Changes in Plant Metabolism and Accumulation of Fungal Metabolites in Response to Esca Proper and Apoplexy Expression in the Whole Grapevine

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ABSTRACT

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Trunk diseases have become among the most important grapevine diseases worldwide. They are caused by fungal pathogens that attack the permanent woody structure of the vines and cause various symptoms in woody and annual organs. This study examined modifications of plant responses in green stem, cordon, and trunk of grapevines expressing Esca proper (E) or apoplexy (A) event, which are the most frequent grapevine trunk disease symptoms observed in Europe. Transcript expression of a set of plant defense- and stress-related genes was monitored by quantitative reverse-transcription polymerase chain reaction while plant phytoalexins and fungal metabolites were quantified by high-performance liquid chromatography-mass spectrometry in order to characterize the interaction between the grapevine and trunk disease agents. Expression of genes encoding enzymes of the phenylpropanoid pathway and *trans*-resveratrol content were altered in the three organs of diseased plants, especially in the young tissues of A plants. Pathogenesis-related proteins and the antioxidant system were severely modulated in A plants, which indicates a drastic stress effect. In the meantime, fungal polyketides 6-MSA, (*R*)-mellein, and (3*R*,4*R*)-4-hydroxymellein, were accumulated in A plants, which suggests their potential effect on plant metabolism during the appearance of foliar symptoms.

Additional keywords: black streaked wood, Chardonnay, fungal metabolites, phenolic compounds, trunk diseases.

Trunk diseases have become among the most important grapevine diseases in the past 10 to 15 years. Considering a replacement of 1% of plants per year—a considerable underestimate in view of the individual regional data found in the literature—the worldwide annual financial cost of the replacement of death plants due to grapevine trunk diseases is more than €1.5 billion (Hofstetter et al. 2012). For example, in France it has been estimated that 13% of vineyards are unproductive due to trunk diseases (Bruez et al. 2013), with an annual cost of €14 million.

Esca disease, Eutypa dieback, and Botryosphaeria dieback are the major grapevine trunk diseases. These diseases are caused by diverse fungal pathogens that attack the woody perennial organs of the vine and ultimately lead to its death (Bertsch et al. 2013; Larignon and Dubos 1997; Mugnai et al. 1999). In regard to etiology, the symptoms that occur in wood and annual organs have been extensively described, revealing that Eutypa dieback symptoms differ from those of Esca disease and Botryosphaeria dieback (Bertsch et al. 2013). Moreover, various fungal species associated with grapevine trunk diseases coexist in the same area of a wood section, *Fomitiporia mediterranea* M. Fischer, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & L. Mugnai) P.W. Crous & W. Gams, *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous, comb. nov., *Botryosphaeriaceae* species, and *Eutypa lata* (Pers; Fr) Tul & C. Tul (Spagnolo et al. 2012). The most frequent symptoms of trunk diseases observed in the Champagne area were assigned to Esca proper (E) and apoplexy (A) (Grosman and Doublet 2012), which have an important impact on French vineyards. Esca proper is the term proposed to indicate the coexistence of two different syndromes, within the Esca disease complex named Esca and Grapevine leaf stripe disease (GLSD), on a same plant (Bertsch et al. 2013; Surico 2009). Causal agents of Esca are different wood rot fungi represented mainly by *F. mediterranea* in Europe and the Mediterranean Basin (Surico et al. 2008). GLSD is a tracheomycotic widespread syndrome in which major causal agents are considered to be *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (Surico 2009; Surico et al. 2008). Symptoms of GLSD occur in the wood of trunk and cordons, as well as in leaves and berries. Apoplexy, consisting in the partial or complete sudden wilting of the crown, is regarded as the acute form of GLSD and/or E (Bertsch et al. 2013; Letousey et al. 2010; Surico et al. 2008). Thus, affected plants die within a few years (Larignon et al. 2009). Recently, physiological changes in *Vitis* plants affected by E (GLSD) or A were reported especially on leaves, and at a lower extent also in green stem and trunk (Agrelli et al. 2009; Fontaine et al. 2015; Letousey et al. 2010; Magnin-Robert et al. 2011, 2014; Petit et al. 2006; Spagnolo et al. 2012). Both leaves and green stems of affected *Vitis vinifera* L. plants showed physiological and metabolic changes related to the external symptoms, although no pathogens associated with E or other trunk diseases have been isolated from these organs (Lima et al. 2010; Magnin-Robert et al. 2011; Spagnolo et al. 2012).

It has been hypothesized that external symptoms of Esca disease complex are caused by toxins produced by fungi in the woody tissues and then translocated to the leaves via the transpiration

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*The *e-X*tra logo stands for "electronic extra" and indicates that one supplementary figure is published online.

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stream (Mugnai et al. 1999). Polyketides represent an important category of fungal secondary metabolites commonly produced by causal agents of grapevine trunk diseases (*Phaeoacremonium minimum*, *Phaeomoniella chlamydospora*, and *Neofusicoccum parvum*). These compounds have been identified and have phytotoxic activity on grapevine protoplasts, calli, and leaves (Abou-Mansour and Tabachi 2004; Abou-Mansour et al. 2015; Andolfi et al. 2012, 2014; Djoukeng et al. 2009; Evidente et al. 2000; Tabacchi et al. 2000). Their impacts on plant physiology remain a crucial step to appreciate the mechanisms leading to disease emergence. A differential response related to symptoms of E and A was reported on leaves and green stems, while no relevant differences were recorded in the trunk. In details, the primary metabolism and the defense response were more or less modified according to the form of symptom expression in leaves and in green stems (Magnin-Robert et al. 2011; Petit et al. 2006; Spagnolo et al. 2014). In the trunk, proteins involved in cell growth and defense response are down expressed in asymptomatic wood. Oppositely, proteins related to defense were overexpressed in the black streaked wood, characterized by the large presence of trunk diseases agents (Magnin-Robert et al. 2014). All of these findings are useful to better understand the host–pathogen interactions but they were obtained on separate plants which is not appropriate to provide a reliable overview of symptom development in the case of E and A.

For all of these reasons, the goal of this work was to investigate plants affected by E and A, through analyzing physiological perturbations on both herbaceous and woody samples in the same plant. We focused on phenylpropanoid pathway by analyzing the total phenolic compounds, the stilbene content, and the expression of nine related genes. The expression of 11 stress defense response genes and two water stress-related genes as well as the abscisic acid quantification were also performed. Moreover, known fungal metabolites such as 6-methylsalicyclic acid, terremutin, scytalone, isosclerone, (*R*)-mellein, and (3*R*,4*R*)-4-hydroxymellein were quantified to characterize the fungus–plant interaction.

MATERIALS AND METHODS

Plant material. Fifteen standing vines (Chardonnay/41B) were uprooted in both summer 2010 and 2011 from a vineyard located in the province of Epernay (Champagne-Ardenne region, France) owned by the company Moët & Chandon and planted in 1984. Five plants represented external leaf symptoms (GLSD) or A, respectively, and five asymptomatic plants were collected each year. Asymptomatic plants were chosen among those that had shown neither GLSD nor A symptoms since 2001, and were thus regarded as visually unaffected plants (control plants, C). Typical wood symptoms of GLSD and Esca were noted in all plants examined, including control plants. Therefore, plants showing foliar and wood symptoms of GLSD were considered as E plants. Four groups of samples were defined for green stems: C (stems from control plants), A and E (symptomatic stems from apoplectic [A] and Esca proper [E]-affected plants), and aS (asymptomatic stems from A and E plants) (Fig. 1). In woody tissues, two types of samples were studied: asymptomatic and black streaked wood. Black streaking consists of single or more xylem vessels gathered into individual blackish brown bundles (Surico et al. 2008). Both woody tissues were sampled in young annual rings (estimated less than 5-year-old

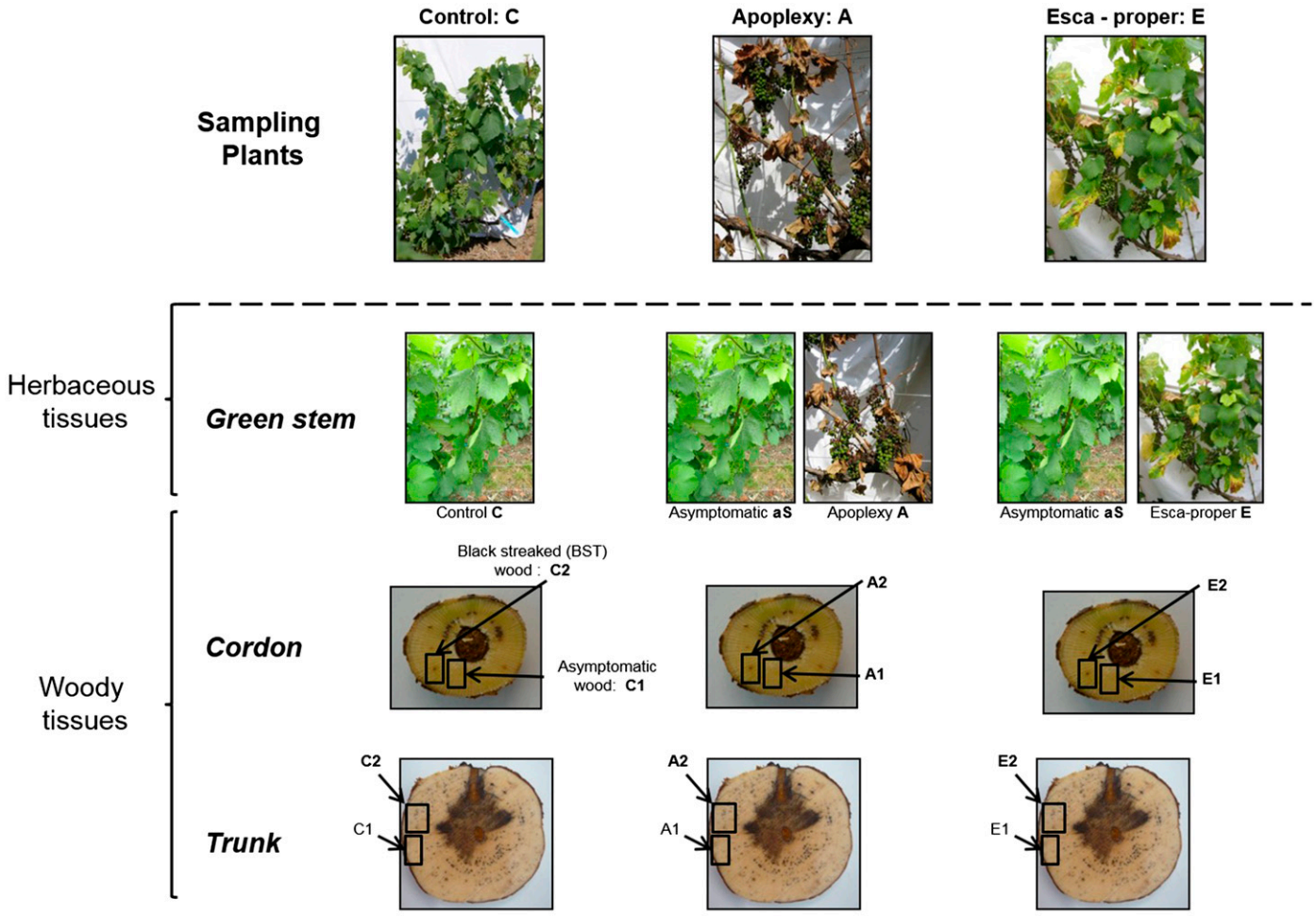

Fig. 1. Description of sampling plants and sample codes used.

wood, Fig. 1). Finally, six groups of samples were defined for woody organs (cordon and trunk): asymptomatic wood of control (C1), Esca proper-affected (E1), and apoplectic (A1) plants; and black streaked wood of control (C2), Esca proper-affected (E2), and apoplectic (A2) plants (Fig. 1). Plant tissues were frozen in the field with liquid nitrogen to halt enzymatic activities and stored at −80°C prior to use. Before each analysis, the amount of biological sample needed was ground to a fine powder in liquid nitrogen with a Mixer Mill MM 400 (Retsch, Haan, Germany). During the 2010 season, various organs of the sampled vines (trunk, cordon, and green stems) were inspected internally for the presence of discolorations associated with trunk diseases, and subsequently subjected to fungal isolation as described by Spagnolo et al. (2012). The major causal agents of Esca proper (*Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, and *F. mediterranea*) were isolated from discolored woody tissues of the three groups of plants (E, A, and control plants). *F. mediterranea* was directly linked to white rot. Other fungi associated with grapevine trunk diseases, such as *Botryophaeriaceae* species and *E. lata* were also isolated. In the opposite, no fungi were detected from either nondiscolored wood of trunk and cordons, or discolored and nondiscolored woody tissues of 1-year-old stems, as well as from green stems of control or diseased plants (Spagnolo et al. 2012).

RNA extraction. Total RNA was isolated from 2 × 50 mg of powdered green stem tissues and 3 × 50 mg of woody tissues (cordon and trunk) using the Plant RNA Purification Reagent (Invitrogen, Cergy Pontoise, France). The RNA pellet was resuspended in 20 µl of RNase-free water, then treated with RQ1 DNase enzyme (Promega) and quantified by measuring the absorbance at 260 nm following manufacturer's instructions.

Real-time RT-PCR analysis of gene expression. In total, 150 ng of total RNA was reverse-transcribed using the Verso SYBR 2-step QRT ROX enzyme (ABgene, Surrey, UK) according to the manufacturer's protocol. PCR conditions were those described by Bézier et al. (2002). Expressions of 22 targeted genes selected from previous published studies, whose expression and/or accumulation of corresponding proteins are altered either in field-grown grapevine affected by trunk diseases or in vitro conditions in response to fungal extracts (Magnin-Robert et al. 2011, 2014; Ramírez-Suero et al. 2014; Spagnolo et al. 2012, 2014), were tracked by quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) using the primers indicated in Table 1. Reactions were carried out in a real-time PCR detector Chromo 4 apparatus (Bio-Rad) using the following thermal profile: 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension) for 40 cycles. Efficiency of the primer sets was estimated by performing real-time PCR on several dilutions. PCR reactions were performed in duplicate. Results correspond to means ± standard deviation of 10 plants sampled, five in 2010 and five in 2011. The data were analyzed using CFX Manager software, and the relative levels of gene expression were determined following the method of Hellemans et al. (2007) with *EF1-α* and *39SRP* serving as the two internal reference genes. The results represent the relative expression in grapevine tissues of diseased plant (A or E) versus those corresponding to control (C).

Control samples consisted of plant tissues (green stem, C; cordon, C1 and C2; and trunk, C1 and C2) collected from control plants. The analyzed genes were considered significantly up- or down-regulated when change of their expression was >2× or <0.5×, respectively.

Quantification of total polyphenols and phytoalexins. *Sample extraction.* Methanolic extracts were prepared from 50 mg of powdered herbaceous and woody tissues mixed with 1 ml of methanol (MeOH) and 25 µl of the internal standard *trans*-4-hydroxystilbene (0.5 mg ml^{-1}) according to Spagnolo et al. (2014).

Quantification of total phenolic compounds. Total phenolics were determined by using the Folin-Ciocalteu method (Singleton and Rossi 1965) downscaled to 96-well-plate (E. Abou-Mansour, *personal communication*). An aliquot (30 µl) of appropriate dilution (green stem 1:10 [vol/vol]; woody tissues, 1:20 [vol/vol]) of methanolic extract was mixed with 150 µl of Folin-Ciocalteu reagent (diluted by 10) and after 5 min of incubation at room temperature, 120 µl of sodium carbonate solution (10%, wt/vol) was added. After incubation at room temperature for 2 h the absorbance of the mixture was read against the prepared blank at 750 nm. For each sample, three independent extractions were quantified by three technical replicates. Total phenolics were expressed as milligram of gallic acid equivalents (GAE) per gram of plant tissues. Values presented in the table correspond to means ± standard deviation of one representative experiment out of two (n_{2011} = 5 plants per condition).

Quantification of stilbenes. Standards such as *trans*-piceid, *trans*-resveratrol, and *trans*-pterostilbene were purchased from Extrasynthèse (Genay-France). The *trans*-ε-viniferin, *trans*-vitisin A, and *trans*-vitisin B (Supplementary Fig. S1) were extracted from lignified canes of Syrah as described by Spagnolo et al. (2014). Sixty microliters of the samples was analyzed on an MN Nucleosil C18 analytical column (250 mm × 4 mm i.d., 100-5) (Machery-Nagel, Duren, Germany) using a flow rate of 0.7 ml min^{-1} at 27°C. The mobile phase consisted of water/formic acid (0.5%) (solvent A) and acetonitrile (solvent B). The linear gradient started with 5% of B for 5 min and increased to 55% within 25 min reaching 80% at 28 min and 100% at 32 min. Spectral data for all peaks were accumulated in the range between 220 and 600 nm. The quantification of stilbenes was performed at λ320 nm using internal standard calibration methods. For each sample, three independent extractions were analyzed. The data are reported as micrograms per gram of fresh weight. Values for stilbenes correspond to means ± standard deviation of one representative experiment out of two (n_{2011} = 5 plants per condition).

Quantification of fungal phytotoxins. *Screening of fungal toxins.* Fungal phytotoxins scytalone, 4-hydroxyscytalone, and isosclerone were obtained from a culture of *Phaeoacremonium minimum* as described in Abou-Mansour and Tabachi 2004, (*R*)-mellein, (3*R*,4*R*)-4-hydroxymellein, 6-methylsalicylic acid (6-MSA), and (-)-terremutin from a culture of *N. parvum* according to the method described in Abou-Mansour et al. (2015).

Identification and quantification of fungal metabolites. In total, 500 µl from the methanolic extract prepared for the stilbene analysis was concentrated to 100 µl and extracted with *n*-hexane 3 × 100 µl to recover (*R*)-mellein, (3*R*,4*R*)-4-hydroxymellein, and 6-MSA. The *n*-hexane extracts were pooled and dried under nitrogen and dissolved in 50 µl of MeOH, and finally 10 µl was analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). The remaining methanolic extracts were dried under a stream of nitrogen and dissolved in 80 µl of MeOH, and 50 µl was injected in the HPLC-MS. Analyses were performed on a HPLC-DAD (Agilent 1100) coupled to a quadrupole mass spectrometer Agilent MSD/SL G1956B. The column used for the analysis of (*R*)-mellein, (3*R*,4*R*)-4-hydroxymellein, and 6-MSA was an MN Nucleodur Phenyl-Hexyl 2.7 µm (150 mm × 4.6 mm i.d.) (Machery-Nagel GmbH) with a mobile phase of water 0.1% formic acid (solvent A) and acetonitrile 0.1% formic acid (solvent B). The gradient started with 5% B for 2 min, and B reached 40% at 12 min, 60% at 23 min, and 100% at 26 min, until 30 min. The column is then re-equilibrated to 5% B during 5 min. The temperature of the column was 30°C and the flow rate 0.4 ml min^{-1}. Scytalone, 4-hydroxyscytalone, isosclerone, and (-)-terremutin were analyzed on a Kromasil C18 column 5 µm (250 mm × 4.6 mm i.d.) with a mobile phase of water 0.1% formic acid (solvent A) and acetonitrile 0.1% formic acid (solvent B). The temperature of the column was 35°C, the flow rate 1 $ml.min^{-1}$ and the split between the DAD and the MSD of 20% with the same gradient as reported in Abou-Mansour et al. (2015) for (-)-terremutin analysis. The MS detector consisted of a simple quadrupole mass detector operated in ESI negative ionization mode for scytalone, 4-hydroxyscytalone, isosclerone, (3*R*,4*R*)-4-hydroxymellein, (-)-terremutin, and 6-MSA and in positive mode for (*R*)-mellein. The source was operated with drying gas N_2 at 12 liters min^{-1}. The MS parameters were optimized by injection of the individual solution of the phytotoxins: fragmentor voltage varying from 80 V to 125 V. The capillary voltage was 3.5 kV in ESI+ and

–3.5 kV in ESI–, the nebulizer pressure 35 psig, and the drying gas temperature 300°C. Analyses were carried in SIM mode. The precursor ion and the most abundant fragment were chosen for quantification of the phytotoxins, the fragmentation pattern of 6-MSA *m/z* 151/107, (-)-terremutin 155/113, scytalone 193/113, 4-hydroxyscytalone 209/113, isosclerone 177/113, (*R*)-mellein *m/z* 179/161, and (3*R*,4*R*)-4-hydroxymellein 193/149. The limit of detection was 0.02 ng for 6-MSA, (*R*)-mellein, (3*R*,4*R*)-4-hydroxymellein, and (-)-terremutin. At 0.1 ng, scytalone and 4-hydroxyscytalone were clearly detected and isosclerone was at the limit of detection. Compounds were identified according to their fragmentation pattern coupled to their retention time. Quantification was performed using external standard calibration method. For each sample, three independent extractions were analyzed. The data are reported as nanograms per gram fresh weight. Values for fungal metabolites correspond to means ± standard deviation of one representative experiment out of two (n_{2011} = 5 plants per condition).

Quantification of abscisic acid (ABA). The method described by Schmelz et al. (2004) was used with some modifications. Sample preparation: 300 mg of grapevine powdered tissues was transferred to screw cap tubes and homogenized twice with 1 ml of extraction buffer (1-propanol/H_2O/HCl: 2/1/0.005) at 70°C. Samples were transferred to a glass tube and 100 ng of internal standard abscisic acid-d6 (Santa Cruz Biotechnology, www.scbt.com) was added. Two milliliters of methylene chloride was added to each sample and mixed for 15 s with a vortex and centrifuged at 2,000 × *g* for 20 min. The lower organic phase was transferred into a 4-ml glass vial and dried by the addition of anhydrous Na_2SO_4. Before derivatization, the volume of $MeCl_2$:1-propanol solvent was reduced until approximately 400 µl. Derivatization: carboxylic acids including ABA were methylated to their corresponding methyl esters by addition of 50 µl of methanol and 20 µl of 2 M bis-trimethylsilyldiazomethane (Sigma-Aldrich) at room temperature for 30 min. Excess of bis-trimethylsilyldiazomethane was quenched by adding 20 µl of 2 M acetic acid during 30 min at room temperature. Vapor phase extraction (VOC): extraction of the vapor phase was performed using a VOC column (www.ars-fla.com) conditioned with 3 × 1 ml of $MeCl_2$. The VOC column and a nitrogen needle were fixed on the screw cap of the tube and solvent was evaporated under a nitrogen stream at 70°C, and then the tubes were heated for 2.5 min at 200°C. The VOC column was eluted with 1 ml of $MeCl_2$. Finally, the eluate was evaporated and samples were

TABLE 1. Primers of genes analyzed by real-time reverse-transcription polymerase chain reaction

	Genes	Primer sequences	GenBank or NCBI accession numbers
Housekeeping genes	*EF1-α* (elongation factor 1-α)	5′-GAACTGGGTGCTTGATAGGC-3′ 5′-AACCAAAATATCCGGAGTAAAAGA-3′	GU585871
	39SRP (39S ribosomal protein L41-A)	5′-GACTGACTTCAAGCTTAAACC-3′ 5′-GATATAACAGGGAATACAGCAC-3′	XM_002285709.1
Phenylpropanoid metabolism	*PAL* (phenylalanine ammonia lyase)	5′-TCCTCCCGGAAAACAGCTG-3′ 5′-TCCTCCAAATGCCTCAAATCA-3′	X75967
	CCoAOMT (caffeoyl-CoA O-methyltransferase)	5′-GTGGACGCAGACAAGGACAAT-3′ 5′-CCCGTTCCAGAGGGTGTTG-3′	XM_002285070
	STS (stilbene synthase)	5′-AGGAAGCAGCATTGAAGGCTC-3′ 5′-TGCACCAGGCATTTCTACACC-3′	FJ851185
	CHI (chalcone isomerase)	5′-GCAGAAGCCAAAGCCATTGA-3′ 5′-GCCGATGATGGACTCCAGTAC-3′	XM_002282072
	IFRhom (isoflavone reductase homolog-like)	5′-GCAACATACATCAAAACAATTGA-3′ 5′-CCGTGAGCTTTTCCCATGTC-3′	XM_002266111
	IFR-L4 (isoflavone reductase like protein 4)	5′-GGATCGTGTTAATGCGGTTGA-3′ 5′-GCCTGGCTGGACCAATGTAG-3′	BN000709
	F′3H (flavonoid-3′-hydroxylase)	5′-CGCTTGTTCATGCGTTCAAC-3′ 5′-CTATTTTAATCATGGGCAAACAACCT-3′	XM_002284115
	leucoAND (leucoanthocyanidin dioxygenase)	5′-ATGAGGGCAAGTGGGTGACA-3′ 5′-TTGACCAGTCCCCTGTGAAGA-3′	X75966
	POX4 (peroxidase 4)	5′-AACATCCCCCCTCCCACTT-3′ 5′-TGCATCTCGCTTGGCCTATT-3′	XM_002269882
Defense protein	*Chit1b* (class I basic chitinase)	5′-ATGCTGCAGCAAGTTTGGTT-3′ 5′-CATCCTCCTGTGATGACATT-3′	Z54234
	Chi4c (class IV chitinase)	5′-TCGAATGCGATGGTGGAAA-3′ 5′-TCCCCTGTCGAAACACCAAG-3′	AY137377
	CHV5 (class V chitinase)	5′-CTACAACTATGGCGCTGCTG-3′ 5′-CCAAAACCATAATGCGGTCT-3′	AF532966
	GLUC (β-1,3-glucanase)	5′-TCAATGGCTGCAATGGTGC-3′ 5′-CGGTCGATGTTGCGAGATTTA-3′	DQ267748
	endoglu (glucan endo-1,3-beta-glucosidase)	5′-AGATGGGCAGCTTGGTTACAA-3′ 5′-TGAAGGCCAACCACTCTCTGA-3′	XM_002277410
	PR6 (serine proteinase inhibitor)	5′-AGGGAACAATCGTTACCCAAG-3′ 5′-CCGATGGTAGGGACACTGAT-3′	AY156047
Detoxification and stress tolerance	*epoxH2* (epoxide hydrolase 2)	5′-TCTGGATTCCGAACTGCATTG-3′ 5′-ACCCATGATTAGCAGCATTGG-3′	XM_002270484
	GST1 (glutathione-S-transferase, tau form)	5′-TGCATGGAGGAGGAGTTCGT-3′ 5′-CAAGGCTATATCCCCATTTTCTTC-3′	AY156048
	SOD (superoxide dismutase)	5′-GTGGACCTAATGCAGTGATTGGA-3′ 5′-TGCCAGTGGTAAGGCTAAGTTCA-3′	AF056622
	HSP70 (heat shock protein 70 kDa)	5′-CAACATGAGGAACACTGTCAAAGAC-3′ 5′-TGCAAGCTGGTTACTGTCCAA-3′	XM_002283496
	HSP (alpha crystalline heat shock protein)	5′-TCGGTGGAGGATGACTTGCT-3′ 5′-CGTGTGCTGTACGAGCTGAAG-3′	XM_002272382
Water stress	*TIP1* (tonoplast intrinsic aquaporin)	5′-ATCACCAACCTCATTCATATGC-3′ 5′-GTTGTTGTCTCAACCCATTTCC-3′	AF271661
	NCED2 (9-cis-epoxycarotenoid dioxygenase 2)	5′-CTCTTGGCCATGTCGGAAGA-3′ 5′-CGGAGCTGCTTGTCGAAGTC-3′	XM_003632982.1

dissolved in 60 µl of hexane before injecting 5 µl on a capillary column HP1 (25 m × 0.25 mm) GC column (Agilent Technologies) fitted to a Hewlett Packard 5980 GC coupled to a 5970 mass specific detector. The methyl esters of ABA and ABA-d6 were detected and quantified by selective ion monitoring at *m/z* 190 and 194, respectively. The amount of ABA (measured as methyl ABA) was calculated by reference to the amount of internal standard. The results are expressed in micrograms per gram fresh weight of plant tissue. For each sample, three independent extractions were analyzed. ABA values correspond to means ± standard deviation of one representative experiment out of two (n_{2011} = 5 plants per condition).

Statistical analysis. Results from quantification of metabolites (total polyphenols, phytoalexins, ABA, and fungal metabolites) correspond to means ± standard deviation of one representative experiment out of two (n_{2011} plants = 5). For relative expression of targeted genes, each value represents the mean of 10 plants per condition, five plants sampled in 2010 and five plants in 2011 (n_{2010} + n_{2011}). Error bars represent the standard deviation of the mean. A Kruskall Wallis test followed by the Dunn's multiple comparisons test (Prism 5, GraphPad Software) were performed to compare relative genes expression and metabolite content between various conditions. Differences at $P < 0.05$ were considered to be significant.

RESULTS

Expression of genes involved in the phenylpropanoid metabolism. We designed primers for enzymes involved in the phenylpropanoid metabolism, including the proteins leading to the synthesis of phytoalexins (stilbenoids) and phytoanticipins (flavonoids). The expression analysis of nine genes revealed a perturbation in the transcripts accumulation in the three organs (green stems, cordons, and trunks) of E and A plants. The amounts of their transcripts in green stems of E plants were similar to those observed in the control plants (Fig. 2). In A plants, the analysis in green stems highlighted an up-regulation of a stilbene synthase (*STS*) and a down-regulation of four genes namely a caffeoyl-CoA O-methyltransferase (*CCoAOMT*), 2 isoflavone reductase (*IFRhom* and *IFRL4*), and a peroxidase (*POX4*) (Fig. 2). In woody tissues, the transcript profile was most perturbed in the black streaked wood of diseased plants (Fig. 2). In cordons, a down-regulation of *IFRhom* and *IFRL4* expression, two genes involved in flavonoid pathway, was observed in the black streaked wood of plants affected by Esca proper (E2) and apoplexy (A2), respectively. In the opposite, transcript accumulation of *STS* was stimulated in the black streaked wood of diseased plants (A2 and E2; Fig. 2), while that of phenylalanine ammonia lyase gene (*PAL*) was solely increased in the black streaked wood of A plants (A2). Only *CCoAOMT* expression was repressed in the asymptomatic cordon wood of E plants (Fig. 2). Even if the transcript accumulation of the *STS* gene was slightly stimulated in the trunk of A (A1, A2) and E (E1) plants, no significant modification of the transcript profile was noticed for *PAL* (Fig. 2). *IFRL4* and flavonoid-3′-hydroxylase (*F'3H*) genes were up-regulated in asymptomatic wood of A and E plants, respectively (Fig. 2). In the meantime, leucoanthocyanidin dioxygenase (*leucoAND*) was up-regulated in the trunk of A plants (A1 and A2; Fig. 2).

Total polyphenolic and stilbene content. The amount of phenolic compounds was analyzed in the three organs (green stems, cordons, and trunk) of control and diseased plants, by monitoring

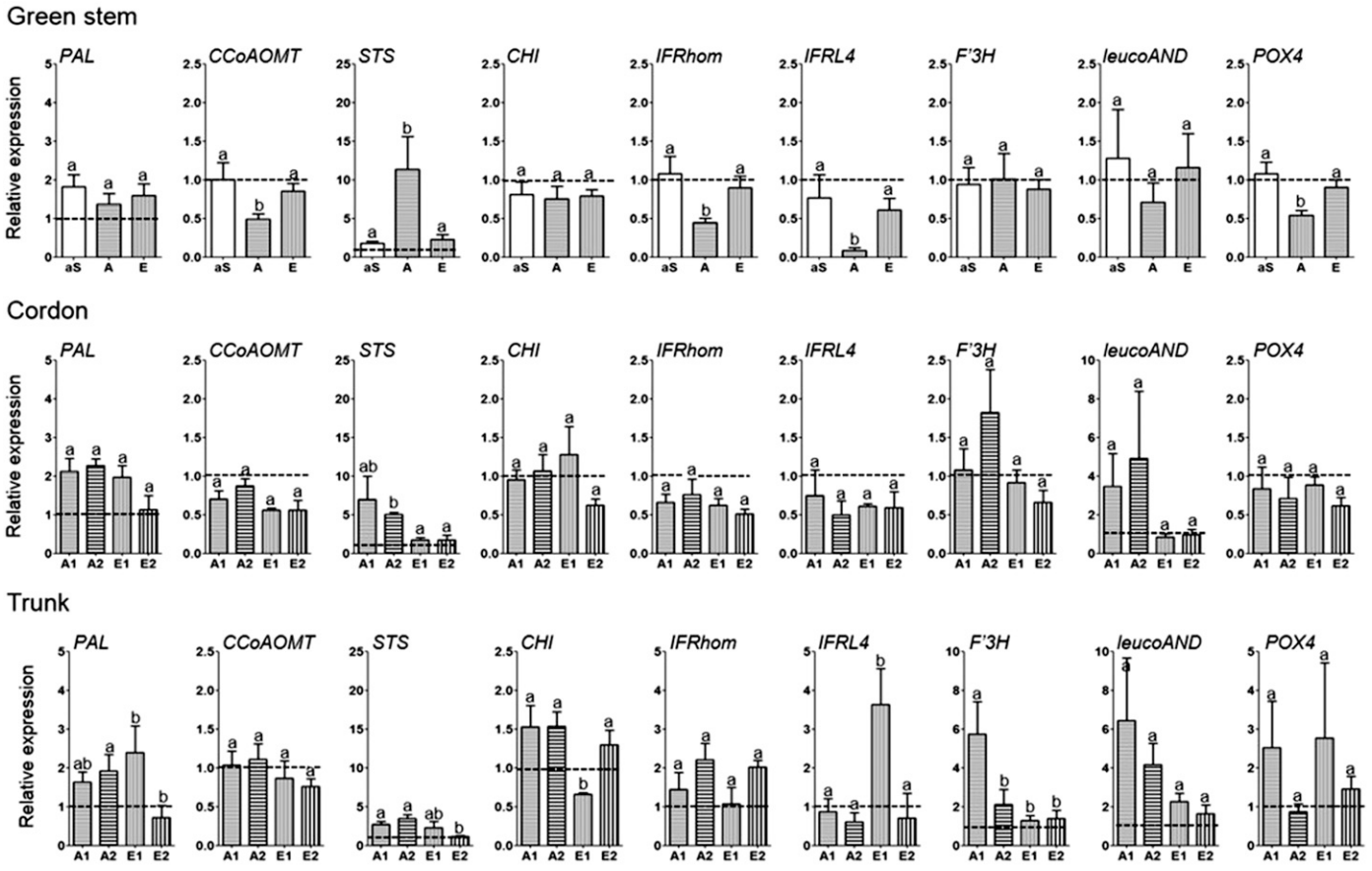

Fig. 2. Expression level of nine selected genes involved in phenylpropanoid metabolism determined by quantitative reverse-transcription polymerase chain reaction were observed in green stems, symptomatic (A and E) and asymptomatic (aS) green stems, cordon, and trunk, asymptomatic (A1 and E1) and black streaked (A2 and E2) wood of apoplectic (A) and Esca proper-affected (E) 26-year-old standing vines of Chardonnay. Gene expression was considered significantly up- or down-regulated to the 1× appropriate controls (dotted lines), when changes in relative expression were >2× or <0.5×, respectively. Results correspond to means ± standard deviation (10 plants, n_{2010} = 5 and n_{2011} = 5). Columns headed by the same letter are not significantly different (Dunn's multiple comparison test, $P < 0.05$).

total phenolics and stilbenoids that are key molecules in vine defense responses. The content of *trans*-piceid, *trans*-resveratrol, *trans*-ε-viniferin, *trans*-vitisin A, and *trans*-vitisin B was quantified in various samples (Table 2). The accumulation of the *trans*-piceid was quantified as it is a nontoxic glycosylated derivate of *trans*-resveratrol, which could be a form of *trans*-resveratrol storage in the plant (Belhadj et al. 2006).

In green stems, no difference was recorded in total phenol content between control and diseased plants (Table 2). Focusing on stilbenes, a slight content of *trans*-piceid was detected in green stems with apparent foliar symptoms (A and E). Moreover, *trans*-resveratrol and *trans*-vitisin B were significantly accumulated in green stems of A plants (Table 2). In wood, the content of phenolics fluctuated depending on the samples. In the cordons, contents of *trans*-piceid, *trans*-ε-viniferin, and *trans*-vitisin A were higher in the black streaked wood when compared with the asymptomatic wood, whatever the control or diseased plants (Table 2). Moreover, *trans*-vitisin B was quantified in A plants and in the black streaked wood (E2) of E plants (Table 2). In the trunk, total polyphenolic compounds were more important in the black streaked wood than in asymptomatic wood, in both control and diseased plants. Total polyphenolic compounds may indicate an accumulation of phenols related to the age of the wood, the level of phenols being higher in the trunk than in the cordon (Table 2). An increase of stilbenes related to the age of the wood was also observed in the black streaked wood (C2, A2, and E2). Nevertheless, we noted a lower level of stilbenes in the asymptomatic wood of trunk when compared with cordons (C1 and A1) (Table 2). These results may suggest that the accumulation of phenolic compounds in the asymptomatic wood of the trunk cannot be explained by the accumulation of stilbenes but by other phenols such as flavonoids. Moreover, the accumulation of total polyphenolic compounds seemed to be affected in the asymptomatic wood of the trunk from diseased plants, with statistical significance for E1 (Table 2).

Pathogenesis-related (PR) protein transcript accumulation in green stems and woody tissues. The expression of six genes encoding PR proteins was investigated: a basic chitinase class I (*Chit1b*), a chitinase class IV (*Chi4c*), a chitinase class V (*CHV5*), a β-1,3-glucanase (*GLUC*), a glucan endo-1,3-β-glucosidase (*endoglu*), and a serine proteinase inhibitor (*PR6*). In green stems, the expression of the genes encoding PR proteins was higher in A than in E and aS (Fig. 3), although no perturbation of *endoglu* expression was noted in diseased plants. For the three genes encoding chitinase, the accumulation of *CHV5* transcripts was the highest in response to the appearance of foliar symptoms. Moreover, the transcript accumulation of *GLUC* was at least 50-fold higher in A than in E and aS (Fig. 3).

In wood, the transcript analysis indicated a perturbation of gene expressions with higher amplitude in cordons, "young" tissues, than in trunks. For *CHV5*, *Gluc*, and *PR6* expression, the highest induction was recorded in cordon of A plants (A1 and A2; Fig. 3). Apart from a slight induction of *Chi4C* gene expression in A1, no significant alterations for *Chi4C* (A2, E1, and E2), *Chit1b*, and *endoglu* expression were observed in cordons (Fig. 3). In trunks of diseased plants, the expression of *CHV5*, *Gluc*, and *PR6* were increased. For both *Gluc* and *CHV5*, the relative expression was 35-fold higher in asymptomatic wood of A1 and E1 plants (Fig. 3). For *endoglu*, its transcript accumulation was only observed in asymptomatic trunks of A1 and E1, whereas the expression of both *Chi4C* and *Chit1b* genes was not affected in the trunk of diseased plants.

Expression pattern of stress-related genes in green stems and woody tissues. To determine whether stress responses were triggered in A or E grapevines, the expression of three detoxification and stress tolerance genes were followed in green stems and woody tissues of diseased plants (Fig. 4): an epoxide hydrolase (*epoxH2*), a glutathione-S-transferase (*GST1*), and a superoxide dismutase (*SOD*). Two genes encoding heat shock protein (HSP) were also investigated, 70 kDa HSP (*HSP70*) and a small chloroplastic HSP (*HSP*).

In green stems, no significant changes of expression of these targeted genes occurred in aS of diseased plants. *GST1* gene was up-regulated in symptomatic stems by 20- and 4.5-fold higher in A and E plants, respectively. Moreover, *epoxH2* was only and slightly up-regulated in symptomatic green stems of A plants (Fig. 4). A repression of *HSP* gene expression was observed in symptomatic stems and was higher in A than in E plants (Fig. 4).

Except for the *GST1* gene, no significant changes occurred in cordons for the stress-related genes (Fig. 4). *GST1* expression was 40- and 18-fold stimulated in A1 and A2 plants, respectively, whereas it was repressed by fourfold in E1 and E2 plants. In the trunk, *GST1* was up-regulated in diseased plants by fivefold in A1, E1, and E2 and by 10-fold in A2 (Fig. 4). The expression of *epoxH2* was only induced in asymptomatic wood of diseased plants (A1 and E1). In the meantime, a general repression of *HSP* was observed in the trunk of diseased plants (Fig. 4). Nevertheless, the relative expression of *HSP* was significantly induced in E1 (Fig. 4). *SOD* was down-regulated in asymptomatic wood of A1, while no significant change occurred in A2, E1, and E2 (Fig. 4).

TABLE 2. Total phenolics and stilbene compounds concentrations in green stem: control stem (C), asymptomatic (aS), and symptomatic stems (A and E), and in both cordon and trunk: asymptomatic (C1, A1, and E1) and black streaked (C2, A2, and E2) wood of apoplectic (A) and Esca proper-affected (E) 26-year-old standing vines of Chardonnay[z]

Samples		Total phenolic compounds (mg GAE g^{-1} FW)	Stilbene compounds (mg g^{-1} FW)				
			trans-piceids	*trans*-resveratrol	*trans*-ε-viniferin	*trans*-vitisin A	*trans*-vitisin B
Green stem	C	8.11 ± 0.79 a	nd a	0.003 ± 0.002 a	0.093 ± 0.085 a	0.025 ± 0.019 a	nd a
	aS	9.72 ± 4.45 a	nd a	0.03 ± 0.02 a	0.29 ± 0 0.28 a	0.014 ± 0.013 a	0.008 ± 0.007 a
	A	9.33 ± 2.41 a	0.004 ± 0.002 a	0.55 ± 0.10 b	0.15 ± 0.06 a	0.064 ± 0.017 a	0.191 ± 0.048 b
	E	9.79 ± 1.23 a	0.006 ± 0.004 a	0.06 ± 0.04 a	0.44 ± 0.33 a	0.051 ± 0.011 a	0.013 ± 0.011 a
Cordon	C1	4.90 ± 0.61 a	0.13 ± 0.03 a	0.04 ± 0.02 a	0.17 ± 0.08 a	0.02 ± 0.01 a	nd a
	C2	8.61 ± 1.69 b	0.27 ± 0.02 b	0.52 ± 0.20 a	2.31 ± 0.76 b	0.35 ± 0.12 a	nd a
	A1	6.33 ± 2.28 ac	0.17 ± 0.06 ac	0.85 ± 0.43 a	0.55 ± 0.23 a	0.07 ± 0.04 a	0.07 ± 0.04 a
	A2	8.04 ± 1.37 bc	0.28 ± 0.09 bc	0.97 ± 0.23 a	1.47 ± 0.35 ab	0.16 ± 0.01 a	0.09 ± 0.03 a
	E1	4.58 ± 0.32 a	0.15 ± 0.02 a	0.07 ± 0.01 a	0.17 ± 0.1 a	0.02 ± 0.01 a	nd a
	E2	9.00 ± 1.89 b	0.41 ± 0.15 b	0.68 ± 0.35 a	1.6 ± 0.4 ab	0.21 ± 0.05 a	0.007 ± 0.005 a
Trunk	C1	24.81 ± 4.16 a	0.04 ± 0.02 a	0.004 ± 0.002 a	0.06 ± 0.03 a	0.008 ± 0.008 a	nd a
	C2	29.09 ± 7.30 ab	0.42 ± 0.04 b	0.65 ± 0.11 b	2.80 ± 0.14 b	0.69 ± 0.05 b	nd a
	A1	20.85 ± 2.72 ac	0.16 ± 0.04 a	0.12 ± 0.04 a	0.19 ± 0.08 a	0.02 ± 0.01 a	0.045 ± 0.030 a
	A2	34.25 ± 12.79 bc	0.38 ± 0.05 b	0.98 ± 0.29 b	4.00 ± 1.79 b	1.00 ± 0.50 b	nd a
	E1	16.16 ± 4.88 c	0.12 ± 0.01 a	0.09 ± 0.02 a	0.15 ± 0.03 a	0.04 ± 0.02 a	0.005 ± 0.003 a
	E2	32.81 ± 3.66 b	0.40 ± 0.04 b	1.69 ± 0.66 b	3.54 ± 1.19	1.06 ± 0.54 b	nd a

[z] Lowercase letters a, b, and c indicate significant difference ($\alpha = 0.05$) for the concentrations found for each organs (Dunn's multiple comparison test, $P \leq 0.05$). nd indicates not detected. Total phenolics were expressed as milligram of gallic acid equivalents (GAE) per gram of plant tissue.

Analysis of fungal metabolite contents. The role of fungal metabolites in causing symptoms on diseased plants is still unclear. Whereas the internal inspection of trunk described the presence of major causal agents of trunk diseases (*Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *F. mediterranea*, *E. lata*, and Botryophaeriaceae species) in our experimental vineyard (Spagnolo et al. 2012), we screened the different samples looking for phytotoxins from these various fungi. We checked fungal metabolites, such as scytalone, 4-hydroxyscytalone for the presence of *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora* (Abou-Mansour and Tabachi 2004; Evidente et al. 2000), isosclerone for the presence of *Phaeoacremonium minimum*, *Phaeomoniella chlamydospora*, and *N. parvum* (Abou-Mansour and Tabachi 2004; Evidente et al. 2000), (*R*)-mellein, (3*R*,4*R*)-4-hydroxymellein for *D. seriata* along with 6-MSA, and (-)-terremutin for *N. parvum* (Abou-Mansour et al. 2015; Djoukeng et al. 2009). Scytalone, 4-hydroxyscytalone, and isosclerone were not detected in grapevine tissues of control and diseased plants. (*R*)-mellein, (3*R*,4*R*)-4-hydroxymellein, and 6-MSA statistically increased especially (3*R*,4*R*)-4-hydroxymellein content in green stems, cordons (A1), and trunks (A1 and A2) of A vines in comparison with control and E plants (Fig. 5) and (-)-terremutin was only detected but not quantified in green stems of A (data not shown).

Hormone accumulation and expression of genes involved in water stress-related responses. As the apoplexy is characterized by a sudden wilting of berries and leaves, we further investigated the impact/participation of the hydric stress in symptom expression.

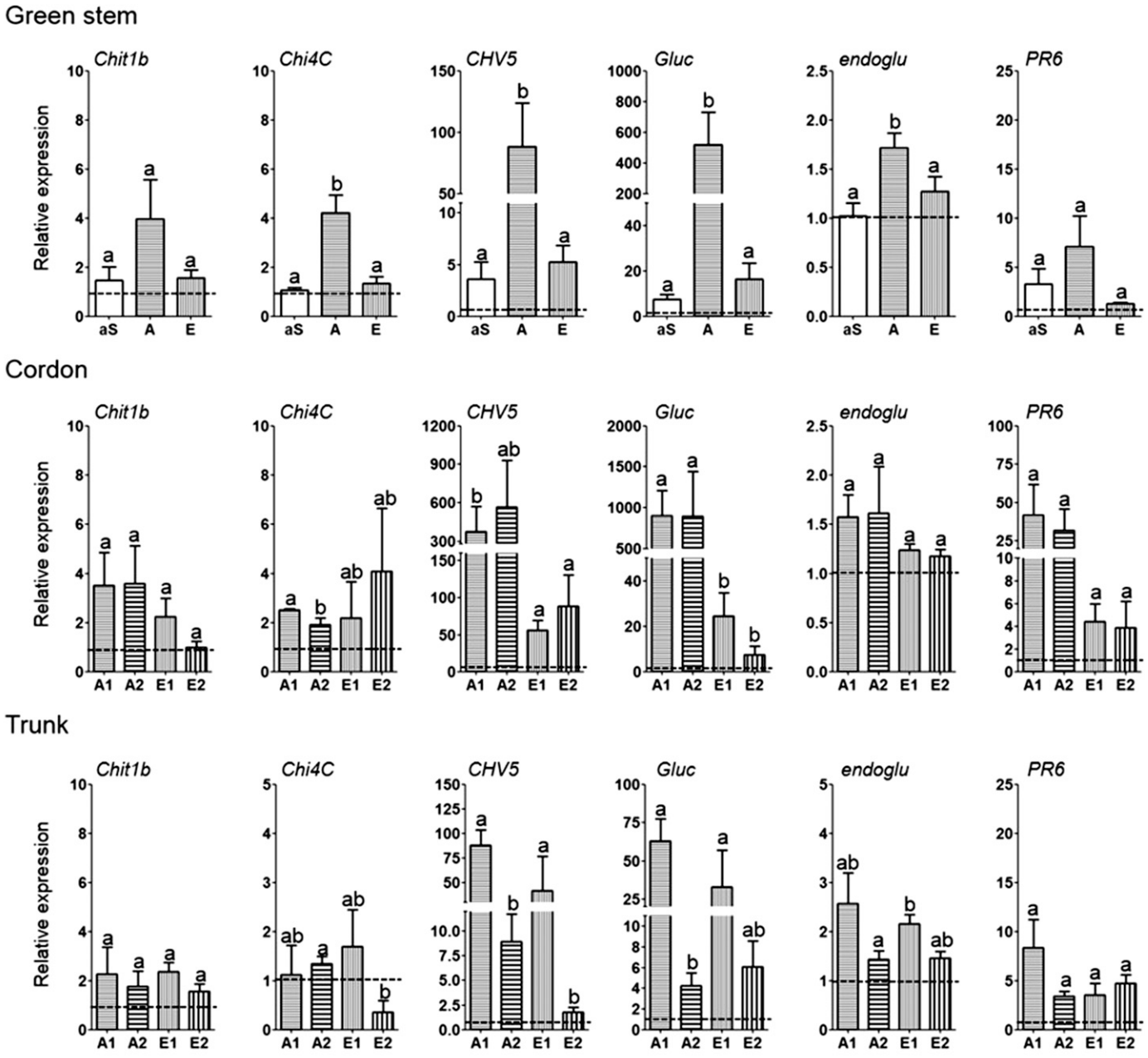

Fig. 3. Expression level of six selected genes encoded for pathogenesis-related proteins determined by quantitative reverse-transcription polymerase chain reaction were observed in green stems, symptomatic (A and E) and asymptomatic (aS) green stems, cordon, and trunk, asymptomatic (A1 and E1) and black streaked (A2 and E2) wood of apoplectic (A) and Esca proper-affected (E) 26-year-old standing vines of Chardonnay. Gene expression was considered significantly up- or down-regulated to the 1× appropriate controls (dotted lines), when changes in relative expression were >2× or <0.5×, respectively. Results correspond to means ± standard deviation (10 plants, $n_{2010} = 5$ and $n_{2011} = 5$). Columns headed by the same letter are not significantly different (Dunn's multiple comparison test, $P < 0.05$).

In this optic, we designed specific primers for an aquaporine, tonoplast intrinsic proteins (*TIP1*) involved in water transport and a 9-*cis*-epoxycarotenoid dioxygenase 2 (*NCED2*) involved in the biosynthesis of the water stress hormone, abscisic acid (ABA). Moreover, the content of ABA was analyzed in green stems, cordons, and trunks. Expression of *TIP1* was up-regulated in green stems (A) and cordons (A1 and A2) of A plants, but not in E plants (Fig. 6). No change of *NCED2* expression profile was observed in diseased plants, except for a down-regulation of *NCED2* in trunks of E2 plants (Fig. 6). The basal content of ABA was 1 ng mg^{-1} FW in green stems. The content was twofold (0.50 ng mg^{-1} FW) and fourfold lower (0.25 ng mg^{-1} FW) in cordons and trunks, respectively (Fig. 7), but no difference was noticed in green stems and cordons between control and diseased plants. For trunks, only a slight accumulation of ABA was detected in black streaked wood of E plants (Fig. 7).

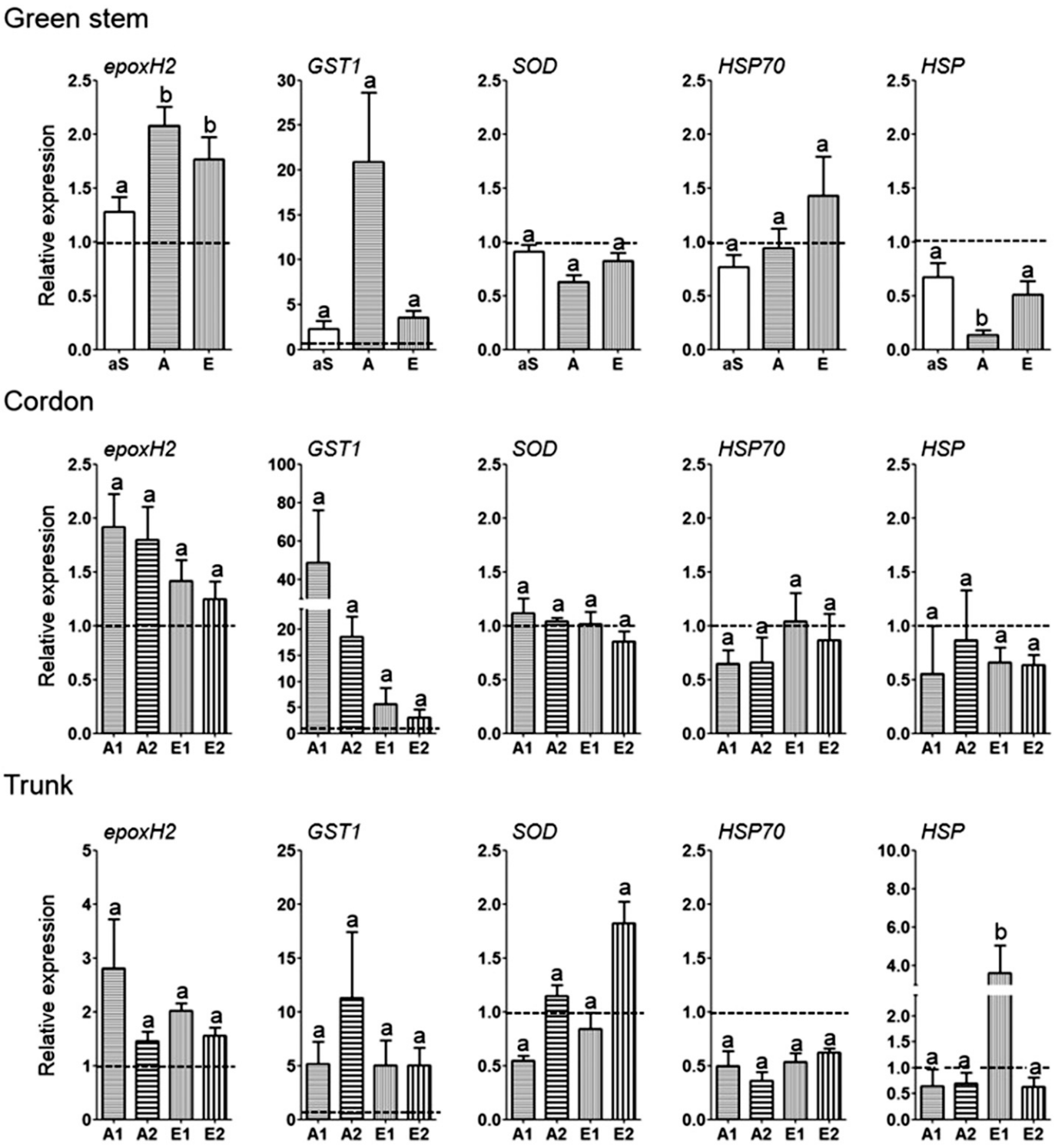

Fig. 4. Expression level of five selected genes implicated in detoxification and stress tolerance determined by quantitative reverse-transcription polymerase chain reaction were observed in green stems, symptomatic (A and E) and asymptomatic (aS) green stems, cordon, and trunk, asymptomatic (A1 and E1) and black streaked (A2 and E2) wood of apoplectic (A) and Esca proper-affected (E) 26-year-old standing vines of Chardonnay. Gene expression was considered significantly up- or down-regulated to the 1× appropriate controls (dotted lines), when changes in relative expression were >2× or <0.5×, respectively. Results correspond to means ± standard deviation (10 plants, $n_{2010} = 5$ and $n_{2011} = 5$). Columns headed by the same letter are not significantly different (Dunn's multiple comparison test, $P < 0.05$).

DISCUSSION

No information was previously reported about the whole dynamic process of defense response in the entire plant during external symptom emergence of trunk diseases. The characterization of the stress responses observed in the whole plant in relation with the presence of foliar symptoms is essential to appreciate the impact of these vascular diseases on plant physiology. Moreover, little information is available on the ability of the various plant organs of different ages to activate and develop efficient defenses. In this context, our study clearly demonstrates an alteration of polyphenols contents as well as a modification of stress responses in green stems and in woody of A and E grapevines, simultaneously at the onset of foliar symptom development.

Alteration of the phenylpropanoid pathway, especially in A plants. To compare the defense responses of grapevine affected by either E or A, we decided to target the best characterized active defense mechanism, namely the phenylpropanoid pathway. Preliminary studies described the phenolics accumulation in annual and in woody tissues of plants affected by trunk disease agents (leaves and berries [Calzarano et al. 2008; Lima et al. 2010]; trunks [Amalfitano et al. 2000; Del Rio et al. 2004; Graniti et al. 2000; Martin et al. 2009]) and also by other pathogenic agents, such as the xylem-infecting bacterium *Xylella fastidiosa* (Pierce's disease) (Wallis and Chen 2012) or fungal agents involved in grapevine foliar diseases (*Botrytis cinerea*, *Plasmopara viticola*, or *Erysiphe necator*) (Bavaresco et al. 1997; Dercks and Creasy 1989; Langcake and Pryce 1976; Romero-Perez et al. 2001). Indeed, a reaction to pathogen attacks is the formation of papillae (poly-phenol rich reactions) in secondary xylem to compartmentalize pathogens in woody tissues. In this work, alteration of gene expression involved in phenylpropanoid metabolism included significant accumulations of *trans*-resveratrol and *trans*-vitisin B, in various tissues of diseased vines, especially in green stems of A plants. These results indicate a higher effect on the phenylpropanoid pathway in young

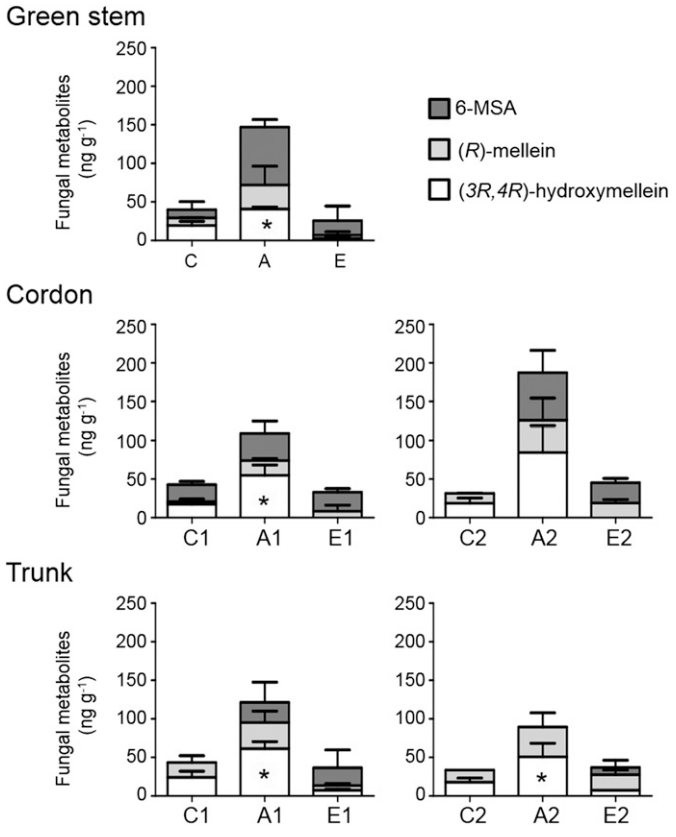

Fig. 5. Fungal metabolites (6-MSA, (*R*)-mellein and (3*R*,4*R*)-4-hydroxylmellein) contents expressed in nanograms per gram fresh weight (ng g^{-1}) were determined in green stems: control stems (C) and symptomatic stems (A and E), both cordon and trunk: asymptomatic (C1, A1, and E1) and black streaked (C2, A2, and E2) wood of apoplectic (A) and Esca proper-affected (E) 26-year-old standing vines of Chardonnay. Results correspond to means ± standard deviation (n_{2011} = 3). Data correspond to the fungal metabolites content evaluated in stems sampled in vineyard during the 2011 season. Asterisk indicates a statistically significant difference to the respective control sample (Dunn's multiple comparison test, $P < 0.05$).

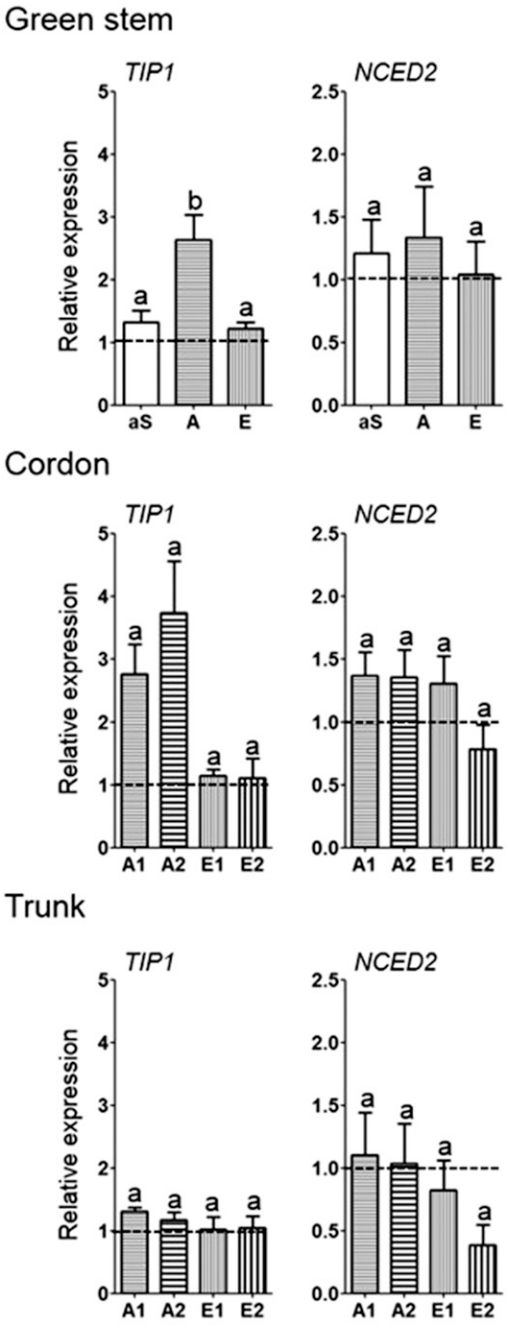

Fig. 6. Expression level of two selected genes implicated in water transport and ABA synthesis determined by quantitative reverse-transcription polymerase chain reaction were observed in green stems, symptomatic (A and E) and asymptomatic (aS) green stems, cordon, and trunk, asymptomatic (A1 and E1) and black streaked (A2 and E2) wood of apoplectic (A) and Esca proper-affected (E) 26-year-old standing vines of Chardonnay. Gene expression was considered significantly up- or down-regulated to the 1× appropriate controls (dotted lines), when changes in relative expression were >2× or <0.5×, respectively. Results correspond to means ± standard deviation (10 plants, n_{2010} = 5 and n_{2011} = 5). Columns headed by the same letter are not significantly different (Dunn's multiple comparison test, $P < 0.05$).

grapevine tissues caused by the stress provoked by apoplexy event in comparison with the onset of E symptoms.

Regarding woody tissues, we observed strong variations of phenolic contents between asymptomatic and black streaked wood, for both control and diseased plants. These differences can be explained by the presence/absence of trunk diseases agent inocula. Actually, *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *F. mediterranea*, and Botryosphaeriaceae species were isolated from black streaked wood, while no fungi were isolated from asymptomatic wood (Spagnolo et al. 2012). Thus, the black streaked wood corresponds to the zone of interaction between the plant and the fungal agents but no correlation has been observed between the emergence of these lesions in the trunk and the capacity of the plant to slow down the colonization by the fungi. Lambert et al. (2012) described in vitro the tolerance of *Phaeomoniella chlamydospora*, *F. mediterranea*, and *Phaeoacremonium minimum* to various phenolics. For Botryosphaeriaceae species, *D. seriata* was described to be more susceptible than *N. parvum*. All together, these results indicate that the accumulation of phenolics in woody tissues may participate in plant defense reactions to limit wood colonization by trunk disease agents; however, their fungicidal activity depends on the pathogenic agents (Lambert et al. 2012). In our study, the content of phenolic compounds was lower in asymptomatic wood of diseased plants, except for stilbenes. This suggests that another group(s) of phenolic compounds can be altered in diseased plants (both A and E) infected by trunk disease causal agents. Expression of three targeted genes involved in flavonoid and anthocyanin biosynthesis, *leucoAND* (X75966, also referenced VIT_02s0025g04720), *CHI* (XM_002282072, also referenced VIT_13s0067g03820), and *F'3H* (XM_002284115, also referenced VIT_17s0000g07210), were altered in leaves of grapevine plantlets infected with *N. parvum* under greenhouse conditions (Czemmel et al. 2015). Alteration of *leucoAND* (X75966, also referenced Vv_10000352) was monitored in grapevine leaves infected by *Eutypa lata*; its up-regulation was associated to the lack of leaf symptoms (Camps et al. 2010). At metabolite level, the anthocyanin content was strongly affected in cell cultures exposed to eutypine, a toxin from *E. lata* (Afifi et al. 2003). Moreover, an interesting proteomic analysis revealed that proteins involved in isoflavonoid and anthocyanin biosynthesis decreased in asymptomatic wood of diseased vines (A and E) (Magnin-Robert et al. 2014). Future research is now required to evaluate the shift between stilbenes and flavonoids in various organs connected with the vine susceptibility to trunk diseases.

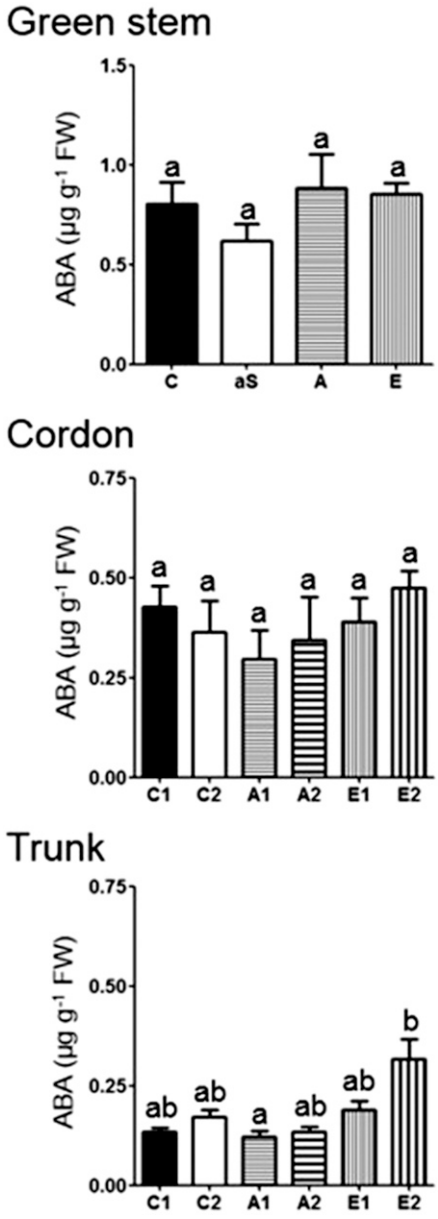

Fig. 7. ABA contents expressed in micrograms per gram fresh weight (µg g^{-1} FW) were determined in green stems, control stems (C), asymptomatic (aS), and symptomatic stems (A and E), cordon, and trunk, asymptomatic (C1, A1, and E1) and black streaked (C2, A2, and E2) wood of apoplectic (A) and Esca proper-affected (E) 26-year-old standing vines of Chardonnay. Results correspond to means ± standard deviation from one representative ($n_{2011} = 5$) out of two. Columns headed by the same letter are not significantly different (Dunn's multiple comparison test, $P < 0.05$).

Modulation of specific defense responses as a consequence of disease expression. In addition to its antimicrobial activity, *trans*-resveratrol can also act as a signaling molecule by the activation of defense-related responses on *Vitis* cell cultures: alkalinization, mild elevation of reactive oxygen species (ROS), and PR protein transcript accumulation (Chang et al. 2011). In this study, the induction level of three out of six PR proteins, *CHV5*, *Gluc*, and *PR6*, was higher in the three organs tested. This suggests that tissues perceive some elicitor signal associated with the presence of symptoms and also that these genes are inducible.

During xylem infection, typical metabolite changes lead to the accumulation of PR proteins in xylem sap (Basha et al. 2010; Rep et al. 2002). Similar to our study, the expression of the three genes was induced in the black streaked of cordons and trunks where fungi live. Nevertheless, their induction was also detected in asymptomatic wood, with a higher level for both *Gluc* and *CHV5* in trunks. These observations may reveal a preventive strategy by the plant to limit future fungal colonization in asymptomatic wood of the trunk. Despite an up-regulation of PR protein gene expression in diseased plants, the grapevine expressed external symptoms (A or E). Hence, PR protein gene induction in our plant pathosystem was not sufficient to avoid symptoms expression. The protection of cellular functions can also be provided by proteins presenting a chaperone role, like the small heat shock proteins. A general down-regulation of *HSP* and *HSP70* expression was observed in the organs of A plants. In this sense, a low accumulation of these proteins was already observed in the brown stripe, a typical wood discoloration of Botryosphaeria dieback (Spagnolo et al. 2014). Their differential level in Pierce's disease (PD)-resistant and in PD-susceptible grapevine genotypes supports the idea that HSPs might be implicated in resistance (Yang et al. 2011). These results suggest that HSPs are likely related to some cellular dysfunctions associated with the presence of symptoms. During stress conditions, cellular structure is compromised by the formation of excessive ROS due to disruption of cellular homeostasis. The scavenging or detoxification of ROS excess is achieved by efficient enzymatic antioxidant system, like SOD and GST (Bowler et al. 1992; Marrs 1996). Here, *SOD* expression was down-regulated in trunk of A vines. Reductions of SOD protein content and of transcript accumulation were also reported in various organs of A and E plants (Letousey

et al. 2010; Magnin-Robert et al. 2011, 2014; Spagnolo et al. 2012), which indicates a lack of oxidative stress control. The decrease of SODs expression could therefore be considered as a potential marker of the onset of disease symptom emergence. GSTs are a large superfamily of enzymes, with five classes defined *Theta*, *Zeta*, *Lambda*, *Phi*, and *Tau*, the two latter being specialized in the conjugation of xenobiotics such as toxins (Frova 2003). Focusing on the *tau* class, *GST1* expression was up-regulated in the three tested organs (this study) and also in the visually healthy leaves of E plants (Magnin-Robert et al. 2011). Moreover, Valtaud et al. (2009) showed an induction of leaf glutathione metabolism simultaneously with the onset of E foliar symptoms appearance. Together, these results suggest that the perturbation of the antioxidant system could be provoked by the presence of fungal metabolites. Phytotoxic metabolites secreted by Esca disease pathogens are considered toxins circulating in plant tissues and translocated to the leaves via the transpiration stream (Mugnai et al. 1999).

Activation of detoxification process and accumulation of specific fungal metabolites. The major wood-infesting fungi *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *F. mediterranea*, *E. lata*, and *Botryosphaeriaceae* sp. are known to produce diverse toxins (Andolfi et al. 2011; Bertsch et al. 2013; Tey-Rulh et al. 1991). We focused on the epoxHs, which catalyzes the detoxification of xenobiotics by the conversion of epoxides to the corresponding diols (Morisseau and Hammock 2005). Interestingly, several toxins produced by trunk disease agents are characterized by the presence of epoxides in their chemical structure (Abou-Mansour et al. 2015; Andolfi et al. 2011, 2012). Our study revealed an up-regulation of the *epoxH2* expression in both green stems and trunks of A plants. In the meantime, identification of various fungal metabolites reported as phytotoxins (Abou-Mansour et al. 2015) were undertaken in green stems, cordons, and trunk. Our study revealed that the levels of (*R*)-mellein, its derivative (3*R*,4*R*)-4-hydroxymellein, along with 6-MSA were present at similar high content in tissues of A plants whereas the other toxins were not detected. (*R*)-mellein and its derivatives are widespread in fungi (Chooi et al. 2015) and were reported for an antigerminative activity, suggesting that they may interfere with the cellular pathway involved in germination or hormone signaling in plants (Chooi et al. 2015). In addition, (*R*)-mellein slows down the cell cycle, extending the mitotic phase (Essad and Bousquet 1981). Interestingly, 6-MSA, a precursor of (-)-terremutin, is known to activate disease resistance in tobacco inducing accumulation of defense proteins and virus resistance, probably by mimicking SA hormone (Yalpani et al. 2001). High level of 6-MSA in A plants may thus explain the strong activation of defense response, such as PR protein accumulation. 6-MSA may act primarily by modulating the plant defense responses and then the polyketide toxins may affect the antioxidant system, suggesting a coordinated and dramatic effect which compromises the establishment of an appropriate and effective defense response able to avoid disease expression.

A strange relationship between water stress and disease expression. Grapevine xylem is an extreme case of efficiency/sacrificial strategy for water transport and is therefore particularly vulnerable to drought stress-induced xylem cavitation. Fungal vascular pathogens are able to use wood polymers as energy sources and may alter the xylem structure, which leads to a loss of xylem function. Infected grapevines may have a greater vulnerability with regard to water stress. Contrary to both defense and antioxidant response alteration, the establishment of the typical responses to water stress in diseased plants and especially in A ones seems to be less obvious. In response to water stress, Galmès et al. (2007) observed a rapid down-regulation in *TIP1* transcript accumulation. On the contrary, the less turgid leaves of A vines displayed an up-regulation of *TIP1*. Similar responses were observed in green stems and cordons. Another typical reaction of grapevine to drought stress is the increase of ABA biosynthesis on stems that regulate stomata opening (Christmann et al. 2007). Moreover, a great deal of evidence highlights the importance of ABA as a root-sourced signal transported via the xylem and involved in stomatal regulation of drought plants (for review, Dodd et al. 1996). Except for the black streaked wood of trunks from E plants, no significant change in the ABA content was observed in diseased plants. In agreement with Christen et al. (2007), our results suggest that the appearance of foliar symptoms cannot be simply considered a water-deficit-inducing alteration but that other physiological mechanisms are involved.

To conclude, our results confirm a slight discrimination of plant responses between vines affected by A events and those by E symptoms. A drastic impact on phenylpropanoid pathway (*STS*, *IFRL4*, and *IFRhom*), transcript accumulation of PR proteins (*CHV5*, *Gluc*, and *PR6*) and antioxidant system (*GST1*) was observed in herbaceous and woody tissues of A plants. This work also described the down-regulation of genes such as *HSP* and *SOD*, which suggested that these proteins may likely be related to cellular dysfunctions leading to the onset of foliar symptoms. As previously cited by Djoukeng et al. (2009), the (*R*)-mellein and more particularly the hydroxylated derivatives, (3*R*,4*R*)-4-hydroxymellein, were efficient diagnostic markers of plants affected by the apoplectic form. Whether the detected fungal metabolites play a role in this plant–fungus interaction is a question that warrants future investigation.

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