 4 °C. This last step was repeated twice. The aqueous phases containing the total RNA were precipitated in 0.25 volume of 10 M LiCl, with the reaction left to proceed overnight at 4 °C. The samples were then centrifuged at 10000*g* for 30 min at 4 °C, and the pellets were resuspended in 70% ethanol and centrifuged again at 10000*g* for 5 min at 4 °C, before being dried at 37 °C for 5 min. The RNA was resuspended in 50 μL of double-distilled DEPC water. The RNA integrity was verified by resolving the samples in 1.5% agarose gels and subsequent ethidium bromide staining. RNA purity was assessed on the basis of an absorbance ratio of 1.80−1.90 at 260/280 nm using BioPhotometer plus (Eppendorf Inc., Westbury, NY).

Reverse Transcription. A total of 40−50 ng of RNA was used for cDNA synthesis with reverse transcription PCR using QuantiTect Reverse Transcription kits (Qiagen), according to the manufacturer's instructions. From each RNA extraction, cDNA synthesis was performed twice, and the products were mixed before the gene expression studies.

Primers and Reference Gene Selection. To obtain accurate and reproducible results using SYBR Green technology, specific primer sets were designed using Primer3 software (http://biotools.umassmededu/bioapps/primer3_www.cgi) from the specific sequence of *V. vinifera* deposited in the NCBI GenBank (Table 1). The primer pairs were chosen and validated in silico using primer BLAST-specific analysis (http://www.ncbi.nlm.nih.gov/Blast.cgi) and then according to the melting profiles obtained from the quantitative real-time PCR conditions (qPCR) described later.

Table 1. Primers Selected for the Gene Expression Analyses of the Recovered, Symptomatic, and Control Grapevines

gene	NCBI accession no.	sequence	TM[a]	amplicon (bp)
β-1,3-glucanase	AJ277900	TGCTGTTTACTCGGCACTTG	60.1	224
		CTGGGGATTTCCTGTTCTCA	60.1	
class III chitinase	DQ406693	AAACTTATCAGCGCCTGGAA	59.8	162
		ACCTCCATACTTGGGGGAAG	60.2	
phenylalanine ammonia-lyase	X75967	GAAATTGCCATGGCTTCCTA	60.1	215
		TCCTCCAAATGCCTCAAATC	60.0	
chalcone synthase	EF192464	TCTCGTGTTCTGGTCGTCTG	60.0	162
		GAACAGTGGGCGTTCAATTT	59.9	
flavanone 3-hydroxylase	EF192467	TGCTGGAGGTGTTGTCTGAG	60.0	236
		AGGTTGAACGGTGATCCAAG	59.9	
NADPH dehydrogenase	EE253759	CGCTGAAGCTGCTATTGGAC	61.0	212
		TCATGAGAGAGGGCCAAGAT	59.7	
class III peroxidase	AY348574	CAAGATCGGGTGGAGATGAT	59.8	194
		GGCCACATCCGTAAAGAAAG	59.6	
catalase	AF236127	GCAAGAGCGTTTCATCAACA	60.0	184
		TTGCTGCAGCTTCTTCTCAA	60.0	
superoxide dismutase	AS213456	TTGAGGATGACCTTGGGAA	60.0	207
		GCTGTGCCACTACATGGGATG	59.9	
18S rRNA[b]	AJ421474	CTGTTTGATGAGCCTG	60.1	202
		ATTGCCTTCTTCACTCACG	60.3	
β-tubulin[b]	AF196485	AGATGTGGGATGCCAAGAAC	59.9	238
		GAATTCCCAATGAAGGTGGA	59.7	
actin[b]	AY84762	TCCTTCGTCTTGACCTTGCT	59.9	246
		AACGGAATCTCTCAGCTCCA	59.9	

[a] Melting temperature. [b] Reference genes.

The constitutive 18S rRNA, β-tubulin, and actin reference genes were validated, using the *geNorm* software program available at http://medgen.ugent.be/genorm,[36] and used to normalize the gene expression analysis.

Quantitative Real-Time PCR Conditions. qPCR reactions were performed in 96-well clear multiplate PCR plates (Bio-Rad, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ real-time PCR detection system (Bio-Rad) under the following conditions: an initial denaturing cycle (8 min at 95 °C), followed by 40 cycles of three steps of denaturation, annealing, and polymerization (20 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C). PCR amplification was carried out in a total volume of 22 μL, containing 9 μL of diluted (1:10) cDNA (duplicate), 0.5 μM of each primer, and 11 μL of iQ SYBR Green Supermix. All of the assays included no RT and no-template controls, to verify nonspecific amplification. At the end of each qPCR, a melting curve analysis was performed over the range from 55 to 98 °C to determine the specificity of the amplicons. The qPCR efficiency (E) of each primer pair was set up initially using standard curves generated according the equation $E = 10^{-1/\text{slope}}$ of six duplicate 10-fold serial dilutions (10^{-3}—10^{-8} ng of DNA) of PCR fragments of the target and reference genes. However, the specific calibration curves used for the analysis were generated according to cDNA pool dilutions (undiluted, 1:10, and 1:100).

Enzyme Activities. Spectrophotometric assays were used to determine β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase, and superoxide dismutase activities of the leaf tissue of the symptomatic and symptomless leaves collected from BN symptomatic plants and symptomless recovered and control leaves collected in September 2007. Frozen leaves (250 mg fresh weight) were ground, and β-1,3-glucanase, chitinase, and superoxide dismutase were extracted with 1% (w/w) PVPP and 50 mM sodium acetate buffer (pH 5.0), containing 1 mM DTT and 0.2% (w/v) PMSF. The phenylalanine ammonia-lyase was obtained using 100 mM potassium phosphate buffer (pH 8.0) containing 1% (w/w) PVPP and 1.4 mM β-mercaptoethanol. The homogenates were centrifuged at 15000*g* for 15 min at 4 °C, and the resulting supernatants were used as the crude enzyme extracts. The protein content in the enzyme extracts was determined according to the Bradford assay[37] (Sigma-Aldrich), using bovine serum albumin as standard.

β-1,3-Glucanase activity was assayed according to the procedure described by Derckel et al.,[38] using 225 μL of 0.5 mg mL $^{-1}$ laminarin (dissolved in 50 mM sodium acetate buffer, pH 5.0). The samples were

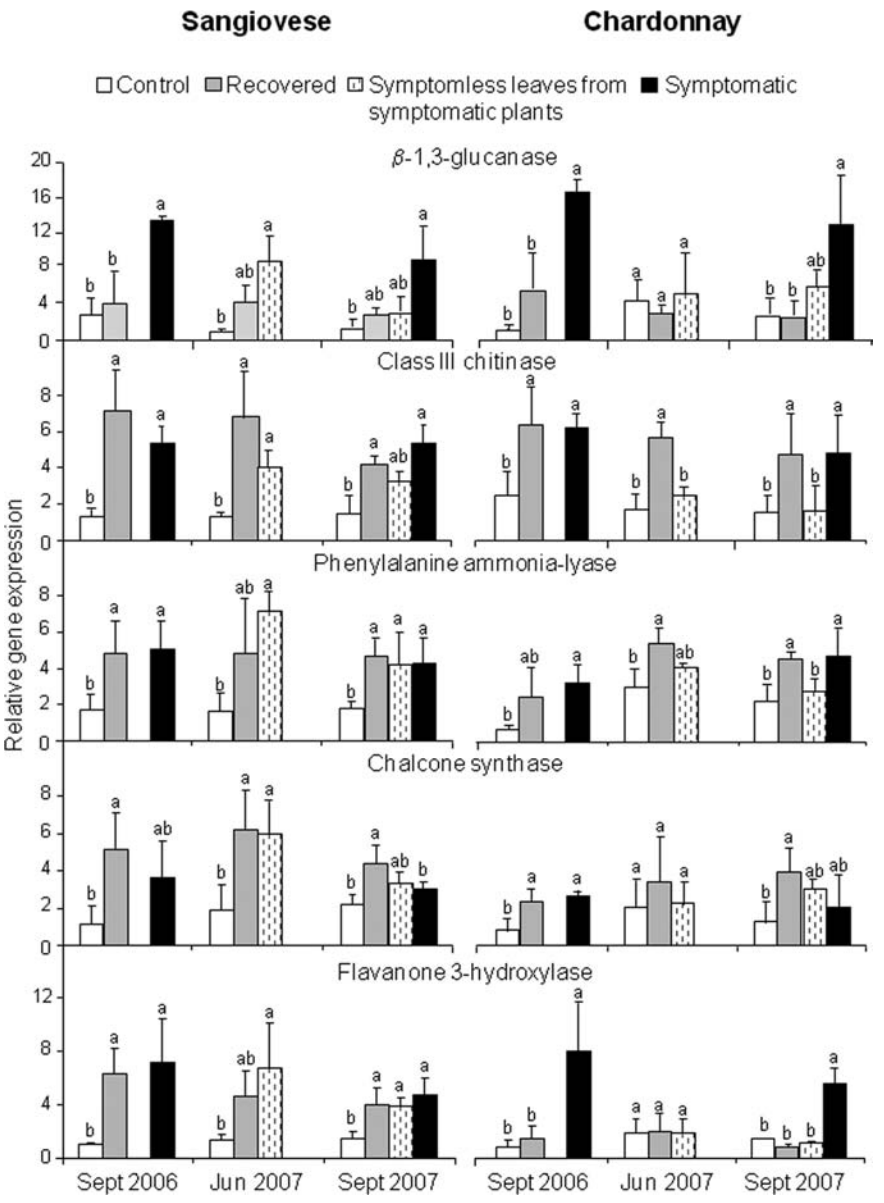

Figure 1. Relative gene expression of hydrolytic pathogenesis-related proteins and enzymes of the phenylpropanoid pathway (as indicated) in leaf tissue from control, recovered, symptomatic leaves, and symptomless leaves from symptomatic plants (see key) of Sangiovese (left) and Chardonnay (right) at the different sampling times. Data are the mean ± SD, and values with the same letter are not statistically different, according to Duncan's multiple-range test, at $P \leq 0.05$.

incubated at 37 °C for 3 h and stopped by the addition of 500 μL of a solution containing 16 g L^{-1} glycine, 450 mg L^{-1} $CuSO_4 \cdot 5H_2O$, and 500 μL of 1.2 mg mL^{-1} neocuproine−HCl, before the whole mixture was boiled for 3 min. The absorbance of reducing sugars released from laminarin was measured at 450 nm. Chitinase and phenylalanine ammonia-lyase activities were measured according to the method of Trotel-Aziz et al.[39] For chitinase activity, 100 μL of extracted protein was mixing with 200 μL of chitin azure as substrate (1 mg mL^{-1} in 100 mM sodium acetate buffer, pH 5.0). The samples were incubated for 2 h at 37 °C before the reactions were stopped with 0.4 mL of 0.3 N HCl and the samples were placed on ice for 10 min. The tubes were then centrifuged at 5000*g* for 10 min. The absorbance of the supernatant was measured at 550 nm.

Phenylalanine ammonia-lyase activity was measured by mixing 0.45 mL of Tris−HCl buffer (100 mM, pH 8.8), 0.2 mL of 40 mM phenylalanine (Sigma Aldrich), and 0.15 mL of enzyme extract. The mixture was incubated for 2 h at 37 °C, and the reaction was stopped by the addition of 0.2 mL of 25% trichloroacetic acid (TCA). The assay mixture was centrifuged at 10000*g* for 15 min at 4 °C, and the absorbance of the supernatant was measured at 289 nm.

Superoxide dismutase activity was assayed by monitoring the inhibition of the photochemical reduction of nitro-blue tetrazolium, according to the method of Giannopolitis and Ries.[40] Three milliliters of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitro-blue tetrazolium, 2 mM riboflavin, and 0.1 mM EDTA, with 100 μL of enzyme extract, was illuminated for 30 min at a light intensity of 3000 lx. The absorbance was measured at 560 nm. All of the enzyme activities were analyzed using a UV 1800 spectrophotometer (Shimadzu Corp., Tokyo, Japan) from leaves from five different plants for each typology. For all of the assays, the absorbance was measured against a blank (crude protein extract in incubation mixture). The enzyme activities were expressed as units of milligrams per minute per gram of protein.

Data Analysis. In the gene expression study, for each data set, the expression levels of each target gene in all of the samples were quantified according to the comparative $\Delta\Delta C_t$ method.[34] Using this method, the levels of expression were given as the mean fold-changes in gene expression normalized to an endogenous reference gene and relative to the untreated control. For the present study, control plant 1 (Sangiovese) and control plant 2 (Chardonnay) were selected. For each individual sample, four technical replicates were analyzed. The gene expression calculations were performed using the Excel program "Gene expression analysis for iCycler iQ Real Time PCR detection system" (Bio-Rad). The calculations in this spreadsheet were derived from the algorithms outlined by Vandesompele et al.[36] for each sample of interest, the normalization factor was the geometric mean of the relative quantities for all of the reference genes that had the same identifier as that of the sample.

For each plant typology, the results are shown as the mean ± SD of $n = 10$ gene expression analyses obtained from the cDNAs from two different RNA extractions and of $n = 10$ enzyme activities obtained from five different plants from two crude protein extractions. The data from each sampling point were statistically evaluated by ANOVA, followed by individual comparisons using Duncan's multiple-range test at $P \leq 0.05$. The square-root transformation was used to normalize the expression means prior to statistical analysis.

RESULTS

Phytoplasma Identification. The detection of the phytoplasma on grapevine leaves was achieved by nested PCR, which confirmed the presence of the expected specific fragments in all of the samples from symptomatic leaves. No phytoplasma fragments were detected in leaf samples from the control, recovered, and symptomless leaves. The PCR-RFLP analysis performed using *Mse*I endonuclease allowed identification of the phytoplasma as belonging to the stolbur group (16SrXII-A subgroup) (data not shown). All plants tested negative for the presence of the main grapevine viruses (data not shown).

Gene Expression Analysis. Specific primer sets (Table 1) were identified for all of the genes analyzed. The melting curve analyses for all of the amplicons showed a single peak (see the Supporting Information, Figure S1), and no nonspecific products or primer−dimer formation was detected with the amplification of the samples and in the template control reactions (data not shown). Agarose gel electrophoresis separation of each of the amplicons showed a single cDNA fragment of the expected size (data not shown). The standard curves generated using a 10-fold serial dilution of cDNA pool for each gene showed the high efficiency of each specific primer pair for RT-qPCR ranging from 94.7 to 103.1%, with a linear correlation coefficient (R^2) ranging from 0.990 to 0.999 (see the Supporting Information, Figure S2). The expression stability of the reference gene was analyzed using the *geNorm* algorithm of three putative reference genes (Table 1). This showed high expression stability for both of the cultivars according to the control, recovered, and symptomatic samples and had M values lower than 1.3, below the default limit of 1.5 suggested by the *geNorm* program (see the Supporting Information, Figure S3). All of the reference genes were used in RT-qPCR analysis.

Defense-Related Genes. For Sangiovese, the analysis performed in September 2006 showed that usually the defense-related genes were significantly up-regulated in symptomatic plants compared to the control plants. In particular, significantly enhanced gene expression was seen for β-1,3-glucanase (4.8-fold), class III chitinase (4.2-fold), phenylalanine ammonia-lyase (2.9-fold), and flavanone 3-hydroxylase (6.9-fold) (Figure 1). From the same sampling period, in the recovered Sangiovese plants, significantly increased gene expression was seen for class III chitinases (5.4-fold), phenylalanine ammonia-lyase (2.7-fold), chalcone synthase (4.5-fold), and flavanone 3-hydroxylase (6.0-fold), compared to the control plants (Figure 1). For Chardonnay in September 2006, in general, similar gene expression patterns were observed across both the symptomatic and recovered plants with respect to Sangiovese. Thus, for Chardonnay, compared to the control plants, there was significantly enhanced gene expression in symptomatic plants for β-1,3-glucanase (15.2-fold), class III chitinase (2.6-fold), phenylalanine ammonia-lyase (4.5-fold), chalcone synthase (3.1-fold), and flavanone 3-hydroxylase (10.5-fold) (Figure 1). In the recovered Chardonnay plants, significantly enhanced gene expression was seen only for class III chitinase (2.6-fold) and chalcone synthase (2.8-fold) (Figure 1).

In June 2007, when the symptoms were still not evident, the gene expression values changed according to cultivar. In Sangiovese, significantly increased gene expression for symptomless leaves from symptomatic plants was found for β-1,3-glucanase (10.3-fold), class III chitinase (3.2-fold), phenylalanine ammonia-lyase (4.3-fold), chalcone synthase (3.1-fold), and flavanone 3-hydroxylase (5.2-fold). In the recovered plants, only class III chitinase (5.3-fold) and chalcone synthase (3.2-fold) showed significantly increased gene expression, compared to the control plants. In Chardonnay, the analyses performed in June 2007 did not show significant differences in the expression of any of these genes between the symptomless leaves from symptomatic and control plants. In this phenological phase, the recovered Chardonnay plants showed significant enhancement of cDNA transcripts for class III chitinase (3.4-fold) and phenylalanine ammonia-lyase (1.8-fold) (Figure 1).

In September 2007, the gene expression studies involved all of the leaf typologies: recovered, symptomatic, and symptomless leaves collected from symptomatic plants. At this last sampling time, the symptomatic Sangiovese leaf tissue analysis confirmed the gene expression trend observed in September 2006, and significant gene up-regulation was seen for β-1,3-glucanase (4.9-fold), class III chitinase (3.7-fold), phenylalanine ammonia-lyase (2.4 fold), and flavanone 3-hydroxylase (3.4-fold). In the symptomless leaves collected from symptomatic Sangiovese plants, phenylalanine ammonia-lyase and flavanone 3-hydroxylase were overexpressed 2.3- and 2.8-fold, respectively, compared to the control plants (Figure 1). In the recovered Sangiovese plants, significantly increased gene expression was seen for class III chitinase (2.8-fold), phenylalanine ammonia-lyase (2.6-fold), chalcone synthase (2.0-fold), and flavanone 3-hydroxylase (2.9-fold) (Figure 1). Again in September 2007, compared to the control plants, Chardonnay showed significantly enhanced gene expression in the symptomatic leaf tissue for β-1,3-glucanase (4.6-fold), class III chitinase (3.2-fold), phenylalanine ammonia-lyase (2.1-fold), and flavanone 3-hydroxylase (3.9-fold). No significant differences were seen in the symptomless leaves collected from symptomatic Chardonnay plants. At the same sampling time, the recovered Chardonnay plants showed significantly enhanced expression of cDNA transcripts for the class III chitinases (3.3-fold), phenylalanine ammonia-lyase (2.1-fold), and chalcone synthase (2.7-fold) genes (Figures 1 and 3).

Electron Transport and Oxidative Stress Genes. For both cultivars, the analyses performed in September 2006 and June 2007 showed no differences between control plants and both the recovered and symptomatic plants. Some differences were seen in September 2007. In symptomatic leaves of both cultivars, NADPH dehydrogenase was down-regulated, at −2.8-fold for Sangiovese

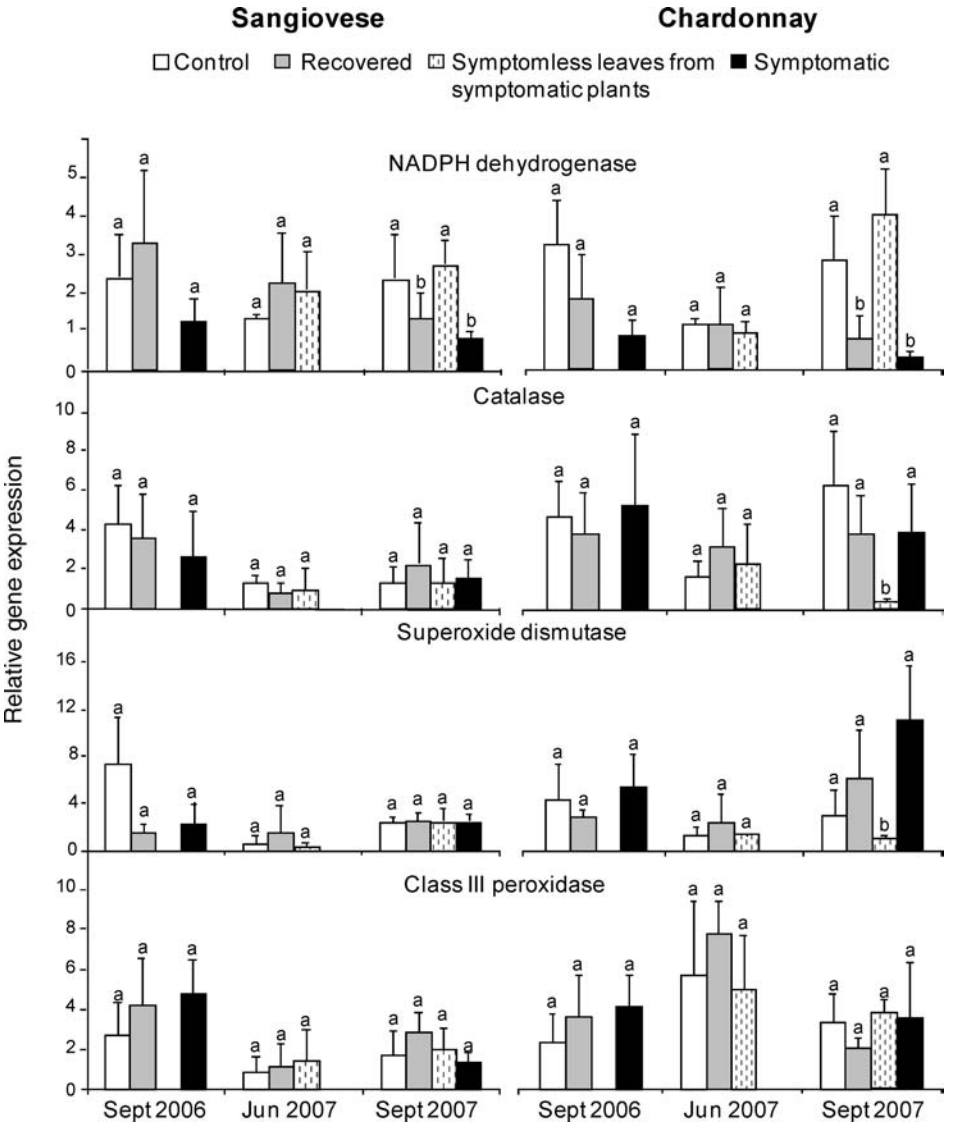

Figure 2. Relative gene expression of enzymes of electron transport and defense mechanisms (as indicated) in leaf tissue from control, recovered, and symptomatic leaves and symptomless leaves from symptomatic plants (see key) of Sangiovese (left) and Chardonnay (right) at the different sampling times. Data are the mean ± SD, and values with the same letter are not statistically different, according to Duncan's multiple-range test, at $P \leq 0.05$.

and −8.7-fold for Chardonnay. At the same time, the recovered plants of both cultivars showed NADPH dehydrogenase down-regulation, at −1.8-fold for Sangiovese and −3.5-fold for Chardonnay. Again, in September 2007, the symptomless Chardonnay leaves collected from symptomatic plants showed down-regulated gene expression for superoxide dismutase and catalase, at −2.97- and −17.4-fold, respectively, compared to the control plants. For all of the sampling times, no differences were seen in the gene expression of class III peroxidase between symptomatic, recovered, and control plants, for both cultivars (Figures 2 and 3).

Enzyme Activities. In September 2007, the whole leaf extracts from the symptomatic and symptomless leaves from the symptomatic, recovered, and control plants were examined for β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase, and superoxide dismutase activities. As illustrated in Figure 4, in symptomatic leaves from symptomatic plants of the Chardonnay cultivar, compared to control plants, there were significant increases in the enzyme activities of β-1,3-glucanase (5.0 units mg^{-1} greater protein), chitinase (7.4 units mg^{-1} protein), phenylalanine ammonia-lyase (7.7 units mg^{-1} protein), and superoxide dismutase (20.5 units mg^{-1} protein). Similarly, the symptomatic leaves showed significantly enhanced enzyme activities compared to symptomless leaves collected from symptomatic plants, for chitinase (6.2 units mg^{-1} greater protein), phenylalanine ammonia-lyase (7.8 units mg^{-1} protein), and superoxide dismutase (17.2 units mg^{-1} protein) (Figure 4). Recovered plants showed significantly increased phenylalanine ammonia-lyase activity (8.5 units mg^{-1} greater protein) compared to control plants (Figure 4). In Sangiovese, symptomatic leaves showed significant increases compared to control plants in the enzyme activities of β-1,3-glucanase (4.9 units mg^{-1} greater protein), chitinase (4.7 units mg^{-1} protein), and phenylalanine ammonia-lyase (4.9 units mg^{-1} protein) (Figure 4). No differences were seen between the symptomatic and symptomless leaves of the Sangiovese cultivar for any of the enzymes analyzed. In Sangiovese recovered plants, there was significantly increased phenylalanine ammonia-lyase activity (9.7 units mg^{-1} greater protein), compared to control plants (Figure 4).

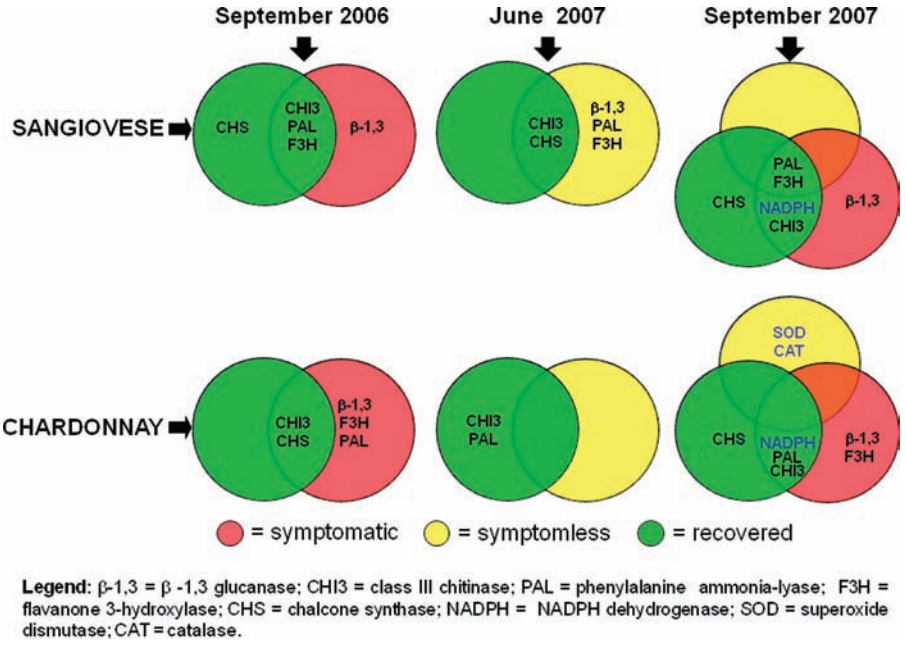

Figure 3. Venn diagrams of genes up-regulated (black font) and down-regulated (blue font) in leaf tissue from control, recovered, and symptomatic leaves and symptomless leaves from symptomatic plants. β-1,3, β-1,3 glucanase; CHI3, class III chitinase; PAL, phenylalanine ammonia-lyase; F3H, flavanone 3-hydroxylase; CHS, chalcone synthase; NADPH, NADPH dehydrogenase; SOD, superoxide dismutase; CAT, catalase.

DISCUSSION

Plant−pathogen interactions can result in the activation of numerous mechanisms of local and systemic defenses. In the present study, we have defined a procedure for the study of relative gene expression and enzyme activities in leaf tissues from symptomatic and recovered BN-infected plants of the two grapevine cultivars Sangiovese and Chardonnay that show different levels of field resistance to BN.[7] We thus report for the first time a gene expression study of BN symptomatic and BN recovered plants analyzed at different phenological stages according the presence of BN symptoms.

In central Italy, BN-infected Chardonnay and Sangiovese grapevines usually show visible symptoms from July to October, which appear as yellow (Chardonnay) or reddish (Sangiovese) bands along the main veins and which gradually extend over large parts of the leaves. In the present study, the occurrence of phytoplasma in leaf tissues was associated with the presence of disease symptoms (data not shown). Leaf veins from the recovered plants were found to be free of the phytoplasma, confirming previous studies.[11,12,23] Here we show that in BN symptomatic leaves, there are increased expression levels of genes that are related to some hydrolytic enzymes that are known as pathogenesis-related proteins (PR-2 group, e.g., β-1,3-glucanase; PR-8 group, e.g., class III chitinase) and of genes that are related to the key enzymes in phenylpropanoid metabolism, such as phenylalanine ammonia-lyase and flavanone 3-hydroxylase, in both Sangiovese and Chardonnay cultivars.

An involvement of pathogenesis-related proteins in grapevine−stolbur interactions has been reported,[20,27] and different studies have indicated that class III chitinase can be considered as an indicator of the SAR response in *Vitis* species.[41,42] The up-regulated gene expression of β-1,3-glucanase in BN symptomatic plants can be associated with the need to make callose-degrading products that can be used to facilitate their spread through the plant, as has been suggested for planthopper attacks.[20,43] We found high expression levels of several genes associated with the biosynthesis of secondary metabolites, such as phenolic compounds and flavonoids, which could be the result of the plant defense responses to the stolbur phytoplasma or to a response associated with symptom development. Compounds of the phenylpropanoid pathway have biological activities against a wide range of pathogens, and they are involved in plant disease resistance.[44,45] Phenylalanine ammonia-lyase catalyzes the first step in the metabolic pathway of phenylpropanoid synthesis, and it participates in the synthesis of precursors of salicylic acid (required for both local defense and SAR), phytoalexins (antimicrobial compounds), and lignin monomers (required to strengthen the mechanical and chemical barriers of plant cells).[46] In grapevine cultivars resistant to fungi, the constitutive up-regulation of phenylalanine ammonia-lyase was suggested as one of the factors responsible for protection against a pathogen attack.[47] Chalcone synthase is a further key branch-point enzyme in the phenylpropanoid pathway, which leads to phytoalexin biosynthesis in grapevine.[48] Flavanone 3-hydroxylase is a pivotal enzyme in the synthesis of the anthocyanin, proanthocyanidin, and flavonol biosynthetic branches, the last of which is known to have a role as a scavenger of reactive oxygen species.[49] On *Vitis* spp., the gene encoding flavanone 3-hydroxylase has been shown to be constitutively expressed, and its expression can also be induced after pathogen inoculation.[28] The phytoplasma symptomatology impairs photosynthesis and accumulation of carbohydrates in mature leaves.[3,19,20] Moreover, Choi et al.[4] proposed the use of secondary metabolic pathways, such as the phenylpropanoids, for the consumption of the accumulated carbohydrates.

In the present study, the gene expression profiling reveals clear differences between the cultivars that depend on the plant phenology and, therefore, according to the appearance of symptoms on the leaves. In symptomatic plants of Sangiovese, the gene expression patterns showed similar trends for all of the genes analyzed, independent of the presence of BN leaf

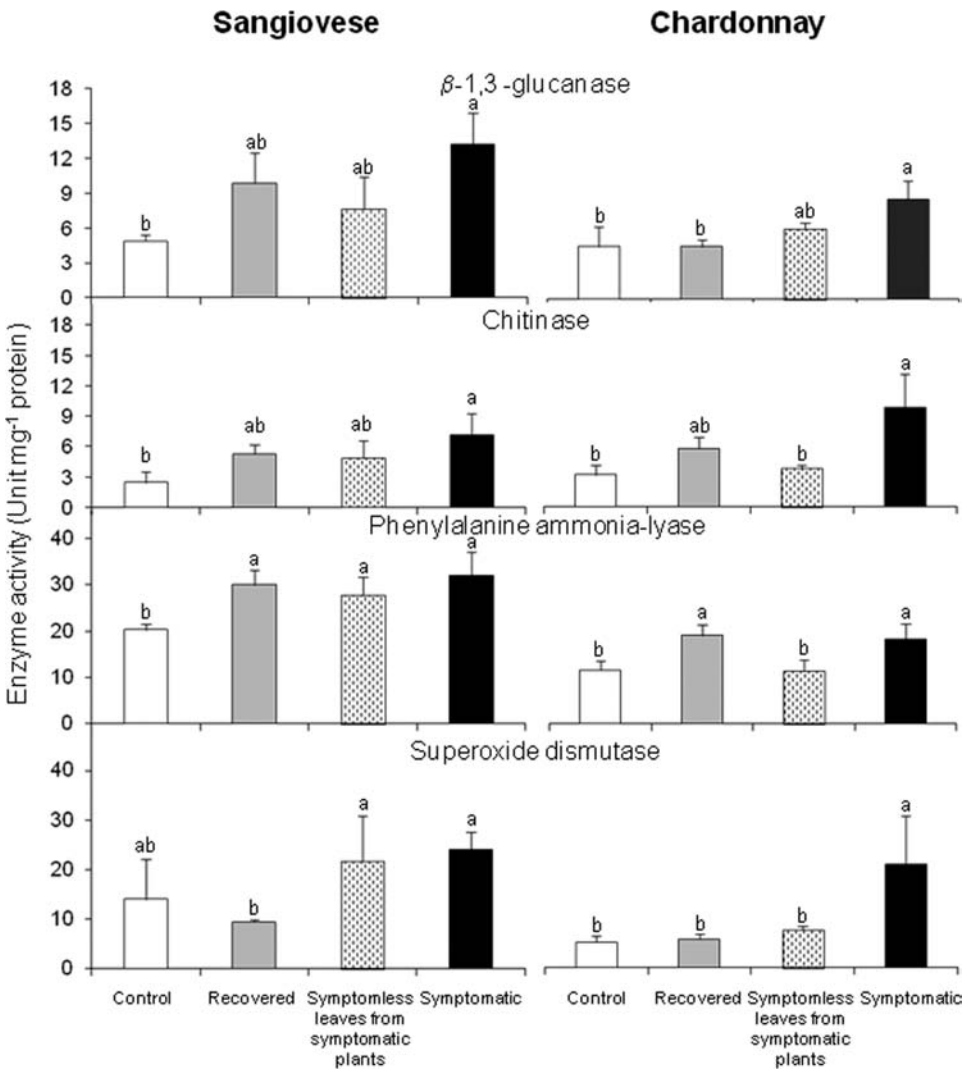

Figure 4. Enzyme activities for β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase, and superoxide dismutase (as indicated) in grapevine leaf tissues from control, recovered, symptomatic plants, and symptomless leaves from symptomatic plants (see key) of Sangiovese (left) and Chardonnay (right) collected in September 2007. Data are the mean ± SD, and values with the same letter are not statistically different, according to Duncan's multiple-range test, at $P \leq 0.05$.

symptoms, for all of the sampling periods; in contrast, in Chardonnay, the increase in gene expression was associated with a seasonal phenology and the appearance of symptoms on leaves. Moreover, the importance of the presence of BN symptoms associated with the cultivar characteristics was confirmed by comparing the gene expression profiles obtained in symptomatic and symptomless leaves from infected plants analyzed in September 2007. In our study, a specific cultivar response was observed for class III chitinase, phenylalanine ammonia-lyase, and flavanone 3-hydroxylase. These cDNA transcripts were up-regulated only in the symptomatic leaves in the Chardonnay cultivar, whereas in the Sangiovese cultivar these genes were up-regulated in both the symptomatic and symptomless leaves from the symptomatic plants. In these plants, the gene expression results generally correlated with the enzyme activities. The only enzyme expression that did not correlate with its activity was superoxide dismutase. In particular, the high superoxide dismutase activity detected for symptomatic leaves from Chardonnay plants compared to control might be associated with post-transcription control or explained by assuming that the superoxide dismutase activity analyzed in this study involved more superoxide dismutase isoforms. However, the activity of the enzymes analyzed in this work confirmed the differences among the cultivars on defense mechanism involvement between symptomless and symptomatic leaves from the same plants. Previous investigations have shown that the abundance of some PR proteins can correlate with cultivar susceptibility to pathogens.[29] Moreover, it is known that these plant responses depend not just on recognition mechanisms but mainly on the biology of the interactions.[50] Nevertheless, the plasticity of the systemic response appears to depend on both the inducer and the challenge. Tolerant plants show that pathogen growth and symptom development can be uncoupled.[51] Indeed, an important aspect to be considered here relates to the different susceptibilities of these cultivars to BN and the characteristics of the constitutive cultivars that can affect local and systemic defense processes. This aspect needs to be further investigated and should include biochemical studies of the molecules affected in the signal

transduction pathways that are suggested to be involved in the responses to phytoplasma infection, such as salicylic acid.[27] In this study, with NADPH dehydrogenase as a component of the plant respiratory chain that is important for the transfer of electrons from NADPH to ubiquinone, its expression was down-regulated in symptomatic leaves, in both cultivars. This shows that the BN symptomatology correlates with disturbances to electron transport during the photosynthetic cycle.

In recovered plants, the genes encoding chalcone synthase, phenylalanine ammonia-lyase, and class III chitinase appeared to be more consistently involved in the recovery phenomenon in both cultivars, and their gene expression was not affected by plant phenology. Previous studies in apricot, apple, and grapevine have suggested that recovery from phytoplasma disease is associated with an accumulation of H_2O_2, and in the grapevine, the down-regulation of enzymatic H_2O_2 scavengers was seen.[21,23] Recently in apple plants recovered from phytoplasma disease, callose synthesis/deposition and phloem−protein aggregation were observed in the plant phloem.[22] In our study, β-1,3-glucanase was not significantly induced in recovered plants, according to both its gene expression and its enzymatic activity. The lack of increase of this enzyme, which is associated with callose degradation, might thus reflect the callose deposition in BN recovered grapevines.[20] In this study, the involvement of class III chitinase in recovered plants that was seen by the gene expression study was not confirmed by the total chitinase activity. This has thus revealed a possible role of inducible class III chitinase compared to the other constitutive defense chitinases, as was seen in proteomic studies carried out in grapevine in response to elicitors,[42] and for SAR induction.[41] In the same way, the involvement of genes related to the phenylpropanoid pathway in the recovered plants might indicate that the accumulation of phytoalexins occurs in these plants.

The present study shows the involvement of specific defense molecules, as mainly β-1,3-glucanase, class III chitinase, phenylalanine ammonia-lyase, and flavanone-3-hydroxylase in BN symptomatic plants and as class III chitinase, phenylalanine ammonia-lyase, and chalcone synthase in recovered grapevines. The novelty of this study resides in the recording of the variation in gene expression according to cultivar sensitivity to BN and the presence/absence of symptoms. A different gene expression pattern was seen for Chardonnay (highly susceptible to BN) as compared to Sangiovese (moderately susceptible). Our data suggest that for Sangiovese, the defense genes were usually up-regulated in both the symptomatic and symptomless leaves (collected in June and September). This behavior was not observed in Chardonnay, where the changes in gene expression were linked to the display of the symptoms. Chardonnay is also less prone to recovery, compared to Sangiovese.[13] Therefore, gene expression and enzyme activities that underlie physiological response of the plant to the pathogen infection might provide the reason for the resistance of the cultivar to the disease.

This study improves our knowledge of the metabolic pathways that are activated in grapevine−phytoplasma interactions and in BN recovered grapevine. This has thus provided useful information toward a better understanding of grapevine BN development, and it will contribute toward the drawing up of management strategies, through defining when plants are more reactive, and to the determination of the optimal timing for the application of BN control measures, such as resistance inducers.[8]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Figure S1 reports the melting curve specific for the primer set used; Figure S2 shows the standard curves of the corresponding target and reference genes; and Figure S3 shows the average expression stability values of the reference genes according to *geNorm* calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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