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Running head : Flavonoid changes in phytoplasma infected vines

ABSTRACT

Flavescence dorée phytoplasma (FDp) infections seriously affect production and survival of grapevine. We analysed the changes in the flavonoid pathway occurring in two red cultivars, the highly susceptible Barbera and the less susceptible Nebbiolo, following FDp infection. A combination of metabolic and transcript analyses was used to quantify flavonoid compounds and expression of a set of genes involved in their biosynthesis. Quantification of anthocyanins, flavonols, proanthocyanidins, and related biosynthetic enzymes, was performed over the vegetative season, at four time points, on healthy, infected and recovered plants. A strong activation of anthocyanin accumulation was observed in infected Barbera leaves, while the response was less marked in Nebbiolo. Proanthocyanidins also accumulated mainly in infected Barbera leaves, even if basal proanthocyanidin concentration was higher in healthy and recovered Nebbiolo. Biochemical data were supported by transcript analysis: genes of the stem flavonoid pathway and of the anthocyanin and proanthocyandin branches were expressed at a higher level in infected than in healthy plants, with a different magnitude between the two cultivars. Based on our results, we hypothesize that flavonoid accumulation is a physiological consequence of FD infection without affecting phytoplasma multiplication, although proanthocyaindin accumulation could help repel further infection by the insect vector.

Keywords: Flavescence dorée; recovery; gene expression; anthocyanins; tannins; flavonols; spectrophotometry; HPLC; quantitative PCR

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is cultivated worldwide and plays a pivotal role in the economy of many countries, both for fresh consumption and wine production. Grapevine yield and quality can significantly decrease because of biotic stress; beside well-known fungal pathogens and viruses, recently phytoplasmas have become increasingly important as serious threats to vineyard survival in several European grape growing areas. Taxonomically, phytoplasmas are plant-pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less micro-organisms phylogenetically related to low G+C, Gram-positive bacteria (Weisburg *et al*. 1989; Woese 1987). In the plant host they are restricted to nutrient-rich phloem sieve tubes and are transmitted by phloem-sap feeding leafhoppers or psyllids in a persistent propagative manner. Flavescence dorée phytoplasma (FDp, elm-yellows group) and Bois Noir phytoplasma (stolbur group), are associated with important phytoplasma diseases of grapevine (Osler *et al*. 1993). Due to its epidemic nature, FDp is a quarantine pathogen in Europe. Use of certified phytoplasma-free propagation material, removal of infected plants from the vineyard, and managing insect vectors are the only indirect approaches available to limit its spread. Phytoplasmas are difficult to control and affect grapevine physiology dramatically. The pathogen spreads through the plant tissues without inducing an effective resistance response; infection is associated with alterations of physiological parameters (Bertamini & Nedunchezhian 2001; Bertamini *et al*. 2002; Endeshaw *et al.* 2012; Rusjan *et al.* 2012a; Rusjan, Veberic & Mikulic-Petkovsek 2012b) and of several metabolic pathways, as described at the molecular level by qRT-PCR transcript quantification (Gambino *et al.* 2013; Hren *et al*. 2009b; Landi & Romanazzi 2011; Santi *et al*. 2013), microarray analysis of the transcriptome (Albertazzi *et al*. 2009; Hren *et al*. 2009a), and, very recently, proteomic analysis (Margaria, Abba & Palmano 2013; Margaria & Palmano 2011). These physiological and molecular effects are linked to dramatic symptoms on infected plants, such as reddening/yellowing of the leaves depending on the variety, downward curling of leaves, stunting, lack of shoot lignification, and fruit abortion, finally resulting in severe yield reduction at the end of the season, and even death of the vines.

Following initial infection and symptom expression, the disease can re-appear in the following years, or disappear completely (recovery phenomenon). In the last case the recovered plant may remain asymptomatic if not exposed again to infective vectors.

Flavonoids are a large class of plant secondary metabolites, encompassing more than 10,000 structures, that accumulate in fruit, flowers and leaves (Falcone Ferreyra, Rius & Casati 2012). These molecules play several roles including regulation of plant cell physiology (Mahajan, Ahuja & Yadav 2011), attraction of animal pollinators (Petroni & Tonelli 2011), response to abiotic (Berli 2010; Tohge *et al*. 2011) and biotic stress (Gutha *et al*. 2010; Miranda *et al.* 2007; Vega *et al*. 2011), and defence against herbivores and insects (Misra *et al.* 2010; Mohanta *et al*. 2012). In grapevine, flavonoids are one of the most important determinants of quality due to their involvement in determining fruit colour and taste. This importance is well documented by the vast literature exploring flavonoid biosynthesis and accumulation in grape berries (Ali *et al.* 2011; Azuma *et al.* 2012; Castellarin *et al*. 2011; Ferrandino *et al*. 2012). Previous studies explored the alterations of flavonoid metabolism in grapevine during biotic stress, such as fungal (Rotter *et al.* 2009; Ali *et al.* 2012; Boubakri *et al*. 2013; Latouche *et al*. 2013) and virus infection (Gutha *et al*. 2010; Vega *et al*. 2011). According to phytoplasma infection, a few studies have described changes in expression of some genes of the flavonoid pathway in grapevine leaves and berries during Bois Noir phytoplasma infection (Albertazzi *et al.* 2009; Hren *et al.* 2009a; Landi & Romanazzi 2011; Rusjan *et al.* 2012a; Rusjan *et al.* 2012b). However, so far, an integrated metabolomic and transcriptomic approach has not been applied to study the impact of FD phytoplasma on grape flavonoid biosynthesis and accumulation.

Here, we investigated the changes in the flavonoid pathway occurring in two important red grape varieties from northern Italy, Nebbiolo and Barbera, base cultivars of some of the world's most appreciated and valued red wines such as Barolo, Barbaresco and Barbera. Nebbiolo and Barbera show different sensitivity to FD infection, the latter being more susceptible than the former. In fact, FDp titre has been reported to be higher in Barbera than in Nebbiolo, which actually sustains a

lower multiplication of the pathogen during the season (Roggia *et al*. 2013). In the present study, biochemical and transcript analyses were made for each cultivar over the 2011 season (four time points) under three sanitary conditions (healthy, infected and recovered plants). Quantification of total phenolics, anthocyanins and proanthocyanidins, followed by specific analysis of flavonols were performed by spectrophotometry and HPLC-DAD, respectively. Key target genes involved in the synthesis of these secondary metabolites, and relative regulatory elements, were quantified by quantitative reverse-transcription (qRT)-PCR. The overall results showed temporal and variety-specific patterns of compound accumulation and gene expression.

This is the first integrated biochemical and molecular analysis of several branches of the flavonoid pathway occurring over-time in two grapevine cultivars with a different susceptibility to FD, under two distinct physiological states: during active phytoplasma-induced infection and following recovery from the disease.

MATERIALS AND METHODS

Vineyards, plant collection and phytoplasma detection

Two vineyards, located in Cocconato and Monteu Roero, Piemonte, in northern Italy, were used for material collection in 2011. The choice of two different vineyards was necessary as our experimental work was performed on healthy, phytoplasma-infected and stable recovered plants (two years old recovery) of two varieties. Therefore we needed to sample from vineyards where the sanitary situation was well known, in our case since 2007. The two vineyards have the same exposure and altitude conditions (350m); vine spacing is 1.0 m x 1.2 m; rows are oriented North-South; electronic meteorological stations of the Phytosanitary Service, Agrometeorological Sector, Piemonte Region, were available for each vineyard and used to monitor climatic parameters. The cultural practices adopted in the two vineyards were those traditionally managed by grape-growers in the cultivation areas in Piemonte, following the requirements for the production of 'Controlled Denomination of Origin' (DOC) wines. In particular neither irrigation nor chemical fertilization

were applied during the year; in both vineyards the grass between the rows was periodically mowed, cut up and left on the ground. The vines were trained to the vertical shoot system and Guyot pruned; before the vegetative season plants were mechanically and manually pruned, while at the beginning of the season young shoots were mechanically pruned to ensure an homogenous vine canopy architecture.

Four time points, spanning from June to September, were selected for the time-course experiment. Collection dates were June 16, July 20, August 11 and September 8. Leaves from healthy and infected plants were sampled at each date, whereas leaves from recovered plants were collected starting from July. Plants sampled as healthy and recovered did not show any symptom of phytoplasma infection, whereas infected plants showed typical symptoms (Margaria *et al*. 2007). The leaves were collected for each plant from both sides of the row to attenuate the influence of light exposure on certain classes of flavonoids, and flavonols in particular (Downey, Harvey & Robinson, 2004). All sampled leaves were detached from the external vegetation layer between the 5th and the 8th node of the vegetative shoot, to ensure the collection of samples of the same age over the season. We performed molecular assays on each single sampled plant to detect FDp (Margaria, Turina & Palmano 2009; Margaria & Palmano, 2013), and exclude the presence of any other known phytoplasmas in infected grapevines, as well any phytoplasma infection in healthy and recovered plants. The phytoplasma strain was characterized (Martini *et al*. 1999) as belonging to subgroup FD-C. All sampled plants were free of symptoms related to other known bacterial, fungal or viruses. Finally, we selected for the experimental work 15 healthy plants, 15 FDp-infected plants and 12 recovered plants for each variety. All the recovered plants considered in this work were stably recovered since two years.

Experimental design

For each sanitary state, we constituted three independent pools in each date (biological samples I, II, III), each one composed by 5 different independent plants for the infected and healthy status and by 4 independent plants for the recovery status. The plants considered and grouped at the first sampling date, were then sampled in the same way at the following dates. In total we had 33 biological samples for each variety over the season, consisting in 12 healthy, 12 infected and 9 recovered biological samples, as shown in Supporting information Fig S1. From each pool, we used 10 g of plant material for metabolite analysis, which were stored at -20 °C in a pH 3.2 ethanolic buffer (Ethanol 120mL, tartaric acid 5g/L, sodium bisulfate 2 g/L, neutralized with 3 vol of NaOH 1N) until analysis. At the same time, 1 g of each pool was frozen in liquid nitrogen and kept at -80° C until RNA extraction. Another aliquot of 1 g of the FD positive samples, was stored at -80° C and used for DNA extraction and phytoplasma titer quantification (Roggia *et al.* 2013).

Extraction and quantification of total phenolics, total anthocyanins and total proanthocyanidins

Leaves were thawed with an Ultraturrax dispersing machine (IKA, Staufen, Germany), equipped with a dispersing element, 22 cm of length and 1.2 cm of diameter and centrifuged at 4000 rpm. The supernatant was stored; the pellet was re-suspended and centrifuged again; the supernatant from the second centrifugation was added to the first supernatant and the mixture was brought to a final volume of 100 ml. Samples were stored at -20 °C until analysis.

For determining the total phenolics and total anthocyanin content, leaf extracts were diluted (from 12.5 to 25 fold, depending on the expected anthocyanin concentration of samples) with ethanol chloride (CH_3CH_2OH: H_2O: HCl, 70:30:1) (Di Stefano, Cravero & Gentilini 1989). Spectrophotometric indexes of total phenolics, with major component of flavonoids (absorbance read at 280 nm) and total anthocyanins (absorbance read at 520 nm) were calculated and quantified using (+)-catechin hydrate (Fluka) and malvidin 3-O-glucoside chloride (Extrasynthèse, Genay Cedex, France) as external standard reference, respectively. Total phenolics were expressed as

grams of (+)-catechin hydrate equivalents per kg of fresh leaf with ε at 280 nm of 3487 L mol^{-1} cm^{-1} for (+)-catechin hydrate dissolved in ethanol chloride. In order to avoid possible interferences by SO2-rich solvent [38] at 280 nm spectrophotometric measures, a blank was used containing the extractant SO2 rich buffer and chloridric ethanol in the same concentration as the samples. Total anthocyanins data were expressed as grams of malvidin 3-O-glucoside chloride equivalents per kg of fresh leaf; the molar extinction coefficient (ε) at 542 nm of for malvidin 3-O-glucoside chloride dissolved in ethanol chloride was 29997 L mol^{-1} cm^{-1}. Total proanthocyanidins (PA) were measured by the protein precipitation method (Harbertson, Kennedy & Adams, 2002), adapted to analyze amounts of PAs in grape leaf extracts. A protein solution (bovine serum albumin, BSA) with a final concentration of 1 mg/mL was prepared in a pH 4.9 buffer (200 mM acetic acid and 170 mM NaCl); a 1 mL aliquot of the protein solution was dispensed into a 1.5 mL microfuge tube, then 500 µL of the extract of grape leaves were added and the mixture was incubated at room temperature for 15 min with slow agitation. After incubation the sample was centrifuged for 5 min at 13500 g to pellet the PA-protein precipitate. The liquid solution was poured off and the pellet was washed with 250 µL of the same buffer used to dissolve the BSA (200 mM acetic acid and 170 mM NaCl adjusted to pH 4.9 with NaOH). Sample was centrifuged again to re-pellet the PA-protein precipitate. The wash solution was discarded, and 875 µL of a buffer containing 5 % of triethanol amine (TEA, v/v) and 10 % of lauryl sulphate sodium salt (SDS, w/v) were added, and the tube was allowed to stand at room temperature for 10 min. The tube was then vortexed to completely dissolve the PA-protein pellet. The solution was allowed to stand at room temperature for 10 min and the background absorbance at 510 nm was read. Subsequently, 125 µL of a ferric chloride reagent (10 mM $FeCl_3$ in HCl 0.01 N) was added, and after 10 min, the absorbance at 510 nm was recorded again. The absorbance was determined by subtracting the background absorbance from the final reading at 510 nm. A zero epicatechin sample was prepared by adding 125 µL of the $FeCl_3$ reagent to 1.875 µL of the TEA/SDS buffer and absorbance at 510 nm of this solution was subtracted from each of the points on the standard curve. PA concentration in leaves were expressed

as grams of (-)-epicatechin gallate equivalents per kg of fresh tissues. A standard curve was prepared using (-)-epicatechin gallate in the range of 25 to 500 mg/L.

Extraction and quantification of individual flavonols

Leaf extracts were diluted 1.1-fold with phosphoric acid 1 M; flavonols were separated and detected by HPLC/DAD (Perkin Elmer series 200-L pump) equipped with a Licrosphere 100 RP-18 5 mm column (25 x 0.4 cm ID) (Merck, Darmstadt, Germany). The chromatographic method, peak identification and quantification were performed accordingly to a previous published method (Ferrandino & Guidoni, 2010). Total flavonols were expressed as milligrams of quercetin 3-O-glucoside equivalents per kilogram of fresh leaves. The percentages of individual flavonols were also calculated.

RNA extraction from grapevine leaves

Total RNA from *V. vinifera* leaves was extracted following a published protocol (Reid *et al.* 2006) with some modifications. One g of leaf tissues was ground in a mortar with liquid nitrogen and immediately transferred to 50 mL tubes containing 20 mL of pre-warmed (65°C) extraction buffer [2% hexadecyltrimethylammonium bromide (CTAB), 2% polivinilpirrolidone 40 (PVP40), 10 mM Tris-HCl pH8, 25 mM EDTA, 2M NaCl, 2% β-mercaptoethanol), vortexed and incubated for 10 min at 65°C. Tubes were added of 1 vol chlorophorm-isoamilic alcohol 24:1, vortexed for 2 min and centrifuged at 3000 g for 30 min at 4°C. Supernatant was transferred to a new tube. One volume of chlorophorm-isoamilic alcohol 24:1 was added, vortexed and centrifuged at 3000 g for 45 min. The supernatant was further centrifuged at 30,000g for 10min at 4°C, and next added with 0.1 volume 3M NaOAC (pH 5.2) and 0.6 volume isopropanol. Following incubation at -80°c for 1 h, tubes were centrifugated at 3500 g for 30min at 4°C. Pellet was dissolved in 1mL of 1X TE pH7.5; 0.3 volume of LiCl 9 M was added and left overnight at 4°C. RNA was precipitated by centrifugation at 20,000 g for 30min at 4°C, washed with 70% cold ethanol and resuspended in 200

µL of 0.1% DEPC-treated sterile water. RNA samples were treated with RNase-free DNase I (Applied Biosystems, Foster City, CA, USA) in the supplied buffer to avoid residual DNA contamination: 1µg was digested for 1h at 37°C with 2 DNase units in 40 µL final volume. DNase was removed by phenol/chloroform extraction following the manufacturer's instructions. RNA was resuspended in 30 µL of RNase-free water treated with 0.1% DEPC, and quality was analysed by a spectrophotometer to evaluate integrity, purity and concentration of the extracts. The RNA aliquots were stored at −80 °C.

Selection of target and reference genes

Thirteen genes were considered in this study. Eight target genes coded for key enzymes of the flavonoid pathway: chalcone synthase isoforms II (*CHS II*, GSVIVT01032968001) and III (*CHSIII*, GSVIVT01000521001), flavanone-3-hydroxylase 2 (*F3H2*, GSVIVT00014419001), leucoanthocyanidin dioxygenase (*LDOX*, GSVIVT00001063001), UGT-glucose:anthocyanin 3-Oglucosyltransferase (*UAGT*, GSVIVT00014047001), flavonol synthase 1 (*FLS*, GSVIVT00015343001), anthocyanidine reductase (*ANR*, GSVIVT00005344001), and leuco-anthocyanidine reductase (*LAR*, GSVIVT00030282001). Two CHS isogenes (*CHS II* and *CHSIII*) were considered, in order to monitor for possible changes in the contribution of each isoform to the pathway, as observed previously in the Grapevine-leafroll-associated virus-3 (GLRaV-3) interaction (Zabala *et al*. 2006). Further reactions involving *F3H*, *LDOX* and *UAGT* enzymes lead to the synthesis of anthocyanidin pigments. Other branches include *FLS*, for the synthesis of flavonols, and *LAR* and *ANR* for synthesis of flavan-3-ols, the precursors of proanthocyanidin (PA) polymers. In a few cases, a specific gene isoform was selected on the basis of previous literature reports showing significant changes during biotic infection in grapevine.

Two genes coding for transcription factors were also chosen as targets: *VvMYBA1* (GSVIVT01022659001), which is involved in regulation of *UAGT* and anthocyanin biosynthesis

(Cutanda-Perez *et al*. 2009), and *VvMYBF1* (GSVIVT00028082001), which regulates flavonol synthesis (Czemmel *et al.* 2009).

Three endogenous reference genes, actin (*ACT*, GSVIVT00034893001), ubiquitin (*UBI*, GSVIVT01038617001) and ribosomal gene *18S* were used for evaluation of the stability of gene expression in our conditions.

All primer pairs used in this study were derived from the literature and references are included in Supporting Information Table S1, together with gene accession number and genome based gene identifier. In order to exclude differences in the gene sequences between the two genotypes, that could negatively impair the ability of the primers to amplify the target DNA, the efficiency of amplification was determined for each gene in each variety, by construction of dilution curves, as described in the following paragraph.

Real-time qRT-PCR assays

For qRT-PCR, first-strand cDNA synthesis was made in duplicate in two independent runs using l00 ng of total DNase treated RNA and random examers as primers, according to the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Two µL of cDNA were used as template in the qPCR reactions, containing 12.5 µL of IQ^{TM} Supermix (Bio-Rad, Life Science Research, Hercules, CA, USA) and 200 nM primers (Supporting Information Table S1), to a final volume of 25 µL. Reaction conditions for all primer pairs were as follows: 3 min at 95 ◦C, 40 cycles of 15 s at 95 °C, 10 sec at 56 °C and 30 sec at 72°C. At the end of each qPCR, melting curve analysis was performed over the range 56-95°C to determine the specificity of amplicons. Reactions were carried out in the StepOne Plus RealTime PCR System (Applied Biosystems).

The sample maximization approach (Derveaux, Vandesompele & Hellemans 2010) was used for plate design, i.e. all samples for each variety (33 total) were simultaneously analyzed in the same plate for each target gene, in duplicate. In order to estimate qPCR efficiency, a standard curve consisting of at least four 1:5 fold dilution points of the cDNA obtained from a Master sample was

included in each plate; preliminary assays were performed in order to evaluate the optimal dilutions for each gene target. The Master sample was prepared by mixing all the 33 DNase-digested RNAs of each variety, divided in aliquots stored at -80°C, and used for cDNA synthesis and standard curve points in all the plates, except those used for *FLS* and *VvMYBF1* target genes. The low concentration of these transcripts in the analyzed samples, in fact, hampered the construction of a dilution curve from cDNA. Thus, for *FLS* and *VvMYBF1* RNAs quantification, standard curves were prepared by using plasmids containing the fragment of interest. Amplified fragments according to Gutha *et al*. 2010 and Czemmel *et al*. 2009, were purified and cloned in the pGEM-T vector (Promega). Liquid cultures were set up and plasmids purified and sequenced. Ten-fold dilutions starting from 60 fg/µL were used for determination of standard curves in qPCR reactions. Standard curves were derived by linear regression analysis from plot of the threshold cycle values of each standard dilution point against the Log of the arbitrary concentration of the DNA dilutions. In order to evaluate the stability of the cDNA used in serial plate runs, two dilutions (1:5; 1:125) of the Master sample were used to amplify the actin gene in all plates and Ct values were analyzed to exclude cDNA degradation in serial qPCR reactions. In each plate, we included sterile distilled water as template instead of RNA as no-template control, and a sample of RNA from the master sample to monitor for possible DNA residuals in the samples.

Validation of reference genes and relative gene expression analysis

The stability of reference genes was determined by using three software tools commonly used for reference gene selection: geNorm (Vandesompele *et al*. 2002), Norm Finder (Andersen, Jensen & Orntoft 2004), and Best Keeper (Pfaffl *et al*. 2004) (Supporting Information Table S2). As input, the mean of the Ct values of each sample reported as ΔCt value to the Ct of the lowest concentrated sample and corrected for PCR efficiency was used. The weighted mean of the expression ratios of the two best genes was used as the normalization factor for relative quantities (RQ) calculation according to the $2^{-\Delta\Delta Ct}$ method.

For each gene, the mean Ct value of healthy samples was taken as reference to calculate the relative expression levels of each sample, in each cultivar. $\Delta\Delta Ct_{weighted}$ was calculated, according to (Yuan, Wang & Stewart, 2008), using the PCR amplification efficiency (PAE) to adjust the ΔΔCt values of each gene. As weight we used w=0.5, since we used two reference genes, RG1 and RG2 (w=1 divided by the number of reference genes). The resulting equation for calculating $\Delta\Delta Ct_{weighted}$ of each sample i for each target gene j was:

$$\Delta\Delta Ct_{weighted\ j,i} = [Ct_{j,i} * PAE_j - (Ct_{RG1i}*PAE_{RG1}*w + Ct_{RG2i}*PAE_{RG2}*w)]+$$
$$- [meanCt_{j,h}* PAE_j - (meanCt_{RG1h}*PAE_{RG1}*w + meanCt_{RG2h}*PAE_{RG2}*w)]$$

where the suffix $_h$ indicates the total healthy plants of the four sampling dates.

Relative quantities values ($RQ_{j,i} = 2^{\wedge} -\Delta\Delta Ct_{weighted\ j,i}$) were calculated for each sample. From these, the mean relative quantity and its standard deviation was calculated for the three biological replicates of each sanitary status and of each sampling date, and used for further comparisons.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means were separated by the Duncan's test at $P \leq 0.05$ and ≤ 0.01 with SAS 8.2 for Windows (SAS Institute, Cary, NC, USA). Multiple regression analysis for correlating metabolites and gene transcripts was done using the software GenStat rel. 15 (VSN International, Hemel Hempstead, U.K.).

RESULTS

Symptoms and FDp quantification in the two cultivars

Symptoms severity varied between the two cultivars, with Barbera vines generally showing more severe symptoms, similar to what was observed in surveys carried out in previous years in the same vineyards (Roggia *et al.* 2013). Starting form the first sampling date, differences were observed: Nebbiolo was characterized by leaf downward rolling, withering of the inflorescence and mild yellows around the veins, while infected Barbera plants showed marked reduced growth of new

shoots, withering of the inflorescence, short internodes and leaf reddening. In late summer, Nebbiolo plants showed lack of shoot lignification, with reddening around veins and limited to leaf sectors; Barbera plants showed lack of shoot lignification, evident brown pustules on canes and marked reddening of the whole leaf often resulting in the whole plant turning purple (Fig. 1). No symptoms were observed on healthy and recovered plants, the last being classified as infected in the previous years. Each single plant was tested for phytoplasma infection, and FDp was found only in the grapevines showing typical symptoms, excluding the presence of any other phytoplasma in all samples. FDp titre was quantified in the two cultivars: mean FDp concentrations in infected Barbera samples were one order of magnitude higher than in infected Nebbiolo samples (Fig. 2a). A seasonal trend in FDp concentration was observed with peak in July for Nebbiolo and in August for Barbera, followed by a decrease during the rest of the season. Amplification was never observed neither in healthy nor in the recovered plants.

Regarding the climatic parameters, average temperatures in the two vineyards were very similar all over the year (Supporting Information Fig. S2), and especially during summer, when temperatures reached 20.4, 21.7, 24, 21 °C in Monteu Roero and 20.4, 21.4, 24.1, 21.2 °C in Cocconato, spanning from June to September. The growing degree days base 10 °C were 2210 in Cocconato and 2123 in Monteu Roero. The relative humidity during the year was 66.8 % in Cocconato and 73.7 % in Monteu Roero), with specific values of 79, 72, 65, 71% in Monteu Roero and 74, 68, 61, 69 % in Cocconato, from June to September.

Total phenolics

Total phenolic concentrations did not show differences between the three sanitary states (healthy, infected and recovered), until the second sampling (July) when infected vines accumulated more phenolics compared to healthy ones, in both varieties. In general, recovery did not affect the phenolic accumulation (Fig. 2b). The seasonal phenolic concentration increase in infected leaves compared to healthy ones, was higher in Barbera than in Nebbiolo; also the timing of the response

to FD infection was different between the two varieties. In fact, in Barbera (Fig. 2b), a peak was detected at the third sampling, when phenolic concentration in leaves from infected vines was almost double compared to that of healthy vines (+ 95 %), whilst, in Nebbiolo (Fig. 2b), the phenolic maximum concentration was concomitant with the second sampling (+ 45 % in infected *vs* healthy leaves).

In general, the trend of phenolic accumulation (Fig. 2b) reflected that of the phytoplasma titre during the season (Fig. 2a) in both cultivars.

Total anthocyanins, proanthocyanidins, and flavonols

As leaf reddening is a typical symptom of FD in red-coloured grape varieties, we measured the concentration of different classes of flavonoids, including anthocyanins, flavonols, and proanthocyanidins in healthy, FDp-infected and recovered plants.

Anthocyanin concentration in leaves of both healthy and recovered plants was low and overall similar in both varieties during the season, in agreement with lack of observable reddening. In infected Barbera leaves, the reddening was visible since the first sampling time, when anthocyanin accumulation was already statistically significant. The peak of anthocyanin accumulation was detected at the third sampling, in coincidence with berry full véraison, and the concentration was up to 10 times higher in infected leaves compared to healthy ones (Fig. 3a; Supporting Information Table S3). In infected Nebbiolo, anthocyanin concentration was limited, likely reflecting the localized and patchy redness generally observed in symptomatic leaves, and the concentration was not more than twice that of healthy leaves (Fig. 3a; Supporting Information Table S3).

Nebbiolo grapes tend to accumulate more proanthocyanidins (PA) than Barbera, and this was also evident in leaves, where basal PA concentrations (at first sampling) in healthy and recovered plants, were higher in Nebbiolo. Proanthocyanidin concentration increased in infected samples of both varieties, but with less evidence in Nebbiolo than in Barbera (Fig. 3b; Supporting Information Table S4).

No difference in total flavonol concentration was detected between the three sanitary conditions in both cultivars (Fig. 3c). In healthy leaves of both genotypes, the main flavonol aglycone was quercetin, followed by kaempferol and myricetin, as reported in leaves of other *Vitis vinifera* varieties (Fernandes *et al*. 2013), and this profile did not change in recovered and infected plants. Neither in Nebbiolo nor in Barbera leaves differences were detected in the percentage concentration of individual flavonols, irrespective of their sanitary status (Supporting Information Table S5). However, some differences were detected between the two genotypes: Nebbiolo leaves were characterized by a higher percentage of quercetin glycosides compared to Barbera, and lower percentages of kaempferol and myricetin glucoside.

Selection of reference genes for qRT-PCR analysis and primer validation

The expression pattern of eight biosynthetic and two regulatory genes was monitored in grapevine leaves collected from healthy, recovered, and infected plants. As the selection of appropriate reference genes is critical for qRT-PCR data analysis, and considering the complexity of our experimental design (two cultivars, three sanitary conditions, four time points), we decided to monitor the stability of expression of three reference genes (actin, ubiquitin and 18S). Three statistical tools were used, i.e. Best Keeper, geNorm and Norm Finder. Output values from the three softwares for each reference gene are reported in Supporting information Table S2. In Barbera, actin was selected as the best reference gene by all programs. In Nebbiolo, geNorm selected actin, while Norm Finder and Best Keeper selected the ubiquitin gene, although also actin was highly stable. In this study, we finally decided to use the weighted mean of the expression ratios of actin and ubiquitin as the normalization factor for relative quantities (RQ) calculation in both varieties. All primers used in the study showed very similar amplification efficiency and very high R^2 values in Barbera and Nebbiolo (Supporting information Table S1), and were then used in the Real-Time RT-PCR assays as described.

Expression patterns of flavonoid biosynthetic genes

Gene expression in Barbera leaves

Early genes of flavonoid biosynthesis (*CHS* and *F3H*) contribute to the production of dihydroflavonols, which are then further processed to flavonols, anthocyanins and proanthocyanidins. In Barbera, a marked difference in the expression of two *CHS* isogenes was observed (Fig. 4): RQs of *CHS3* showed a large increase in infected leaves, with a peak in August followed by decrease afterwards. A similar profile was not observed for *CHS2* which, moreover, showed high variability among biological replicates. *F3H2* mRNA concentration levels in infected tissues also increased during the season, reaching RQ values up to 7 times those observed in healthy samples in August, followed by a decrease (Fig. 4).

An expression trend similar as for the early biosynthetic genes *CHS3* and *F3H2* was observed for the *LDOX* and *UAGT* genes, which are addressed to anthocyanin biosynthesis (Fig. 5). As expected, based on the anthocyanin concentration data, expression of these genes in infected Barbera plants showed an increase of 35 and 170 fold respectively above healthy plants, at the peak of expression. Accordingly, the expression of the *UAGT*-transcription factor *VvMYBA1* (Fig. 5), was significantly higher in infected plants in July (about 80 times the healthy samples), and later decreased.

LAR and *ANR* are involved in proanthocyanidin synthesis, respectively producing catechin and epi-catechin from di-hydroxylated substrates. No significant differences in the RQ values were observed for the single-copy gene *ANR* (Fig. 6). In grapevine, *LAR* is present in two isoforms, *LAR1* being more expressed in leaves (Bogs *et al.* 2005). *LAR1* transcripts showed increased accumulation during the season in phytoplasma-infected leaves, starting in August till the last sampling point (Fig. 6). The flavonol branch of the flavonoid biosynthetic pathway was probed analysing expression of the enzyme-coding gene *FLS1*, and of the cognate transcription factor *VvMYBF1*. Both failed to show significant changes among the four time points and, accordingly, no differences were observed among the sanitary states (Fig. 7).

Gene expression trends in recovered plants were similar to healthy plants and not significantly different among sampling dates (Fig. 4,5,6,7).

Gene expression in Nebbiolo leaves

Differential expression of flavonoid biosynthetic genes in infected plants during the season was also observed in Nebbiolo plants. As observed in Barbera, *CHS3*, *F3H2*, *LDOX*, *UAGT* and *VvMYBA1* were up-regulated in infected plants (Fig. 4, 5), but their RQ values at the peak of expression were markedly lower than in Barbera: about 5, 4, 7, 44, and 25 times than healthy samples. The first three of the above mentioned genes showed the peak of expression in July, and no further transcript accumulation was observed later in the season.

Within the proanthocyanidin pathway, *ANR* did not show increased expression in infected samples, while *LAR1* reached the peak in July, with RQ value about 2 times those observed in healthy samples (Fig. 6). No significant differences were observed for the expression of genes of the flavonol branch, *FLS* and *VvMYBF1* (Fig. 7). Also in Nebbiolo, no differences were observed between RQ values of healthy and recovered plants (Fig. 4,5,6,7).

Correlations between metabolite concentration and transcript abundance

Relationships between anthocyanins and UAGT transcripts

Significant correlations were found between the Log-transformed values of *UAGT* RQ and the Log-transformed concentrations (g/kg) of anthocyanins, in infected samples for both the Barbera and Nebbiolo vines (Log_Anthocyanins= 0.4556 Log_RQ - 0.6662 for infected Barbera and Log_Anthocyanins= 0.1761 Log_RQ – 0.57322 for infected Nebbiolo; percentage variance accounted for: 97.4; F probability of regression < 0.001). Healthy and recovered plants of both cultivars, at all sampling dates, had levels of transcripts and anthocyanins so close to 0 that it was not possible to correlate the two variables. In infected plants, the anthocyanin concentration was related to transcript quantities in both cvs. As shown in Figure 8, the line slope was higher in

Barbera than Nebbiolo, indicating that in presence of equal *UAGT* RQ values, the repercussion of transcript accumulation on anthocyanins concentration is stronger, in particular double, in Barbera.

Relationships between proanthocianidins and LAR and ANR transcripts

We have studied the relationships of the concentration of proanthocyanidins [PA] with the RQ of both *LAR* and *ANR* transcripts for the two pooled cultivars. The model considered is:

[PA] = a*Log_RQ_*LAR* + b*Log_RQ_*ANR*+ c.

The fitted models expressing the PA concentration in relation to *LAR* and *ANR* RQ was:

[PA] = 0.3888*Log_RQ_*LAR* – 0.675*Log_RQ_*ANR* + 0.9168 (% variance accounted for 36.3 ; F probability of regression < 0.001). No significant changes to the regression are introduced separating the values according either to the cultivar or to the sanitary state (Fig. 9a).

Figure 9b show the distribution of the three sanitary states separately for Barbera and Nebbiolo for an easier visualization. In Barbera, it is evident the clustering of infected samples toward the high values of PA; instead, in Nebbiolo the single values of different sanitary states are not evidently grouped in levels of PA.

Relationships between flavonols and FLS transcripts

No significant correlation was found between *FLS* RQ and flavonols concentration. In fact FD infection did not affected flavonol accumulation (Fig. 3c), neither the expression of the related biosynthetic gene (Fig. 7).

DISCUSSION

This study is the first analysis of changes in different branches of the flavonoid biosynthetic pathway, in FDp-infected, recovered, and non-infected grapevine plants, based on an integrated metabolic and transcriptomic approach. Barbera and Nebbiolo are two traditional and economically important Italian grapevine cultivars. The differential susceptibility of the two cultivars to FDp

infection is well known: Barbera is highly susceptible to FDp and shows severe symptoms, already visible in early summer, while symptoms on Nebbiolo vines are milder and not so evident until middle summer (Morone *et al.* 2007). Two vineyards in Piedmont were considered for sampling; this was necessary because we needed to sample from fields where the sanitary situation was known since many years, in order to sample stable recovered plants, i.e. plants that were recovered since 2 years. The symptomatology described for the two cultivars in the two vineyards reflected the situation which is commonly observed anywhere Barbera and Nebbiolo are planted and Flavescence dorée infection insists, with Barbera always more sensitive than Nebbiolo (Morone *et al.* 2007; Roggia *et al.* 2013). Moreover, the vineyards were located in a specific growing location which is representative of the denomination of origin area (Cocconato for Barbera d'Asti DOCG and Monteu Roero for Nebbiolo d'Alba) and where the cultural practices are the traditional ones requested for the production of 'Controlled Denomination of Origin' (DOC) wines, thus reflecting the real situation we can find in Piemonte with field-grown material. Even though we are aware of the important effects exerted by climatic conditions on grape quality (Ferrandino & Lovisolo 2013; Jakoola & Hohna 2010; Tossi *et al*. 2012), the main climatic parameters (temperature and relative humidity) were very similar. It has also been shown that light intensity and type (high or low solar UV-B) influenced the antioxidant capacity of leaves (Berli *et al.* 2013). However, it is known that the cultural practice management in the vineyard can be used to regulate the vine canopy, and thus the leaf exposure to light. In the two vineyards the cultural practices adopted were the same (see Materials and Methods for detail), so the influence exerted by the type of canopy management on the light interception (and, consequently, on the accumulation of secondary metabolites) could reasonably be excluded. As previously stated, biological observation based on symptom severity, indicated Barbera as a more susceptible cultivar to FDp infection than Nebbiolo; a recent study has shown that the two cultivars also support different phytoplasma titres. In particular, FDp concentration in Barbera was always higher than in Nebbiolo in two successive years, although no linear correlation between FDp titre and symptoms severity could be assessed (Roggia *et al.* 2013).

The phytoplasma titre in our samples confirmed the higher concentration in Barbera than Nebbiolo. Absolute values (Fig. 2a) are comparable to those assessed in the report of Roggia *et al.* 2013, when it is taken into account the variability in phytoplasma titres among years and that, in this study, we extracted whole leaf samples, instead of using only veins, whose weight amounts to 5-10% of total leaf tissue, consequently resulting in a diluted concentration of the phloematic pathogen. The erratic phytoplasma distribution in woody hosts (Berges, Rott & Seemuller 2000), may have also played a role in the quantitation results, as during the sampling we took material representative of the whole plant without focusing just on symptomatic branches, commonly used for diagnosis.

Expression of flavonoid biosynthetic genes and accumulation of flavonoids is a common hallmark of pathogen infection of plant leaves. Flavonoid biosynthesis in grapevine requires the stepwise action of "stem" biosynthetic genes, which produce precursors used for the synthesis of specific flavonoids in "branch" reactions. Within the "stem" biosynthetic genes, we analyzed expression of two isoforms of *CHS* (*CHS2* and *CHS3*) and of *F3H2*.

Three *CHS* genes were described in grape: *CHS1* and *CHS2* are expressed in leaves and unripe berries, while *CHS3* was reported to be limited to fruits (Goto-Yamamoto *et al*. 2002) and its expression was correlated with anthocyanin accumulation in berry skins (Ageorges *et al*. 2006). In our leaf samples, however, no significant accumulation of *CHS2* transcripts took place in infected leaves, while *CHS3* expression markedly increased. This suggests that *CHS3* is strictly linked to anthocyanin accumulation also in leaves, and that the other two *CHS* isoforms may be required for accumulation of other types of flavonoids. This could be in agreement with the marked difference in the magnitude of *CHS3* activation between the two varieties (Fig. 4), with a strong increase in Barbera (800 times the healthy at the mean peak) with respect to Nebbiolo (5 times compared to healthy plants) likely promoting the massive accumulation of anthocyanin only in Barbera (Fig. 3a). In the leaf, *CHS3* may respond specifically to biotic stress. A pivotal role of CHS as key checkpoint enzyme of flavonoid biosynthesis in response to pathogen infection has been reported previously in many plant systems (Dao, Linthorst & Verpoorte 2011), however little is known about the specific

contribution of the single isoforms. Recently, it has been shown that within the *Glycine max CHS* family, *CHS4* did not accumulate, in contrast to the other isogenes, during response to *Pseudomonas siringae* pv. *glycinea*, thus suggesting gene-specific contributions to infection (Zabala *et al.* 2006). This hypothesis is supported by a study on grapevine leaves accumulating anthocyanins as a consequence of GLRaV-3 virus infection, which also showed a marked increase of *CHS3* expression and much smaller changes for *CHS1* and *CHS2* (Gutha *et al.* 2010). Comparing the two cultivars, beside differences in the magnitude of gene expression we also observed a cultivar-specific kinetic of *CHS3* induction, with the mean peak in August for Barbera and in July for Nebbiolo. A similar behaviour was observed for *F3H2*, suggesting a different timing in expression of "stem" biosynthetic genes between the two cultivars, that find a good correspondence with the total phenolics accumulation peak in Barbera and Nebbiolo (Fig. 2b). Interestingly, both upstream biosynthetic gene expression and phenolics accumulation trends, have correlated with that of phytoplasma titre (Fig. 2a), suggesting that flavonoid accumulation does not affect phytoplasma titre, while rather, is a consequence of FDp colonization, without excluding the influence of specific branch metabolites on the disease development.

The three main specific groups of flavonoids in grapevine are anthocyanins, proanthocyanidins and flavonols. Fungal pathogens often activate proanthocyanidin biosynthesis (Kortekamp 2006; Polesani *et al*., 2010; Rotter *et al*. 2009), while expression of genes controlling the anthocyanin branch, such as *UAGT* and *VvMYBA1* (Cutanda-Perez *et al.* 2009), and the flavonol branch, such as *FLS* and *VvMYBF1* (Czemmel *et al*. 2009), is not affected. Some grape viruses and phyoplasmas differently affect the expression of grapevine flavonoid biosynthetic genes and proteins, and, besides activating the proanthocyanidin branch, they markedly affect the anthocyanin branch inducing leaf reddening (Albertazzi *et al.* 2009; Guidoni *et al.* 1997; Gutha *et al.* 2010; Hren *et al.* 2009a; Margaria *et al.* 2013). Viruses and phytoplasmas are retained in the symplast, lack cell walls and could partly escape sensing by the plant cells, thus not inducing the same responses observed in the case of fungal pathogens; many plant defence-related proteins in fact, have an antifungal or

glycan-lytic activity, that may limit the colonization of invading-microbes (van Loon, Rep & Pieterse 2006).

Anthocyanin concentration increased in both cultivars, but the accumulation was dramatic in Barbera, and much lower in Nebbiolo. Integrating metabolic and gene-expression data, we could find a significant correlation between *UAGT*, the anthocyanin-specific biosynthetic gene, and anthocyanin concentration in infected leaves. A mathematical model representing the relationship between these two variables (metabolite and gene) was found for each variety (Fig. 8). The model showed that the effect of *UAGT* accumulation on anthocyanins was double in Barbera (0.4556 Log_RQ versus 0.1761 Log_RQ) possibly suggesting genotype-dependent differences in *UFGT* efficiency. An explanation for the observed activation of anthocyanin biosynthesis may reside in the sugar concentration increase in infected leaves. High sugar level, in particular sucrose, is known to induce anthocyanin biosynthesis in Arabidopsis plants (Solfanelli *et al*. 2006) and in grapevine cell cultures (Gollop *et al*. 2002, Gollop, Farhi & Perl 2001). Accumulation of soluble carbohydrates and starch have been reported in phytoplasma-infected leaves of several hosts, such as periwinkle (Choi *et al*. 2004), coconut (Maust *et al*. 2003), papaya (Guthrie *et al*. 2001) and maize (Junqueira, Bedendo & Pascholati, 2004). In Bois Noir (BN) phytoplasma-infected grapevines cv Chardonnay, symptoms have been related to reduced photosynthetic activity and anomalous accumulation of carbohydrates (Bertamini & Nedunchezhian 2001). Accordingly, gene expression studies showed significant changes in the grapevine charbohydrate transport and metabolism induced by BN phytoplasma (Albertazzi *et al.* 2009, Hren *et al.* 2009a, Santi *et al.* 2013). Phytoplasma infection in grapevine is therefore correlated to sugar increase, which is also related to callose accumulation, that cause physical obstruction of sieve tubes leading to inhibition of phloem loading and transport (Musetti 2010; Musetti *et al*. 2013).

An open question is whether anthocyanin and proanthocyandin accumulation has any direct protective role toward the establishment of phytoplasma infection. The answer to this question is probably no, as flavonoids are accumulated in the wall and in vacuoles and thus spatially separated

from the phloem-restricted phytoplasmas. However, anthocyanin accumulation is important for protecting photosystems from oxygen radicals generated by photon-saturated photosystems in leaf cells. In fact under high light, low temperature, and chlorophyll loss, many plant leaves accumulate anthocyanins (Hoch, Singsaas & McCown 2003). Decreasing photosynthetic rates and photosystem efficiency have been recently observed in leaves of plants displaying FD symptoms (Vitali *et al.* 2013), and anthocyanins could have an antioxidant role, limiting further oxidative damage of leaf cells. However, anthocyanin accumulation could also act as a quencher of defence reactions mounted by the host plant. It has been demonstrated in fact, that peroxide radicals accumulates in the cell wall of phloem cells infected by the FD phytoplasma and it has been proposed that this accumulation can favour the recovery process (Gambino *et al.* 2013; Musetti *et al*. 2007). H_2O_2 can easily cross cell membranes and get in contact with the vacuoles containing anthocyanins, that are well-known antioxidants (Agati *et al*. 2012): consequently, anthocyanin accumulation could dampen the protective effect of oxygen radicals, resulting in increased phytoplasma growth. This observation is in line with the strong accumulation of anthocyanins and the higher phytoplasma titer found in Barbera. Our study showed that Nebbiolo leaves accumulate anthocyanins to a much lower extent than in Barbera (Fig. 3a), in agreement with our view of anthocyanin accumulation as a non-specific effect of FD-induced leaf sugar accumulation. Accordingly, this low anthocyanin concentration in Nebbiolo sustains a lower phytoplasma titer (Fig.2a).

Proanthocyanidins concentration was generally higher in infected Barbera leaves (Fig. 3b). An interesting hallmark of Nebbiolo was the higher basal concentration of proanthocyanidins in healthy and recovered plants (Fig. 3b). This high constitutive proanthocyanidin concentration in the leaves, could explain Nebbiolo's low susceptibility to FD, possibly due to the repelling effect of these substances against feeding insects, as demonstrated in other plants against sucking pests (Mansour *et al.* 1997; Rao 2002).

In agreement with the significant differences in the concentrations of proanthocyanidins in the two cultivars, we also found strong differences in the RQ of *LAR* transcripts, between Barbera and

Nebbiolo, and among the three sanitary states. We were able to fit the accumulation of PA with the transcription levels of biosynthetic genes, and despite the strong differences between the varieties, we found a single model correlating the three variables (Fig. 9).

Given the role of flavonoids as general modulators in stress responses, in particular hormone balance (Pourcel *et al*. 2013), our findings provide a better understanding of the cross-talk between different biochemical pathways and the chain reactions that can be induced by FDp infection, as it was noticed earlier in proteomic studies (Margaria & Palmano 2011; Margaria *et al.* 2013). In particular, the hormone jasmonic acid (JA) is known to be involved in regulating plant response to stress; down-regulation of genes involved in its biosynthesis was observed in FDp- infected grapevine leaves (Gambino *et al.* 2013). Interestingly, JA level was significantly reduced by flavonoid accumulation in Arabidopsis (Pourcel *et al.* 2013). The flavonoid trend we have observed in response to FDp infection did fit with these observations, suggesting that flavonoid increase could have an effect on JA accumulation in grapevines as well. Moreover, extending the analysis to other phenolic compounds other than flavonoids, would likely furnish new data on the complex reaction of grapevine to phytoplasma infection: in this view, stilbenoids would be a good candidate for future experimental works, given their documented role in other biotic interactions in grapevine (Malacarne *et al*. 2011; Mattivi *et al*. 2011; Toffolatti *et al*. 2012; Vannozzi *et al.* 2013).

In conclusion, the results presented here, besides providing a molecular and biochemical description of the flavonoid pathway in response to FDp infection in different grapevine cultivars, raise intriguing hypothesis on its involvement in phytoplasma pathogenesis and on the response of different grapevine genotypes to the disease. We tried our best to minimize the climatic and agronomic differences between the different sampling sites, but of course, as we decided to study the two cultivars in their original field-grown conditions, and due to the heterogeneity of vineyards, we cannot exclude other stress factors that may have influenced the flavonoid pathway in the two cultivars. However the similarity in light exposure, temperature, humidity and culture conditions between the two sampling vineyards, together with the general biological behaviour of the two

cultivars in terms of symptom reactivity to FDp infection and supported phytoplasma titre, made us confident that our speculations have a real confirmation in nature, and that the dramatic changes observed between the two cultivars cannot be exclusively attributed to a restricted environmental factor, but instead are a biological characteristic of the different grapevine genotypes in response to phytoplasma infection.

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Figure legends

Figure 1. Different symptoms of Flavescence dorée infection on Barbera (a,b) and Nebbiolo (d,e) leaves, and typical withering of grape berries (c).

Figure 2. Phytoplasma titer and total phenolic accumulation. a) Quantification of Flavescence dorée phytoplasma in infected Barbera and Nebbiolo grapevines, reported as phytoplasma cells/ng leaf DNA. b) Quantification of total phenolic concentration as equivalents of (+)-catechin hydrate in healthy, FDp-infected and recovered leaves of Barbera and Nebbiolo during the 2011 vegetative season.

Figure 3. Relative levels of: (a) total anthocyanin concentration (g kg-1 as equivalents of malvidin 3-O-glucoside chloride); (b) total proanthocyanidin concentration (g kg-1 as equivalents of (-)-epicatechin gallate); (c) total flavonol concentration (as equivalents of quercetin 3-O-glucoside), in the leaves of healthy, FDp-infected, and recovered plants of Barbera and Nebbiolo, at four sampling dates expressed as days of the year (x axes). Means (three replicates ± standard errors) were separated by the Duncan's test (capital letters indicate significant differences for $P \leq 0.01$; lower case letters for $P \leq 0.05$).

Figure 4. Expression of genes of the stem flavonoid pathway in healthy, Flavescence dorée phytoplasma- infected and recovered grapevine leaves, at four sampling dates expressed as days of the year (x axes). Relative quantities values ($RQ_{j,i} = 2^{\wedge} -\Delta\Delta Ct_{weighted\ j,i}$) were calculated for each sample using the mean Ct value of all the healthy samples as reference. *CHS*: chalcone synthase; *F3H*: flavanone-3-hydroxylase. Vertical bars represent standard errors.

Figure 5. Expression of genes of the anthocyanin branch in healthy, Flavescence dorée phytoplasma- infected and recovered grapevine leaves, at four sampling dates expressed as days of

the year (x axes). Relative quantities values ($RQ_{j,i} = 2^{\wedge} -\Delta\Delta Ct_{weighted\ j,i}$) were calculated for each sample using the mean CT value of all the healthy samples as reference. *LDOX*: leucoanthocyanidin dioxygenase; *UAGT*: UGT-glucose:anothocyanin 3-Oglucosyltransferase; *VvMYBA1*: *UAGT*-transcription factor. Vertical bars represent standard errors.

Figure 6. Expression of genes of the proanthocyanidin branch in healthy, Flavescence dorée phytoplasma- infected and recovered grapevine leaves, at four sampling dates expressed as days of the year (x axes). Relative quantities values ($RQ_{j,i} = 2^{\wedge} -\Delta\Delta Ct_{weighted\ j,i}$) were calculated for each sample using the mean CT value of all the healthy samples as reference. *ANR*: anthocyanidine reductase; *LAR*: leuco-anthocyanidine reductase. Vertical bars represent standard errors.

Figure 7. Expression of genes of the flavonol branch in healthy, Flavescence dorée phytoplasma-infected and recovered grapevine leaves, at four sampling dates expressed as days of the year (x axes). Relative quantities values ($RQ_{j,i} = 2^{\wedge} -\Delta\Delta Ct_{weighted\ j,i}$) were calculated for each sample using the mean CT value of all the healthy samples as reference. *FLS*: flavonol synthase; *VvMYBF1*: *FLS*-transcription factor. Vertical bars represent standard errors.

Figure 8. Relationship between Relative Quantity (RQ) of UGT-glucose:anothocyanin 3-Oglucosltransferase (*UAGT*) gene and g/kg of anthocyanins (Log scale) and their regression lines. Infected plants of both cultivars showed a relatioship between the anthocyanins concentration and transcript quantities, but the RQ in Barbera was significantly higher than in Nebbiolo and its effect on the anthocyanins concentration was almost double.

Figure 9. Representation of the relationship between proanthocyanidins (PA) accumulation and *LAR* and *ANR* relative quantities (RQ). (a) Fitted model representing the relationships of the concentration of proanthocyanidins [PA] with both the RQ of *LAR* and *ANR* transcripts for the two

cultivars together, without considering the sanitary states. (b) Distribution of the three sanitary states separately for Barbera and Nebbiolo. Clustering of the infected samples toward the high values of PA is evident in Barbera, in contrast to Nebbiolo, where the sanitary states are dispersed along the levels of PA.

SUPPORTING INFORMATIONS

Figure S1. Experimental design of the study. The picture shows the complete work-flow, from sampling in the field to biochemical and gene-expression analysis.

Figure S2. Climatic data obtained from meteorological stations for the two vineyards considered in the study. (a) Mean Temperatures. (b) Growing degree days base 10 °C. (c) Relative humidity. The red squares highlights the months considered for sampling.

Table S1. Reference source of the primers used in RealTime qRT-PCR reactions, for quantification of three reference genes, and ten candidate genes, together with GeneID, Genoscope gene accession, and primer amplification efficiencies in Barbera and Nebbiolo.

Table S2. Output values of the validation analysis of candidate reference genes, according to three software tools commonly used for reference gene selection: geNorm, Norm Finder, and Best Keeper.

Table S3. Quantification of total anthocyanins by spectrophotometer analysis (absorbance read at 520 nm), using malvidin 3-O-glucoside chloride as external standard reference. H: healthy sample; I: infected sample; R: recovered sample.

Table S4. Quantification of total proanthocyanidins as equivalents of (-)-epicatechin gallate by spectrophotometer analysis. H: healthy sample; I: infected sample; R: recovered sample.

Table S5. Quantification of flavonols by HPLC-DAD, in Barbera (A) and Nebbiolo (B) healthy, Flavescence dorée phytoplasma-infected and recovered leaves.

Means of three replicates ± standard errors; TF = total flavonol (g/kg), Myr mG (Myricetin 3-O-glucoside), Q glr (quercetin 3-O-glucuronide), Q mG (quercetin 3-O-glucoside), K glr (kaempferol 3-O-glucuronide), K mG (kaempferol 3-O-glucoside). M = percentage of myricetin 3-O-gluoside over total flavonols; Q = percentage of quercetins over total flavonols; K = percentage of kaempferols over total flavonols.

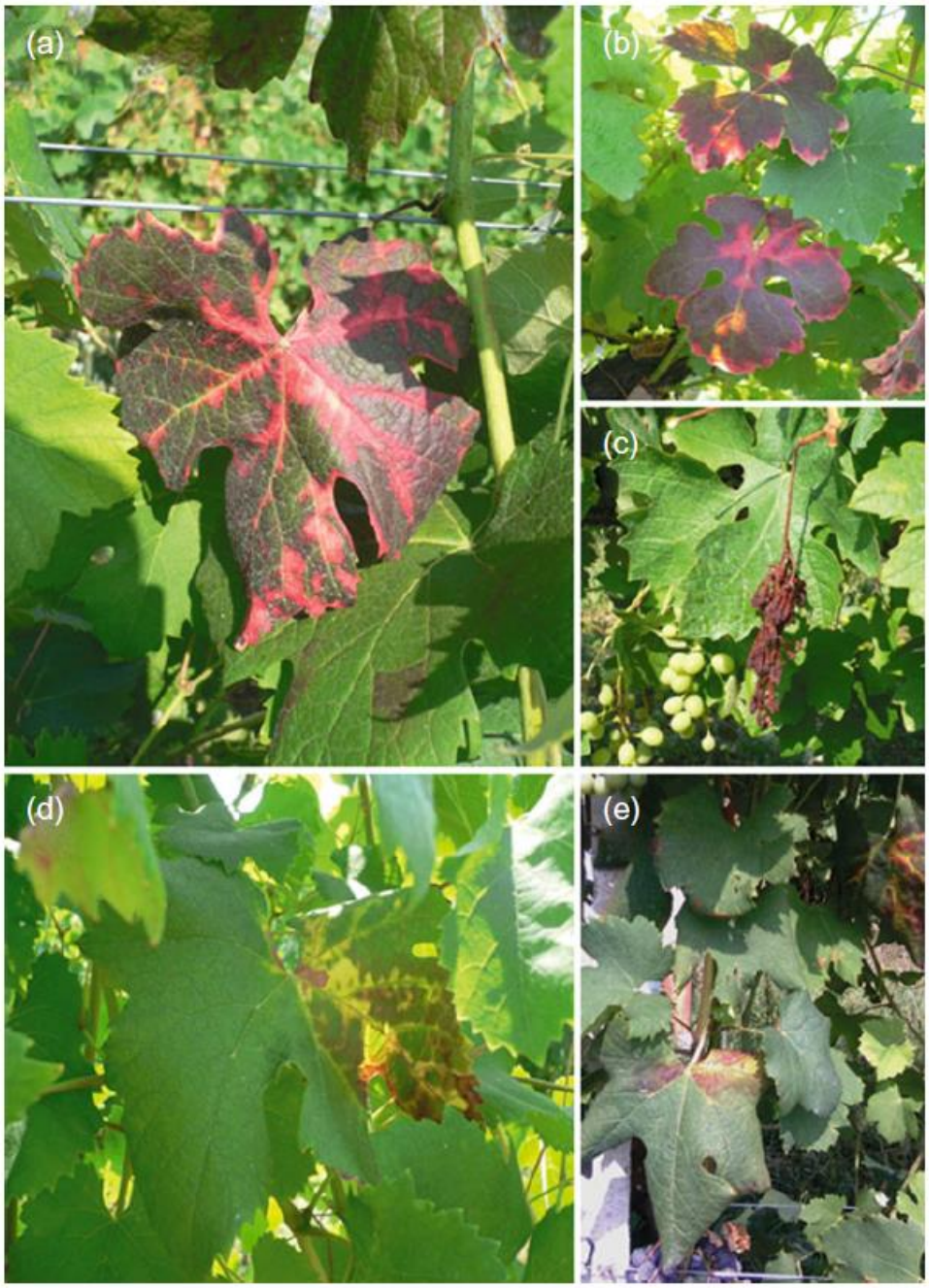


Figure 1

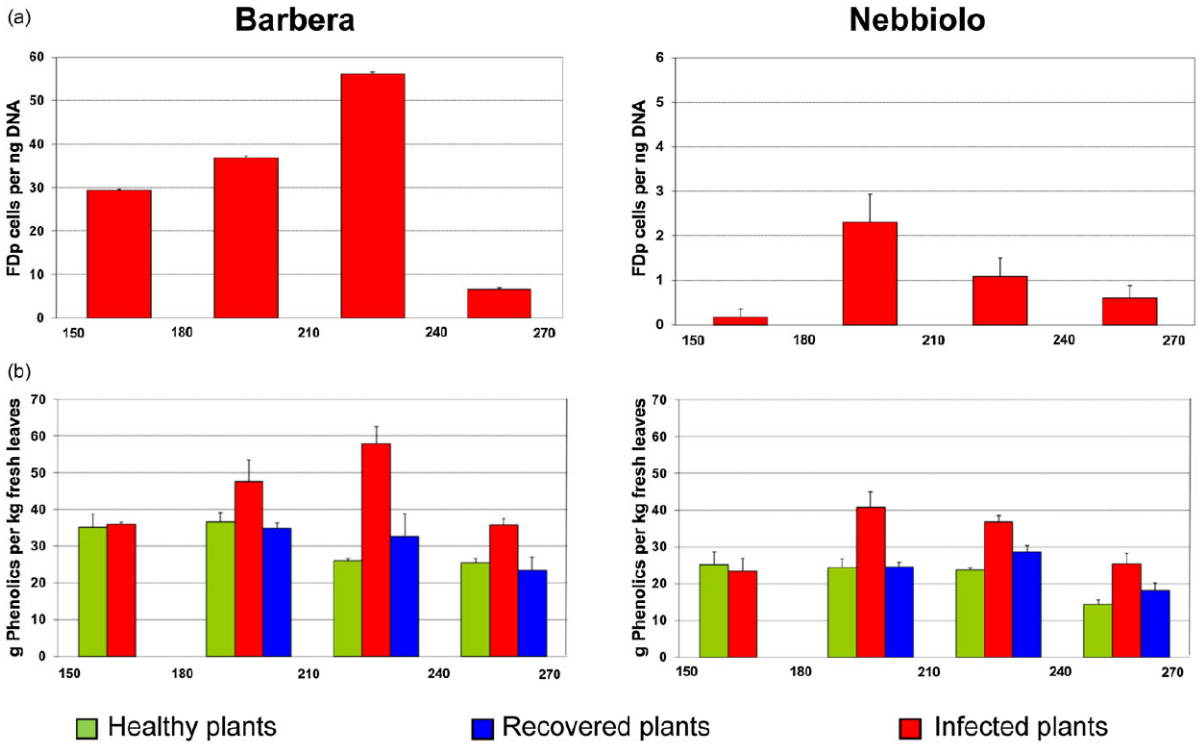

(a)
Barbera
Nebbiolo
FDp cells per ng DNA
(b)
g Phenolics per kg fresh leaves
Healthy plants
Recovered plants
Infected plants

Figure 2

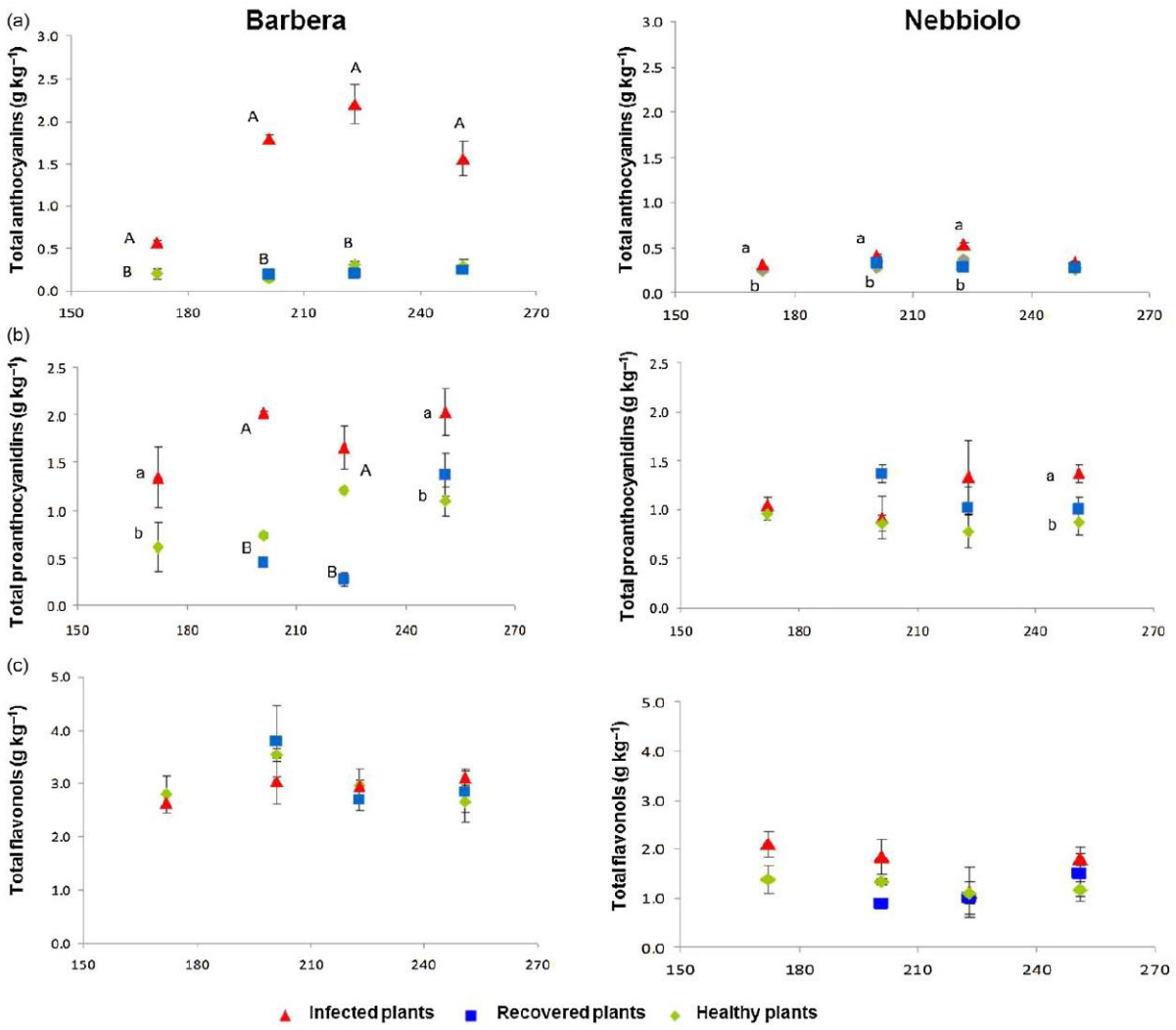

(a)
Barbera
Nebbiolo
Total anthocyanins (g kg⁻¹)
(b)
Total proanthocyanidins (g kg⁻¹)
(c)
Total flavonols (g kg⁻¹)
Infected plants
Recovered plants
Healthy plants

Figure 3

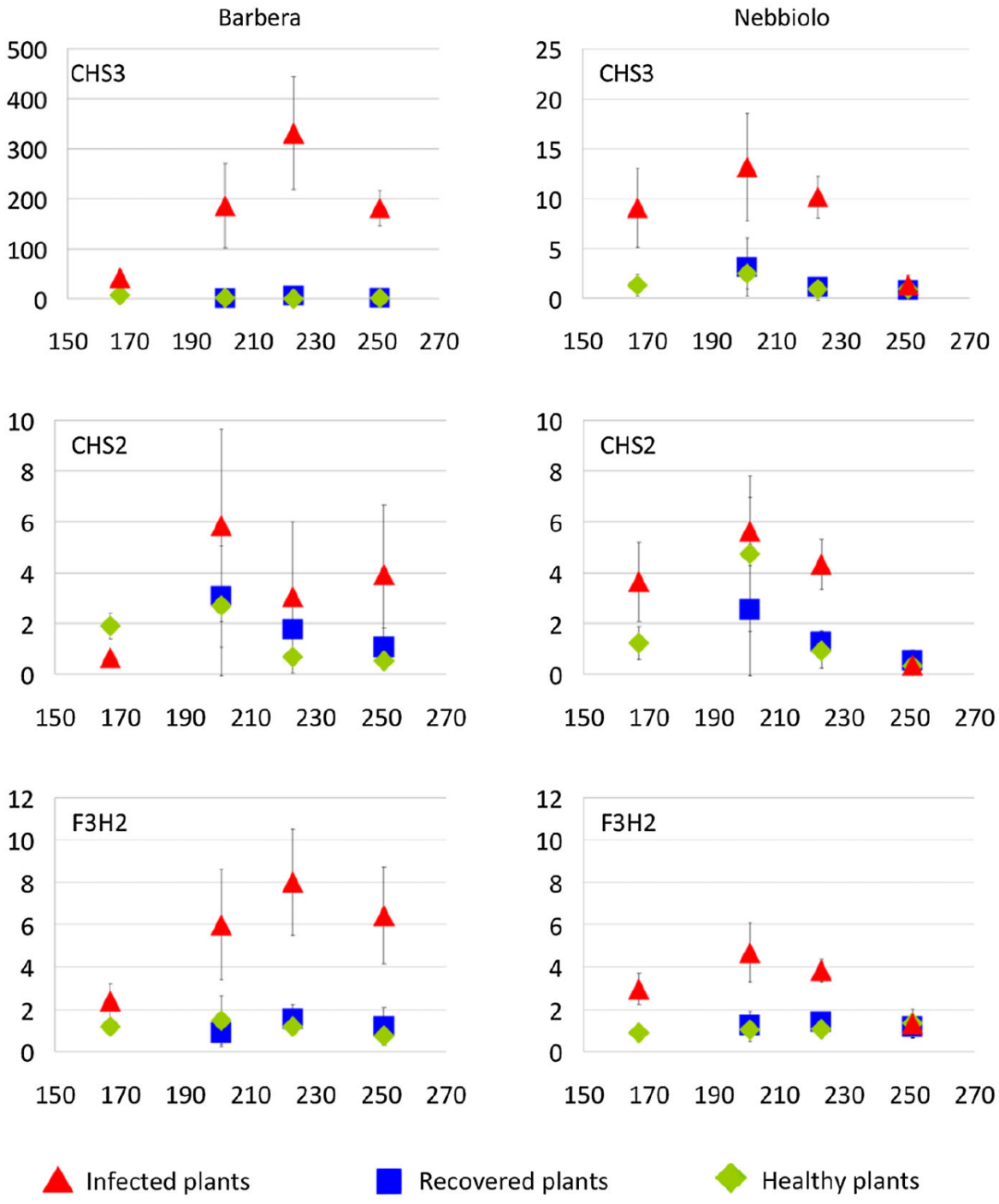

Barbera
Nebbiolo
CHS3
CHS2
F3H2
Infected plants
Recovered plants
Healthy plants

Figure 4

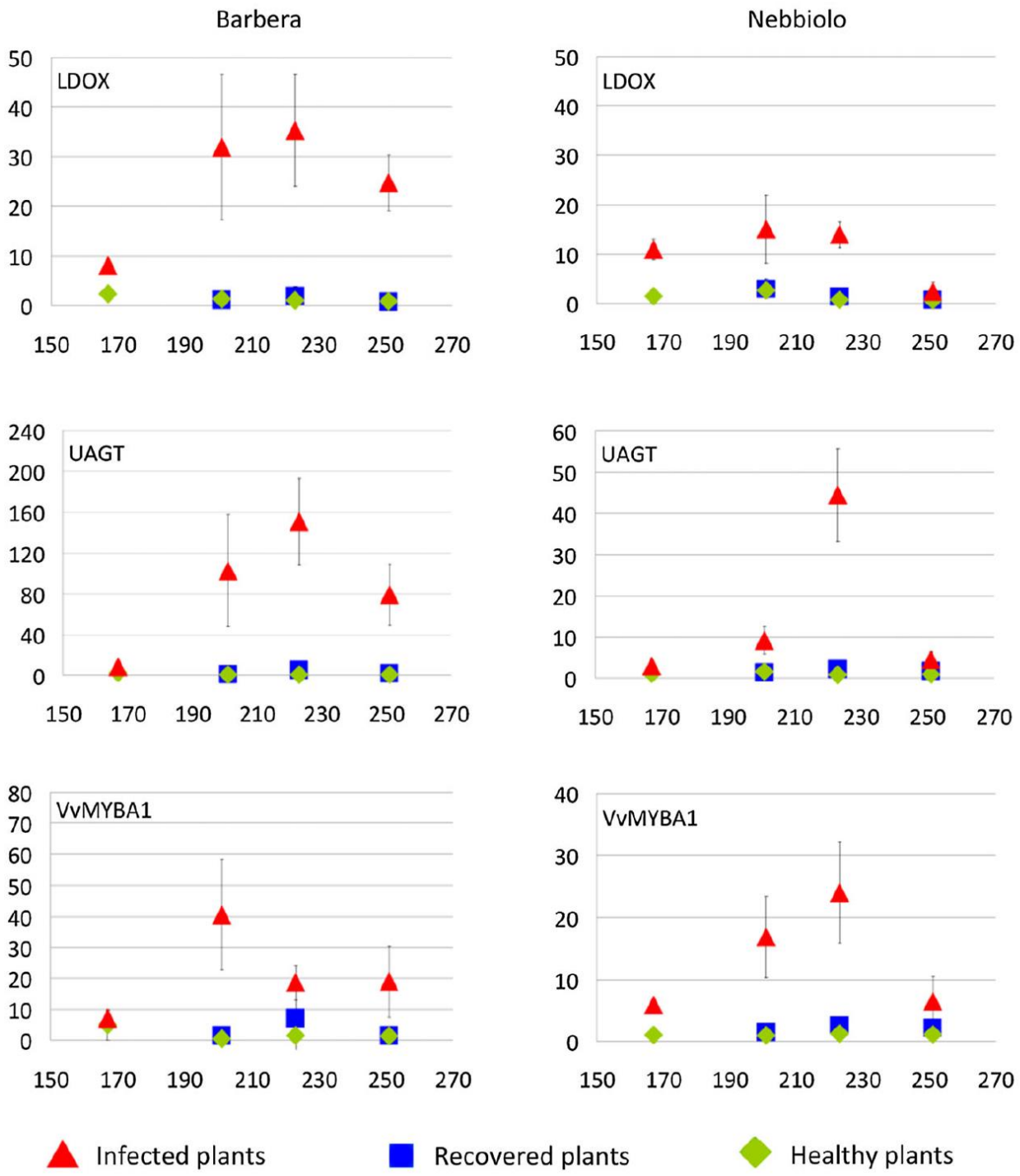

Barbera
Nebbiolo
LDOX
UAGT
VvMYBA1
Infected plants
Recovered plants
Healthy plants

Figure 5

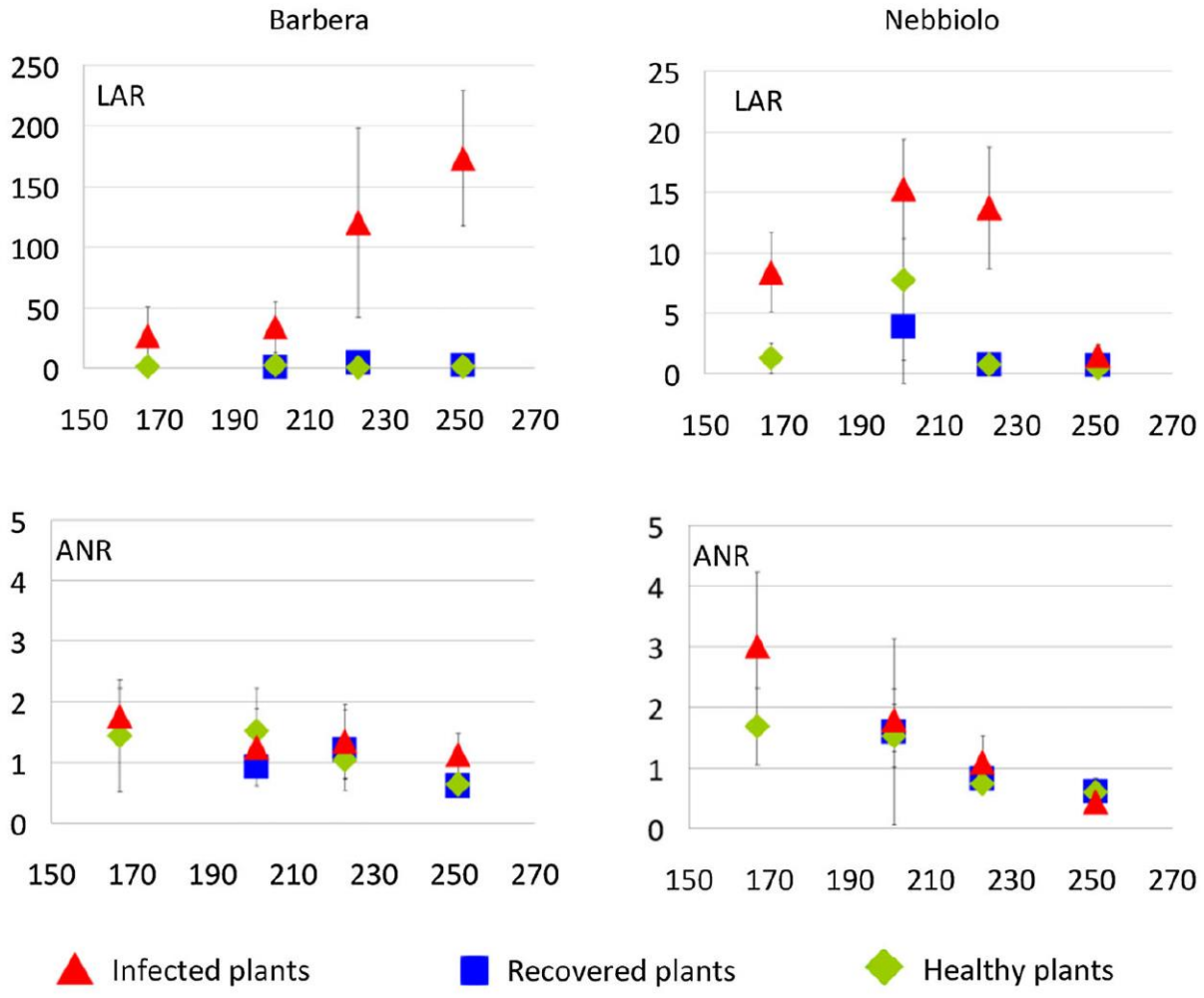

Barbera
Nebbiolo
LAR
LAR
ANR
ANR
250
200
150
100
50
0
25
20
15
10
5
0
5
4
3
2
1
0
150 170 190 210 230 250 270
Infected plants
Recovered plants
Healthy plants

Figure 6

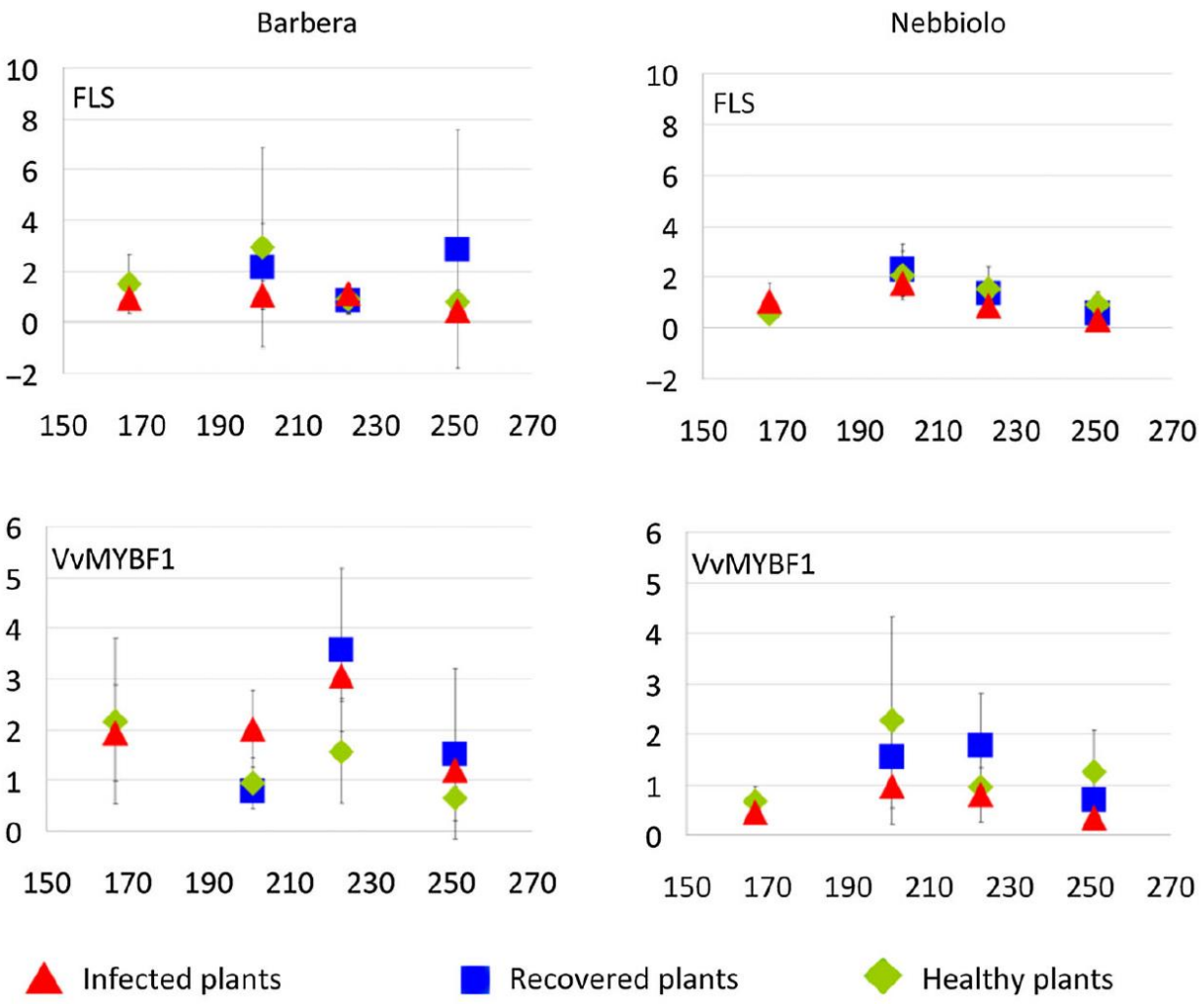
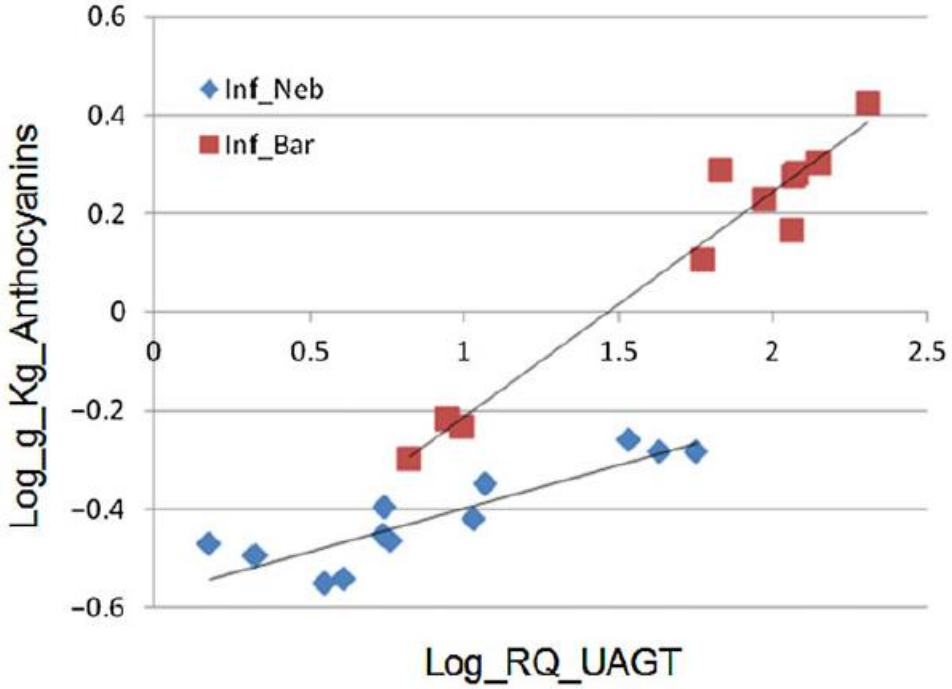

Barbera
Nebbiolo
FLS
FLS
VvMYBF1
VvMYBF1
10
8
6
4
2
0
−2
6
5
4
3
2
1
0
150
170
190
210
230
250
270
Infected plants
Recovered plants
Healthy plants

Figure 7


0.6
0.4
0.2
0
−0.2
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0
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1
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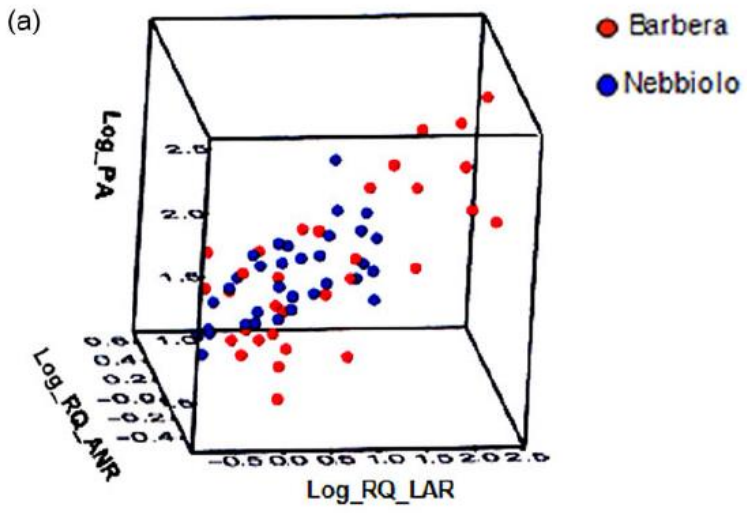
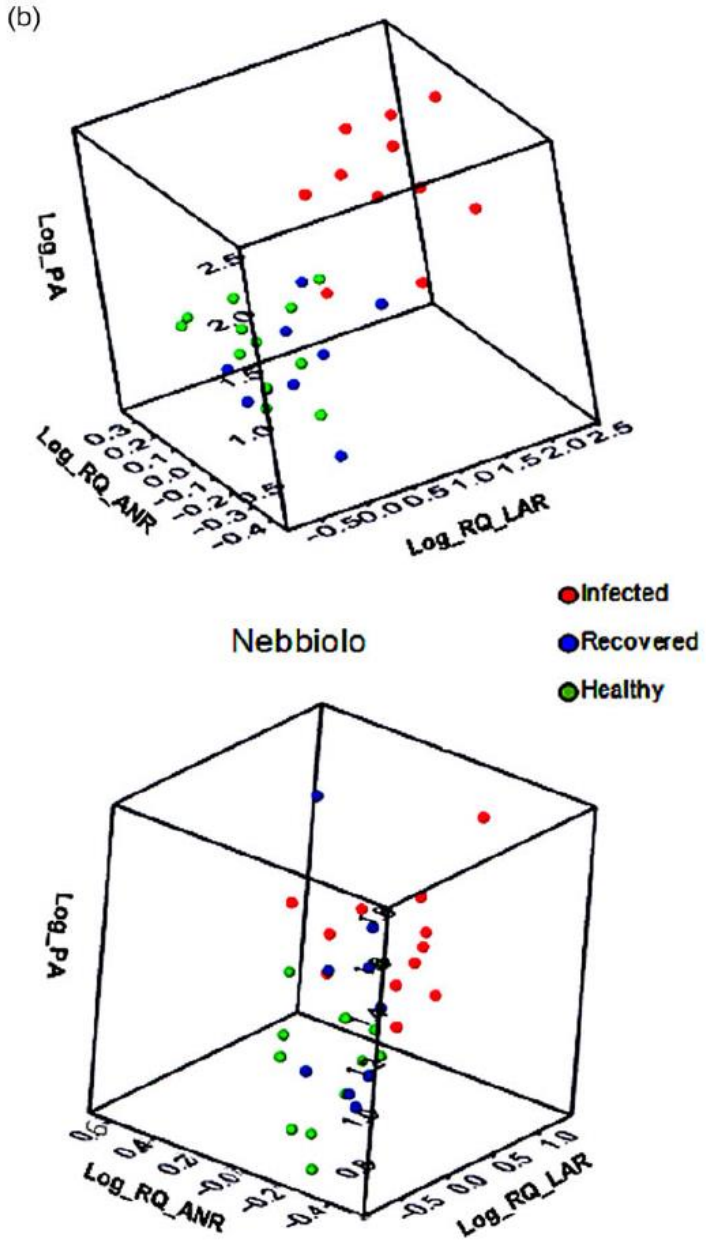
2
2.5
Inf_Neb
Inf_Bar
Log_g_Kg_Anthocyanins
Log_RQ_UAGT

Figure 8



Barbera



Nebbiolo

1053

Figure 9