Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Physiological responses of 'Italia' grapevines infected with Esca pathogens‡

Giovanni Luigi BRUNO[1,*], Maria Paola IPPOLITO[2], Francesco MANNERUCCI[1], Luca BRAGAZZI[1], Franca TOMMASI[2]

[1] *Department of Soil, Plant and Food Sciences (Di.S.S.P.A.) - Plant pathology Unit, University of Bari Aldo Moro, Via G. Amendola, 165/A; 70126 - Bari, Italy*
[2] *Department of Biology, University of Bari Aldo Moro, Via Orabona, 4; 70125 - Bari, Italy*
*Corresponding author. E-mail: giovanniluigi.bruno@uniba.it
‡ Dedicated to the memory of Antonio Graniti (1926-2019), Emeritus Professor of Plant Pathology at the University of Bari, Italy.

Summary. Physiological features were examined of a 20-year-old *Vitis vinifera* 'Italia' table grape vineyard cropped in Apulia, Italy. Healthy vines with no foliar symptoms and any indications of wood or berry alterations, vines with natural wood infections by *Phaeoacremonium minimum* (syn. *P. aleophilum*) and *Phaeomoniella chlamydospora* showing brown wood streaking symptoms, and vines naturally infected with *P. minimum*, *P. chlamydospora* and *Fomitiporia mediterranea* with brown wood streaking and white rot symptoms, were surveyed. Bleeding xylem sap, collected at bud-break from healthy vines showed the greatest total ascorbic acid level, while vines with brown wood streaking and white rot had the greatest viscosity coefficient, glutathione concentration, and plant growth regulator activities. Compared to healthy vines, leaves of wood affected vines, sampled during the unfolded leaf, fruit setting, cluster closing and bunch ripening vine growth stages, had reduced fresh and dry weights, total chlorophyll concentrations, and increased leaf surface area. Low ascorbic acid and reduced glutathione concentrations, weak redox state, and moderate levels of dehydroascorbic acid and oxidized glutathione were also detected in these vines. Analyses also detected reduced activities of dehydroascorbate reductase, ascorbate free radical reductase and glutathione reductase in diseased vines. The cell membrane damage, associated with lipid peroxidation, was coupled with high hydrogen peroxide concentrations. These changes could contribute to the cell death of leaves and foliar symptom development. The ascorbate-glutathione cycle supports grapevine susceptibility to Esca complex-associated fungi.

Keywords. Antioxidant systems, ascorbic acid, glutathione, membrane lipid peroxidation, redox state, hydrogen peroxide.

OPEN ACCESS

Citation: G.L. Bruno, M.P. Ippolito, F. Mannerucci, L. Bragazzi, F. Tommasi (2021) Physiological responses of 'Italia' grapevines infected with Esca pathogens. *Phytopathologia Mediterranea* 60(2): 321-336. doi: 10.36253/phyto-12171

Accepted: March 1, 2021

Published: September 13, 2021

Data Availability Statement: All relevant data are within the paper.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Hanns-Heinz Kassemeyer, Staatliches Weinbauinstitut Freiburg, Germany.

INTRODUCTION

The disease commonly called "Esca" is one of the longest recognized and most destructive diseases of grapevines (*Vitis vinifera*), and is associated with wood discolouration and decay, and sudden wilting of whole vines

or individual vine arms within a few days (apoplexy or apoplectic symptoms), as well as leaf tiger stripe leaf symptoms (Ravaz, 1898; de Rolland, 1873; Marsais, 1923). Esca is now considered to be a complex of different diseases overlapping in the same vine or developing at different stages of a vine life. Esca-complex comprises brown wood streaking of grapevine cuttings, Petri disease, grapevine leaf stripe disease (GLSD, previously "young esca"), and white rot (which is at the origin of the name Esca). GLSD affects young and old vines, which show wood streaking and discolouration. The association between GLSD and white rot was described as a condition called "Esca proper". Within the Esca complex, white rot, and Esca proper can also show apoplectic symptoms (Surico, 2009; Mondello *et al*., 2018). Brown wood streaking, Petri disease and GLSD are also grouped under the name phaeotracheomycosis complex (Bertsch *et al*., 2012).

Members of the basidiomycetous genus *Fomitiporia* (*F. mediterranea* mainly in Europe and the Mediterranean area) are associated with wood decay of white rot (Fischer, 2002; 2006; Ciccarone *et al*., 2004). *Phaeoacremonium minimum* (syn. *P. aleophilum*) and *Phaeomoniella chlamydospora* are the most important etiological agents of brown wood streaking, Petri disease and GLSD (Mugnai *et al*., 1999; Surico, 2009; Baranek *et al*., 2018). Other species of *Phaeoacremonium* and *Cadophora* are present in the wood of grapevines affected by Esca complex diseases (Moreno-Sanz *et al*., 2013; Elena *et al*., 2018; Jayawardena *et al*., 2018).

No pathogens have been isolated from leaves or berries of infected plants. Leaf tiger stripe and berry symptoms are considered linked to cultivar susceptibility, vine age, the microorganisms involved, pedoclimatic conditions and other physiological factors (Graniti *et al*., 2000; Surico *et al*., 2006; Bertsch *et al*., 2012; Guérin-Dubrana *et al*., 2013; Claverie *et al*., 2020). The substances originating in the discoloured woody tissues of affected trunks and branches contribute to development of the typical symptoms on leaves (Andolfi *et al*., 2011; Bertsch *et al*., 2012; Lecomte *et al*., 2012). These materials could be reaction products of the diseased wood, phytotoxic metabolites excreted by Esca-associated fungi, or a combination of these (Mugnai *et al*., 1999; Bruno *et al*., 2007). *P. minimum* and *P. chlamydospora* produce two naphthalenone pentaketides, scytalone and isosclerone, and exopolysaccharides including the α-glucan pullulan (Evidente *et al*., 2000; Tabacchi *et al*., 2000; Bruno and Sparapano 2006a; 2006b; 2007). Macro- and micro-nutrients also play roles in Esca complex symptom progression (Calzarano *et al*., 2009, 2014; Calzarano and Di Marco, 2018).

Stomata closure and changes in the photosynthetic apparatuses affect the pre-symptomatic leaves of Esca diseased grapevines (Petit *et al*., 2006; Magnin-Robert *et al*., 2011). Low density cytoplasm, plastids with small starch grains, underdeveloped grana, and elongated thylakoids occur in grapevine leaves before tiger stripes develop (Lima *et al*., 2010; Fontaine *et al*., 2016). Glutathione pools, PR-proteins, and phenolic compounds are also affected (Magnin-Robert *et al*., 2011; Valtaud *et al*., 2011; Lambert *et al*., 2013; Calzarano *et al*., 2016; 2017a; 2017b; Fontaine *et al*., 2016). Xylem dysfunction also influences water transport and leaf water potential (Bruno *et al*., 2007; Fontaine *et al*., 2016), and NMR metabolomics data suggest increased phenylpropanoid compound production and decreased glucose and fructose contents occur in leaves of Esca complex diseased vines (Lima *et al*., 2010).

The aim of the present study was to gain insight into variations of physiological features of bleeding xylem sap and leaves of 'Italia' grapevine plants that were either healthy or naturally infected with *P. minimum* and *P. chlamydospora* showing brown wood streaking, or infected with *P. minimum*, *P. chlamydospora* and *F. mediterranea* showing brown wood streaking and white rot. Special emphasis was placed on the differences in hydrogen peroxide, lipid peroxidation, and antioxidant defence responses associated with the ascorbate-glutathione cycle. The changes in the physiology of diseased plants were assessed as possible factors in development of foliar symptoms.

MATERIALS AND METHODS

Vineyard

A 20-year-old *V. vinifera* 'Italia' table grape vineyard (1600 vines) cropped in the countryside of Bari (Apulia, southern Italy) was used for sample collection. The vines, grafted onto 140-Ru rootstock, were trained by the Tendone system, and grown under irrigation in an alkaline clay soil. Since 2006, each vine had been under observation for symptom development of diseases within the Esca complex, i.e., foliar symptoms, or sudden wilting of GLSD or esca proper.

Wood core sampling and vine characterization

To assess the presence of symptoms and fungi in the grapevine trunks, two wood cores per vine were taken with a 95% ethanol pre-sterilised Pressler increment borer at 30 and 110 cm above the ground level. All the 1600

vines were surveyed. To prevent further wood contamination, wood core sampling holes were disinfected with copper oxychloride solution (20% in water) and filled with 2.5% copper oxychloride in linseed oil. Each core was cut to fragments 1.5–2 cm in length. Slices were surface-sterilized for 1 min in 70% ethanol, soaked for 1-2 min in a sodium hypochlorite solution (3% active chlorine), and rinsed three times in sterile distilled water. The slices were then aseptically cut into pieces that were seeded onto 90 mm diam. Petri dishes (five per plate) containing agar media. Malt extract (2%) agar (MEA), MEA amended with 0.25% chloramphenicol (MEAC) and MEA amended with 0.25% benomyl (MEAB) were used as the isolation media. MEAC and MEAB were used as semi-selective media for detection, respectively, of phaeotracheomycotic and basidiomycete fungi. Inoculated plates were incubated at 25±1°C in the dark. The isolation frequency (IF) of each fungus taxon was calculated as $IF = 100 \times (N_i/N_t)$, where N_i was the number of wood-fragments from which the fungus was isolated and N_t the total number of seeded wood tissue pieces.

At completion of characterizations of the vine wood, associated with leaves and berries symptoms surveyed during 12 years, 15 vines were selected, including five with brown wood streaking, five with brown wood streaking and white rot, and five healthy (symptomless) that did not show any foliar symptoms, and any indications of wood or berry alterations.

Bleeding xylem sap sampling and characterization

To evaluate bleeding sap quantity, viscosity, and ascorbate, glutathione and hormone concentrations, bleeding xylem sap was collected from five vines with brown wood streaking, five with brown wood streaking and white rot, and five healthy vines. Bleeding xylem sap was collected at the bud-break vine growth stage (Baggiolini, 1979; Wilcox *et al*., 2015). From each selected plant, four vine shoots were cut and the end of each spur was surface-treated with sodium hypochlorite solution (3% active chlorine), then with 95% ethanol, and then rinsed twice with sterile distilled water. The sap exuded during the first 15 min was discarded. A sterile plastic bottle covered with aluminium foil was secured at the end of each bleeding spur to collect the liquid over the following 4 d. All sap samples were filtered through 0.45 mm membranes (Millipore).

The dynamic viscosity (η_x) of each sap sample was calculated as $\eta_x=[(\rho_s \times t_x)/(\rho_w \times t_w)] \times \eta_w$, where ρ_s = sap density, ρ_w = water density, η_w = water dynamic viscosity ($0.8937'10^{-3}$ Poiseuille), t_s = flow time of sap in sec, and t_w = water flow time (sec). Measurements of t_s and t_w were carried at 25±0.1°C using an Ostwald glass capillary viscometer (Cannon-Fenske Instruments).

For each sap sample, 2 mL were lyophilized, and the resulting powder was treated with 5% metaphosphoric acid (6 mL). After centrifugation (20,000*g*, 15 min, 4°C), the supernatant was used for total ascorbate and total glutathione determinations following the method of Zhang and Kirkham (1996).

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

Leaf sampling and characterization

To evaluate leaf fresh and dry weights, and areas, as well as total chlorophyll, hydrogen peroxide, lipid peroxidation, ascorbic acid, dehydroascorbic acid, reduced and oxidized glutathione concentrations and the activity of enzymes involved in ascorbate regeneration, leaves were collected from the selected 15 vines. Leaves (ten per vine) were randomly picked from each vine during the unfolded leaf, fruit setting, cluster closing, and bunch ripening vine growth stages (Baggiolini, 1979; Wilcox *et al*., 2015). At the cluster closing and bunch ripening vine growth stages of the diseased vines, symptomless and symptomatic leaves were sampled. The leaf petioles were removed, and the leaves were photographed.

Each leaf was weighed with a Sartorius BP 210S analytical balance (Data Weighing Systems, Inc.) to assess leaf fresh weight.

Leaf area was estimated using ImageJ open-source image-processing software (National Institutes of Health).

Leaf dry weight was measured by drying 100 mg of leaves for 20 min at 105°C using an infrared LP 16-M desiccator (Mettler-Toledo SpA). Leaf moisture was calculated as a percentage (%) of fresh weight.

Total chlorophyll concentration was verified by Harborne's method (1973) using leaf lamina samples (2 g each) and 80% acetone (16 mL) as extraction solvent in an ice bath. Absorbance at 645 and 663 nm were measured using a Beckman DU 640 spectrophotometer (Beckman Coulter Inc.).

Hydrogen peroxide content was determined as reported by Lee and 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 assessed as malondialdehyde (MDA) quantity in 200 mg of leaf lamina samples (Heath and Packer, 1968).

Ascorbic acid, dehydroascorbic acid, and reduced and oxidized glutathione were quantified in 2 g of ground leaf lamina samples, at 4°C, in 5% metaphosphoric acid (6 mL). After centrifugation (20,000*g*, 15 min, 4°C), the supernatant was used as proposed by Zhang and Kirkham (1996). The ascorbate redox state (A-RS) was calculated as A-RS = [AsA / (AsA + DHA)], where AsA represents ascorbic acid and DHA represents dehydroascorbic acid. The glutathione redox state (G-RS) was calculated as G-RS = [GSH / (GSH + GSSG)], where GSH represents reduced glutathione, and GSSG represents oxidized glutathione.

The activity of enzymes involved in ascorbate regeneration was detected on 2 g of leaf lamina homogenized 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. After centrifugation (25,000*g*, 15 min, 4°C), the supernatant was dialyzed against 50 mM Tris-HCl (pH 7.8) and used to determine activities of ascorbate peroxidase (EC 1.11.1.11), dehydroascorbate reductase (EC 1.8.5.1), glutathione reductase (EC 1.6.4.2), and ascorbate free radical reductase (= monodehydroascorbate reductase, EC 1.6.5.4), according to Paciolla *et al.* (2008).

Statistical analyses

Data were subjected to general linear analysis of variance models using the SAS/STAT version 9.0 (SAS Institute Inc.). Normal distributions were assessed using the Shapiro-Wilk tests, and homoscedasticity was assessed using Bartlett's tests. Means were compared using Fisher's LSD test at $P \leq 0.05$. Data of morphological and physiological features of the grapevine leaves were analysed for vine typology (brown wood streaking, brown wood streaking and white rot, or healthy), vine growth stages (unfolded leaf, fruit setting, cluster closing, or bunch ripening), symptoms on leaves (presence or absence), and their interactions.

RESULTS

Wood core and symptom examination

The wood core examinations showed that 562 vines had brown wood streaking, 176 had brown wood streaking and rotted wood in the trunks, and 862 vines were healthy. *P. chlamydospora* and *P. minimum* were always associated with brown wood streaking, while *F. mediterranea* was isolated only from rotted wood (Table 1). Other micromycetes, including species of *Penicillium*, *Alternaria*, *Paecilomyces*, *Trichoderma*, *Chaetomium*, *Cladosporium*, and *Paraconiothyrium*, and sterile fungi, were also isolated from diseased vines. Species of *Penicillium* and *Alternaria* were the only fungi isolated from cores from the healthy vines.

Table 1. Isolation frequency (%) of fungus species obtained from 'Italia' vines that were healthy (H), affected with brown wood streaking (BWS) or with brown wood streaking and white rot (BWSWR).

Fungus	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 fungi [b]	3±1	6±1	4±1
No isolations	97±2	10±2	9±2

[a] For each group of vines, data are means for 900 woody chips ± standard deviation.
[b] *Penicillium* spp., *Alternaria* spp., *Paecilomyces* spp., *Trichoderma* spp., *Chaetomium* spp., *Cladosporium* spp., *Paraconiothyrium* spp. and sterile fungi.

During the survey, symptoms were recorded on leaves and berries of diseased vines (Figure 1). The typical tiger stripe symptoms were first observed in July on vines with brown wood streaking and with brown wood streaking and rotted wood in the trunks. On plants also infected with *F. mediterranea*, trunk cracking was recorded. No apoplexy was viewed on selected vines throughout the survey.

Bleeding xylem sap characterization

Healthy vines discharged the lowest quantities of xylem sap. Both typologies of diseased vines discharged four- and five-fold more sap than the healthy vines, and the sap from these vines had the greatest dynamic viscosity coefficients, total ascorbic acid and glutathione concentrations, and auxin-like and kinetin activities (Table 2).

Leaf characterization

A selection of leaves sampled from the 15 selected vines is illustrated in Figure 1G. Morphological and physiological features were strongly affected by the vine growth stages, vine typology, symptom development, and their interactions (Table 3).

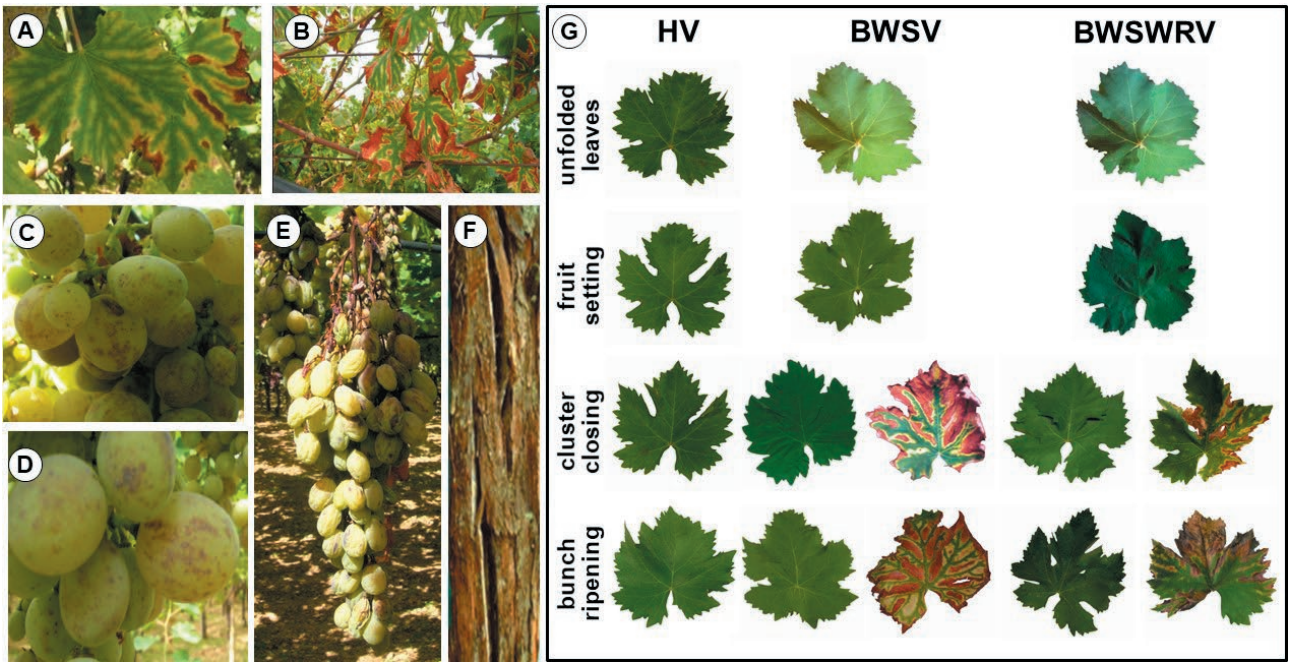

Figure 1. Symptoms developed in the 20-year-old *Vitis vinifera* 'Italia' vineyard. Chlorotic areas (A) and 'tiger stripes' (B) on leaves; spots on the skin (C-D) and wilt (E) of berries and cracking of the trunk (F) of the diseased vine. A selection of leaves (G) collected during the vine growth stages of unfolded leaves, fruit set, cluster closing and bunch ripening, from healthy vines (HV), vines with brown wood streaking (BWSV) or vines with brown wood streaking and white rot (BWSWRV).

Table 2. Flux, viscosity coefficient (h_x), concentrations of total ascorbate (T-ASC), glutathione (T-GLU), and growth regulator activity (GRA) of bleeding xylem sap collected from 'Italia' vines[a] that were healthy (H), or had brown wood streaking (BWS) or brown wood-streaking and white rot (BWSWR).

Vines	Flux[b] (mL vine^{-1})	h_x[d] (Poiseuille)	T-ASC[c] (μg mL^{-1})	T-GLU[c] (μg mL^{-1})	GRA (μg mL^{-1})	
					Auxin [e]	Kinetin [e]
H	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 ± 3.5 B	22.25 ± 2.45 A
BWSWR	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 accompanied by the same letters are not significantly different ($P < 0.05$), Fisher's LSD test.
[b] Each value is the means of five vines ± standard deviation.
[c] Data are means of 15 replicates ± standard deviations.
[d] Values are means of 50 replicates ± standard deviations.
[e] Data are means of 20 replicates ± standard deviations.

Only symptomatic leaves collected from vines with brown wood streaking and white rot at cluster closing stages showed a small decrease (29.6%) in fresh weight compared with symptomless leaves collected from the same vines. At bunch ripening, leaves from healthy vines and symptomless leaves from vines with brown wood streaking and white rot reached maximum fresh weight. Symptomless leaves of vines with brown wood streaking had reduced mean leaf fresh weight compared with healthy vines. Symptomatic leaves from vines with both disease typologies had leaves with fresh weights that were further 35–40% less.

The greatest leaf dry weight (Figure 2B) was recorded at the bunch ripening phase from symptomatic leaves of vines with brown wood streaking and white rot.

No statistically significant differences were found in leaf moisture content for the different vine disease categories. Leaf moisture contents were in the range 90 to 95% of leaf fresh weight.

Surface areas of leaves sampled at unfolded leaf and

Table 3. Results of general linear analyses of variance considering effects of sampling time (ST), vine typology (VT), symptoms (SY) and their interactions on leaf fresh (Lfw) and dry (Ldw) weights, moisture content (WC), surface area (LSA), and contents of chlorophyll (Chlo), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), ascorbic acid (AsA), dehydroascorbic acid (DHA), reduced (GSH) and oxidized (GSSG) glutathione, and activities of ascorbate peroxidase (APX), dehydroascorbate reductase (DHA-R), glutathione reductase (G-R), or ascorbate free radical reductase (AFR-R).

Sources of variation	df	F values[a]												
		Lfw	Ldw	WC[b]	LSA	Chlo	H_2O_2	MDA	AsA	DHA	GSH	GSSG	APX	DHA-R	AFR-R	G-R
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	22.45**	27.85**	28.19**
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*	89.61**	83.32**	168.52**
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*	3.40	26.45**	49.21**
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	59.59**	39.94**	2.36
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	6.62	1.65	0.32
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	52.59**	38.39**	3.10*
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	5.98	5.54	1.02

[a] * and ** indicate, respectively, $P = 0.05$ and 0.01.
[b] ln (Y) transformation used to stabilize variances.

fruit set stages did not show any statistically significant differences, while the leaves of healthy vines were significantly smaller than those of diseased plants during the other two vine growth stages (Figure 2C). At cluster closing stage, the surface areas of symptomless leaves from diseased vines were 2-fold greater than for leave from the healthy vines. At bunch ripening, symptomatic and symptomless leaves of diseased vines had leaf surface areas that were three times greater than leaves from the healthy vines.

During the four sampling times, total chlorophyll concentration in healthy vines was in the range of 160-165 mg g^{-1} leaf fresh weight (Figure 3A). At the cluster closing and bunch ripening stages, diseased plants had less total chlorophyll. At cluster closing, in comparison with healthy vines, symptomless leaves from vines with brown wood streaking had 20% less total chlorophyll. This loss in symptomatic leaves was greater than 40%. Symptomless leaves from vines with brown wood streaking and white rot had 31% less total chlorophyll compared with healthy vines, and symptomatic leaves had a further 36% less total chlorophyll.

During the assessed four vine growth stages, healthy vines showed the least H_2O_2 concentrations (Figure 3B). Symptomless leaves collected from vines with brown wood streaking had increased H_2O_2 content compared with the healthy vines. Symptomatic leaves collected during cluster closing and bunch ripening had 20% more H_2O_2 than healthy vines. Leaves from vines with brown wood streaking and white rot, had more H_2O_2 than healthy vines at all four growth stages. Symptomatic leaves collected during cluster closing or bunch ripening had 3% more H_2O_2 than symptomless ones.

MDA concentrations were different between healthy vines and the two categories of disease at the four vine growth stages (Figure 3C). Healthy vines reached minimum MDA contents in the unfolded leaf stage, and the greatest MDA concentration was occurred during bunch ripening. A similar trend was recorded in symptomless leaves from vines with brown wood streaking, but the final MDA concentrations were greater than in healthy vines. Symptomless leaves from vines with brown wood streaking and white rot, compared to healthy vines, had greater MDA content at all the sampling times. At the cluster closing and bunch ripening stages, symptomatic leaves from the same vines showed, respectively, further 43% and 11% increases in MDA contents.

During the four sampling times, ascorbic acid concentrations in healthy vines were always greater than in leaves from diseased vines (Figure 4A). At each assessed growth stage, ascorbic acid contents were reduced by up to 78% in leaves collected from vines with brown wood

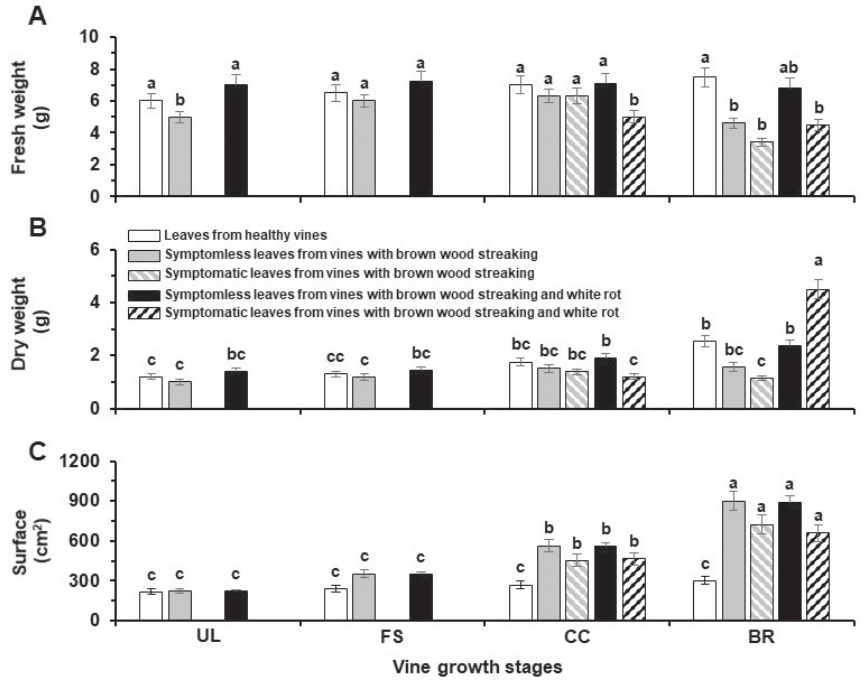

Figure 2. Fresh (A) and dry (B) weights, and surface areas (C) of 'Italia' leaves collected during the unfolded leaf (UL), fruit set (FS), cluster closing (CC) and bunch ripening (BR) vine growth stages from infected or healthy vines. Data are the means of 50 replicates ± standard deviations. For each parameter, values accompanied by the same letters are not significantly different ($P \leq 0.05$) according to Fisher's LSD test.

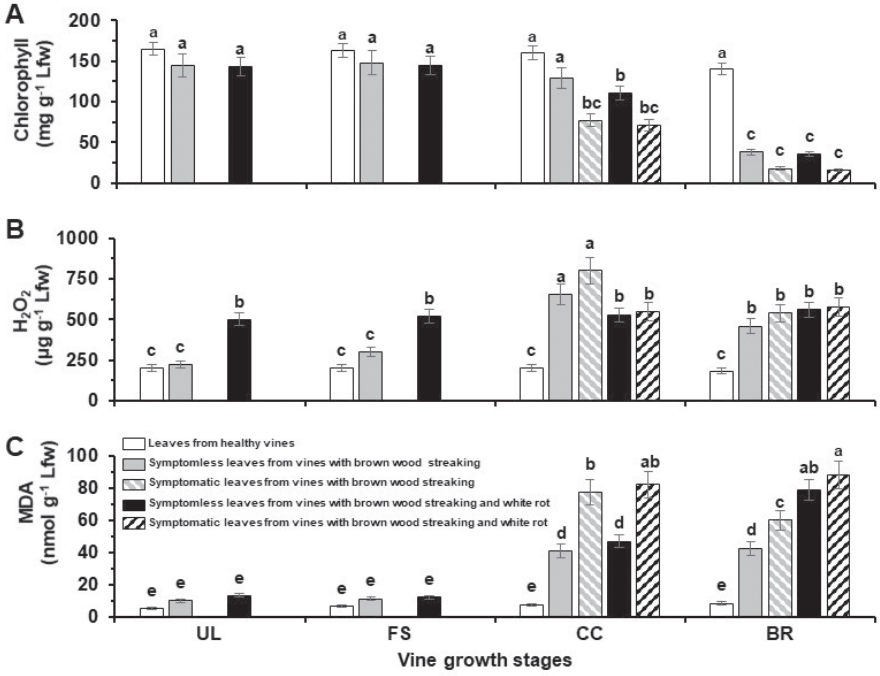

Figure 3. Total chlorophyll (A), hydrogen peroxide (H_2O_2; B) and malondialdehyde (MDA; C) concentrations of 'Italia' leaves collected during the unfolded leaf (UL), fruit set (FS), cluster closing (CC) and bunch ripening (BR) vine growth stages, from infected or healthy vines. Data are the means of 50 replicates ± standard deviations. For each parameter, values accompanied by the same letters are not significantly different ($P \leq 0.05$) according to Fisher's LSD test.

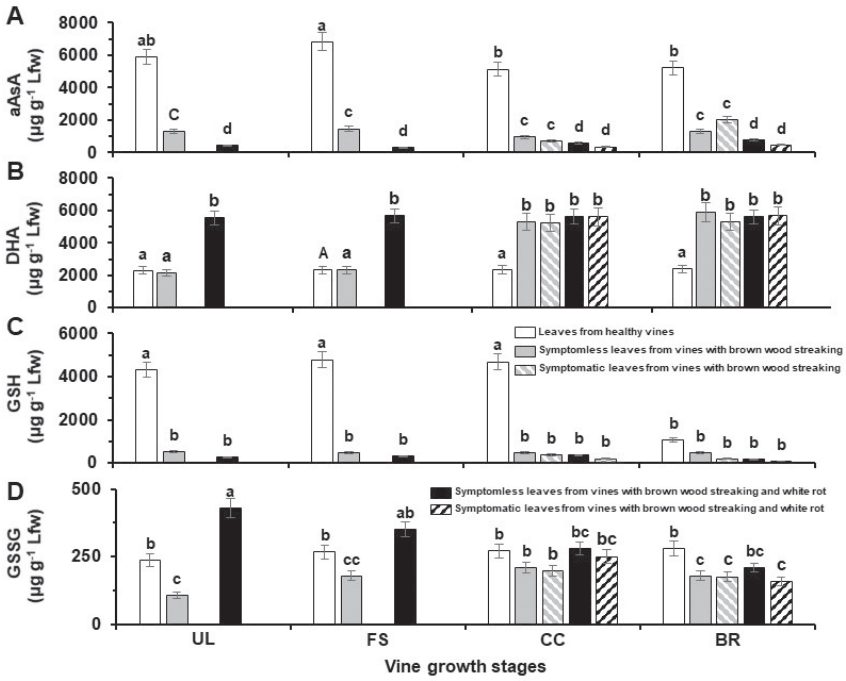

Figure 4. Ascorbic acid (aAsA; A), dehydroascorbic acid (DHA; B), reduced (GSH; C) and oxidized (GSSG; D) glutathione concentrations of 'Italia' leaves collected during the unfolded leaf (UL), fruit set (FS), cluster closing (CC) and bunch ripening (BR) vine growth stages, from infected or healthy vines. Data are the means of 50 replicates ± standard deviations. For each parameter, values accompanied by the same letters are not significantly different ($P \leq 0.05$) according to Fisher's LSD test.

streaking, and 91% in leaves from vines with brown wood streaking and white rot.

Leaves from healthy vines showed similar levels of dehydroascorbic acid during the four assessed growth stages (Figure 4B). In the unfolded leaf and fruit set stages, leaves collected from vines with brown wood streaking and white rot showed the greatest dehydroascorbic acid concentrations.

The reduced glutathione contents of leaves collected from healthy vines were greater than in leaves collected from diseased plants (Figure 4C).

The oxidized glutathione amounts (Figure 4D) in symptomless leaves collected from vines with brown wood streaking, during the four considered growth stages, were approx. 38% less than in leaves from the healthy vines.

The lowest redox states (Table 4) were recorded in all symptomatic leaves tested.

No significant differences were detected between healthy and diseased vines in ascorbate peroxidase activity (Figure 5A) during the four growth stages.

Dehydroascorbate reductase activity (Figure 5B) in leaves from healthy vines and symptomless diseased vines reached minima in the unfolded leaf growth stage and were greatest at the bunch ripening stage.

Ascorbate free radical reductase activity (Figure 5C) in leaves from healthy vines was the least at the unfolded leaf stage, increased during the next two growth stages, and reached a maximum at the bunch ripening stage. Compared to healthy vines, at the cluster closing and bunch ripening stages, leaves from diseased vines had reduced ascorbate free radical reductase activities.

Glutathione reductase activity (Figure 5D) in leaves from healthy vines was least during the unfolded leaf stage and reached a maximum at bunch ripening. Compared to healthy vines, leaves from diseased vines had reduced glutathione reductase activity. No significant changes in glutathione reductase activity were detected in symptomless compared to symptomatic leaves from diseased vines.

DISCUSSION

In this study, in a 20-year-old 'Italia' vineyard surveyed since 2006 for symptoms of diseases within the Esca complex, we selected vines with brown wood streaking, vines with brown wood streaking and white rot, and healthy vines. The fungus isolation procedure confirmed the presence of *P. minimum* and *P. chlamydo-*

Table 4. Ascorbate and glutathione redox states in symptomless or symptomatic grapevine leaves collected from 'Italia' grapevines during the vine growth stages of unfolded leaves (UL), fruit set (FS), cluster closing (CC) or bunch ripening (BR), from healthy vines (HV), or vines with brown wood streaking (BWSV) or brown wood streaking and white rot (BWSWRV).

Leaves from	Vine growth stages			
	UL	FS	CC	BR
Ascorbate redox state[a]				
HV	0.72 ± 0.05	0.75 ± 0.05	0.69 ± 0.05	0.69 ± 0.05
BWSV symptomless	0.36 ± 0.02	0.38 ± 0.02	0.15 ± 0.01	0.18 ± 0.02
symptomatic	n.p.[b]	n.p.	0.12 ± 0.01	0.28 ± 0.01
BWSWRV symptomless	0.07 ± 0.05	0.13 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
symptomatic	n.p.	n.p.	0.10 ± 0.01	0.10 ± 0.01
Glutathione redox state[a]				
HV	0.95 ± 0.05	0.95 ± 0.05	0.95 ± 0.05	0.79 ± 0.06
BWSV symptomless	0.83 ± 0.04	0.73 ± 0.04	0.69 ± 0.04	0.73 ± 0.04
symptomatic	n.p.	n.p.	0.66 ± 0.04	0.52 ± 0.02
BWSWRV symptomless	0.38 ± 0.01	0.55 ± 0.04	0.56 ± 0.04	0.45 ± 0.02
symptomatic	n.p.	n.p.	0.45 ± 0.02	0.37 ± 0.01

[a] Data are means of 15 replicates ± standard deviations.
[b] n.p. leaf typology not present.

spora in grapevine wood with brown wood streaking, and *F. mediterranea* was always associated with white rotted tissues. No *Botryosphaeriaceae* fungi or other grapevine trunk disease pathogens were isolated from diseased vines. No symptoms on foliage, wood, or berries were observed on healthy vines. Vines with brown wood streaking and vines with brown wood streaking and white rot showed 'tiger-stripe' symptoms on leaves, and spots, shrivelling and wilt of berries. These observations agreed with those previously described for vines affected by Esca complex pathogens (Bruno and Sparapano, 2007; Mondello *et al*., 2018).

The present study recorded differences in the contents of bleeding xylem sap and leaves between healthy and diseased vines. Bleeding of xylem sap is a process that characterizes grapevines and many other perennial plants as an effect of positive root pressure that transports water upward. Bleeding occurs in the springtime because increasing soil temperatures stimulated root pressure. Water fills the xylem vessels, dissolves, and pushes out air bubbles formed during the winter and restores xylem activity (Sperry *et al*., 1987). Cavitation reduces sap flux density and sap surface tension and induces xylem dysfunction (Hammond-Kosack and Jones, 2015). To bypass obstructions or cavitation, and restore vertical water conductivity, plants respond by producing new xylem conduits or refill cavitated vessels (Nardini *et al*., 2008).

Xylem-colonising pathogens, including *P. minimum* and *P. chlamydospora*, cause xylem dysfunction associated with imbalances between water absorption and transpiration. Permanent xylem blockage results from fungus presence and plant defence responses. Xylem-associated pathogens produce conidia, hyphae, high molecular weight substances and large molecules from cell wall breakdown. Host plants affected by tracheomycoses develop tyloses or gummoses as barriers that limit fungus invasion (Tyree and Zimmermann, 2002). Pathogen metabolites could develop thin films on host vessel inner wall surfaces, minimize sap frictional pressure and change xylem hydraulics, or increase transpiration flux (Yoder, 1980; Bruno *et al*., 2007). *F. mediterranea*, *P. minimum* and *P. chlamydospora* produce phytotoxic metabolites (Evidente *et al*., 2000; Tabacchi *et al*., 2000; Bruno and Sparapano, 2006a; Luini *et al*., 2010; Andolfi *et al*., 2011) and enzymes (Chiarappa, 1959; Mugnai *et al*., 1999; Marchi *et al*., 2001; Bruno and Sparapano, 2006c). Polyphenol-rich zones, tyloses and gels in xylem (Yadeta and Thomma, 2013), cell wall chemical modifications with suberin deposition (Pouzoulet *et al*., 2013), and the accumulation of pathogenesis-related-proteins contribute to impeding the spread of xylem-inhabiting pathogens in host wood. Oxidative burst, peroxidases, superoxide dismutase, glutathione S-transferase, phenolic compounds, stilbenes and phytoalexins are also activated by these pathogens (del Rio *et al*., 2004; Calzarano *et al*., 2016; 2017a; 2017b).

Presence of *F. mediterranea* and its wood-degrading action could alter xylem conductivity, and thus, the quantity of bleeding sap. As in previous studies (Bruno

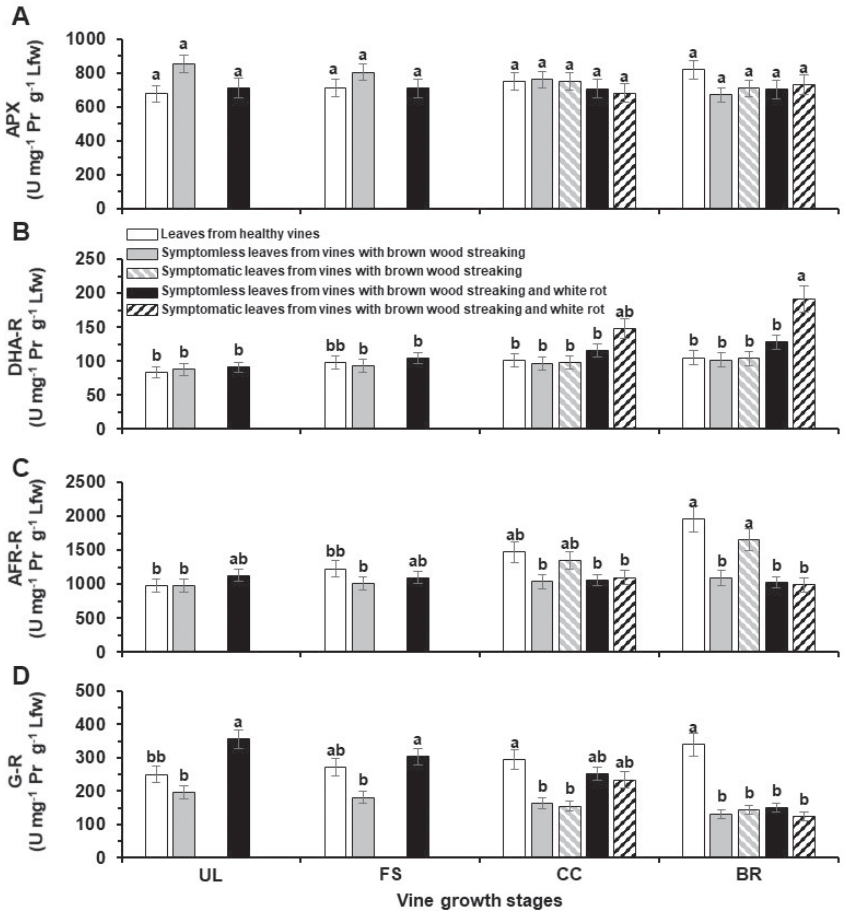

Figure 5. Activities of the redox enzymes: A) ascorbate peroxidase (APX), B) dehydroascorbate reductase (DHA-R), C) ascorbate free radical reductase (AFR-R), D) glutathione reductase (G-R), D) ascorbate free radical reductase (AFR-R) in cv. Italia grapevine leaves, collected during unfolded leaf (UL), fruit set (FS), cluster closing (CC) and bunch ripening (BR) vine growth stages, from infected or healthy grapevines. Data are means of 50 replicates ± standard deviations. For each parameter, values accompanied by the same letters are not significantly different ($P \leq 0.05$) according to Fisher's LSD test.

and Sparapano, 2006b; Bruno *et al*., 2007), diseased vines here analysed bled more abundantly than healthy vines.

Viscosity is the capacity of a fluid layer to run with an adjacent layer. In the present study, the dynamic viscosity coefficient increased from healthy vines to the vines with brown wood streaking or with white rot and brown wood streaking. These results suggest that substances produced by fungal pathogens, and molecules resulting from cell component degradation by pathogen lytic enzymes, added to vine response molecules (such as phenols, tannins, flavonoids), could affect dynamic viscosity coefficients, and, thus, the xylem sap flow.

The presence of several plant hormones in host xylem sap has been assessed in herbaceous and woody plants, including grapevine (Niim and Torikata, 1978). In the present study the greatest auxin- and kinetin-like activities were detected in diseased vines. Auxin activity increased when the vines showed white rot symptoms associated with *F. mediterranea*.

This study also demonstrated that grapevine leaf surface area, leaf fresh and dry weights, and chlorophyll contents varied according to the host growth stages, but were significantly affected by the behaviour of the pathogens inside the woody tissues and, consequently, by altered physiological functions. Symptomatic leaves always had the least fresh and dry weights, and total chlorophyll concentrations. Diseased plants had physiological dysfunctions related to photosynthesis (i.e., reduced photosynthetic pigments) similar to those reported for Esca affected 'Cabernet Sauvignon' and 'Merlot' grapevines (Christen *et al*., 2007). Decreased gas exchange and chlorophyll fluorescence, and repression of photosynthesis-related genes have been detected

for presymptomatic leaves of Esca-affected vines (Magnin-Robert *et al*., 2011). Chlorophyll decline leads to decreased photosynthesis efficiency, organic carbon production, host growth, and general plant health. Symptomatic leaves showed further reductions in fresh and dry weights associated with lamina necrosis and wilt. The main aetiological agents of the Esca complex produced phytotoxic metabolites involved, *in vitro* and *in planta*, with symptom development on leaves (Evidente *et al*., 2000; Bruno and Sparapano, 2006a, 2006b; Luini *et al*., 2010; Andolfi *et al*., 2011). Chlorophyll decline could also explain the decrease in leaf weight because of low photosynthesis efficiency. Activation of plant defence mechanisms possibly modified host sugar metabolism, moving towards production of new molecules (Jeandet *et al*., 2002) and reducing carbohydrates used for plant growth and reproduction.

The experiments carried out in the present study have shown increases in leaf surface area in diseased plants. These results are similar to those where growth regulator activities have been measured for bleeding xylem sap from diseased vines. Hormone-like substances with auxin activity, produced by *P. minimum*, *P. chlamydospora* and especially by *F. mediterranea*, could contribute to host cell hyperplasia, hypertrophy, and leaf lamina expansion.

The most prominent features of plant responses to pathogens and, in general, against stresses, is the 'oxidative burst', i.e. the rapid increase in the cellular concentration of Reactive Oxygen Species (ROS) and mainly H_2O_2 (De Gara *et al*., 2003; Torres *et al*., 2009). In the present study, leaves collected from healthy vines had the lowest H_2O_2 concentrations during all the considered growth stages. In leaves of diseased plants, H_2O_2 increased about 3-fold in symptomless leaves compared to healthy ones, regardless of growth stage, and reached greatest amounts in symptomatic plants. This evidence suggests strong correlation between the metabolic activity of pathogens and H_2O_2 production and accumulation in host leaves. H_2O_2 in leaves of diseased plants may act as an antimicrobial to counteract pathogens, or as a strengthener of cell wall polymers, a promoter of phytoalexin synthesis, or in triggering of programmed cell death. However, infected vines failed their defence upgrades and eventually suffered the effects of oxidative stress. H_2O_2 increased oxidative stress and damaged integrity and functionality of cell membranes (Pérez *et al*., 2002), by lipid peroxidation of unsaturated fatty acids. The lipid peroxidation levels clearly showed changes in cell membranes. Levels of MDA, a product of lipid peroxidation, were correlated to membrane damage (Heath and Packer, 1968; Soares *et al*., 2019).

Plants produce ROS-scavenging mechanisms under biotic and abiotic stresses. Enzymes, including superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, and glutathione reductase (Zhang and Kirkham, 1996; Lee and Lee, 2000), and non-enzymatic antioxidants such as tocopherols, ascorbic acid, and glutathione (Noctor and Foyer, 1998), functioned as ROS detoxifiers. Ascorbic acid is considered a key molecule for H_2O_2 elimination. Ascorbic acid reacts with H_2O_2 directly or by ascorbate peroxidase, a Class I heme-peroxidase that uses ascorbic acid as an electron donor and is the main peroxidase involved in H_2O_2 detoxification (Asada, 1999). Monodehydroascorbate reductase, dehydroascorbate reductase and reduced glutathione regenerate ascorbic acid. Glutathione controls the redox state in plant cells under abiotic and biotic stresses, and this compound is involved in ascorbic acid regeneration through the Ascorbate-Glutathione cycle (Noctor and Foyer, 1998; Asada, 1999; Mittler, 2002; Hung *et al*., 2005). If the Ascorbate-Glutathione cycle operated well, ascorbic acid content and ascorbate peroxidase activity increased in host leaves of infected vines as expected (De Gara *et al*., 2003; Hung *et al*., 2005). However, leaves of diseased vines, during all the four growth assessed, showed ascorbic acid, and reduced glutathione concentrations that were less than in healthy vines. This trend was also confirmed for total ascorbate presence in bleeding xylem sap. In contrast, presence of pathogens in the grapevine trunks stimulated total glutathione contents.

L-Ascorbic acid (2,3-didehydro L-threo-hexano-1,4-lactone, the well-known functional form of vitamin C) is a multifunction molecule (Gallie, 2013). Ascorbic acid also plays indirect roles in plant responses against pathogens, changes in gene expression and resistance against biotic and abiotic stresses (Khan *et al*., 2011).

The results from the present study allow development of the hypothesis that *P. minimum* and *P. chlamydospora*, or these fungi in association with *F. mediterranea*, can affect host antioxidant defences based on both glutathione and ascorbic acid. This change could be correlated with an unbalanced host oxidative state, damage to membrane integrity and appearance leaf necrosis symptoms.

Leaves of diseased vines showed significant decreases in redox state, and shift of ascorbic acid and glutathione towards oxidized forms. Ascorbate and glutathione redox states provide reliable estimation of cellular oxidative stress (Munné-Bosch and Alegre, 2003). In the present study, diseased vines were more stressed than healthy vines. To explain this change in physiological status, we suggest that the cause was the metabolic complex produced by *F. mediterranea*, *P. minimum* and

P. chlamydospora (Bruno and Sparapano, 2006a, 2006b; Bruno *et al*., 2007). The accumulation of resveratrol, benzoic acid derivatives and flavonols as host defence compounds (Amalfitano *et al*., 2000; Jeandet *et al*., 2002; Bruno and Sparapano, 2006b; Calzarano *et al*., 2016, 2017a, 2017b) were also suggested as causes. Phenols or flavonoids contribute to ascorbic acid oxidation in the scavenging of H_2O_2 in grape leaves (Yamasaki *et al*., 1997), i.e., phenoxy or flavonoxy radicals accept electrons from ascorbic acid and produce the monodehydroascorbate radical (Pérez *et al*., 2002).

To prevent oxidative stress, following the glutathione-ascorbate metabolic pathway, ascorbate peroxidase reduced H_2O_2 to water converting ascorbic acid to monodehydroascorbate that spontaneously disproportionate into ascorbic and dehydroascorbic acids. Using reduced glutathione, dehydroascorbate reductase reduced dehydroascorbic acid to ascorbate and produced oxidized glutathione. Finally, glutathione reductase reduced oxidized glutathione using NADPH as an electron donor (Asada, 1999). Under our conditions, the activities of enzymes regenerating ascorbic acid, also active in diseased as well as healthy vines, did not show any marked differences. This implied that ascorbate peroxidase, dehydroascorbate reductase, ascorbate free radical reductase, and glutathione reductase made a non-significant contribution to increasing ascorbic acid regeneration in diseased vines. Therefore, our data on ascorbate peroxidase, the key-enzymes of Ascorbate-Glutathione cycle, were in contrast with the low concentrations in cv Sultanina protoplasts (Papadakis *et al*., 2001) and the absence in 'Sultana' leaves (Pérez *et al*., 2002). This difference could be due to grape varieties, stress considered, plant materials and assay procedure applied.

In conclusion, the results of the present study suggest that *F. mediterranea*, *P. minimum* and *P. chlamydospora* interfere with several morphological, physiological, and biochemical functions in 'Italia' grapevines. Alterations affected bleeding xylem sap and leaves. Flux, dynamic viscosity, and growth regulator activity distinguished between bleeding xylem sap of vines infected with *P. minimum* and *P. chlamydospora* and those infected with *P. minimum*, *P. chlamydospora* and *F. mediterranea*. Leaf surface area, fresh, and dry weights, chlorophyll, hydrogen peroxide contents, lipid peroxidation, and redox states were altered in leaves of all assessed diseased vines. The presence of *F. mediterranea* in wood tissues of infected vines further debilitated the host physiological status. These alterations were detected in symptomatic leaves and, at low intensity, in symptomless leaves of diseased vines. These deleterious effects marked a presymptomatic stage, for irreversible changes inducing symptoms appearance. In diseased vines, low concentrations of ascorbic acid, reduced glutathione, and moderate levels of dehydroascorbic acid and oxidized glutathione were also associated with increased amounts of H_2O_2 and MDA, and considerable oxidative stress. Under these conditions, scavenging enzymes were not able to sufficiently restore the balance between ROS and the antioxidants managing host stress conditions. The stresses caused by oxidative unbalance increased lipid peroxidation of unsaturated fatty acids of host membranes, damaged membrane integrity, and contributed to cell death and development of leaf symptoms. The present study has indicated that Ascorbate-Glutathione cycle is likely to be involved in grapevine susceptibility to fungi associated with the Esca complex.

ACKNOWLEDGEMENTS

Mr L. Scarola (University of Bari Aldo Moro - Di.S.S.P.A.) gave technical support in this study. Ms Tonia Gemma and Ms Sylvia J. Stastny provided helpful discussions and skilful proof reading of the manuscript of this paper.

LITERATURE CITED

Amalfitano C., Evidente A., Surico G., Tegli S., Bertelli E., Mugnai L., 2000. Phenols and stilbene polyphenols in the wood of esca-diseased grapevine. *Phytopathologia Mediterranea* 39: 178–183. https://oajournals.fupress.net/index.php/pm/article/view/4799/4797.

Andolfi A., Mugnai L., Luque J., Surico G., Cimmino A., Evidente A., 2011. Phytotoxins produced by fungi associated with grapevine trunk diseases. *Toxins* 3: 1569–1605. https://doi.org/10.3390/toxins3121569.

Asada K., 1999. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology* 50: 601–639. https://doi.org/10.1146/annurev.arplant.50.1.601.

Baggiolini M., 1979. Stades repères de la vigne. In: *La Défence des Plantes Cultivées* (R. Bovey, ed.), Payot, Lausanne, CH, 456–461.

Baránek M., Armengol J., Holleinová V., Pečenka J., Calzarano F., … Eichmeier A., 2018. Incidence of symptoms and fungal pathogens associated with grapevine trunk diseases in Czech vineyards: first example from a north-eastern European grape-growing region. *Phytopathologia Mediterranea* 57:

449–458. https://doi.org/10.14601/Phytopathol_Mediterr-22460.

Bertsch C., Ramírez-Suero M., Magnin-Robert M., Larignon P., Chong J., … Fontaine F., 2012. Grapevine trunk diseases: complex and still poorly understood. *Plant Pathology* 62: 243–265. https://doi.org/10.1111/j.1365–3059.2012.02674.x

Bruno G., Sparapano L., 2006a. Effects of three esca-associated fungi on *Vitis vinifera* L.: I. Characterization of secondary metabolites in culture media and host responses to the pathogens in calli. *Physiological and Molecular Plant Pathology* 69: 209–223. https://doi.org/10.1016/j.pmpp.2007.04.008.

Bruno G., Sparapano L., 2006b. Effects of three esca-associated fungi on *Vitis vinifera* L.: II. Characterization of biomolecules in xylem sap and leaves of healthy and diseased vines. *Physiological and Molecular Plant Pathology* 69: 195–208. https://doi.org/10.1016/j.pmpp.2007.04.007.

Bruno G., Sparapano L., 2006c. Effects of three esca-associated fungi on *Vitis vinifera* L.: III. Enzymes produced by the pathogens and their role in fungus-to-plant or in fungus-to-fungus interactions. *Physiological and Molecular Plant Pathology* 69: 182–194. https://doi.org/10.1016/j.pmpp.2007.04.006.

Bruno G., Sparapano L., 2007. Effects of three esca-associated fungi on *Vitis vinifera* L.: V. Changes in the chemical and biological profile of xylem sap from diseased cv. Sangiovese vines. *Physiological and Molecular Plant Pathology* 71: 210–229. https://doi.org/10.1016/j.pmpp.2008.02.005.

Bruno G., Sparapano L., Graniti A., 2007. Effects of three esca-associated fungi on *Vitis vinifera* L.: IV. Diffusion through the xylem of metabolites produced by two tracheiphilous fungi in the woody tissue of grapevine leads to esca-like symptoms on leaves and berries. *Physiological and Molecular Plant Pathology* 71: 106–124. https://doi.org/10.1016/j.pmpp.2007.12.004.

Calzarano F., Amalfitano C., Seghetti L., Cozzolino V., 2009. Nutritional status of vines affected with esca proper. *Phytopathologia Mediterranea* 48: 20–31. https://doi.org/10.14601/Phytopathol_Mediterr-2872

Calzarano F., Di Marco S., D'agostino V., Schiff S., Mugnai L., 2014. Grapevine leaf stripe disease symptoms (esca complex) are reduced by a nutrients and seaweed mixture. *Phytopathologia Mediterranea* 53: 543–558. https://doi.org/10.14601/Phytopathol_Mediterr-15253.

Calzarano F., D'Agostino V., Pepe A., Osti F., Della Pelle F., … Di Marco S., 2016. Patterns of phytoalexins in the grapevine leaf stripe disease (esca complex)/grapevine pathosystem. *Phytopathologia Mediterranea* 55: 410–426. https://doi.org/10.14601/Phytopathol_Mediterr-18681.

Calzarano F., Osti F., D'Agostino V., Pepe A., Di Marco S., 2017a. Mixture of calcium, magnesium and seaweed affects leaf phytoalexin contents and grape ripening on vines with grapevine leaf stripe disease. *Phytopathologia Mediterranea* 56: 445–457. https://doi.org/10.14601/Phytopathol_Mediterr-22023.

Calzarano F., Osti F., D'Agostino V., Pepe A., Della Pelle F., … Di Marco S., 2017b. Levels of phytoalexins in vine leaves with different degrees of grapevine leaf stripe disease symptoms (Esca complex of diseases). *Phytopathologia Mediterranea* 56: 494–501. https://doi.org/10.14601/Phytopathol_Mediterr-22055.

Calzarano F., Di Marco S., 2018. Further evidence that calcium, magnesium and seaweed mixtures reduce grapevine leaf stripe symptoms and increase grape yield. *Phytopathologia Mediterranea* 57: 459–471. https://doi.org/10.14601/Phytopathol_Mediterr-23636

Chiarappa L., 1959. Extracellular oxidative enzymes of wood-inhabiting fungi associated with the heart rot of living grapevines. *Phytopathology* 49: 578–582.

Christen D., Schönmann S., Jermini M., Strasser R.J., Défago, G., 2007. Characterization and early detection of grapevine (*Vitis vinifera*) stress responses to esca disease by in situ chlorophyll fluorescence and comparison with drought stress. *Environmental and Experimental Botany* 60: 504–514. Doi:10.1016/j.envexpbot.2007.02.003.

Ciccarone C., Graniti A., Schiaffino A., Marras F., 2004. Molecular analysis of Fomitiporia mediterranea isolates from esca-affected grapevines in southern Italy. *Phytopathologia Mediterranea* 43: 268–272. https://oajournals.fupress.net/index.php/pm/article/view/5056

Claverie M., Notaro M., Fontaine F., Wery J., 2020. Current knowledge on Grapevine Trunk Diseases with complex etiology: a systemic approach. *Phytopathologia Mediterranea* 59: 29–53. Doi: 10.14601/Phyto-11150

De Gara L., de Pinto M., Tommasi F., 2003. The antioxidant systems via-á-via reactive oxygen species during plant-pathogen interaction. *Plant Physiology and Biochemistry* 41: 863–870. https://doi.org/10.1016/S0981–9428(03)00135-9.

de Rolland G., 1873. Considérationes physiologiques sur l'apoplexie de la vigne. *Bulletin de la Société d'Agriculture de France* 5: 539–545.

del Rio J.A., Gómez P., Báidez A., Fuster M.D., Ortuño A., Frías V., 2004. Phenolic compounds have a role in the defence mechanism protecting grapevine against

the fungi involved in Petri disease. *Phytopathologia Mediterranea* 43: 87–94. DOI: 10.14601/Phytopathol_Mediterr-1736.

Elena G., Bruez E., Rey P., Luque J., 2018. Microbiota of grapevine woody tissues with or without esca-foliar symptoms in northeast Spain. *Phytopathologia Mediterranea* 57: 425–438. Doi: https://doi.org/10.14601/Phytopathol_Mediterr-23337.

Evidente A. Sparapano L., Andolfi A., Bruno G., 2000. Two naphthalenone pentaketides isolated from liquid cultures of *Phaeoacremonium aleophilum*, a fungus associated with esca disease syndrome. *Phytopathologia Mediterranea* 39: 162–168. https://oajournals.fupress.net/index.php/pm/article/view/4807/4805.

Fischer M., 2002. A new wood-decaying basidomycete species associated with esca of grapevine: *Fomitiporia mediterranea* (*Hymenochaetales*). *Mycological Progress* 1(3): 315–324. https://doi.org/10.1007/s11557–006-0029-4.

Fischer M., 2006. Biodiversity and geographic distribution of basidiomycetes causing esca-associated white rot in grapevine: a worldwide perspective. *Phytopathologia Mediterranea* 45(S): 30–42. https://oajournals.fupress.net/index.php/pm/article/view/5187.

Fontaine F., Pinto C., Vallet J., Clément C., Gomes A.C., Spagnolo A., 2016. The effects of grapevine trunk diseases (GTDs) on vine physiology. *European Journal of Plant Pathology* 144: 707–721. https://doi.org/10.1007/s10658–015-0770-0.

Gallie D.R., 2013. L-Ascorbic acid: a multifunctional molecule supporting plant growth and development. *Scientifica* 795964. https://doi.org/10.1155/2013/795964.

Graniti A., Surico G., Mugnai L., 2000. Esca of grapevine: a disease complex or a complex of diseases? *Phytopathologia Mediterranea* 39: 16–20. https://oajournals.fupress.net/index.php/pm/article/view/4787/4785.

Guérin-Dubrana L., Labenne A., Labrousse J.C., Bastien S., Rey P., Gégout-Petit A., 2013. Statistical analysis of grapevine mortality associated with esca or Eutypa dieback foliar expression. *Phytopathologia Mediterranea* 52: 276–288.

Hammond-Kosack K.E., Jones J.D.G., 2015. Responses to plant pathogens. In: *Biochemistry and Molecular Biology of Plants*, 2nd ed. (Buchanan B.B., Gruissem W., Jones, R.L., ed.), John Wiley & Sons Ltd., New Jersey, USA, 984–1050.

Harborne J., 1973. Nitrogen compounds. In: *Phytochemical Methods. A guide to modern techniques of plant analysis* (J.B. Harborne, ed.), Chapman & Hall, London, UK, 176–221.

Heath R.L., Packer L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125: 189–198. https://doi.org/10.1016/0003–9861(68)90654-1.

Hung S.H., Yu C.W., Lin C.H., 2005. Hydrogen peroxide functions as a stress signal in plants. *Botanical Bulletin of Academia Sinica* 46: 1-10. https://ejournal.sinica.edu.tw/bbas/content/2005/1/Bot461–01.pdf.

Jayawardena R.S., Purahong W., Zhang W., Wubet T., Li X., … Yan J., 2018. Biodiversity of fungi on *Vitis vinifera* L. revealed by traditional and high-resolution culture-independent approaches. *Fungal Diversity* 90: 1–84. https://doi.org/10.1007/s13225–018-0398-4

Jeandet P., Douillet-Breuil A.C., Bessis R., Debord S., Sbaghi M., Adrian M., 2002. Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *Journal of Agricultural and Food Chemistry* 50: 2731–2741. https://doi.org/10.1021/jf011429s.

Khan T.A., Mazid M., Mohammad F., 2011. Role of ascorbic acid against pathogenesis in plants. *Journal of Stress Physiology & Biochemistry* 7: 222–234. http://www.jspb.ru/issues/2011/N3/JSPB_2011_3_222–234.pdf.

Lambert C.K.K.I., Lucas S., Télef-Micoleau N., Mérillon J.M., Cluzet S., 2013. A faster and stronger defence response: one of the key elements in grapevine explaining its lower susceptibility to esca? *Phytopathology* 103: 1028–1034. http://dx.doi.org/10.1094/PHYTO-11–12-0305-R.

Lecomte P., Darrieutort G., Liminana J. M., Comont G., Muruamendiaraz A., … Fermaud M., 2012. New insights into esca of grapevine: The development of foliar symptoms and their association with xylem discoloration. *Plant Disease* 96: 924–934. DOI: 10.1094/PDIS-09–11-0776-RE.

Lee D.H., Lee C.B., 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. *Plant Science* 159: 75–85. https://doi.org/10.1016/s0168–9452(00)00326-5.

Lima M.R.M., Felgueiras M.L., Graca G., Rodrigues J.E.A., Barros A., … Dias, A.C., 2010. NMR metabolomics of esca disease-affected *Vitis vinifera* cv. Alvarinho leaves, *Journal of Experimental* Botany 61: 4033–4042. https://doi.org/10.1093/jxb/erq214.

Luini E., Fleurat-Lessard P., Rousseau L., Roblin G., Berjeaud J., 2010. Inhibitory effects of polypeptides secreted by the grapevine pathogens *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* on plant cell activities. *Physiological and Molecular Plant Pathology* 74: 403–411. https://doi.org/10.1016/j.pmpp.2010.06.007.

Magnin-Robert M., Letousey P., Spagnolo A., Rabenoelina F., Jacquens L., … Fontaine F., 2011. Leaf strip of

esca induces alteration of photosynthesis and defence reactions in presymptomatic leaves. *Functional Plant Biology* 38: 856–866. http://dx.doi.org/10.1071/FP11083.

Marchi G., Roberti S., D'Ovidio R., Mugnai L., Surico G., 2001. Pectic enzymes production by *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* 40: S407-S416. https://oajournals.fupress.net/index.php/pm/article/view/4921/4919.

Marsais P., 1923. Maladie de l'esca. *Revue de viticulture* 59: 8-14.

Mittler R., 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* 7: 405–410. https://doi.org/10.1016/s1360–1385(02)02312-9.

Mondello V., Songy A., Battiston E., Pinto C., Coppin C., … Fontaine F., 2018. Grapevine Trunk Diseases: a review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Disease* 102: 1189–1217. https://doi.org/10.1094/PDIS-08–17-1181-FE.

Moreno-Sanz P., Lucchetta G., Zanzotto A., Loureiro M.D., Suarez B., Angelini E., 2013. Fungi associated to grapevine trunk diseases in young plants in Asturias (Northern Spain). *Horticultural Science (Prague)* 40: 138–144.

Mugnai L., Graniti A., Surico G., 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83: 404–418. https://doi.org/10.1094/PDIS.1999.83.5.404.

Munné-Bosch S., Alegre L., 2003. Drought-induced changes in the redox state of a-tocopherol, ascorbate and diterpene carnosic acid in chloroplast of Labiatae specie differing in carnosic acid contents. *Plant Physiology* 131: 1-10. https://doi.org/10.1104/pp.102.019265.

Nardini A., Ramani M., Gortan E., Salleo S., 2008. Vein recovery from embolism occurs under negative pressure in leaves of sunflower (*Helianthus annuus*). *Physiologia Plantarum* 133: 755–764. https://doi.org/10.1111/j.1399–3054.2008.01087.x

Niim Y., Torikata H., 1978. Changes in endogenous plant hormones in the xylem sap of grapevines during development *Journal of the Japanese Society for Horticultural Science* 47 181–187. https://www.jstage.jst.go.jp/article/jjshs1925/47/2/47_2_181/_pdf.

Noctor G., Foyer C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control, *Annual Review of Plant Physiology and Plant Molecular Biology* 49: 249–279. https://doi.org/10.1146/annurev.arplant.49.1.249.

Paciolla C., Ippolito M.P., Logrieco A., Dipierro N., Mulè G., Dipierro S., 2008. A different trend of antioxidant defense responses makes tomato plants less susceptible to beauvericin than to T-2 mycotoxin phytotoxicity. *Physiological and Molecular Plant Pathology* 72: 3-9. https://doi.org/10.1016/j.pmpp.2008.06.003.

Papadakis A.K., Siminis C.I., Roubelakis-Angelakis K.A., 2001. Reduced activity of antioxidant machinery is correlated with suppression of totipotency in plant protoplasts. *Plant Physiology* 126: 434–441. https://doi.org/10.1104/pp.126.1.434.

Pérez F.J., Villegas D., Mejia N., 2002. Ascorbic acid and flavonoid-peroxidase reaction as a detoxifying system of H_2O_2 in grapevine leaves. *Phytochemistry* 60: 573–580. https://doi.org/10.1016/S0031–9422(02)00146-2.

Petit A.N., Vaillant N., Boulay M., Clement C., Fontaine F., 2006. Alteration of photosynthesis in grapevines affected by esca. *Phytopathology* 96: 1060–1066. https://doi.org/10.1094/phyto-96–1060.

Pouzoulet J., Jacques A., Besson X., Dayde J., Mailhac N., 2013. Histopathological study of response of *Vitis vinifera* cv. Cabernet sauvignon to bark and wood injury with and without inoculation by *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* 52: 313–323. doi:10.14601/Phytopathol_Mediterr-12581

Ravaz L., 1898. Sur le folletage. *Revue de Viticulture* 10: 184–186.

Soares C., Carvalho M.E.A., Azevedo R.A., Fidalgo F., 2019. Plants facing oxidative challenges—A little help from the antioxidant networks. *Environmental and Experimental Botany* 161: 4–25. https://doi.org/10.1016/j.envexpbot.2018.12.009.

Sperry J.S., Holbrook N.M., Zimmermann M.H., Tyree M.T., 1987. Tyree, spring filling of xylem vessels in wild grapevine. *Plant Physiology* 83: 414–417. https://doi.org/10.1104/pp.83.2.414.

Surico G., 2009. Towards a redefinition of the diseases within the esca complex of grapevine. *Phytopathologia Mediterranea* 48: 5–10. https://oajournals.fupress.net/index.php/pm/article/view/5262/5260.

Surico G., Mugnai L., Marchi G., 2006. Older and more recent observations on esca: A critical review. *Phytopathologia Mediterranea* 45: S68–S86. DOI: 10.14601/Phytopathol_Mediterr-1847.

Tabacchi R., Fkyerat A., Poliart C., Dubin G.M., 2000. Phytotoxins from fungi of esca of grapevine. *Phytopathologia Mediterranea* 39: 156–161. https://oajournals.fupress.net/index.php/pm/article/view/4800/4798.

Torres M.A., Jonathan D.G., Dangl J.L., 2006. Reactive oxygen species signaling in response to pathogen. *Plant Physiology* 141: 373–378. https://doi.org/10.1104/pp.106.079467.

Tyree M.T., Zimmermann M.H., 2002. *Xylem structure and the ascent of sap.* 2nd ed. Springer, Berlin, D, 284 pp.

Valtaud C., Thibault F., Larignon P., Berstch C., Fleurat-Lessard P., Bourbouloux A., 2011. Systemic damage in leaf metabolism caused by esca infection in grapevines. *Australian Journal of Grape and Wine Research* 17: 101–110. https://doi.org/10.1111/j.1755–0238.2010.00122.x.

Wilcox W.F., Gubler W.D., Uyemoto J.K., 2015. *Compendium of Grape Diseases, Disorders, and Pests*. 2nd ed. APS Press, St. Paul, MN, 5–14.

Yadeta, K., Thomma, B.P.H.J., 2013. The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant Science* 4: Article 97. https://doi.org/10.3389/fpls.2013.00097. doi: 10.3389/fpls.2013.00097. eCollection 2013.

Yamasaki H., Sakihama Y., Ikehara N., 1997. Flavonoid-peroxidase reactions as a detoxification mechanism of plant cells against H_2O_2. *Plant Physiol*ogy 115: 1405–1412. https://doi.org/10.1104/pp.115.4.1405.

Yoder O.C., 1980. Toxins in pathogenesis. *Annual Review of Phytopathology* 18, 103–129. https://www.annualreviews.org/doi/pdf/10.1146/annurev.py.18.090180.000535

Zhang J., Kirkham M.B., 1996. Antioxidant responses to drought in sunflower and sorghum seedlings. *New Phytologist* 132: 361–373. https://doi.org/10.1111/j.1469–8137.1996.tb01856.x.

Zhao Z.R., Wu Z.L., Huang G.Q., Li G.R., 1992. An improved disk bioassay for determining activities of plant growth regulators. *Journal of Plant Growth Regulation* 11: 209–213. https://doi.org/10.1007/BF02115479.