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Physiological response of 'Italia' grapevine to some "Esca complex"-associated fungi

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Abstract

This study investigated some physiological features in a 20-year-old 'Italia' vineyard cropped in Apulia, Italy. Five vines with brown-wood-streaking associated to Phaeoacremonium minimum (sin. Pm. aleophilum) and Phaeomoniella chlamydospora (BWSV), five with brown-wood-streaking and white-rot caused by Fomitiporia mediterranea (BWSWRV) and five healthy vines (HV) were surveyed. Bleeding xylem sap (BXS) collected at bud-break, symptomless and symptomatic leaves taken during stretched-out leaves, fruit setting, cluster closing and bunch ripening phenological phases were characterized. BXS from HV showed the highest total ascorbic acid level, while BWSWRV had the highest viscosity coefficient, glutathione concentration and growth regulators activity. Low fresh and dry weight, total chlorophyll concentration and the hight leaf surface, hydrogen peroxide and cell membranes damage were detected in leaves of diseased vines. Symptomless and symptomatic leaves of BWSV and BWSWRV exhibited low concentrations of ascorbic acid, reduced glutathione and redox state; moderate levels of dehydroascorbic acid and oxidized glutathione. Higher dehydroascorbate reductase and low ascorbate free radical reductase and glutathione reductase activities were showed by leaves collected from diseased vines. However, no differences were detected in ascorbate peroxidase activity. The decrease of oxidative status alters cell membranes integrity and could contribute to cell death and symptoms development on leaves.

1 INTRODUCTION

"Esca" is one of the oldest diseases of grapevine (*Vitis vinifera* L.) known wherever grapes are grown, characterised by wood decay, "apoplexy" (sudden wilting of the vine) and leaf "tiger-stripes" (Mugnai, Graniti & Surico, 1999; Graniti, Surico & Mugnai, 2000; Surico, 2009). The most recent proposal considers "Esca" as a complex of different diseases overlapping in the same vine or developing the vine life. Following this tendency, the old "Esca" is a combination of five diseases: brown wood streaking, Petri disease, grapevine leaf stripe disease, white rot and Esca proper. Brown wood streaking affects vines in the nursery. Petri disease interferes with new vine plantations and coupled vascular discolouration with chlorosis and decline. The grapevine leaf stripe diseases involve on young and adult vines, with wood streaking or/and wood decay. Esca proper is referred to adult vines with the simultaneous presence of white rot, dark wood streaking and leaf "tiger-stripes" (Graniti, Surico & Mugnai, 2000; Surico, 2009; Mondello et al., 2018). Different fungi are involved in these diseases. Species of *Fomitiporia* (*F.mediterranea* M. Fisch. mainly in Europe) are associated with wood decay of white rot and Esca proper. *Phaeoacremonium minimum* (Tul. & C. Tul.) Gramaje, L. Mostert & Crous ([?] *Pm. aleophilum* W. Gams, P.W. Crous, M.J. Wingf. & L. Mugnai) and the anamorphic*Phaeomoniella chlamydospora* (W. Gams, P.W. Crous, M.J. Wingf. & L. Mugnai) P.W. Crous & W. Gams, are the etiological agents of brown wood streaking and Petri disease (Mugnai, Graniti & Surico,1999; Surico, 2009; Mondello et al., 2018). Other *Phaeoacremonium* and*Cadophora* species have been isolated from wood of grapevines affected by "Esca complex" diseases (Surico, 2009; Moreno-Sanz et al., 2013; Mondello et al., 2018; Elena et al., 2018; Jayawardena et al., 2018).

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The leaf stripe symptoms are considered as the result of cultivar susceptibility, age of the vines, microorganisms involved and pedoclimatic conditions (Graniti, Surico & Mugnai, 2000). The substances originate in the discoloured woody tissues of the trunk and branches, moved to the leaves in the transpiration stream and are associated to leaf "tiger-stripes" development. These substances can be reaction products of the wood, phytotoxic metabolites excreted by "Esca complex" fungi, or their combination (Bruno, Sparapano & Graniti, 2007). *Pa. chlamydospora* and *Pm. minimum* produce two naphthalenone pentaketides (scytalone and isosclerone) and exopolysaccharides including the α-glucan named pullulan (Evidente et al., 2000; Tabacchi et al., 2000; Bruno & Sparapano 2006a, 2006b, 2007). Macro and micronutrient have a role in "Esca complex" symptom progression (Calzarano et al., 2006).

Stomata closure and change of the photosynthetic apparatuses were associated with grapevine leaf stripe occurrence (Petit et al., 2006; Magnin-Robert, 2011). The presence of less dense cytoplasm, plastids with small starch grains, underdeveloped grana and elongated thylakoids describe grapevine leaves before developing "tiger-stripes" (Lima et al., 2010; Fontaine et al., 2016). Glutathione pool, PR-proteins and phenolic compounds are also affected (Calzarano et al., 2006; Magnin-Robert, 2011; Lima et al., 2010; Fontaine et al., 2016; Valtaud et al., 2011; Lambert, 2013). Xylem dysfunction also influenced water transport and leaf water potential (Bruno, Sparapano & Graniti, 2007; Fontaine et al., 2016). NMR data suggests an increment of phenylpropanoid compounds production and a decrement of glucose and fructose content in "Esca" diseased vines (Lima et al., 2010).

In the present paper, the goal is an increase of knowledge on the variations of some physiological features of bleeding xylem sap and leaves of grapevine cv Italia. However, this study gives a great deal of attention to the differences in hydrogen peroxide, lipid peroxidation and antioxidant defence responses associated with the ascorbate-glutathione cycle between health and diseased vines.

2. MATERIALS AND METHODS

2.1 Plant material

A 20-year-old *V. vinifera* cv. Italia vineyard (1,600 vines) located in the countryside of Bari (Apulia, southern Italy), was used for symptoms survey and sample collection. The vines, grafted onto 140-Ru, were trained by the overhead system ('tendone') and grown under irrigation on alkaline-clayey soil. Since 2006, the vineyard was surveyed for incidence and development of "Esca complex" symptoms.

Wood cores were taken with a 95% ethanol pre-killed Pressler increment borer from the trunk at 30 and 110 cm above the ground, to assess internal infections. Holes were disinfected with copper oxychloride solution (20% in water) and filled with a mixture of 2.5 % copper oxychloride in linseed oil. For each core, slices of wood were cut, surface sterilised for 1 min in 70% ethanol, soaked 1-2 min in a sodium hypochlorite solution (3% active chlorine) and rinsed thrice in sterile distilled water. The pieces were then axenically cut to chips and seeded (five per plate) on 90-mm diameter Petri dish. Malt extract (2%) agar (MEA), MEA amended with 0.25% chloramphenicol (MEAC) and MEA adjusted with 0.25% benomyl (MEAB) were used. Isolation plates were incubated at 25±1°C, in the dark, for 4-6 weeks. The isolation frequency (IF) of each fungal taxon was calculated as $IF = N_i/N_t \times 100$, where N_i is the number of wood-fragments from which the fungus was isolated and N_t the total number of seeded chips.

Fifteen vines were selected: five with brown wood-streaking (BWSV), five with brown wood-streaking and white rot (BWSWRV) and five healthy (HV) without internal and external symptoms.

2.2 Bleeding xylem sap sampling and characterization

Bleeding xylem sap (BXS) was collected at bud-break phenological phase (Baggiolini, 1979) from the 15 selected vines. The end of each spur was surface-treated with a diluted solution of sodium hypochlorite (3% active chlorine), then with 95% ethanol and finally rinsed twice with sterile distilled water. The sap exuded for the first 15 min was discarded. A sterile plastic bottle covered with aluminium foil was secured to the end of each bleeding spur to collect the liquid over the following four days. After harvesting, the sap collected

from each vine was kept in an ice bag and transported to the laboratory, where it was filtered on 0.45 µm Millipore membranes (Millipore, Bedford, MA, USA).

Dynamic viscosity (η_x) of each BXS was calculated as $\eta_x=[(\rho_x \times t_x)/(\rho_w \times t_w)] \times \eta_w$ where ρ_x = sap density, ρ_w = water density, η_w = water dynamic viscosity (0.8937×10^{-3} Poiseuille), t_x = flow time of sap, t_w = water flow time. Measurements were carried at 25±0.1 °C using an Ostwald glass capillary viscometer (Cannon-Fenske Instruments, State College, PA, USA). For each BXS sample, 10 measurements were recorded.

About 2 ml of freshly collected BXS were lyophilized and the resulting powder was treated with 5% metaphosphoric acid (6 ml). After centrifugation (20,000 $\times g$, 15 min, 4°C), total ascorbate (T-ASC) and total glutathione (T-GSH) concentrations were measured as described by Zhang & Kirkham (1996).

The auxin and kinetin content of xylem sap was evaluated with the filter paper disk method (Zhao et al, 1992) using the excised cucumber (Cucumis sativus L.) cotyledon root formation (auxin) and the cucumber cotyledon expansion (kinetin) bioassays. Indole-3-acetic acid and 6-furfurylaminopurine were dissolved in 95% ethanol and tested in the range 0.3-50.0 µg ml^{-1}; 95% ethanol was performed as a control.

2.3 Leaves sampling and characterization

Leaves (10 per vine) were randomly picked (as replications) from the 15 selected vines at stretched-out leaves (SL), fruit setting (FS), cluster closing (CC) and bunch ripening (BR) phenological phases (Baggiolini, 1979). At CC and BR collections, symptomless and symptomatic leaves were taken from diseased vines.

Leaves were transported to the laboratory in an icebox, deprived of the petiole, photographed and subjected to morphological and physiological characterization.

Each leaf was weight with a Sartorius BP 210S analytical balance (Data Weighing Systems, Inc., Wood Dale, IL, USA) to assess fresh weight (Lfw).

Leaf size was measured with ImageJ (National Institutes of Health, MD, USA) an open-source image-processing program.

Leaf dry weight (Ldw) was estimated by drying 100 mg of leaves for 20 min at 105 °C with the infrared Mettler LP 16-M desiccator (Mettler-Toledo SpA., Milan, I). Leaf moisture (Lm) was calculated on a wet-weight basis.

Chlorophyll was determinate as described by Harborne (1973) using about 2 g of leaf samples and 80% acetone (16 ml) in ice bath. Total chlorophyll concentration (mg g^{-1} Lfw) was calculated as: $[((20.2 \times A_{645}) + (8.02 \times A_{663})) / (100 \times W)] \times V$, where A_{645} and A_{663} = absorbance at 645 and 663 nm respectively, V = volume (ml), W = Lfw (g).

Hydrogen peroxide concentration was determined as previously described (Lee & Lee 2000) using 1 g of leaf lamina ground with 4 ml of sodium phosphate buffer (0.1 M; pH 6.5).

Lipid peroxidation was estimated as malondialdehyde (MDA) on 200 mg of the lamina (Heath & Packer, 1968). The results were expressed as nmol MDA per gram of Lfw.

The levels of ascorbic acid (AsA), dehydroascorbic acid (DHA), reduced (GSH) and oxidized (GSSG) glutathione were quantified on 2 g of leaf lamina ground, at 4°C, in 5% metaphosphoric acid (6 ml). After centrifugation (20,000 $\times g$, 15 min, 4°C) the supernatant was used as proposed by Zhang & Kirkham (1996).

The ascorbate (A-RS) and glutathione (G-RS) redox state were calculated as A-RS = [AsA / (AsA + DHA)] and G-RS = [GSH / (GSH + GSSG)].

For enzymes involved in ascorbate regeneration, leaf lamina (2 g) was homogenised in 6 ml of extraction buffer (50 mM Tris-HCl pH 7.8, 0.3 mM mannitol, 10 mM $MgCl_2$, 1 mM EDTA, 0.05% cysteine) at 4°C. The homogenate was centrifuged (25,000 $\times g$, 15 min, 4°C). The supernatant was dialysed against 50 mM Tris-HCl (pH 7.8) and used for ascorbate peroxidase (EC 1.11.1.11, APX), dehydroascorbate reductase (EC 1.8.5.1, DHA-R), glutathione reductase (EC 1.6.4.2, G-R) and ascorbate free radical reductase ([?]

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monodehydroascorbate reductase, EC 1.6.5.4, AFR-R) activities determination according to Paciolla et al. (2008).

2.4 Statistical analysis

Data were subjected to general linear analysis of variance models using the SAS/STAT version 9.0 (SAS Institute Inc., Cary, NC, USA). Normal distribution and homoscedasticity were tested using the Shapiro-Wilk and Bartlett's tests, respectively. Pair-wise comparisons of means were performed according to the Tukey test at *P* [?]0.05. Data of leaves morphological and physiological features were analysed for vine typology (BWSV, BWSWRV and HV), phenological phases (SL, FS, CC and BR), symptoms (presence, absence) and their interactions.

3. Results

3.1 Plant material

The cores allowed assessing the presence of brown wood-streaking and rotted wood in the trunk. The fungal taxa listed in table 1 were isolated. In particular, from woody tissue with brown wood-streaking were always isolated *Pa. chlamydospora* and *Pm. minimum* , while *F. mediterranea* was associated only with rotted wood. Other micromycetes, including *Penicillium* spp., *Alternaria* spp., sterile fungi and few no identified species, were also detected from diseased vines. Species of *Penicillium* and *Alternaria* were 'contaminant' from cores of HV.

Symptoms were recorded on leaves and berries (Fig. 1) of diseased vines. Leaf symptoms start as light green or chlorotic, irregular areas between the main veins or along the leaf margin. The chlorotic areas gradually expand and coalesce, becoming in part necrotic. At the CC and BR phenological phases, the typical "tiger-stripes" patterns were noted on leaves of BWSV and with BWSWRV. Berries symptoms include minute dark brown, violet or purple spots on the skin, shrivelling and sometimes wilt (Fig. 1C-E). On plants infected even with *F. mediterranea*cracking of trunk (Fig. 1F) were recorded. Healthy vines, used as a control, show neither foliar symptoms nor any sign that they might have wood or berries alterations. No apoplexy was recorded on selected vines during the survey.

3.2 Xylem sap

As summarized in table 2, during the bleeding period, HV discharged the lower quantity of BXS.

The dynamic viscosity coefficient of the BXS collected from HV was lower than the values of the samples spurted from diseased vines. Among diseased vines, the presence of white rot increases dynamic viscosity (Table 2).

The highest concentration of T-ASC was recorded in sap bleed from HV. The lowest was in BWSV (Table 2). BXS collected from HV had the lowest quantity of T-GLU, whereas the highest was in BWSWRV (Table 2).

The auxin-like activity was detected in all the tested BXS samples. Sap collected from diseased vines showed auxin activity 2-3 times greater than HV (Table 2). The lowest kinetin activity was recorded on BXS bleed from HV (Table 2).

3.3 Leaves

A selection of leaves harvested from the 15 selected vines is given in figure 1G. Morphological and physiological features were strongly affected by phenological phases, fungal presence, symptoms development and their interactions (Table 3).

Fresh and dry weight, moisture content and surfaces of leaves collected during the four phenological phases are presented in figure 2.

During the collections, Lfw (Fig 2A) increase from HV and BWSWRV symptomless leaves. Symptomatic leaves showed always the lowest Lfw.

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The highest Ldw (Fig 2B) was recorded from symptomatic leaves collected from BWSWRV at BR phenological phase.

No significant differences were verified on the moisture content of the leaves that range from 89.53 to 94.56% of Lfw.

No differences were shown on leaf surfaces (Fig. 2C) at SL phenological phases, while leaves of HV were significantly smaller than those of diseased plants during the other phenological phases.

Chlorophyll, H_2O_2 and MDA concentrations in leaves collected during the four phenological phases are given in figure 3. During the SL and FS phases, no significant differences were recorded on chlorophyll content (Fig. 3A) among diseased and HV. At CC and BR phenological phases, diseased plants reduce the chlorophyll concentrations. Symptomatic leaves collected from BWSV and BWSWRV showed the lowest chlorophyll content.

During the four phenological phases, HV vines showed the lowest H_2O_2 concentrations (Fig. 3B).

Diseased plants increased MDA level (Fig. 3C) in leaves. At CC and BR phases, diseased plants presented a sharp rise in lipid peroxidation level. A further increment in MDA levels also occurred in symptomatic leaves (Fig. 3C).

The ranges of AsA, DHA, GSH and GSSG on leaves collected during the four phenological phases are reported in figure 4.

During the four phenological phases (Fig. 4A), AsA level in leaves collected from HV was similar and was always higher to those found in leaves from diseased vines.

The DHA concentrations were related to vine phenology and fungal presence (Fig. 4B, Tab. 3). At SL and FS phases, leaves collected from BWSWRV showed the highest concentrations of DHA. During CC and BR, symptomless leaves from diseased vines reached the highest DHA levels (Fig. 4B). The content of GSH on leaves collected from HV was higher than diseased plants (Fig. 4C). The GSSG levels on leaves collected from BWSV were lower than HV (Fig. 4D). The GSSG levels on leaves collected from BWSWRV showed the highest concentration at SL, then decrease and reached the lowest-level at BR.

A-RS and G-RS (Tab. 4) decrease in the leaves collected from diseased vines. The lowest redox states were recorded in all symptomatic leaves.

The activities of enzymes involved in ascorbate regeneration are shown in Figure 5. No significant changes were noted in APX activity (Fig. 5A) during the four phenological phases and between healthy and diseased vines. DHA-R activity increase in symptomatic leaves collected from BWSWRV at CC and BR phases (Fig. 5B). AFR-R (Fig. 5C) and G-R (Fig. 5D) activities were lower in leaves collected from diseased vines.

4. Discussion

In this study, from a 20-year-old *V. vinifera* cv. Italia plants surveyed for "Esca complex of diseases" symptoms development, we select five vines with brown wood-streaking, five with brown wood-streaking and white rot and five healthy vines. The isolation procedure applied on wood cores taken with a Pressler increment borer, associate *Pm. minimum* and *Pa. chlamydospora* to brown wood-streaking and *F. mediterranea* to rotted white tissues. Species of *Penicillium* and *Alternaria* but also*Aureobasidium* , *Chaetomium* , *Cladosporium* ,*Epicoccum* , *Fusarium* , *Gibberella* , *Nectria* ,*Phoma* , *Paraconiothyrium* and *Trichoderma* have been reported as endophytic mycota associated with vines as secondary invaders or saprophytes (Moreno-Sanz, et al., 2013; Jayawardena, et al., 2018; Elena et al., 2018).

No alterations were viewed on HV, while symptoms were recorded on leaves and berries of *Pm. minimum* and *Pa. chlamydospora* or*Pm. minimum* , *Pa. chlamydospora* and *F.mediterranea* infected plants. BWSV and BWSWRV display the so-called 'tiger stripes' pattern on leaves and spots, shrivelling and wilt of berries. These observations agree with other Authors on this cultivar (Bruno & Sparapano, 2006b, 2007) and in

general for vines affected by “Esca complex” (Mugnai, Graniti & Surico,1999; Graniti, Surico & Mugnai, 2000; Surico, 2009; Mondello et al., 2018).

The selected vines allow us to collect xylem sap during the bleeding period and leaves at the phenological phases of stretched-out leaves, fruit setting, cluster closing and bunch ripening. Bleeding is a physiological process that characterizes vine and many perennial plants as an effect of positive root pressure that upward transport water and solute. Bleeding occurs in the springtime since soil temperature stimulates root growth and leads xylem vessels, dissolves and pushes out air bubbles formed during the winter and restores xylem works (Sperry, Holbrook & Zimmermann, 1987). During vascular diseases, permanent xylem blockage is the result of both the fungal presence (conidia, hyphae and high molecular weight substances secreted by the pathogen) and development of tyloses and gummosis as plant-barriers that limiting the fungal invasion. To bypass obstructions, plants respond with a reduction of leaf potential. Polyphenol-rich zones formation, the accumulation of pathogenesis-related-proteins and the activation of oxidative burst contribute to inhibit pathogens progression in the wood. *Pm. minimum* and *Pa. chlamydospora* phaeo-tracheomycosis are characterized by streaking and necrosis of varying extent in the wood of the trunk and principal branches of affected vines. The presence of*F. mediterranea* results in lignin degradation, the occurrence of areas consists mainly of cellulose and suggests increases xylem dysfunction. In these conditions, bleeding sap collected was reduced. In agreement with previous papers, diseased vines bleed more abundantly than HV (Bruno & Sparapano, 2006b; Bruno, Sparapano & Graniti, 2007).

Analysis of the bleeding sap provides useful perceptions of internal physiological functions. *V. vinifera* bleeding xylem sap composition included oxalic, tartaric, malic, glutamic and succinic acids, chlorides, sulphates, nitrites, nitrates, silicates and phosphates of sodium, potassium, calcium, iron, magnesium, manganese and aluminium, diastase, peroxidase and catalase (Wormall, 1924). Plant hormones (Skene, 1967), phenols, sucrose, glucose, fructose and, on vines infected by *Pa. chlamydospora* and *Pm. minimum* , isosclerone, scytalone and pullulans (Bruno & Sparapano, 2006b, 2007) were also present.

In this study, BXS was characterized by total ascorbate and glutathione concentrations, dynamic viscosity coefficients and growth regulators activity.

Viscosity is the ability of a fluid layer to run with the adjacent one. Our study reports an increment of the dynamic viscosity coefficient from HV to BWSV or BWSWRV. These results suggest that substances produced by the fungal ”endophytes” as well as molecules resulting from cell-components degradation by the lytic enzymes formed by these pathogens and vine-response molecules (phenols, tannins, flavonoids, and other) could influence dynamic viscosity coefficients, and as consequence xylem flow and potential.

The filter paper disk bioassay (Zhao et al, 1992) correlates the concentrations of growth regulators substances to their physiological effects on cucumber cotyledon root formation (auxin) and fresh weight variations (kinetin). The presences of several plant hormones in the xylem sap have been displayed on herbaceous and woody plants including grapevine (Niim & Torikata, 1978). In our research, the higher auxin and kinetin like activity was detected on diseased vines. Auxin activity increases when vines show white-rot associate to *F.mediterranea* .

Plant disease is the result of a constant chemical cross-talk among pathogen actions and plant defence reactions. Pathogens interfere on the plant with their pathogenicity or virulence tools, including chemical weapons (i.e. enzymes, phytotoxins, polysaccharides, plant-growth-regulators). These substances interfere with the different physiological function(s), cell organelles and molecules of the plant and lead to developing morphological changes. Plants react to pathogens penetration and spread with different weapons. “Esca complex”, and each of the diseases Esca-associated, can be read in this contest.*F. mediterranea* , *Pm. minimum* and *Pa. chlamydospora* , closely associated to wood tissues of “Esca complex” affected grapevines, produce phytotoxic metabolites (Evidente et al., 2000; Tabacchi et al., 2000; Bruno & Sparapano, 2006a, 2006b; Andolfi et al., 2011; Luini et al., 2010) and enzymes (Mugnai, Graniti & Surico, 1999; Andolfi et al., 2011; Chiarappa, 1959; Marchi et al., 2001; Bruno & Sparapano, 2006c). Vines defence responses included physical responses or metabolic changes, such as the formation of tyloses and gels in xylem (Yadeta &

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Thomma, 2013), cell wall chemical modifications with suberin deposition (Pouzoulet et al., 2013), production and accumulation of peroxidases, superoxide dismutases, glutathione S-transferases, phenolic compounds, stilbenes, and phytoalexins (del Rio et al., 2004).

Here, differences were recorded on leaves about fresh and dry weight, leaf surfaces and chlorophyll concentrations. These features varied with the phenological phases but are significantly affected by the behaviour of the pathogens inside the woody tissues and, as a consequence, of physiological function(s) altered. Symptomatic leaves always showed the lower fresh and dry weight and total chlorophyll concentration. Diseased plants show a general decline of photosynthetic pigments even with the absence of visible changes. As reported for Cabernet Sauvignon and Merlot (Christen et al., 2007), our data suggest that physiological dysfunctions related to photosynthesis are presents. A decrease of gas exchange and chlorophyll fluorescence and the repression of photosynthesis-related genes were registered on pre-symptomatic leaves of esca affected vines (Magnin-Robert et al., 2011). Chlorophyll decline leads to a decrease in photosynthesis efficiency, organic carbon production, growth and, in general, plant health. Symptomatic leaves show a further reduction on fresh and dry weight associate to lamina necrosis and wilt. Leaf chlorosis, necrosis and wilting are due to water stress caused by vascular occlusion, but also to toxic metabolites produced by pathogens (Van Alfen, 1989; Pennisi & Graniti, 1987). The main aetiological agents of "Esca complex" produce phytotoxic metabolites involved, in vitro and in planta, with symptoms development on leaves (Evidente et al., 2000; Tabacchi et al., 2000; Bruno & Sparapano, 2006a, 2006b; Luini et al., 2010; Andolfi et al., 2011). Chlorophyll decline could also explain leaves weight decrement because of low photosynthesis efficiency. Further, the activation of plant defence mechanisms modifies sugars metabolism, moving towards the production of new molecules (Jeandet et al., 2002) and reducing the carbohydrates used for plant growth and reproduction. In contrast with these data, we recognize leaf surfaces increase on diseased plants. These findings agree with the growth regulators activity registered from bleeding xylem sap by diseased vines. Hormone-like substances with auxin activity could be produced by *Pm. minimum* , *Pa. chlamydospora* and especially by *F. mediterranea* and contribute to cell hyperplasia, hypertrophy and leaf lamina expansion.

It is well-known that grapevine reacts with powerful defence mechanisms to cope with pathogenic microorganisms. In particular, production and accumulation of glycolic acid, stilbenes, viniferins and other phenolic phytoalexins (Jeandet et al., 2002; Amalfitano et al., 2000); rapid and localized cell death (Chang et al., 2011) synthesis of pathogenesis-related proteins (Jeandet et al., 2002), and production of Reactive Oxygen Species (ROS) such as superoxide radical O_2^- and hydroxyl radical HO^* (Hung, Yu & Lin, 2005) were reported. The most prominent features of the plant response to pathogens and, in general, against stresses, are the 'oxidative burst': a rapid increase in the cellular concentration of ROS and mainly hydrogen peroxide (De Gara, de Pinto & Tommasi, 2003). This compound works as an antimicrobial and as an intracellular and intercellular signalling compound to activate further defence responses as a mechanical and chemical defence. The modification of cell walls consisting in cross-linking, lignifications and incorporation of phenolic compounds and the synthesis of secondary metabolites (phytoalexins) are defence responses activated by hydrogen peroxide (Torres, Jonathan & Dangl 2006; Almagro et al., 2009). In this work, leaves collected from HV vines showed the lower H_2O_2 concentrations at all considered phenological phases. The H_2O_2 production is a physiological response to light exposure which increased O_2 photo-reduction in photosystem I of chloroplasts and photorespiration (Asada, 1999; Leshem, 1992). Whereas, in leaves of diseased plants, H_2O_2 increases about 3-fold in symptomless leaves independently of phenological phases and reaches the higher values on symptomatic leaves. This suggests a strong correlation between pathogens or their metabolic activity and H_2O_2 production and accumulation in leaves. H_2O_2 in leaves of diseased plants might have been used to counteract pathogens as antimicrobial, as a strengthening of cell wall polymers, as a promoter for phytoalexin synthesis, or in programmed cell death triggering. However, infected vines failed their defence-upgrade and finally suffer the effects of oxidative stress. H_2O_2 by itself increase oxidative stress and damage the integrity of cell membranes (Perez, Villegas & Mejia, 2002). ROS intensify lipid peroxidation of unsaturated fatty acids of membranes, compromising membrane integrity and functionality. Analyses of the lipid peroxidation level clearly show changes in the cell membranes. Levels of MDA, a product of lipid peroxidation, are correlated to membrane damages (Heath & Packer, 1968; Perez, Villegas & Mejia, 2002;

Soares et al., 2019).

Plants scavenge ROS are produced under biotic and abiotic stresses. Enzymes, including superoxide dismutase, catalase, peroxidase, APX and G-R (Zhang & Kirkham, 1996; Lee & Lee, 2000) and non-enzymatic antioxidants such as tocopherols, AsA and glutathione (Noctor & Foyer, 1998) work as ROS detoxifiers. AsA is considered as a molecule-key for H_2O_2 elimination. AsA reacts with H_2O_2 directly or by APX, a Class I heme-peroxidase which uses AsA as electron donor and is considered to be the main peroxidase involved in H_2O_2detoxification (Asada, 1999). Monodehydroascorbate reductase, DHA-R and GSH regenerate AsA. Glutathione control the redox state in plant cells under abiotic and biotic stress and it is involved on AsA regeneration in the AsA-GSH cycle (Hung, Yu & Lin, 2005; De Gara, de Pinto & Tommasi, 2003; Asada, 1999; Noctor & Foyer, 1998; Mittler, 2002). If the AsA-GSH cycle works well, an increase in AsA content and APX activities in leaves of infected vines are expected. However, leaves of vines with BWS and BWS-WR during all the four phenological phases here considered, show AsA and GSH concentrations lower than HV. This trend is also confirmed on of total ascorbate (AsA+DHA) in bleeding xylem sap. On the contrary, total glutathione (GSH+GSSG) was stimulated by the presence of pathogens in the trunk.

L-Ascorbic acid (2,3-didehydro L-threo-hexano-1,4-lactone, the well-known functional form of vitamin C) is a multifunction molecule: redox buffer in coordination with glutathione, cell photo-protector, enzyme cofactor, a regulator of cell division and expansion, cell wall growth and signal transduction (Gallie, 2013). It is involved in oxalate, tartrate ethylene, gibberellins, anthocyanins and hydroxyproline synthesis. AsA is an indirect response of plants against pathogens, changes gene expression in plants and plays a role in resistance against biotic and abiotic stresses (Khan, Mazid & Mohammad, 2011).

In this study, AsA, DHA, GSH and GSSG concentrations and APX, DHA-R, AFR-R and G-R activities were quantified in leaves. The findings here reported signalling that *Pm. minimum* , *Pa. chlamydospora*alone or in association with *F. mediterranea* affect antioxidant defences based on the use and recycling of both GSH and AsA and this could be correlated with an unbalanced oxidative state, damage to membrane integrity and leaf necrosis appearance.

Leaves of diseased vines show a significant decrease of redox state and a shift of both AsA and glutathione towards the oxidised forms. Ascorbate and glutathione redox states providing a reliable estimation of the extent of oxidative stress in the cell and changes in their levels are reported during stress conditions (Munne-Bosch & Alegre, 2003). In our studies, diseased vines seem more stressed than healthy vines.

To explain this physiological status, we invoke the metabolic complex produced by *F. mediterranea* , *Pm. minimum* and*Pa. chlamydospora* (Evidente et al., 2000; Tabacchi et al., 2000; Bruno & Sparapano, 2006a, 2006b, 2007; Bruno, Sparapano & Graniti 2007) and the accumulation of resveratrol, benzoic acid derivatives and flavonols as host defence compounds (Bruno & Sparapano, 2006b; Jeandet et al., 2002; Amalfitano et al 2000). The presences of phenols or flavonoids contribute to AsA oxidation in the scavenging of H_2O_2 in grape leaves (Yamasaki, Sakihama & Ikehara, 1997): phenoxy or flavonoxy radicals can accept electrons from AsA that produce the monodehydroascorbate radical (Perez, Villegas & Mejia, 2002).

To prevent oxidative stress, following the glutathione-ascorbate metabolic pathway, APX reduces H_2O_2 to water converting AsA to monodehydroascorbate that disproportionates into AsA and DHA. Using GSH, DHA-R reduces DHA to ascorbate and produces GSSG. Finally, GSSG is reduced to GSH by G-R using NADPH as the electron donor (Asada, 1999). In our conditions, the activities of these AsA-regenerating enzymes, even active in diseased and healthy vines, did not present marked differences. This implied that APX, DHA-R, AFR-R and G-R make a non-significant contribution to increasing AsA regeneration in diseased vines.

Therefore, our data on APX, the key-enzymes of AsA-GSH cycle, are in contrast with the low concentrations in cv Sultanina protoplasts (Papadakis, Siminis & Roubelakis-Angelakis, 2001) and the absence in cv. Sultana leaves (Perez, Villegas & Mejia, 2002). The difference could be due to grape varieties, stress considered, and assay procedure applied.

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5. Conclusions

The results of this study suggest that *F. mediterranea* ,*Pm. minimum* and *Pa. chlamydospora* disturb various morphological, physiological and biochemical functions in cv Italia grapevine during the vegetative period. Alterations affect both bleeding xylem sap and leaves. Flux, dynamic viscosity and growth regulators activity distinguish bleeding xylem sap of vines infected by *Pm. minimum* and *Pa. chlamydospora* or by *Pm. minimum* ,*Pa. chlamydospora* and *F. mediterranea* . Surface, fresh and dry weight, chlorophyll, hydrogen peroxide, lipid peroxidation and redox state were altered on leaves of diseased vines. The presence of *F. mediterranea* in wood tissues of infected vines further worsens the physiological status. These alterations were detected on symptomatic leaves and, with low intensity, on symptomless leaves of diseased vines. Probably, these damages mark a pre-symptomatic stage that, over time, will cause irreversible alterations which induce symptoms appearance. In diseased vine, low concentrations of AsA, GHS and moderate levels of DHA and GSSG are also associated with higher H_2O_2 and MDA values and remarkable oxidative stress status. On these conditions, the scavenging enzymes are not enough able to restore the balance between ROS and antioxidants level managing stress conditions. The oxidative unbalance stress enhances lipid peroxidation of unsaturated fatty acids of membranes, damages membrane integrity and contributes to cell death and leaf symptoms development. As it is obvious from the present data, AsA-GSH cycle might be involved in grapevine susceptibility of "Esca complex"-associated fungi.

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Authorship

G.L. Bruno Conceptualization, Investigation, Methodology, Writing original draft, Resources. **F. Tommasi**Conceptualization, Investigation, Methodology, Writing - original draft.**L Bragazzi** Investigation, Methodology, Formal analysis.**M.P. Ippolito** Investigation, Methodology, Formal analysis.

Conflict of Interest Statement

The authors declare that they have no known competing for financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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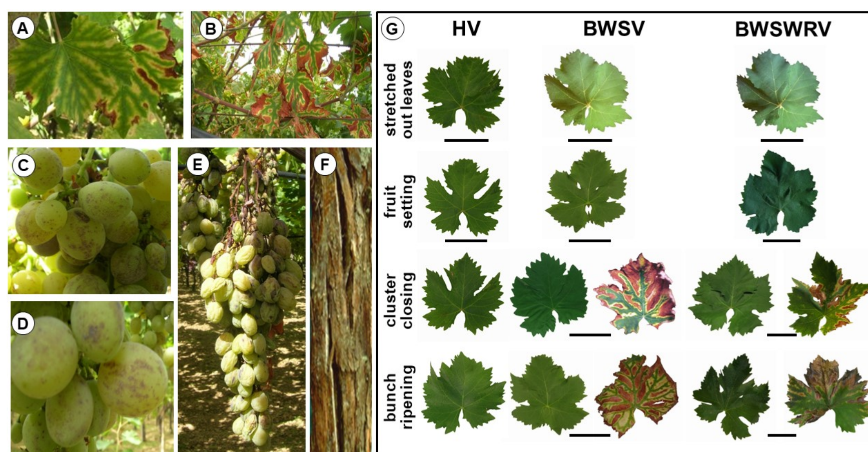

Fig. 1. Symptoms developed on the 20-year-old *Vitis vinifera* cv. Italia vineyard. Chlorotic areas (A) and 'tiger stripes' (B) on leaves; spots on the skin (C-D) and wilt (E) of berries, and cracking of trunk (F) of the diseased vine. A selection of leaves (G) collected during the phenological phases of stretched-out leaves, fruit setting, cluster closing and bunch ripening from healthy (HV), with brown wood-streaking (BWSV) or vines with brown wood-streaking and white rot (BWSWRV). Bars = 10 cm.

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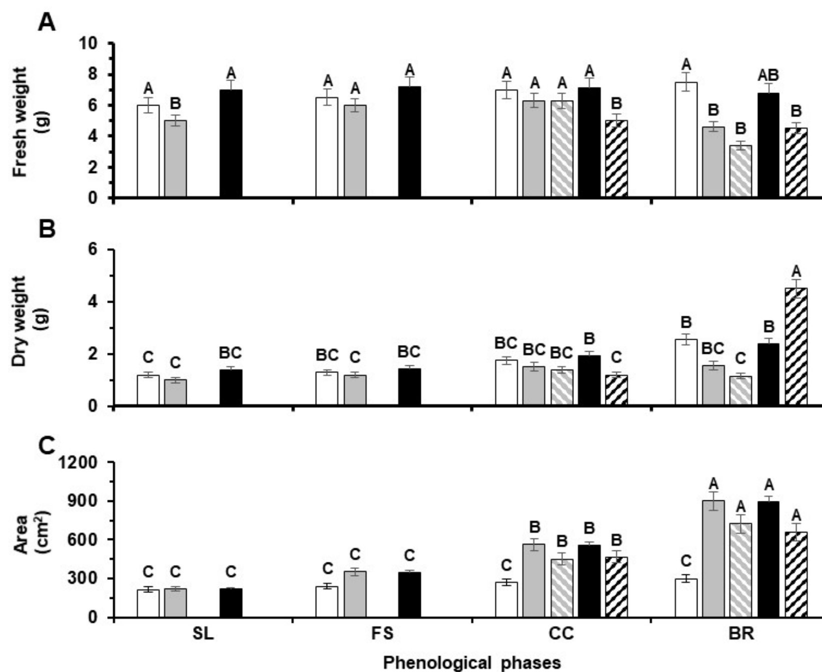

Fig. 2. Fresh (A) and dry (B) weight, and surface (C) of leaves collected during the phenological phases of stretched-out leaves (SL), fruit setting (FS), cluster closing (CC) and bunch ripening (BR) from symptomless (filled bars) or symptomatic (banded histograms) cv. Italia vines healthy (), with brown wood-streaking () or with brown wood-streaking and white rot (). Data are means of fifty replicates ± sd. For each parameter, values with the same letters are not significantly different according to Tukey post-hoc test P?0.05.

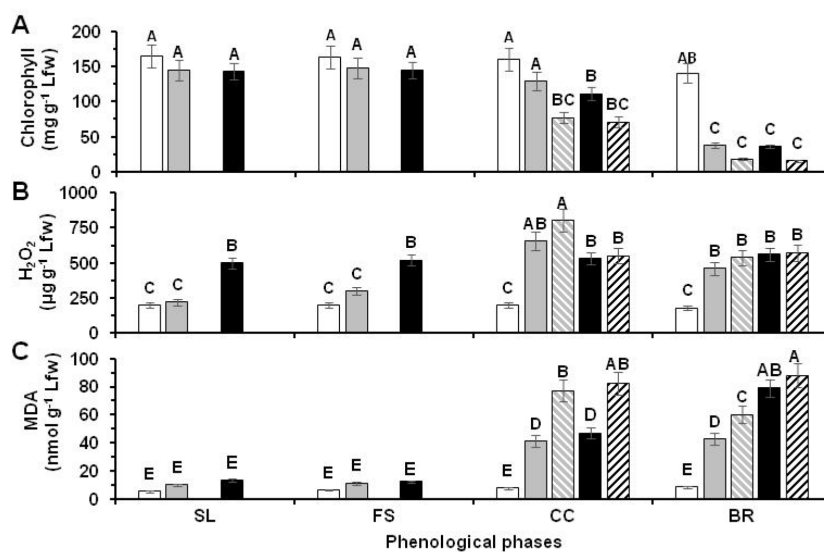

Fig. 3. Total chlorophyll (A), hydrogen peroxide (B) and malondialdehyde (C) concentrations in leaves collected during the phenological phases of stretched-out leaves (SL), fruit setting (FS), cluster closing (CC)

and bunch ripening (BR) from symptomless (filled bars) or symptomatic (banded histograms) cv. Italia healthy vines (), vines with brown wood-streaking () or with brown wood-streaking and white rot (). Data are means of fifteen replicates ± sd. For each parameter, values with the same letters are not significantly different according to Tukey post-hoc test P?0.05.

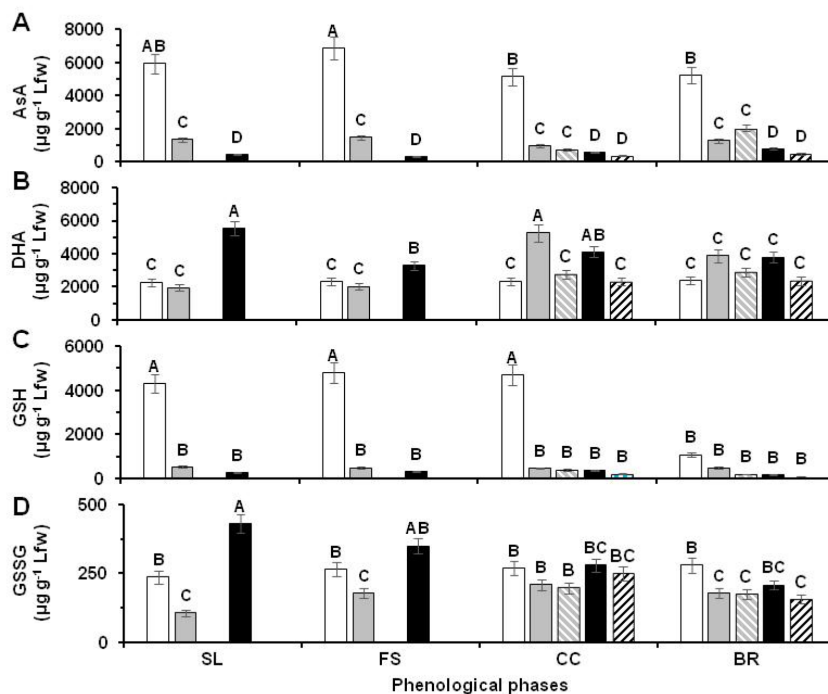

Fig. 4. Ascorbic acid (A), dehydroascorbic acid (B), reduced (C) and oxidized (D) glutathione concentrations of leaves collected during the phenological phases of stretched-out leaves (SL), fruit setting (FS), cluster closing (CC) and bunch ripening (BR) from symptomless (filled bars) or symptomatic (banded histograms) cv. Italia healthy vines (), vines with brown wood-streaking () or with brown wood-streaking and white rot (). Data are means of fifteen replicates ± sd. For each parameter, values with the same letters are not significantly different according to Tukey post-hoc test P?0.05.

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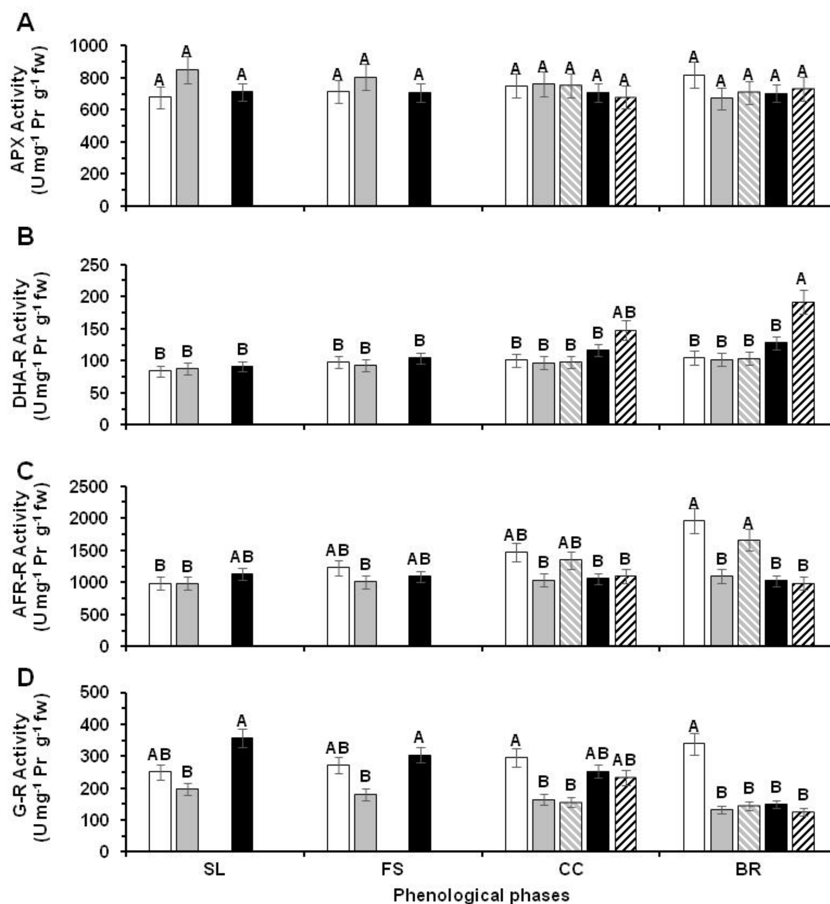

Fig. 5. Activities of the redox enzymes: A) ascorbate peroxidase (APX), B) dehydroascorbate reductase (DHA-R), C) glutathione reductase (G-R), D) ascorbate free radical reductase (AFR-R) on leaves collected during stretched-out leaves (SL), fruit setting (FS), cluster closing (CC) and bunch ripening (BR) phenological phases from symptomless (filled bars) or symptomatic (banded histograms) cv. Italia vines healthy (), with brown wood-streaking () or with brown wood-streaking and white rot (). Data are means of fifteen replicates ± sd. For each parameter, values with the same letters are not significantly different according to Tukey post-hoc test P?0.05.

Table 1. Isolation frequency (%) of fungal species obtained from cv Italia vines healthy (H), with brown wood-streaking (BWS) and with BWS and white rot (BWSWR).

Fungal species	**Vines** [a]	**Vines** [a]	**Vines** [a]
	H	**BWS**	**BWSWR**
Phaeomoniella chlamydospora	0	45±4	38±3
Phaeoacremonium minimum	0	25±2	30±4
Fomitiporia mediterranea	0	0	87±6
Other “contaminant” fungal species [b]	3±1	6±1	4±1
No isolations	97±2	10±2	9±2

[a] For each group of vines, data are means of 900 woody-chips ± sd.

[b] *Penicillium* spp., *Alternaria* spp., sterile fungi, no identified species.

Table 2. Flux, viscosity coefficient ($\eta_{|x}$), concentrations of total ascorbate (T-ASC) and glutathione (T-GLU), and growth regulators activity (GRA) of bleeding xylem sap collected from cv Italia healthy vines (HV), with brown wood-streaking (BWS) and with BWS and white rot (BWSWR)[a]

Vines	**Flux** [b]	η_ξ [d]	**T-ASC** [c]	**T-GSH** [c]	**GRA** (µg ml^{-1})	**GRA** (µg ml^{-1})
	(ml vine^{-1})	(Poiseuille)	(µg ml^{-1})	(µg ml^{-1})	**Auxin** [e]	**Kinetin** [e]
HV	144±9.82 C	0.85±0.04 C	308.6±26.4 A	18.1±3.1 C	10.25±1.7 C	0.91±0.27 B
BWS	677±11.79 A	1.13±0.04 B	211.2±26.6 C	46.4±8.6 B	20.10±3.5B	22.25±2.45 A
BWS-WR	574±9.165 B	1.73±0.07 A	269.1±26.9 B	178.2±38.1 A	30.05±3.9 A	22.45±2.04 A

[a] For each column, values with the same capital letters are not significantly different according to Tukey post-hoc test P<0.05.

[b] Each value is the means of 5 vines ± sd.

[c] Data are means of 15 replicate ± sd.

[d] Values are means of 50 replicate ± sd.

[e] Data are means of 20 replicate ± sd.

Table 3. Results of ANOVA analysis considering sampling time (ST), vine typology (VT), symptoms (SY) and their interactions on leaf fresh (Lwt) and dry (Ldw) weight, moisture (WC), surface (LS), chlorophyll (Chlo), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), ascorbic acid (AsA), dehydroascorbic acid (DHA), reduced (GSH) and oxidized (GSSG) glutathione content, ascorbate peroxidase (APX), dehydroascorbate reductase (DHA-R), Glutathione reductase (G-R), ascorbate free radical reductase (AFR-R) activities

Sources of variation	**df**	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]	**F values** [a]
		Lfw	**Ldw**	**WC** [b]	**LS**	**Chlo**	H_2O_2	**MDA**	**AsA**	**DHA**	**GSH**	**GSSG**	**APX**
ST	3	19.80**	177.11**	75.27	476.46**	208.09**	47.83**	196.30*	22.67**	67.45*	173.96**	15.11**	0.16
VT	2	27.74**	41.28**	35.80**	47.98**	118.98**	279.69**	250.53**	2193.37**	68.80**	2211.61**	141.76**	5.23
ST×VT	6	12.67**	17.70**	22.58**	14.71*	23.60**	41.24**	66.28**	18.45**	127.74**	149.93**	59.85**	5.77
SY	1	84.39**	176.76**	22.65	73.09**	94.52**	22.84**	293.71**	187.21	265.55**	6.51**	12.21**	0.12
ST×SY	1	5.27**	6.90**	12.15	2.91	14.67**	1.81*	62.38**	6.81*	189.80**	0.37	0.26	1.51
VT×SY	1	27.56**	42.73**	11.24	239.69**	0.78	12.68**	2.56	9.08**	12.32**	0.31	6.13**	0.12
ST×VT×SY	1	2.69	0.89	1.27	235.58**	0.78	1.19	1.65	9.08**	26.24**	1.62	1.19	0.02

[a] Levels of probability: * and** = 0.05 and 0.01, respectively.

[b] transformation to stabilize variances: = ln (Y).

Table 4. Ascorbate and glutathione redox state on symptomless or symptomatic leaves collected during stretched-out leaves (SL), fruit setting (FS), cluster closing (CC) and bunch ripening (BR) phenological phases from cv. Italia vines healthy (HV), vines with brown wood-streaking (BWSV) or with brown wood-streaking and white rot (BWSWRV)[a]

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Leaves from	**Phenological phases**	**Phenolo**
	SL	**FS**
Ascorbate redox state = AsA / (AsA + DHA)	**Ascorbate redox state** = AsA / (AsA + DHA)	**Ascorba**
HV	0.72±0.05	0.75±0.05
BWSS symptomless	0.40±0.02	0.42±0.02
symptomatic	n.p.[a]	n.p.
BWSWRS symptomless	0.07±0.05	0.13±0.01
symptomatic	n.p.	n.p.
Glutathione redox state = GSH / (GSH + GSSG)	**Glutathione redox state** = GSH / (GSH + GSSG)	**Glutathi**
HV	0.95±0.06	0.95±0.06
BWSS symptomless	0.83±0.04	0.73±0.04
symptomatic	n.p.	n.p.
BWSWRS symptomless	0.38±0.01	0.55±0.04
symptomatic	n.p.	n.p.

[a] Data are means of 15 replicates ± sd.

[b] n.p. leaves typology not present.