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Differential gene expression in two grapevine cultivars recovered from "flavescence dorée"

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ABSTRACT

The biological bases of recovery of two grapevine cultivars, Nebbiolo and Barbera, showing different susceptibility and recovery ability to "flavescence dorée" (FD) phytoplasma infection were investigated. The expression over one vegetative season, in FD-recovered and healthy grapevines, of 18 genes involved in defence, hydrogen peroxide and hormone production was verified at two time points. Difference (Δ) between the relative expressions of August and July were calculated for each target gene of both cultivars. The significance of differences among groups assessed by univariate and multivariate statistical methods, and sPLS-DA analyses of the Δ gene expression values, showed that control and recovered grapevines of both cultivars were clearly separated. The Barbera-specific deregulation of defence genes supports a stronger response of this variety, within a general frame of interactions among H_2O_2, jasmonate and ethylene metabolisms, common to both varieties. This may strengthen the hypothesis that FD-recovered Barbera grapevines modulate transcription of their genes to cope with potential damages associated to the alteration of their oxidative status. Nebbiolo variety would fit into this picture, although with a less intense response, in line with its lower degree of susceptibility and recovery incidence to FD, compared to Barbera. The results evidenced a scenario where plant response to phytoplasma infection is highly affected by climatic and edaphic conditions. Nevertheless, even after several years from the original FD infection, it was still possible to distinguish, at molecular level, control and recovered grapevines of both cultivars by analyzing their overall-season response, rather than that of a single time point.

1. Introduction

Phytoplasmas are wall-less phytopathogenic bacteria classified in the genus '*Candidatus* Phytoplasma' responsible for economically relevant diseases of several herbaceous and woody plant species (Marcone, 2014). Phytoplasmas are restricted to the phloem tissue and are transmitted by phloem-feeding Hemipteran vectors (leafhoppers, planthoppers, and psyllids) in a persistent propagative manner (Weintraub and Beanland, 2006). All vegetative organs can harbor phytoplasma cells, even though the distribution in the host tissues is erratic and seasonal variations may occur (Berges et al., 2000). Infected plants often show typical symptoms such as witches' broom, dwarfism and phyllody, as consequence of the dramatic alteration of plant hormone pathways induced by phytoplasma effector proteins (Maejima et al., 2014). Some plant species may show mild symptoms and a natural symptom remission may occur in some woody species (Osler et al., 2014), but no resistant plants to phytoplasma infection have been reported so far.

Different '*Ca*. Phytoplasma' species can infect grapevine inducing a complex of diseases commonly referred to as grapevine yellows (GY) (Maixner et al., 2006); the more economically important GY diseases in Europe are "bois noir" (BN) and "flavescence dorée" (FD).

The agent of FD is transmitted to grapevine by the leafhopper vector *Scaphoideus titanus* Ball (Schvester et al., 1963), and it is included in the European list of quarantine organisms (Jeger et al., 2016). Disease symptoms usually appear one year after inoculation, on some or all shoots of infected plants often leading to plant death along the season (Morone et al., 2007). All *Vitis vinifera* cultivars are susceptible to infection although at different degrees (Eveillard et al., 2016; Kuzmanovic et al., 2008). Recent works showed that diverse susceptibility of two economically relevant Italian cultivars Barbera and Nebbiolo supports different pathogen loads (Margaria et al., 2014; Roggia

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et al., 2014).

FD-infected grapevines may show a spontaneous and stable remission of symptoms associated with a significant restoring of yield and product quality, referred to as recovery (Morone et al., 2007), that has been reported also in some woody crops affected by other phytoplasma diseases such as apple (Musetti et al., 2004) and apricots (Musetti et al., 2005; Osler et al., 2014). These observations have led to a deeper investigation of the phenomenon, in particular on GY-recovered grapevines. Differential expression of genes involved in several metabolic pathways have been evidenced by microarray analysis on BN-recovered grapevines (cv. Montepulciano) (Punelli et al., 2016). Through the combined use of ultrastructural and gene expression analyses of leaf tissues, Santi et al. (2013) show that BN-recovered grapevine (cv Chardonnay) show changes in sugar transport and metabolism, while a down regulation of genes involved in salicylate signaling and the activation of jasmonate pathway has been pointed out by Paolacci et al. (2017). In the case of FD infection, differences between recovered, infected and healthy grapevines have been studied by different approaches: variations at transcriptomic (Gambino et al., 2013) and proteomic (Margaria et al., 2014, 2013) levels of FD-recovered grapevines compared to healthy ones have been underlined, and biochemical analysis on recovered Prosecco plants (cv Glera), showed that hydrogen peroxide (H_2O_2) foliar phloem levels were significantly higher compared to those of healthy and infected plants (Musetti et al., 2007).

In the present work, the biological bases of recovery were further investigated using two Italian grapevine cultivars, Barbera and Nebbiolo, characterized by a different degree of susceptibility to FD infection and recovery abilities (Margaria et al., 2014; Roggia et al., 2014). Barbera is highly susceptible to FD and shows severe symptoms, already visible in early summer, while symptoms on Nebbiolo are milder and not so evident until middle summer (Morone et al., 2007; Roggia et al., 2014). Taking into account all the information acquired so far on the recovery phenomenon from the above cited studies, a list of 18 genes related to pathways possibly involved in grapevine recovery from phytoplasma diseases was created, and their expression levels were evaluated at different time points during the vegetative season and compared to those of respective control plants. It has been demonstrated that FD-recovered grapevines are unable to serve as source of pathogen acquisition for the vector *S. titanus* (Galetto et al., 2014), so a better knowledge about recovery, coupled to insecticide treatments for vector population control, may represent a valuable tool to reduce yield losses in grapevine growing areas where the disease is not eradicable.

2. Materials and methods

2.1. Plant material

In July (Berry pie size, stage 31, Coombe, 1995) and August (Veraison, stage 35, Coombe, 1995) 2015, surveys were carried out in a vineyard located in Cocconato (Asti, Piemonte, IT, gps data: 45°04′58.4″N 8°03′21.1″E, N-S orientation) to collect grapevines of the cultivars Barbera and Nebbiolo belonging to two phytosanitary status: control (symptomless and never reported infected by FD since 2007) and recovered (symptomless, but previously infected by FD). The two cultivars were planted in 1999 on SO4 rootstock. The vineyard were regularly treated with fungicides and insecticides and monitored since 2007 for phytoplasma infection, so a map of the sanitary status (never infected and recovered) of each plant was available at the beginning of the study. Recovered samples were identified among plants that were infected between three to five years before 2015, and asymptomatic since two to four years at the time of sampling. At both dates, 20 control and 20 recovered grapevines were selected for each cultivar, to be further analyzed by molecular assays for the presence of viruses and GY. Five leaves were detached between the fifth and the eighth node of the vegetative shoot, to ensure collection of samples of the same age over the season, and kept at 4 °C until sample preparation, performed on the same collection day. Pools of midrib and leaf tissues were divided into aliquots and stored at -80 °C until DNA and RNA extractions (about 0.5 g and 0.1 g, respectively).

2.2. Nucleic acids extraction

Total DNA was extracted from 0,5 g of frozen plant material following a CTAB protocol (Doyle and Doyle, 1990); the final DNA pellet was dissolved in 50 µL of sterile water and stored at - 20 °C. Total RNA was extracted from 100 mg plant tissue using the PureLink® Plant RNA Reagent (Thermo Fisher Scientific) following the manufacturer's instructions. Following extraction, total RNA samples were treated with TURBO DNA-free™ Kit TURBO™ DNase (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA was finally suspended in 30 µL of RNase-free water containing 0.1% DEPC. Nucleic acid extracts were analyzed in a NanoDrop spectrophotometer to evaluate their concentration and purity, then stored at –80 °C.

2.3. Phytoplasma and virus detection

Molecular assays (nested PCR) were used to exclude the presence of FDp in single infection or in mixed infection with other grapevine phytoplasma species, as described in Marzachi et al. (1999). The absence of the main grapevine viruses reported in the Piemonte region as *Grapevine leafroll associated virus-1and 3* (GLRaV-1, -3), *Grapevine fan leaf* (GFLV), *Grapevine virus A* (GVA) and *Grapevine fleck virus* (GFKV) was also confirmed by RT-PCR driven with virus-specific primers following the protocol reported in Gambino and Gribaudo (2006). Finally, from the initial sampling of 40 plants for each cv, four different categories were identified according to the sanitary status and sampling date for both Barbera (B) and Nebbiolo (N): phytoplasma-free and virus-free control samples (h), collected in July (L) (BhL and NhL) and August (A) (BhA and NhA); phytoplasma-free and virus-free recovered samples (r), collected in July (BrL and NrL) and August (BrA and NrA). At the end of the detection assays, eight samples were assigned to each category.

2.4. cDNA synthesis and mRNA quantification

Eighteen *V. vinifera* genes known to be involved in the molecular response of the plant leading to recovery from phytoplasma infection were selected based on literature (Musetti et al., 2007; Gambino et al., 2013; Margaria et al., 2013, 2014; Osler et al., 2014).The selected genes were classified in four functional groups, which are reported in Table 1. For each gene, the transcript levels at both sampling dates was measured by quantitative reverse transcription PCR reactions (qRT-PCR) with SYBR Green. The relative expression of each target gene was normalized to the expression of two *V. vinifera* reference genes, actin (Genoscope gene accession: GSVIVT00034893001) and ubiquitin 1 (Genoscope gene accession: GSVIVT00037199001), known to be stably expressed during FD phytoplasma infection (Margaria et al., 2014). As first step, 1 µg of total RNA was used for cDNA synthesis with High Capacity cDNA Reverse Transcription Kit® (Applied Biosystem) and random hexamers, following the manufacturer's instructions. Total RNA controls in the absence of reverse transcription were included to confirm the absence of genomic DNA contamination in the RNA samples.

Two µl of 1:10 cDNA dilutions (in sterile distilled water, SDW) were used as template in 10 µL qPCR reactions containing 1X iQ SYBR green Supermix (Bio-Rad), 200 nM (each) primers and SDW. For each target gene, specific primers were selected from the literature or designed for this study from grapevine sequences available in the GenBank, using the Primer Express software v3.0.1 (Applied Biosystems, Branchburg, NJ, USA; Supplementary Table S1). In order to exclude the possibility that genome nucleotide differences between Barbera and Nebbiolo

Table 1
Two-way ANOVA *p* values for testing the effect of sanitary status (S) and collection month (M) on target gene expression in Barbera and Nebbiolo grapevine cultivars. Bold *p* values in the S x M column indicate significant correlations ($p \leq 0.05$).

Metabolism	Target Gene	Barbera			Nebbiolo		
		S	M	S x M	S	M	S x M
H_2O_2 (production/scavenging)	GLO1	0.0670	0.2492	0.1568	0.4786	0.1758	0.7508
	GER1	0.6934	0.9056	0.1236	0.477	0.8452	0.0592
	APX6	0.6884	0.0730	**0.0438**	0.5356	0.0502	0.9078
	CAT1	0.0212	0.0260	0.1874	0.0368	0.0122	0.0904
	APX2	0.4132	0.3742	0.2042	0.778	0.1632	0.3228
Defence	WRKY2	0.0094	0	**0.0044**	0.8706	0.1172	0.2544
	ACO	0.2704	0	**0.0258**	0.492	0.1978	0.5194
	HSP70	0.3996	0	**0.0084**	0.4722	0.1084	0.2248
	PR1	0.6012	0.4390	0.3948	0.0626	0.7004	0.2618
Jasmonate	LOXO	0.0132	0	0.0870	0.1608	0.4636	0.0944
	JAZ5	0.1648	0	0.3604	0.3648	0.1268	0.0714
	JAZ7	0.1094	0.1862	0.1840	0.1758	0.107	0.2020
	JAZ10	0.2140	0.0036	0.5800	0.2572	0.2976	0.7672
	OPR3	0.0866	0.0002	**0.0126**	0.0942	0.4152	0.1534
General metabolism	GPM1	0.8524	0.0022	0.9072	0.1958	0.8748	0.4386
	GS	0.0226	0	**0.0072**	0.8308	0.9186	**0.0212**
	THI1-1	0.4232	0.3110	**0.0164**	0.3124	0	**0.0100**
	AKR	0.0012	0	**0.0046**	0.3084	0.7042	**0.0145**

could affect the primers ability to amplify the target cDNA, specificities and efficiencies of the reactions were determined for each primer pair for both cultivars by qPCR. To evaluate the qPCR efficiency, a standard curve consisting of at least four 1:5 fold dilution points of the cDNA was included in each plate; PCR amplification efficiency was calculated according the equation: Efficiency % = $(10^{[-1/\text{slope}]} - 1) \times 100$ (Bustin et al., 2009). To assess the reaction specificity, a melting analysis was performed at the end of each run. cDNA samples were run in duplicate together with negative controls consisting of complete reaction mixes with total RNA and SDW instead of cDNA. All qRT-PCR reactions were carried out in a CFX Connect real-time PCR detection system (Bio-Rad) supported by CFX Manager Software, version 3.0. The Gene Study function of the same software was used to calculate the relative gene expression; mean relative quantity and its standard deviation were calculated for the eight biological replicates of each sanitary status at each sampling date for the two cultivars, and used for further comparisons.

2.5. Statistical analyses

The relative gene expression (GE) values of recovered and control plants of both cultivars, collected at the two time points (July and August), were compared. To assess the statistical significance of differences among groups, both univariate (one-way and two-way ANOVA) and multivariate methods (PCA, sPLS-DA) were used.

Welch's ANOVA was performed when the normality assumption (checked via Shapiro-Wilks test) was satisfied; otherwise, non-parametric one-way ANOVA was calculated using Kruskal-Wallis method. Principal Component Analysis (PCA) was used mainly as exploratory analysis. The Sparse Partial Least Squares Discriminant Analysis (sPLS-DA), an extension of classical PLS-DA that allows a better selection of really relevant variables for classification problems (Lê Cao et al., 2011), was then applied. As it was necessary to reduce seasonal influence on the relative gene expression (GE) of the analyzed genes, the difference (ΔGE) between the relative GE measured in July (GE_L) and August (GE_A) was calculated for each target gene of both cultivars according to the formula:

$$\Delta GE = GE\_A - GE\_L$$

The computed results were processed for statistical analysis using the R software environment (R Core Team, 2011). sPLS-DA was performed using the R package mixOmics (Lê Cao et al., 2016).

3. Results

3.1. Samples selection

All sampled plants were asymptomatic for known bacteria, fungi or viruses, and in particular, the absence of grapevine yellows (FD and BN), grapevine leafroll associated virus -1 and -3 (GLRaV-1, -3), grapevine fan leaf virus (GFLV), grapevine virus A (GVA), and grapevine fleck virus (GFKV) was confirmed by molecular analyses. These grapevine viruses are the most common reported in the Piemonte region, and cause in grapevine similar symptoms to those associated with phytoplasma presence (Martelli, 2017), suggesting the involvement of common plant metabolic pathway in response to the infection. Moreover, even if no symptoms are evident in the plants, the effects of virus presence can alter the plant physiology (Montero et al., 2017). After detection results, for each cultivar, eight h and eight r phytoplasma-free and virus-free samples were selected from the initial 20 samples of each category.

3.2. Transcript analysis of selected genes

Specific signals were obtained following melting analysis of the qPCR amplicons, while no amplification was obtained from no-reverse transcribed total RNA as well as from water controls. Melting peak temperatures ranged from 76 °C (WRKY2) to 83.5 °C (THI1-1). Efficiencies of qPCR reactions varied between 76.7% (HSP70) and 100% (WRKY2) (Supplementary Table S1).

In general, most of the analyzed genes showed highly variable transcription levels within control and recovered plants, irrespective of the cultivar, the sanitary status and the sampling date (Fig. 1 and 2). Two-way ANOVA analysis carried out for cultivar Barbera evidenced that ACO, AKR, APX6, GS, HSP70, OPR3, THI1-1 and WRKY2 gene transcription levels varied significantly according to both the sanitary status and the sampling date (Table 1). For cultivar Nebbiolo, AKR, GS and THI1-1 gene transcription levels varied significantly according to both the sanitary status and the sampling date (Table 1).

3.3. Transcriptional behavior of selected stress-related genes

According to the exploratory PCA analysis, the total level of variance explained by the first two principal components was higher than 50% for both cultivars (Fig. 3). Considering results obtained for Barbera

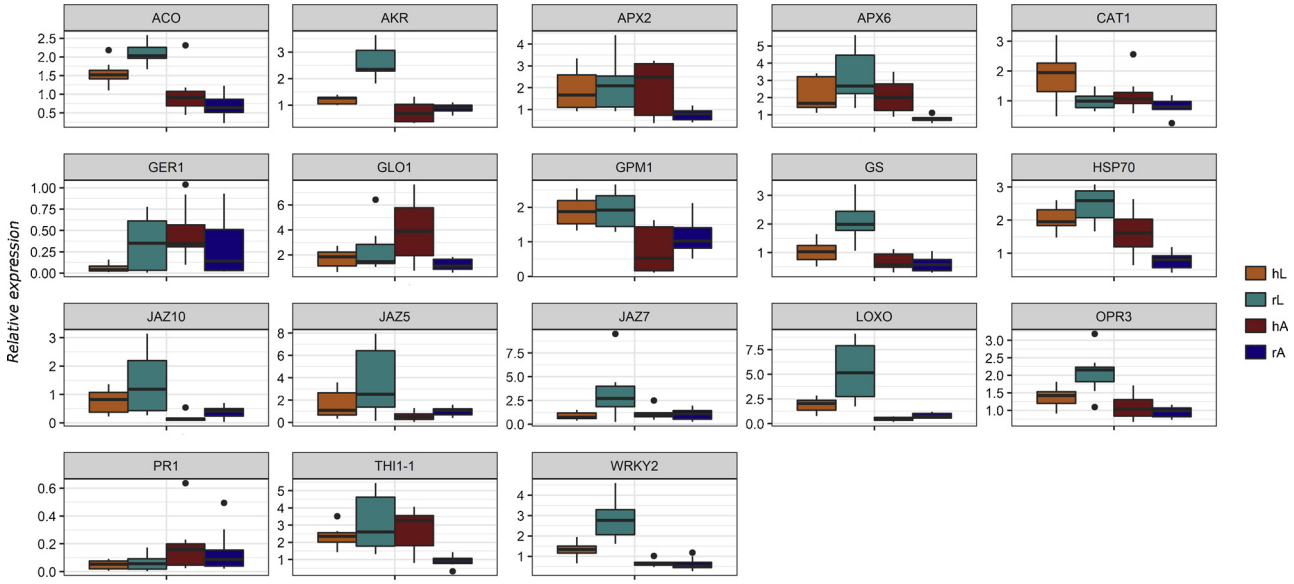

Fig. 1. Box plot of gene expression in Barbera grapevine cultivar. Lines extending vertically from the boxes indicating variability outside the upper and lower quartiles. Outliers are plotted as individual points. hL: control samples collected in July; rL: recovered samples collected in July; hA: control samples collected in August; rA: recovered samples collected in August.

cv (Fig. 3a), it was impossible to distinguish h samples from r grapevines all over the season. Considering separately the two sampling times, August (A) and July (L), samples were better separated according to their collection time rather than their sanitary status, although h and r where slightly distinguishable in July (BhL vs BrL).

In the case of Nebbiolo cv (Fig. 3b), the PCA plot was not able to discriminate the samples in relation to their sanitary status, even when the two sampling time were separately analyzed.

In order to overcome the high variability observed within the same sanitary category (h and r) and reduce the single sampling-point influence on the transcription of the analyzed genes, the difference (Δ) between the relative gene expression (GE) measured in August (GE_A) and July (GE_L) was calculated. and values subject to one-way ANOVA (Figs. 4 and 5). For the cultivar Barbera, ΔGE values of genes coding ACO, AKR, APX6, GS, HSP70, OPR3, WIRKY2 and THI1-1 differed significantly between h and r grapevines (Fig. 4). In the case of Nebbiolo, h and r grapevines showed significant differences for the genes coding AKR, GER1, GS, JAZ5, OPR3 and THI1-1 (Fig. 5). The ΔGE values were further analyzed by multivariate statistical approaches. PCA of ΔGE values of the analyzed genes (Fig. 6), incremented the possibility to discriminate control and recovered grapevines much better than the single time point PCA(Fig. 3), and total level of variance explained by the first two principal components was higher than 50% for both cultivars. Among the 18 genes included in the PCA of ΔGE values for the Barbera cultivar, the main contributors to the first component were genes coding APX6, WRKY2, JAZ7, LOXO, THI1-1 and HSP70, and the main contributors to the second component were GPM1, GER1 and PR1 (Fig. 6a). In the case of Nebbiolo cultivar, the main contributors to the first component of PCA of the ΔGE values, were genes coding JAZ5, APX6, AKR and OPR3, and the main

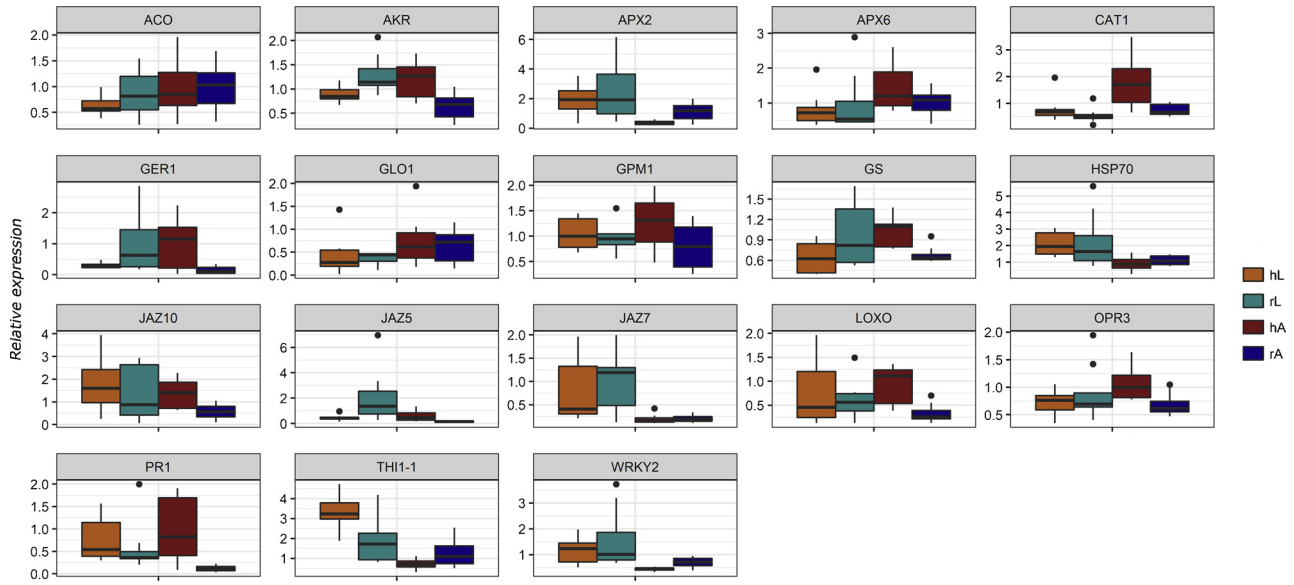

Fig. 2. Box plot of gene expression in Nebbiolo grapevine cultivar. Lines extending vertically from the boxes indicating variability outside the upper and lower quartiles. Outliers are plotted as individual points. hL: control samples collected in July; rL: recovered samples collected in July; hA: control samples collected in August; rA: recovered samples collected in August.

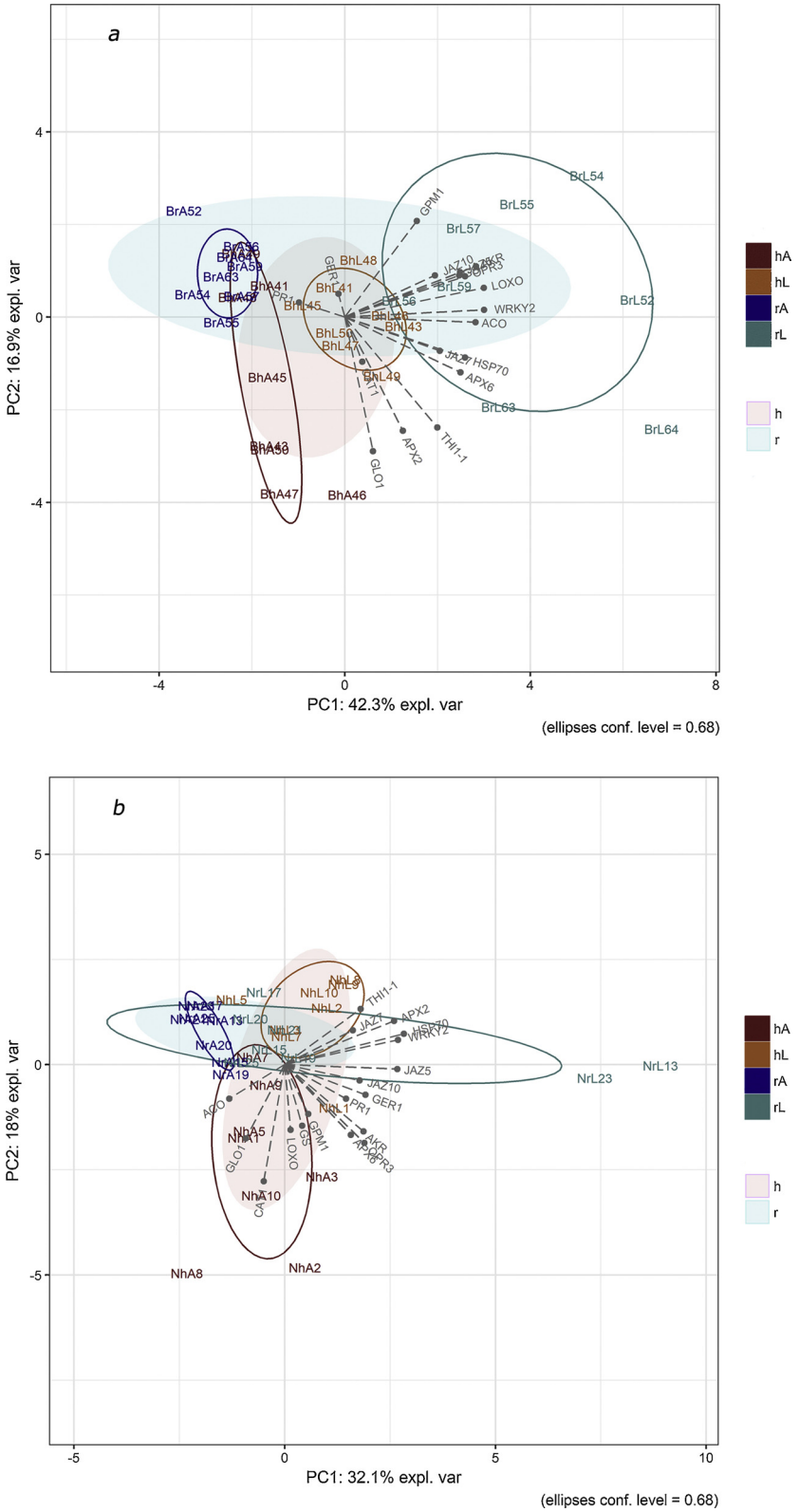

Fig. 3. PCA scatter plots of Barbera (a) and Nebbiolo (b) gene expression values. Distinction between grapevines according to their sanitary status. h: control plants (pink) and r: recovered plants (light blue). Separation according to both sanitary status and sampling date is detailed as: hL: control samples collected in July; rL: recovered samples collected in July ; hA: control samples collected in August; rA: recovered samples collected in August (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

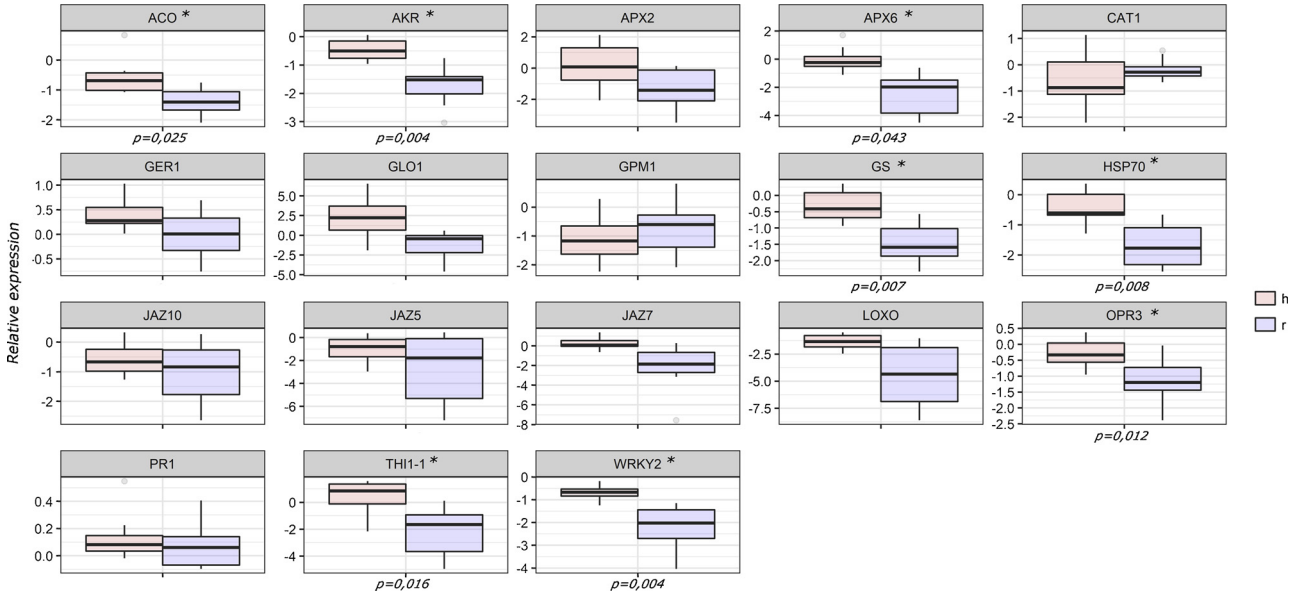

Fig. 4. Box plot of the difference gene expression (ΔGE) values, between the relative GE measured in August and July of each gene, in Barbera cultivar. Lines extending vertically from the boxes indicating variability outside the upper and lower quartiles. ΔGE values that differed significantly by one-way ANOVA ($p \leq 005$) between h and r grapevines are marked with * and the *p* values are reported. h: control plants; r: recovered plants.

contributors to the second component were LOXO, CAT1, GLO1 and THI1-1 (Fig. 6b). Following PCA, sPLS-DA was performed on ΔGE values, to further improve the discrimination between h and r clusters and to identify the most discriminating variables. sPLS-DA plots on ΔGE are shown in Fig. 7 where the separation between h and r for both the cultivars was evident.

For Barbera, the classification error rates of the sPLS-DA model (defined as the ratio between the number of wrong classifications and the total number of classifications performed) were 0 for class h and 0.125 for class r, on both components; for cultivar Nebbiolo the classification error rates were 0.125 for both class h and class r, on both components. The stability values obtained for each gene for each cultivar are reported in Table 2; the most stable genes were consistent with the results obtained by ANOVA on ΔGE values, including the genes coding for ACO, AKR, APX6, GS, HSP70, LOXO, OPR3, WRKY2 and THI1-1 in Barbera and AKR, GS, OPR3, JAZ5, THI1-1 in Nebbiolo. Therefore, the transcript levels of these genes in the time period studied behaved differently between control and recovered grapevines, and the transcriptional behaviour of recovered plants was different from that of controls in both cultivars, even after at least two years from the original infection.

4. Discussion

As the ability of grapevine to recover from FD infection is cultivar specific, the transcriptional behaviour of selected markers of tolerance/resistance to biotic stresses is highly suitable for the development of sustainable management strategies based on field massive screening

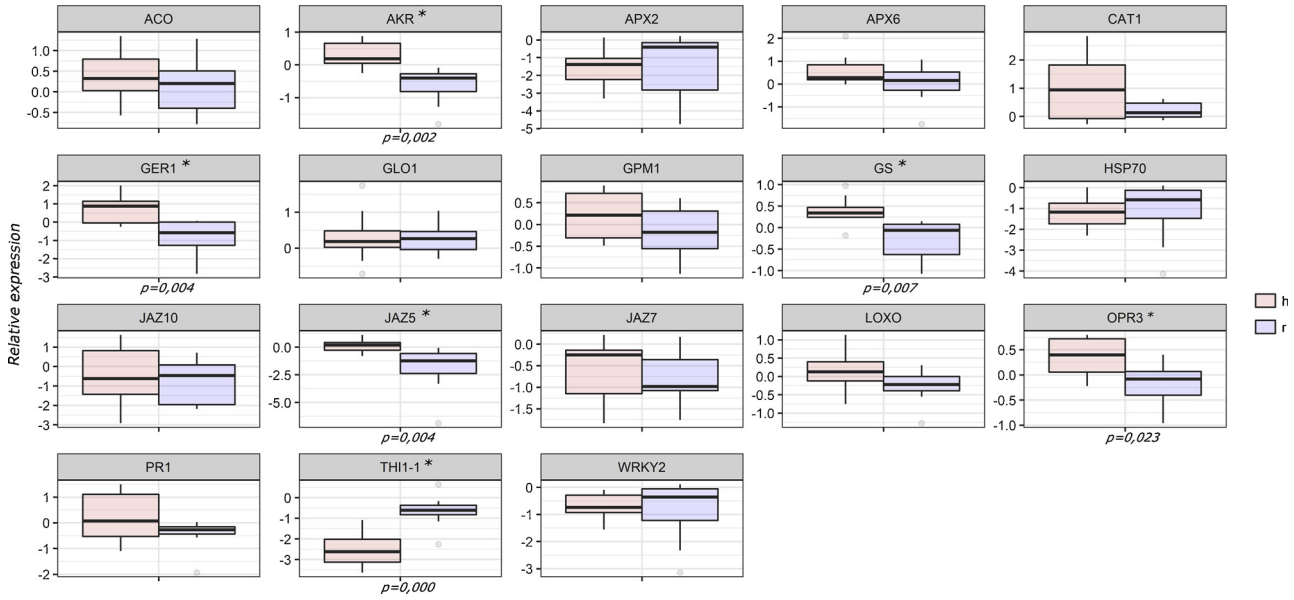

Fig. 5. Box plot of the difference gene expression (ΔGE) values, between the relative GE measured in August and July of each gene, in Nebbiolo cultivar. Lines extending vertically from the boxes indicating variability outside the upper and lower quartiles. ΔGE values that differed significantly by one-way ANOVA ($p \leq 005$) between h and r grapevines are marked with * and the *p* values are reported. h: control plants; r: recovered plants.

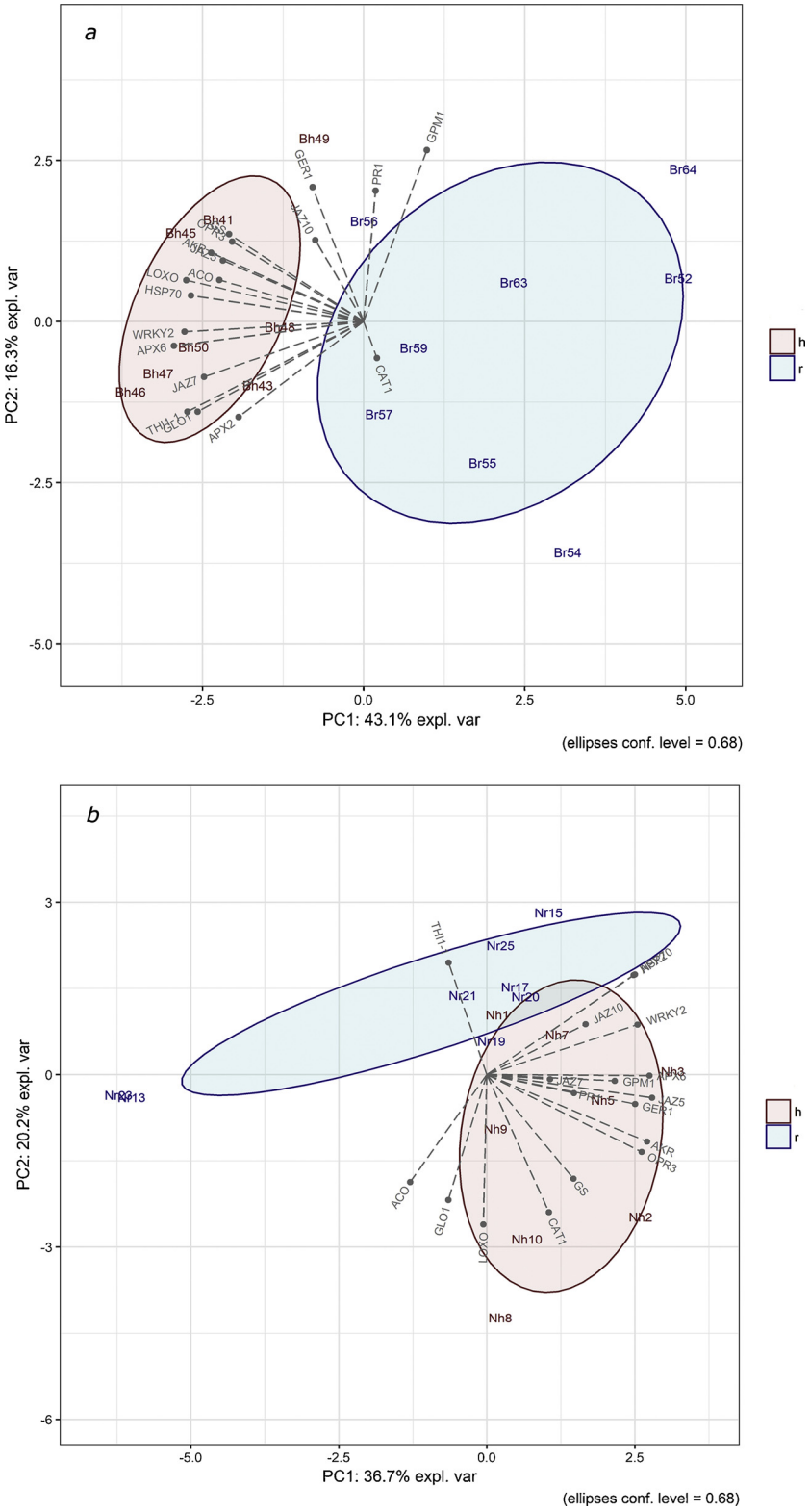

Fig. 6. PCA scatter plot of the difference gene expression (ΔGE) values for each gene, between the relative GE measured in August and July of Barbera (a) and Nebbiolo (b) grapevines. h: control plants; r: recovered plants.

and assisted selection programs. This would be particularly beneficial in case of phytoplasma diseases of perennial crops such as grapevine, for which no direct control of the pathogen can be applied. An important issue was to work under field conditions, as several physiological parameters of grapevine are known to be influenced by their growth in a pot rather than in the vineyard (Rocheta et al., 2016). To provide a method consistent with the field conditions, all outlying points were included in the final statistical analysis. All primers used in the study allowed amplification of their target genes, with comparable amplification efficiencies and R^2 values for both Barbera and Nebbiolo.

The results evidenced a complex scenario, where recovered grapevines of both cultivars could be better characterized at molecular level

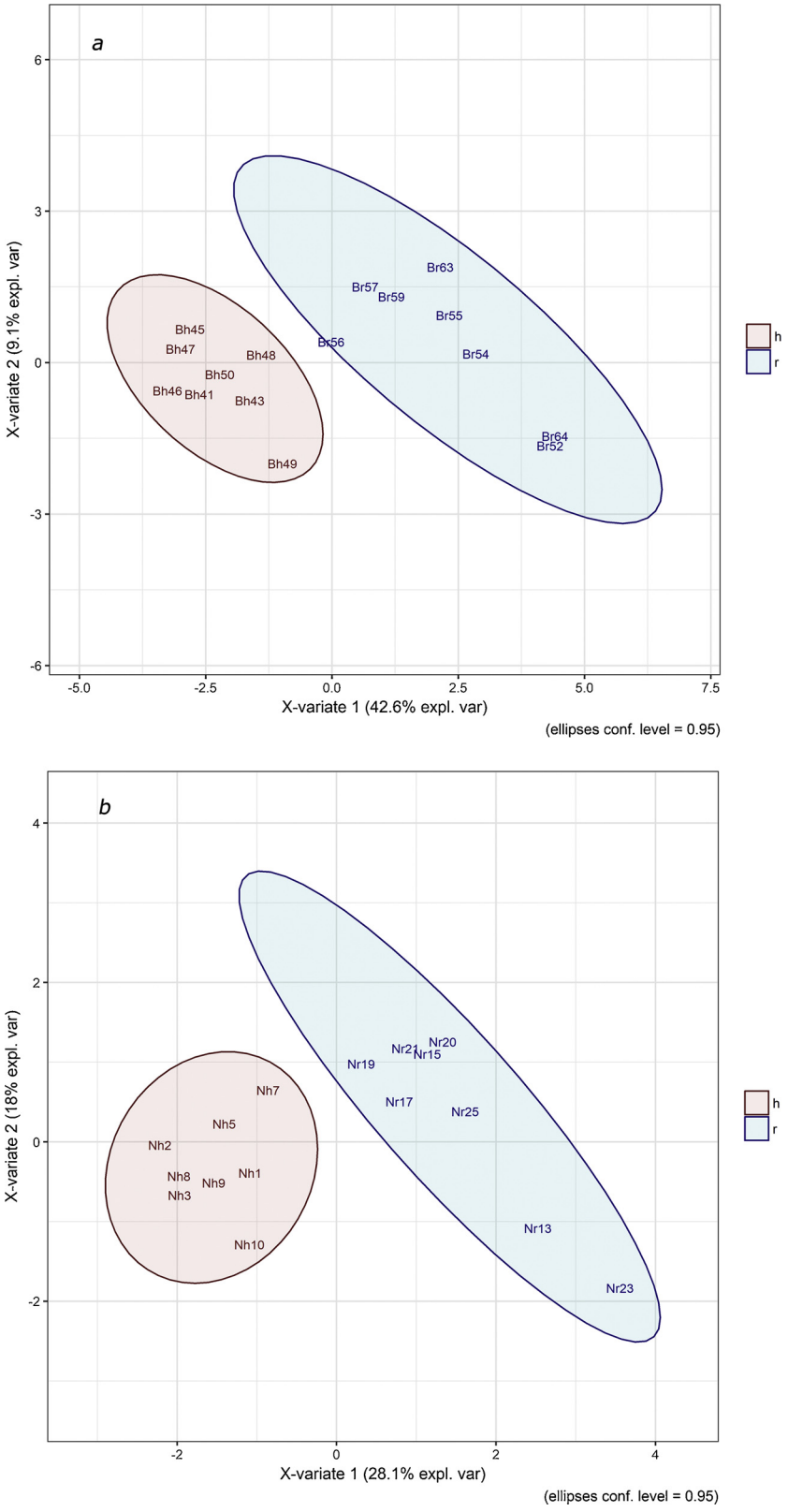

Fig. 7. sPLS-DA scatter plot of the difference gene expression (ΔGE) values for each gene, between the relative GE measured in August and July of Barbera (a) and Nebbiolo (b). h: control plants; r: recovered plants.

in comparison to the respective control plants by their over-season response of several of the analyzed genes, rather than by the snapshot of a single time-point analysis. Despite the possible effects of climatic and edaphic conditions, differential gene expression over the vegetative season showed that seasonal influence on the expression of some of the analyzed genes was clearly different between control and recovered plants of both cultivars.

Indeed, the univariate or multivariate analysis of the selected gene

Table 2
Gene stability values on first and second components of the sPLS-DA on ΔGE values of Barbera and Nebbiolo cultivars.

Genes	Barbera		Nebbiolo	
	1°component	2°component	1°component	2°component
ACO	1	–	–	0936
AKR	1	0,432	1	0,596
APX2	0,996	–	–	0,952
APX6	1	–	–	096
CAT1	0,424	0,956	–	–
GER1	0,988	0,972	0936	–
GLO1	1	–	–	–
GPM1	–	–	–	0,872
GS	1	0,724	0,944	–
HSP70	1	–	–	0,964
JAZ10	–	–	–	–
JAZ5	0,676	1	0,564	0,372
JAZ7	1	–	–	0,54
LOX0	1	0,932	–	0,828
OPR3	1	–	–	0,668
PR1	–	0868	–	066
THI1-1	1	–	1	–
WRKY2	1	–	–	0,984

transcript levels at each sampling point was not sufficient to identify marker genes significantly discriminating among h and r grapevines of the two cultivars. In contrast, both exploratory PCA and subsequent sPLS-DA analyses applied to the differences between the relative gene expression values measured at the two sampling times of the growing season, July and August, clearly indicated different transcriptional behaviours of recovered Barbera and Nebbiolo compared to control plants. The sPLS-DA analyses on seasonal ΔGE values achieved the best separation between control and recovered grapevines of both cultivars.

The distribution of the altered genes was cultivar specific for genes within the defence category, where a seasonal regulation of three (WRKY2, ACO1, HSP70) out of the four analyzed genes was found only in Barbera. The DNA binding protein WRKY2 belongs to a large group of plant transcriptional factors involved in plant responses to pathogen infection, tolerance to abiotic stresses, and responses to hormones like jasmonic and salicylic acids (Rushton et al., 2010). In grapevine, WRKY2 accumulates in responses to both biotic and abiotic stresses such as fungal infection and wounding and provides a broad resistance to necrotrophic fungal pathogens in a salicylate-independent way (Mzid et al., 2007). Over-expression of WRKY2 is known for FD-recovered Barbera and for BN-recovered Chardonnay grapevines (Gambino et al., 2013; Paolacci et al., 2017). Here, the different seasonal gene expression of WRKY2 between recovered and control Barbera plants confirmed the involvement of this gene in the recovery response of this variety to FD infection. The significant difference in the seasonal regulation of ACO1 between control and recovered Barbera plants supports the hypothesis of a possible involvement of the ethylene signaling in the maintenance of the recovery state in this cultivar, in line with the findings of Gambino et al. (2013). ACO1 also participates as molecular signal, together with H_2O_2, in the protection of buds from hypoxia (Vergara et al., 2012), besides being involved during grape berry development (Böttcher et al., 2013), and at the early stages of cold stress response (Sun et al., 2016). Finally, HSP70 belongs to a family of molecular chaperones involved in response to different biotic and abiotic stresses (Mayer and Bukau, 2005; Duan et al., 2011; Zhang et al., 2008). In *Arabidopsis thaliana*, plastidial HSP70 expression is required for protection against ROS accumulation and oxidative stress (Pulido et al., 2017), thus, FD-recovered Barbera plants may modulate transcription of the chloroplast HSP70 to cope with the potential damages associated to the alteration of their oxidative status. Therefore, the Barbera-specific deregulation of genes within the defence category, supports a stronger response of this variety compared to Nebbiolo, within a general frame of interactions among H_2O_2, jasmonate and ethylene metabolisms, common to both varieties under study. Indeed, several genes belonging to the remaining categories under analyses, showed altered seasonal regulation between h and r plants in both varieties. In particular, most genes within the general metabolism group showed altered regulation in both Barbera and Nebbiolo. In this category, a significant seasonal regulation of genes coding for glutamine synthetase (GS), thiamine thiazole synthase (THI1-1), and aldo-keto reductase (AKR), three proteins playing a pivotal role in primary metabolic functions and also in plant defence against pathogens (Sengupta et al., 2015; Turóczy et al., 2011; Seifi et al., 2013; Machado et al., 1997; Ribeiro et al., 2005; Tunc-Ozdemir et al., 2009; Li et al., 2016), was found for both varieties. Higher expression values of AKR and GS were reached in late summer for both varieties, while THI1-1 showed opposite regulation between the two cultivars grown under the same environmental conditions. GS modulation in recovered plants would indicate an increased demand of nitrogen mobilization, possibly in response to molecular mechanisms associated to the recovery status. Regulation of genes within THI family in response to multiple stresses may differ in a variety-dependent way, both under controlled conditions and even in the field (Rocheta et al., 2016). The AKR regulation described in the present work may have been induced by the H_2O_2 accumulation occurring in recovered grapevines (Musetti et al., 2007). Indeed, within the hydrogen peroxide metabolism, we found alteration in gene expression of both cultivars. Transcripts of the H_2O_2 scavenging enzyme APX6 showed a significant difference of seasonal regulation between control and recovered Barbera samples, in accordance with the hypothesis that, reduced expression of H_2O_2-scavenger enzymes can explain the H_2O_2 accumulation observed in leaf tissue of FD-recovered plants (Musetti et al., 2007; Gambino et al., 2013; Margaria et al., 2013). In recovered Nebbiolo, basal defence via H_2O_2 production also showed a seasonal different trend between h and r plants, in this case through altered regulation of the GER1 gene, coding a protein of the germin-like protein (GLP) family with superoxide dismutase activity (Knecht et al., 2010). Indeed, over-expression of the same gene was already reported also for FD-recovered Barbera grapevines (Gambino et al., 2013). As for the jasmonate metabolism, OPR3 gene was differentially regulated over the season in recovered plants of both cultivars, and JAZ5 only in Nebbiolo grapevines. Significant differences in the expression of genes controlling the jasmonate biosynthesis (LOX, OPR) as well as the repressing jasmonate activity (JAZ) between healthy and BN-recovered Chardonnay plants have been reported (Paolacci et al., 2017).

4.1. Conclusions

Many of the genes that were seasonally regulated in FD-recovered grapevines with respect to their control counterparts were involved in several biochemical networks, with a partial overlap of the known plant responses to biotic and abiotic stresses. In such a complex scenario, the snapshot of a single time-point analysis may not be enough for characterizing FD-recovered grapevine modified metabolisms, while the analysis of the plant over-season regulation of selected genes could provide a significant identification of the grapevine behaviour during recovery. For some patho-systems, plants recovered from phytoplasma infection maintain active defence strategies and are less prone to be re-infected, as suggested for European stone fruit yellows infected apricot trees (Osler et al., 2016, 2014) although, in the case of grapevine and FD infection, preliminary results have shown that FD recovered plants are susceptible to new infections, irrespective of the cultivar susceptibility to the pathogen (Pegoraro et al., 2017). The wide metabolic alteration of recovered grapevines, suggests that the profound stress induced by FD infection results in a long-lasting effect of the infectious event. Moreover, the results showed that recovery from FD infection involves alteration of most of the analyzed genes within the general metabolism category, with clear involvement of jasmonate and H_2O_2

pathways in both varieties, although Barbera-specific regulation of analyzed genes within the defence category was evident. This distinct response of FD-recovered grapevines under field conditions was therefore cultivar dependent, probably as consequence of the different degree of susceptibility to the disease of Barbera and Nebbiolo.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.12.005.

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