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Xylem vessel size and indigenous mycorrhizae on grapevine cultivars: relationship with incidence of esca

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ABSTRACT

Xylem vessel size and indigenous mycorrhizae on grapevine cultivars: relationship with incidence of esca

Esca is considered the most destructive grapevine trunk disease, and it is mainly associated with several pathogenic fungi that inhabit plant woody tissues. Field observations have indicated that to date there are no grapevine cultivars with resistant to such vascular diseases. Previous studies have suggested that for grapevine, wide xylem vessel diameter favours development of *Phaeomoniella chlamydospora*, one of the fungi involved in esca, thus affecting disease susceptibility.

The aim of the present study was to analyse the characteristics of the xylem tissue of grapevine cultivars with different susceptibilities to disease, and the relationships between grapevines symptomatic for esca and beneficial indigenous soilborne arbuscular mycorrhizal fungi (AMF) in vineyards in Marche (central Italy).

Initially, the diameters and frequencies of xylem vessels were analysed across 51 grapevine cultivars, as 27 white berried and 24 red berried, for correlation with previous analysed esca incidence. Differences in vessel sizes among cultivars were detected, but no linear relationships were observed between vessel size and esca incidence in the field. Overall, the white-berried cultivars showed greater vessel diameters and disease incidence than the black-berried cultivars.

In the second part of this study, AMF symbiosis was analysed for roots from grapevines symptomatic and asymptomatic for esca that were collected from three vineyards. Total AMF symbiosis showed higher AMF colonisation for plants symptomatic for esca, compared to

nearby asymptomatic plants. Moreover, when analysed using droplet digital PCR assays, abundance of the AMF species *Rhizophagus irregularis* and *Funneliformis mosseae* showed high variability among plants.

These investigations show that the properties of the xylem vessels and the plant-rhizosphere interactions can provide useful information for investigations into the relationships between grapevine genotype structure and esca incidence.

RIASSUNTO

Dimensione dei vasi xilematici e simbiosi micorrizica indigena in cultivar di vite: relazione con l'incidenza del mal dell'esca

Il mal dell'esca è una delle più dannose malattie della vite. Si tratta di una sindrome complessa determinata da patogeni colonizzatori del legno. Attualmente non sono disponibili efficienti mezzi di lotta, né cultivar di vite resistenti. Test in laboratorio hanno evidenziato che l'incremento della dimensione dei vasi xilematici in vite favorisce lo sviluppo di *Phaeomoniella chlamydospora*, fungo coinvolto nella malattia. Lo scopo di questo studio è stato quello di analizzare le dimensioni dei vasi xilematici in cultivar di vite con differente incidenza di mal dell'esca, e la relazione di piante sintomatiche alla malattia con microrganismi benefici del suolo, i funghi arbuscolari micorrizici (AMF), che vivono in simbiosi con le radici delle piante, in vigneti marchigiani. In un primo lavoro è stata misurata la dimensione dei vasi xilematici di 51 cultivar di vite, 27 a bacca bianca e 24 a bacca nera, e correlata con l'incidenza del mal dell'esca, precedentemente analizzata. Differenze nella dimensione dei vasi è stata rilevata tra le cultivar, ma nessuna relazione lineare è stata osservata tra dimensione dei vasi e malattia in campo. Analizzando il dato medio complessivo, le cultivar a bacca bianca hanno mostrato un diametro dei vasi e un'incidenza della malattia maggiore rispetto a quelle a bacca nera.

Nel secondo lavoro è stata analizzata la simbiosi AMF in radici di piante di 3 vigneti senza e con sintomi di mal dell'esca. La simbiosi totale micorrizica ha evidenziato una maggiore colonizzazione di AMF nelle piante sintomatiche rispetto alle adiacenti asintomatiche. Variabile è risultata l'abbondanza delle specie di AMF,

Rhizophagus irregularis e *Funneliformis mosseae*, fra le piante, analizzata mediante la tecnica droplet digital PCR.

L'indagine ha evidenziato che le caratteristiche dei vasi xilematici e l'analisi dell'interazione pianta-rizosfera può fornire informazioni utili alla comprensione delle dinamiche del mal dell'esca in campo.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crop worldwide with a high economic relevance, based on hectares cultivated and economic value. Among limiting factors for the crop, the Grapevine trunk diseases (GTD) are considered one of the most destructive, having devastating consequences in the vineyards of the past three decades and are of rapidly growing concern in all wine producing countries (Bertsch et al., 2009). The worldwide economic cost for the only replacement of dead grapevine plants is estimated to be in excess of 1.5 billion dollars per year (International Organisation of Vine and Wine, 2011). The associated crop losses, together with the high financial and environmental costs to manage these diseases, have made grapevine and its pathogens an increasingly important research area (Borges et al., 2014). The causal agents of GTDs are known to be included several filamentous fungi, with species belonging to Botryosphaeriaceae, Diatrypaceae spp., *Phaeoacremonium minimum* (previously named *P. aleophilum*) (Gramaje et al., 2015; Roblin et al., 2019), *Phaeomoniella chlamydospora* and lignicolous basidiomycetes as *Fomitiporia* spp. and *Inocutis* spp., (Mugnai et al., 1999; Armengol et al., 2001; White et al., 2011). *P. chlamydospora* has been associated with young grapevine decline (Petri disease) and/or esca, *Diplodia seriata* has been associated with *Botryosphaeria* dieback, and *Inocutis* sp. has been associated with yellowish, soft and spongy wood decay (white rot) in adult plants (Mugnai et al., 1999; White et al., 2011).

GTD pathogens can infect propagating material and limit the growth of grapevines in newly planted vineyards (Halleen et al., 2003), or they can infect established vines through pruning wounds, causing relevant economic losses reducing longevity and

productivity of grapevines. The control of these diseases in grapevines is problematic, and for their management it is necessary the use of healthy planting material, as well as the prevention of pruning wound infections. Grapevines can be infected by aerial inoculum of Petri disease pathogens through pruning wounds (Eskalen et al., 2007; Quaglia et al., 2009; Rolshausen et al., 2010), but there are evidences that plants can become infected in nurseries during the propagation phase (Retief et al., 2006; Halleen et al., 2007; Aroca et al., 2010; Gramaje and Armengol, 2011), or in vineyards after planting in infested soils (Agustí-Brisach et al., 2013). The control of GTDs consists in an integrated management strategy that combine the use of preventive, pre-planting and post-planting measures to minimize pathogen dispersion and risk of infection of new plant (Jimenez-Diaz et al., 2012; Yadeta and Thomma, 2013). In the past, only the chemical treatments with sodium arsenite had a potential effect against GTDs (Larignon et al., 2008), but it has been prohibited, beginning in 2000, owing to its toxicity for environment and human health (Bisson et al., 2006). As consequence, the planting of cultivars resistant to trunk diseases remain one of the most durable and economically efficient control measure to prevent the diffusion of fungal pathogens in host tissues (Yadeta and Thomma, 2013). Nowadays, grape cultivars completely resistant to esca are unknown. However, there are degrees of susceptibility ranging from highly susceptible to tolerant, and the incidence of the foliar symptoms vary across cultivars within a given geographic area (Murolo and Romanazzi, 2014). Other studies reported that grape cultivars and rootstocks also differed in their degrees of susceptibility after experimental inoculation, as measured by differences in foliar symptom incidence, streaking in woody tissue, timing of bud breaking and shoot weight (Bertsch et al., 2013; Bruez et al., 2013; Travadon et al., 2013; Murolo

and Romanazzi, 2014). Among GTDs, *Botryosphaeria* dieback, *Eutypa* dieback and esca are the most destructive, causing significant economic losses (Mugnai et al., 1999; Molyneux et al., 2002). These fungal diseases have common properties: colonize the xylem tissues in trunks and cordons producing alterations in wood structure, which more firmly allowed the characterization of the concerned disease. Esca is a complex of at least two diseases: a white rot caused by *Fomitiporia mediterranea* and a tracheomycosis due to *P. chlamydospora* and *P. minimum*, or other species of *Phaeoacremonium* (Di Marco et al., 2011). In esca, wood infection is indicated in longitudinal sections by black streaks and, in further stages, by invasion of the center of the trunk by a white rot limited by a thick brown-red area. Esca is associated with mature grapevines, whose external symptoms are characterized by an interveinal chlorosis or reddening sing of the leaves known as "tiger stripes", shoot tip dieback and gray to brown spots appearing on the berries (black measles). Internal symptoms principally include black streaking of the xylem vessels, which sometimes can be associated with the presence of white rot that gradually transform the hard wood into soft-yellowish wood (Mugnai et al., 1999). Two forms of the esca disease can be observed in the field. The acute form, characterized by a sudden wilt of affected plants (apoplexy), is favored during hot and dry summers, while the chronicle form is characterized by the expression of progressive symptoms on leaves and berries, that can be intermittent from year to year (Mugnai et al., 1999; Romanazzi et al., 2009; Bertsch et al., 2013).

Several studies performed about *P. chlamydospora*, the main agent of esca disease, and xylem colonization evidence the ability of this pathogen to colonize the host xylem vessels, to degrade structural cell wall polymers, and also to produce phytotoxins (Morales-

Cruz et al., 2015). Considering that the loss of capacity for water transport can come about through loss of xylem vessel function by cavitation in a response to drought or freezing, or by occlusion of vessels by tyloses and gels in response to sapwood-dwelling pathogens, the study of xylem can give useful information to prevent or counteract esca.

Recent investigations evidence that the xylem characteristics, and specifically the diameter of vessels, play an important role in the resistance to wilt diseases, as esca, suggesting that this feature could explain the difference in grapevine cultivars resistance to esca disease (Pouzoulet et al., 2014), but the physiological mechanisms linking host resistance to those anatomical differences still remain hypothetical. Investigation on anatomical properties of xylem on grapevine cultivars can provide useful information for a better management of esca disease.

Mycorrhizal symbiosis is the most ancient and widespread form of fungal symbiosis with land plants (Redecker et al., 2000; Bonfante and Genre, 2008). About 80% of plants exchange mutual benefits with a group of root obligate biotrophs as the arbuscular mycorrhizal fungi (AMF) (Smith and Read, 2008). They are considered natural biofertilizers, facilitate mineral nutrient uptake from the soil in exchange for plant-assimilated carbon, promote water-stress tolerance and protection of plants against soilborne pathogens (Smith and Read, 2008; Berruti et al., 2016). Thus, AMF are primary biotic soil components which, when missing or impoverished, can lead to a less efficient ecosystem functioning. The considerable impact on plant growth places the management of these fungi in an integrated role in sustainable agriculture. AMF fulfills the need of host plant and provides support in many ways by induction of attenuated defense signaling against phytopathogenic

fungi. This makes plant more tolerant towards the attack of plant pathogens and pests, and enhances the genetic, biochemical and signaling factors responsible for its defense purpose.

Several studies were performed related to symbiosis of AMF inoculated in the host plants. These studies underline that the establishment of the AMF symbiosis implies remarkable changes in the physiology of the host plant. The changes span from alterations in the hormonal balance and transcriptional profile to modify primary and secondary metabolism (Hause et al., 2007; Liu et al., 2007; Schliemann et al., 2008; López-Ráez et al., 2010). This global reprogramming of plant functions has an impact on the plant interaction with the environment, modifying its responses to biotic and abiotic stresses. As a result, mycorrhizal plants are generally more tolerant to environmental stress. The consequences go beyond the individual level as they may influence plant diversity and productivity in terrestrial ecosystems (van der Heijden et al., 2008). The impact of the symbiosis in terms of resistance/tolerance to biotic stress differs among AMF isolates for a given plant-pathogen interaction. Moreover, such impact can be modulated by environmental conditions. Despite of this variability, general trends emerge from the multiple studies dealing with mycorrhiza in diverse pathosystems. Generally, enhanced resistance to soilborne pathogens has been reported in AMF plants. Furthermore, the symbiosis can also impact plant interactions with above-ground pathogens. In this case, the outcome ranges from enhanced resistance to increased susceptibility, largely depending on the pathogen life-style (Pozo and Azcón-Aguilar, 2007).

AMF can be used as potential biocontrol agents in integrated management programs for disease control (Sharma and Adholeya, 2000; Harrier and Watson, 2004; Whipps, 2004; Mukerji and Ciancio, 2007). A key factor

determining the effect of the symbiosis on interactions with other organisms seems to be the extension of root colonization by the AMF. With some exceptions (Caron et al., 1986; García-Garrido and Ocampo, 1988; St-Arnaud et al., 1997; Kapoor, 2008), reports on mycorrhizal protection against pathogens show the requirement of a well-established symbiosis prior to the challenge with the pathogen (Rosendahl, 1985; Cordier et al., 1998; Slezack et al., 2000; Khaosaad et al., 2007). Mycorrhiza-induced protection, improving plant nutrition, compensate consequently the damages caused by the pathogen. In addition to the nutritional aspects, changes in the plant architecture, root exudation and in the microbial populations in the rhizosphere, and the activation of plant defense mechanisms may all be relevant, depending on the organisms involved and the timing of the interactions (Whipps, 2004).

Many studies revealed that AMF reduce the incidence and/or severity of diseases as root rot or wilting caused by diverse fungi such as *Fusarium*, *Rhizoctonia*, *Macrophomina* and *Verticillium*, bacteria as *Erwinia carotovora*, and oomycetes as *Phytophthora*, *Pythium* and *Aphanomyces*. Similarly, a reduction of the deleterious effects by parasitic nematodes such as *Pratylenchus* and *Meloidogyne* has been reported in mycorrhizal plants (Pinochet et al., 1996; de la Peña et al., 2006; Li et al., 2006). Additional reports showed protection to other soil pathogens as *Armillaria mellea* in grapevine (Nogales et al., 2009), broadening the range of pathosystems in which AMF symbioses may have a protective effect. The effectiveness against such diverse range of pathogens confirms the broad spectrum character of the induced resistance associated to the AMF symbiosis. Studies comparing different fungal species or isolates highlighted that the degree of protection is highly dependent on the AMF involved (Kobra et al., 2009). Interestingly, many

studies point to a higher protector effect of *Glomus mosseae* (now renamed *Funneliformis mosseae*) in comparison to other AMF (Pozo et al., 2002; Utkhede, 2006; Ozgonen and Erkilic, 2007). Several mechanisms may operate simultaneously in the enhanced resistance of mycorrhizal plants to soil pathogens. In addition to a possible competition for photosynthates between the AMF and the pathogens, competition for colonization sites has been demonstrated. For example, in tomato roots, full exclusion of *Phytophthora* spp. from mycorrhized root cells was evidenced (Cordier et al., 1998). Mycorrhizal colonization is also known to induce changes in the root system architecture and morphology (Schellenbaum et al., 1991; Norman et al., 1996). These changes may alter the dynamics of infection by the pathogen, although direct evidences of such correlation are lacking. An altered pattern of root exudation may also impact the development of the pathogen. Mycorrhizal colonization leaded to modifications in root exudates composition that significantly reduced the sporulation of *Phytophthora fragariae* (Norman and Hooker, 2000) and altered the chemotactic response of the zoospores of *Phytophthora nicotianae* (Lioussanne et al., 2010). Since root exudates are key factors in shaping soil microbial communities (Badri and Vivanco, 2009), the changes in exudation into the mycorrhizosphere may result in alteration of the microbial communities including possible antagonistic organisms. This may be the reason underlying the biocontrol of pathogens in non-AMF species by co-culture with mycorrhizal plants (St-Arnaud et al., 1997). Because of the root localization of both pathogen and AMF, it is difficult to discern the local or systemic character of the protection observed. However, the use of split-root experimental systems allowing physical separation between AMF and pathogens has confirmed a reduction of disease symptoms in the non-mycorrhizal parts of the

mycorrhizal root systems. The systemic character of the induced resistance pointed to the involvement of plant defenses.

About the grapevine plants, they are dependent on mycorrhizae, since this plant has low-density roots and few root hairs. In the vineyards, the AMF communities mainly belong to the *Glomerales* group. Indeed, in the vineyard, AMF communities are highly influenced by the soil characteristics but also to a smaller extent by the host plant development stage (Schreiner and Mihara, 2009; Balestrini et al., 2010). Moreover, although AMF from grape roots mainly belong to the *Glomerales* group, members of the *Diversisporales* group are mainly found in sandy vineyard soils (Balestrini et al., 2010). However, little is known about the species composition of AMF communities associated to grapevine roots in the vineyards. Previous studies, based on the identification of AMF spores, reported the genus *Glomus* as being the most represented in vineyards (Karagiannidis et al., 1997). However, the spores were not able to mirror the AMF community present in the soil due to the seasonality and their different production rate.

As regard tracheomycotic diseases, for which actually no resistant grapevine cultivars are known, could be useful to analyze the context in which host plant and pathogen interfaced. In particular, we refer to xylem tissues where the pathogens inhabit, but also to the indigenous microorganisms naturally present in the rhizosphere that, in the plant host compete with pathogens.

With this aim, one experimental work was performed analyzing the size of xylem vessels in 51 white-berried and red-berried grape cultivars. This information was correlated with the incidence of esca disease in the field, previously assessed by Murolo and Romanazzi (2014), for verify that vessel dimension could be predict the esca susceptibility (Pouzolet et al., 2017). Another

experimental trial was achieved for assess the quantitative relationship among asymptomatic and symptomatic grapevines affected by esca and indigenous arbuscular mycorrhizal fungi.

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XYLEM VESSEL SIZE ON GRAPEVINE CULTIVARS: RELATIONSHIP WITH INCIDENCE OF ESCA DISEASE

Abstract

Esca disease is one of the most important grapevine trunk diseases of this crop. It seriously reduces the quality and quantity of grapevine production, and results in a shorter vineyard lifespan. Previous studies have suggested that wide xylem vessel diameter favours development on grapevine of *Phaeomoniella chlamydospora*, one of the fungi involved in esca, thus affecting disease susceptibility. In this study, 27 white-berried and 24 red-berried grapevine cultivars were grown in the same experimental vineyard and were analysed for xylem vessel sizes (as diameter and frequency) for correlation with esca incidence. In this study, the cultivars showed significant differences in the xylem vessel parameters. However, no linear relationship was detected between vessel size and esca incidence in the field. Overall, white-berried cultivars showed wider vessel diameters than red-berried cultivars. The relationship between xylem vessel size in the red-berried and white-berried cultivars and incidence of esca symptoms is discussed. We suggest that vessel anatomy profiles can provide useful information for further investigations on grapevine genotype structure–esca incidence relationships.

Keywords: esca; red-berried grapevines; vessel size; white-berried grapevines; xylem vessel diameter.

2.1 Introduction

In commercial grapevine (*Vitis vinifera*) growing, vascular diseases (e.g., *Eutypa* dieback, *Botryosphaeria* dieback, and esca) are the major factors that can limit crop productivity and vineyard longevity. The causal agents are a set of taxonomically unrelated fungi, which include *Eutypa lata*, *Phaeoacremonium minimum* (syn. *P. aleophilum*), *Phaeomoniella chlamydospora*, *Diplodia seriata* and *Neofusicoccum parvum*, as some of the most virulent and widespread (Gubler et al., 2010; Urbez-Torres, 2011; Travadon et al., 2012; Bertsch et al., 2013; Gramaje et al., 2015). These vascular diseases are detrimental to all viticulture areas worldwide, because they reduce vineyard longevity, cumulative yield and fruit quality (Munkvold et al., 1994; Mugnai et al., 1999; Lorrain et al., 2012; Bertsch et al., 2013).

Grapevines become infected primarily through pruning wounds (Rolshausen et al., 2010), either in nurseries during the propagation phase (Gramaje and Armengol, 2011) or in vineyards after planting in infested soils. These types of infection have been confirmed through *P. chlamydospora*, providing evidence that this pathogen is also soilborne (Agustí-Brisach et al., 2013). Field observations indicate that to date there are no known grapevine cultivars that are resistant to these vascular diseases (Bertsch et al., 2013). Therefore, the degrees of susceptibility range from highly susceptible to low susceptibility (Péros and Berger, 1994; Feliciano et al., 2004; Christen et al., 2007; Landi et al., 2012; Bruez et al., 2013; Travadon et al., 2013; Murolo and Romanazzi, 2014, Borgo et al., 2016).

Usually, in plant genotypes that are more tolerant to vascular diseases, despite pathogen colonisation, the disease symptoms are less severe than in sensitive genotypes. This suggests that tolerant plants can neutralise

the effects of virulence factors produced by these pathogens (Beckman and Roberts, 1995). This affects the colonisation of the host by spores transported in the xylem by the sap flow and hyphal growth. These hosts that are more tolerant to pathogens rapidly compensate for the loss of xylem vessels due to pathogen compartmentalisation, by differentiating new functional vessels to maintain sufficient stem water conductivity, which might also explain the tolerance to wilt diseases (Talboys, 1972; Fradin and Thomma, 2006).

In esca, which involves the woody tissues, the plant host can contain the spread of the pathogen through reduction of the spatial lumen of the plant xylem vessels, or by production of gels and development of tyloses that occlude the vessels and entrap the pathogen (Fradin and Thomma, 2006; Yadeta and Thomma, 2013). A spatio-temporal model of compartmentalisation of vascular wilt fungi in vessels has been hypothesised for annual plants, including tomato (*Solanum lycopersicum*) and cotton (*Gossypium* spp.) (Beckman and Roberts, 1995), and in perennial crops. It was observed that in *Ulmus* genotypes affected by Dutch elm disease, which is a wilt disease caused by *Ophiostoma novo-ulmi* (C.M. Brasier) (Brasier, 1991), the diameter of the xylem vessels has an important role in resistance to this wilt disease, whereby susceptible hosts have higher proportions of vessels of wide diameters, compared to resistant genotypes (Solla and Gil, 2002; Venturas et al., 2014). Recently, in grapevine cultivars, the amounts of inoculated *P. chlamydospora* and the necrotic lesion lengths correlated with vessel size (Pouzoulet et al., 2017). Thus, it was suggested that xylem vessel diameter affects disease susceptibility in grapevine, to potentially explain the differences between the highly susceptible and tolerant hosts to esca disease (Pouzoulet et al., 2014, Pouzoulet et al., 2017). However, the xylem anatomical properties linked to grapevine genotypes need

to be investigated further to confirm such a xylem vessel size–esca relationship.

The goal of this study was to investigate the xylem vessel properties, as vessel size and frequency, in 27 white-berried and 24 red-berried cultivars of *V. vinifera* of Italian national and international interest, and the known average values of disease incidence in the field. The correlation of these anatomical properties with disease incidence was also investigated.

2.2 Materials and methods

2.2.1 Sampling site

The xylem vessels size and density were investigated in 51 cultivars of *V. vinifera*, of which 24 were red berried (**Figure 1**) and 27 white berried (**Figure 2**), and which had been examined for esca incidence previously (Murolo and Romanazzi, 2014).

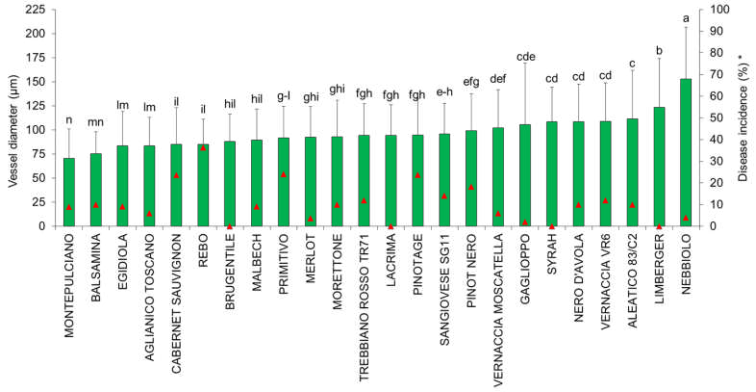

Figure 1 – Quantification of vessel diameter (columns) and esca disease incidence detected in the 24 red-berried grapevine cultivars. Data are means ±standard deviation. Different letters above columns indicate significant differences between cultivars (Fisher's tests; p ≤0.05). *, esca incidence (Murolo and Romanazzi, 2014); ▲, disease incidence.

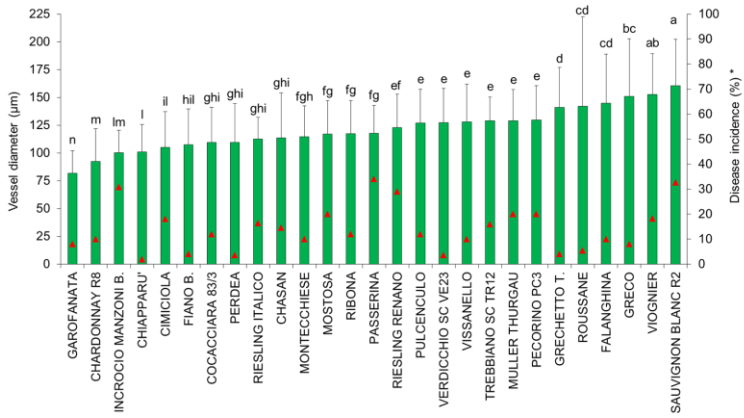

Figure 2 – Quantification of vessel diameter (columns) and esca disease incidence detected in the 27 white-berried grapevine cultivars. Data are means ±standard deviation. Different letters above column indicate significant differences between cultivars (Fisher's tests; p ≤0.05). *, esca incidence (Murolo and Romanazzi, 2014); ▲, disease incidence.

The experimental trial was performed in an experimental vineyard located in Carassai (AP), at 158 m a.s.l., in the central Italy Marche region (43°02'18.07''N; 13°39'39.41''E) of the Agency for Services in the Agro-Food Sector of Marche Region (ASSAM). The vineyard was planted in 1989. The grapevines were grafted onto Kober 5BB rootstock (*Vitis berlandieri* × *V. riparia*) and were grown under the cordon training system. The plants were spaced as 3 m between rows and 1 m within rows. Samples were collected in mid-February 2018. From each cultivar, four different plants were selected, and from each plant, one wood segment was cut from a 1-year-old initial productive spur (cordon). Each segment, contained 2-3 internodes, and measured 8-15 mm in diameter, and 30-50 mm in length.

2.2.2 Assessment of xylem vessel diameter and frequency

Measurements of tangential diameter and frequency of the vessels were performed on stem cross-sections, as described by Scholz et al. (2013). For each plant, 5 mm internode fragments were fixed in a formaldehyde/ acetic acid/ ethyl alcohol (FAA) solution (5/5/9; Sigma–Aldrich, St Louis, MO, USA) for 48 h at 4 °C, and then rinsed in deionised water and stored at 4 °C in 80% ethanol. Three cross-sections per plant, were analysed. Each cross-section (100 µm thick) was made using a cryostat microtome (MICROM HM 505 E) at -25 °C, and then stained on 0.05% Toluidine O solution, pH 4.3 (Sigma–Aldrich). Measurements of vessel diameters and vessel densities were taken under an upright light microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) at 4× magnification, equipped with a digital camera (Leica Microsystems CMS GmbH, Wetzlar, Germany), and using the application ruler of the X-Entry software (Alexasoft, Florence, Italy). For each section, the number of vessels and vessel densities were determined according to an automatic software grid measuring 1 mm^2 (Alexasoft, Florence, Italy) (**Figure 3**).

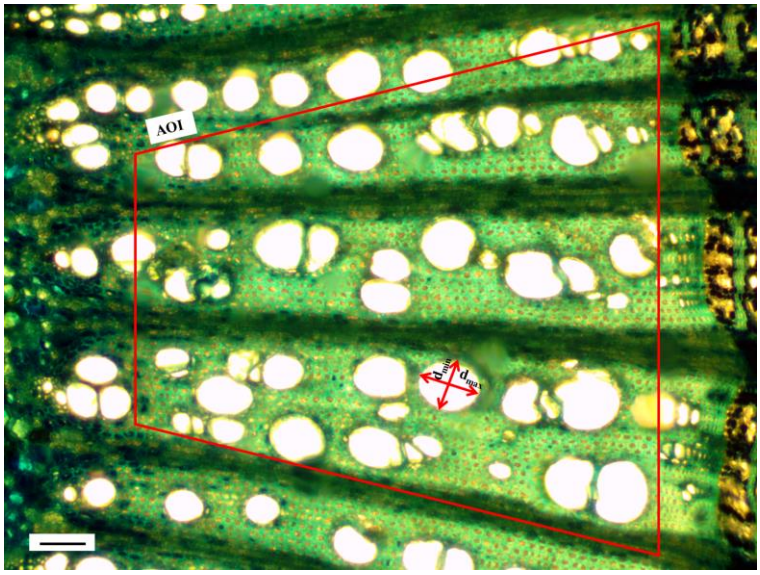

Figure 3 – Illustration of representative vessel dimensions measured under the light microscope (4× magnification) on a transverse section from a grapevine cultivar. The maximum diameter **(d_{max})** and minimum diameter **(d_{min})** are easily measured on transverse sections. To calculate the vessel density, the total numbers of vessels per 1 mm^2 were determined. The red polygon shows the area of interest (AOI). All vessels outside the AOI were excluded. Scale bar, 100 µm.

Tangential vessel diameters were calculated by measuring the average of the minimum and maximum diameters for each vessel, considering at least 120 vessels per cultivar. Vessel density was quantified by counting the average number of vessels per 1 mm^2. Nine diameter classes, at 20 µm steps, were considered: 40-59 µm, 60-79 µm, 80-99 µm, 100-119 µm, 120-139 µm, 140-159 µm, 160-179 µm, 180-199 µm, and finally, >200 µm, as modified from Pouzoulet (2017). The frequency of each diameter class was determined using Excel 2010 (Microsoft Corporation, Redmond, WA, USA). None of the cross-sections considered in the survey showed the presence of gels,

tyloses, gums or other elements that are typical of vessels with occluded lumens.

2.2.3 Statistical analysis

From each cultivar, tangential vessel diameters (n = 120) and vessel densities for the different diameter classes (n = 3) were subjected to one-way analysis of variance (ANOVA) followed by multiple comparisons of means, using Fisher's protected least significant difference (LSD) tests, at $p \leq 0.05$ (Statsoft, Tulsa, OK, USA). Finally, the datasets were tested for correlations using Pearson's coefficient (r), at $p < 0.05$ (Statsoft, Tulsa, OK, USA).

2.3 Results

2.3.1 Vessel diameter

High vessel diameter variability was recorded across all of the grapevine cultivars (**Figure 4**).

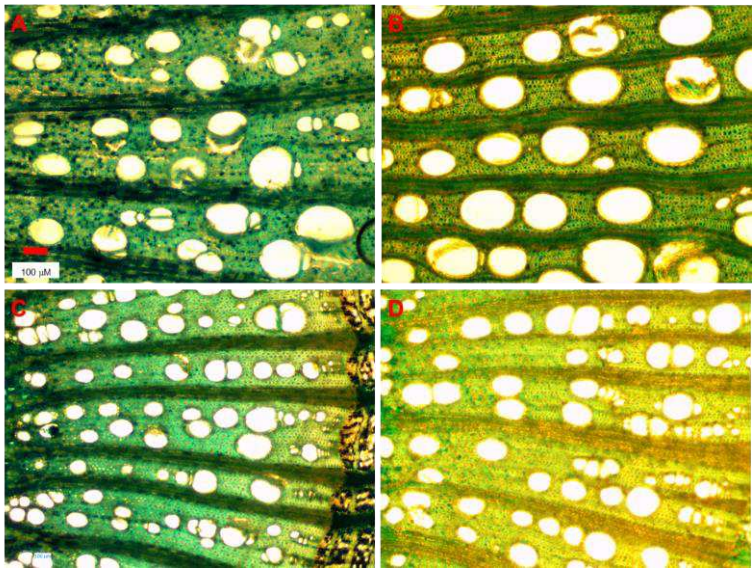

Figure 4 – Representative cross-sections observed under the light microscope (4× magnification) for the 'Sauvignon Blanc' R2 (**A**),

'Garofanata' (**B**), 'Nebbiolo' (**C**) and 'Montepulciano' (**D**) cultivars. Scale bar, 100 μm.

Red-berried cultivars – 'Nebbiolo' showed the largest vessel diameters (152.9 μm), followed by 'Limberger' (123.4 μm). Instead, 'Balsamina' and 'Montepulciano' showed the smallest diameters, at 75.4 μm and 70.6 μm, respectively. All of the other cultivars showed intermediate values (**Figure 1**).

White-berried cultivars - In this group, the vessel diameters ranged from 160.4 μm, for 'Sauvignon Blanc' R2, to 81.9 μm, for 'Garofanata'. All of the other cultivars showed intermediate values (**Figure 2**).

2.3.2 Vessel density and number of vessels per diameter class

The density and frequency of xylem vessel size classes differed according to the cultivars.

Red-berried cultivars - Vessel density was quantified as the average number of vessels per 1 mm^2, and this showed the highest value (60.0 vessels mm^{-2}) for 'Montepulciano', while 'Aleatico 83/c2' showed the lowest value (28.7 vessels mm^{-2}). In the other cultivars, intermediate values were found, with high variability among the cultivars (**Figure 5**).

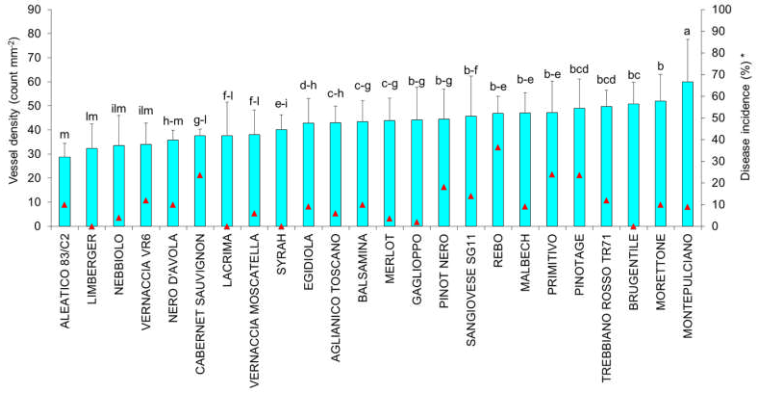

Figure 5 – Quantification of vessel density (columns) and disease incidence detected in the 24 red-berried grapevine cultivars. Data are means ±standard deviation. Different letters above columns indicate significant differences between cultivars (Fisher's tests; $p \leq 0.05$). *, esca incidence (Murolo and Romanazzi, 2014); ▲, disease incidence.

For the vessel size frequency, in the red-berried cultivars, the most representative diameter classes were 60-79 µm and 80-99 µm. However, some cultivars shown the highest diameter classes, such as 'Sangiovese' SG11 (7.3 vessels), 'Trebbiano rosso' TR71 (6.2 vessels), 'Limberger' (4.6 vessels) and 'Syrah' (5.8 vessels) where the vessels were included in the 100-119 µm class, and 'Pinot nero' (5.2 vessels) and 'Nebbiolo' (6.8 vessels), where the vessels were included in the >200 µm class. Other cultivars shown lower diameter classes, such as 'Montepulciano' (7.8 vessels), 'Cabernet Sauvignon' (6.3 vessels), 'Pinotage' (5.2 vessels) and 'Egidiola' (8.2 vessels), which showed vessels in the 40-59 µm class (**Figure 6**).

CULTIVAR	DIAMETER CLASSES									DISEASE INCIDENCE *
	40-59 µm	60-79 µm	80-99 µm	100-119 µm	120-139 µm	140-159 µm	160-179 µm	180-199 µm	> 200 µm	
REBO	5.2	8.6	7.6	5.8	1.6	0.8	0.0	0.2	0.0	36.4
PRIMITIVO	4.0	6.2	8.2	6.0	2.2	0.6	1.0	0.4	0.2	24.0
CABERNET SAUVIGNON	6.3	6.0	4.8	3.8	2.8	1.5	1.0	0.5	0.0	23.6
PINOTAGE	5.2	5.2	5.2	4.0	3.8	3.0	1.2	0.2	0.0	23.6
PINOT NERO	4.8	4.8	4.6	4.0	5.2	3.6	1.2	0.0	0.2	18.2
SANGIOVESE SG11	4.3	4.5	5.8	7.3	3.8	2.0	0.5	0.3	0.0	14.0
TREBBIANO ROSSO TR71	4.0	6.2	5.4	6.2	4.6	1.8	0.2	0.2	0.2	12.0
VERNACCIA VR6	1.4	4.2	8.2	5.4	4.2	2.0	1.8	0.4	1.2	12.0
ALEATICO 83/C2	3.0	8.0	4.2	3.2	3.0	1.4	2.8	1.4	2.4	10.0
BALSAMINA	7.2	8.6	8.4	2.6	0.4	0.4	0.0	0.0	0.0	10.0
MORETTONE	5.4	5.6	5.0	4.4	3.8	2.4	0.8	0.0	0.4	10.0
NERO D'AVOLA	3.2	2.8	5.4	6.0	3.4	4.8	1.6	0.8	0.0	10.0
EGIDIOLA	8.2	5.4	5.0	3.6	3.2	1.0	0.4	0.4	0.0	9.1
MALBECH	4.3	6.3	6.8	6.0	2.5	1.5	0.5	0.0	0.0	9.1
MONTEPULCIANO	7.8	5.2	3.8	5.4	1.4	0.4	0.0	0.0	0.0	8.9
AGLIANICO TOSCANO	6.0	7.6	6.8	4.2	2.4	1.2	0.2	0.0	0.0	6.0
VERNACCIA MOSCATELLA	3.5	4.3	6.8	6.0	4.3	1.8	0.8	1.8	0.3	6.0
NEBBIOLO	0.3	2.5	1.8	3.5	3.0	2.8	4.0	3.5	6.8	4.0
MERLOT	2.4	7.6	7.0	5.0	3.0	2.0	0.8	0.0	0.0	3.6
GAGLIOPPO	5.3	5.5	5.3	3.0	3.3	3.0	2.0	0.8	0.8	2.0
BRUGENTILE	4.5	8.5	7.0	5.3	2.5	0.8	0.8	0.0	0.0	0.0
LACRIMA	3.6	7.6	6.0	5.6	3.2	3.0	0.2	0.2	0.0	0.0
LIMBERGER	2.4	3.6	3.0	4.6	4.2	3.2	3.4	1.0	3.0	0.0
SYRAH	3.2	3.8	4.6	5.8	5.0	4.8	1.8	0.2	0.0	0.0

Minimum — Maximum

Figure 6 – Quantification of vessel distribution per diameter class (vessels/mm^2) for the 24 red-berried grapevine cultivars. The differentiation in colour defines the minimum (light green) to maximum (light red) values. *, esca incidence (Murolo and Romanazzi, 2014).

White-berried cultivars - In this group of cultivars, the highest vessel density was observed in 'Garofanata' (46.8 vessels mm^{-2}), followed by 'Chiapparù' (43.0 vessels mm^{-2}), while the lowest was observed for 'Sauvignon Blanc' R2 (18.3 vessels mm^{-2}). All of the other cultivars had intermediate values (**Figure 7**).

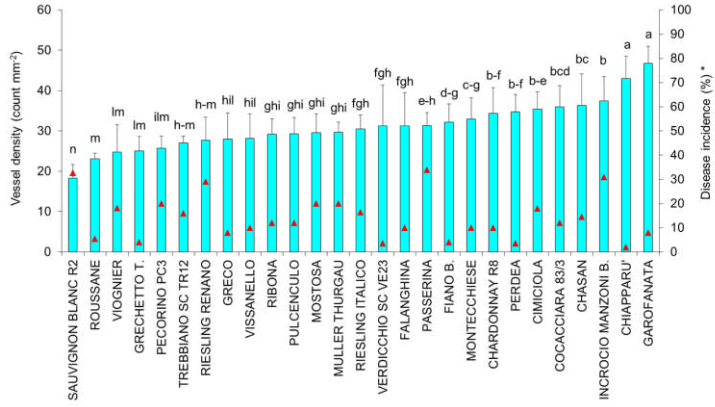

Figure 7 – Quantification of vessel density (columns) and disease incidence detected in the 27 white-berried grapevine cultivars. Data are means ±standard deviation. Different letters above columns indicate significant differences between cultivars (Fisher tests; p ≤0.05). *, esca incidence (Murolo and Romanazzi, 2014); ▲, disease incidence.

For the vessel size distribution, the most representative classes for most of the cultivars ranged from 80-99 µm to 100-119 µm. However, except for 'Garofanata' with 10.3 vessels in the 60-79 µm class, a number of cultivars showed greater vessels sizes corresponding to different classes. In detail, some cultivars showed vessels sizes corresponding to the 120-139 µm class, such as 'Sauvignon Blanc' R2 (5.4 vessels), 'Viognier' (5.6 vessels), 'Trebbiano' SC TR12 (10.3 vessels) and 'Verdicchio' SC VE23 (7.8 vessels). Other cultivars showed vessels sizes that corresponded to the 140-159 µm class, such as 'Sauvignon Blanc' R2 (5.4 vessels), 'Roussane' (9.0 vessels) and 'Grechetto' T (6.2 vessels). In the 160-179 µm class, there were 'Viognier' (5.6 vessels) and 'Falanghina' (5.2 vessels). Finally, in the >200 µm class, 'Sauvignon Blanc R2' (5.4 vessels) and 'Greco' (8.3 vessels) were observed (**Figure 8**).

CULTIVAR	DIAMETER CLASSES									DISEASE INCIDENCE *
	40-59 µm	60-79 µm	80-99 µm	100-119 µm	120-139 µm	140-159 µm	160-179 µm	180-199 µm	> 200 µm	
PASSERINA	0.0	1.2	6.2	10.2	6.6	3.0	2.2	0.4	0.0	34.0
SAUVIGNON BLANC R2	0.0	0.0	0.8	4.8	5.4	5.4	4.6	3.6	5.4	32.7
INCROCIO MANZONI B.	0.0	4.3	11.0	9.0	4.5	1.0	0.0	0.0	0.0	30.9
RIESLING RENANO	0.0	0.7	6.3	10.3	4.0	2.3	3.7	1.3	0.3	29.1
PECORINO PC3	0.0	0.0	5.2	8.0	6.2	4.2	3.8	1.6	0.6	20.0
MOSTOSA	0.2	2.6	6.8	7.8	5.0	4.2	2.0	1.0	0.0	20.0
MULLER THURGAU	0.0	0.3	2.3	9.7	8.7	3.0	3.0	1.3	0.7	20.0
VIOGNIER	0.0	0.4	2.4	2.8	5.6	4.6	5.6	4.8	3.0	18.2
CIMICIOLA	0.8	5.5	8.0	6.3	3.8	2.0	1.8	0.5	0.0	18.0
RIESLING ITALICO	0.0	0.0	8.3	12.3	5.5	2.3	0.8	0.0	0.0	16.4
TREBBIANO SC TR12	0.0	0.3	2.7	7.0	10.3	8.0	1.3	0.0	0.3	16.0
CHASAN	1.4	4.2	6.8	5.2	4.6	3.4	0.6	1.0	1.6	14.5
RIBONA	0.0	2.2	6.2	8.4	6.6	3.6	0.4	1.2	0.6	12.0
PULCENCULO	0.0	0.8	5.4	7.8	5.0	6.4	2.2	1.8	0.2	12.0
COCACCIARA 83/3	0.3	4.8	8.0	7.0	3.3	2.8	2.8	0.5	0.0	12.0
VISSANELLO	0.0	2.2	3.6	7.8	6.2	4.6	2.6	1.8	0.6	10.0
FALANGHINA	0.0	2.0	3.8	5.0	2.6	4.2	5.2	2.8	4.0	10.0
MONTECCHIESE	0.0	2.8	6.8	9.0	4.8	3.0	2.8	0.3	0.0	10.0
CHARDONNAY R8	3.4	5.8	7.6	7.0	2.8	1.6	0.6	0.0	0.0	10.0
GRECO	0.0	1.5	4.5	4.8	3.8	3.3	2.3	1.8	8.3	8.0
GAROFANATA	4.0	10.3	8.8	5.0	1.0	0.0	0.0	0.0	0.0	8.0
ROUSSANE	0.0	0.0	1.8	7.5	6.5	9.0	2.5	2.0	0.3	5.5
GRECHETTO T.	0.0	0.6	3.6	5.0	5.0	6.2	4.4	2.4	2.2	4.0
FIANO B.	1.0	5.0	7.8	5.8	4.2	3.0	2.4	0.2	0.0	4.0
VERDICCHIO SC VE23	0.0	2.4	3.6	4.6	7.8	6.4	3.2	0.6	0.4	3.6
PERDEA	0.0	1.8	10.3	9.5	6.0	1.0	0.8	0.0	0.3	3.6
CHIAPPARU'	1.4	2.6	10.8	6.6	4.8	1.6	0.4	0.0	0.0	2.0

Minimum — Maximum

Figure 8 – Quantification of vessel distribution per diameter class (vessels/mm^2) for the 27 white-berried grapevine cultivars. The differentiation in colour defines the minimum (light green) to maximum (light red) values. *, esca incidence (Murolo and Romanazzi, 2014).

Correlations between vessel anatomical characteristics and disease incidence were investigated. For the red-berried cultivars, a negative correlation coefficient was found between vessel diameter and vessel density (r = -0.67) (**Figure 9A**) and between vessel diameter and disease incidence (r = -0.31) (**Figure 9B**). Otherwise, a positive correlation coefficient was found between disease incidence and vessel density (r = 0.22) (**Figure 9C**). For the white-berried cultivars, a negative correlation coefficient was observed between vessel

diameter and vessel density (r = -0.86) (**Figure 9D**), while a positive correlation coefficient was found between vessel diameter and disease incidence (r = 0.14) (**Figure 9E).** A negative correlation coefficient was detected between disease incidence and vessel density (r = -0.28) (**Figure 9F**).

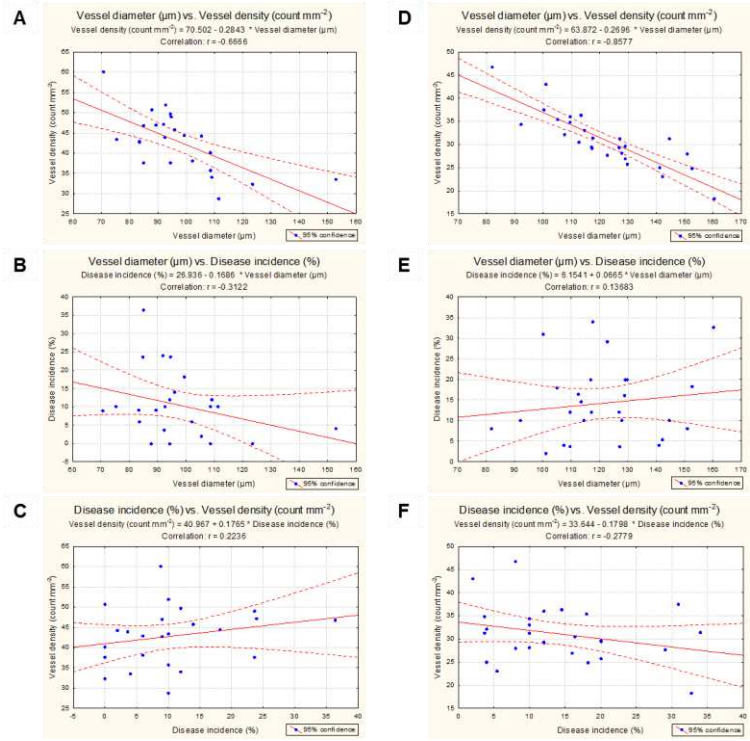

Figure 9 – Pearson's correlations between vessel diameters and vessel densities **(A, D)**, vessel diameters and disease incidence **(B, E)**, and disease incidence and vessel densities **(C, F)** in the red-berried cultivars **(A-C)** and white-berried cultivars **(D-F)**.

2.3.3 Comparisons among red-berried and white-berried cultivars

The comparison between red-berried and white-berried cultivars highlighted significant differences between the two groups. The vessel diameter average values were 121.9 µm for white-berried and 97.0 µm for red-berried cultivars (**Figure 10A**). In addition, regarding the vessel density, red-berried cultivars showed a higher density in comparison with the white-berried cultivars, with 42.8 vessels mm^{-2} and 31.0 vessels mm^{-2}, respectively (**Figure 10B**).

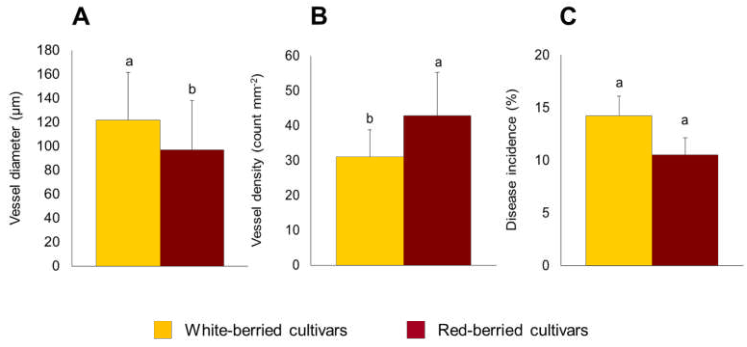

Figure 10 – Comparisons between the xylem characteristics as vessel diameter **(A)**, vessel density **(B)** and disease incidence **(C)** of the red-berried and white-berried cultivars of the grapevines. Different letters above column indicate significant differences between cultivars (Fisher's tests; $p \leq 0.05$).

2.4 Discussion

Esca and other grapevine trunk diseases are one of the major limiting factors for grape production throughout the world (Mohammadi et al., 2013). In esca, which affects the trunk vascular tissues, information about the morphology of the xylem among grapevine cultivars will allow better understanding of the mechanisms of vascular pathogens. Previous studies have shown that the xylem vessel diameter differs among grapevine cultivars, and that these features might predict the degree of susceptibility of any given cultivar to vascular diseases (Pouzoulet et al., 2014).

Applying the methodologies used by Scholz et al. (2013), we investigated the main anatomical traits in xylem vessels in a wide range of grapevine genotypes, as 24 red-berried and 27 white-berried cultivars, with Italian national and international importance in viticulture. Some of these grapevine cultivars are spread worldwide, as reported in the International Organisation of Vine and Wine (OIV) (Focus OIV, 2017). The information gained here provides novel knowledge about the anatomical characteristics of xylem tissue in grapevine cultivars. In particular, our data show high variability according both vessel diameter and vessel density within and among red-berried and white-berried cultivars grown in the field. The differences in vessel sizes and frequencies observed among the grapevine cultivars are in agreement with data reported in previous studies, which have involved small numbers of cultivars growth in greenhouses (Pouzoulet et al., 2014; Pouzoulet et al., 2017). In particular, we observed similar vessel densities for the same cultivars previous analysed, as ‘Merlot’, ‘Chardonnay’ and ‘Cabernet Sauvignon’.

Several studies carried out in a list of species have shown that the variation in xylem vessel size within

genotypes changes across different environments (Fisher et al., 2007; Choat et al., 2011; Schreiber et al., 2013; Medeiros and Pockman, 2014, Palliotti et al., 2014). These investigations showed that smaller vessels are advantageous to avoid freezing-induced embolism and to minimise the impact of drought-induced cavitation. In peach rootstock, larger vessels sizes were found in the more vigorous rootstock than in the most dwarfing one, and these characteristics were associated to the different hydraulic conductances estimated among these rootstock (Tombesi et al., 2010).

The present study analysed a large number of cultivars grown on the same experimental field, grafted onto the same rootstock, and trained with the same trellis system. For this reason, we can assume that external environmental factors, such as agricultural practices and soil and weather conditions, did not affect the results, which can instead be associated to grapevine genotypes.

In this study, different distribution for the diameter classes were observed. In particular, there was a higher average vessel diameter in the white-berried cultivars, compared to the average value detected in the red-berried cultivars. As might be predicted, negative correlation was detected among the vessel diameters and vessel densities in both the white-berried and red-berried cultivars. Moreover, the lowest correlation coefficient observed for the red-berried cultivars, which suggests less uniformity among this group regarding vessel size.

To the best of our knowledge, the xylem anatomical properties associated with red-berried and white-berried cultivars represent new information in the field. However, further studies are needed to confirm these data and understand whether this anatomical feature has an important role in plant physiology or in plant–pathogen interactions. In this regard, we examined the relationship between these characteristics and incidence of esca

symptoms, as previously assessed by Murolo and Romanazzi (2014) for the same grapevine cultivars. For the relationships among vessel sizes and densities with esca incidence previously detected on the same grapevines, no linear relationships was observed for either the red-berried or white-berried cultivars. These data appear to disagree with previous investigations, which have indicated that cultivars with larger vessels might be more susceptible to inoculated *P. chlamydospora* (Pouzoulet et al., 2017), one of the putative agents of esca, according to both pathogen concentration and wood necrosis lesions. However, in the present study, we analysed the esca symptoms observed in the field, which are known to involve several fungal species (Bruno and Sparapano, 2007). On the other hand, sometimes the presence of pathogens involved in esca was not resolved on symptoms manifestation. In addition, grapevine trunk diseases have unpredictable discontinuity in the expression of foliar symptoms year by year (Mugnai et al., 1999; Surico et al., 2000; Romanazzi et al., 2009; Wagschal et al., 2008). Even after their first appearance, foliar symptoms do not develop systematically and cannot be predicted from year to year, which indicates that several factors are probably involved in their development (Bertsch et al., 2013).

The overall average value of vessel diameter detected among the red-berried and white-berried cultivars show that the white berried cultivars have larger vessel sizes than the red-berried cultivars. A similar trend was observed among the red-berried and white-berried cultivars relative to the overall disease incidence observed for the same cultivars, where the average disease incidence, as assessed by Murolo and Romanazzi (2014), was higher in the white-berried cultivars (14.2%) than in the red-berried cultivars (10.5%) (**Figure 10C**). In addition, OIV 2016 reported that in Italy the incidence of

trunk disease depends on the varietal susceptibility: on plants of 15 to 18 years, the average incidence can fluctuate around 12% to 19%, respectively, for the white-berried cultivars, and around 8% to 10%, respectively, for the red-berried cultivars (Borgo et al., 2016; Mondello et al., 2018). This suggests that some relationship among vessel dimension and esca incidence might be possible.

Our data represent a first step that is useful for understanding the anatomical characteristics of the xylem of several grapevine cultivars. In esca, as for other trunk diseases, for the control of the diffusion of fungal pathogens in host tissues, planting of cultivars resistant to wilt diseases remains one of the most durable and economically efficient control measures (Yadeta and Thomma, 2013). For this reason, it becomes increasingly important to better understand the plant defence mechanisms and identify the less susceptible genotypes within the existing germplasms. These are key elements for sustainable agriculture.

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THE MYCORRHIZAL STATUS IN VINEYARDS AFFECTED BY ESCA

Abstract

Plant–microbe interactions show a wide array of relationships ranging from beneficial, neutral or negative effect in the host plant. In this work we have analyzed the correlation among beneficial indigenous arbuscular mycorrhizal fungi (AMF) and grapevine plants affected by esca, a serious grapevine trunk disease, mainly associated with different pathogenic fungi inhabiting the woody tissues. The AMF symbiosis was analyzed in roots from esca symptomatic and asymptomatic grapevine collected in three vineyards of the Marche region (Italy). The total AMF symbiosis, identified by Trypan Blue non-vital staining, showed higher AMF colonization value in esca symptomatic plants compared to asymptomatic plants. The abundance of indigenous *Rhizophagus irregularis* and *Funneliformis mosseae* AMF species in the vineyards, was analyzed developing a droplet digital PCR (ddPCR) assay selecting specific primers targeting 28S rRNA gene and large subunit ribosomal RNA, respectively. Both AMF species were detected in the asymptomatic and symptomatic plants, and a great variability among the analyzed plants was observed. Overall, both species showed a higher average value in symptomatic plants compared to asymptomatic plants in all vineyards. Our data showed that esca affected the mycorrhizal symbiosis in the grapevine roots. The example of mycorrhizal fungi reported here underline the value of a more holistic view that involve the complexities and dynamics of native microbial communities, useful for a better understanding of esca disease in grapevine.

Keywords: esca; droplet digital PCR; mycorrhizal non-vital staining; native AMF; grapevine.

3.1 Introduction

Esca with Eutypa dieback, and Botryosphaeria dieback are considered the most destructive trunk diseases of grapevine and are growing rapidly concern in all wine producing countries (Bertsch et al., 2009). This disease involves several xylem-inhabiting fungi (Larignon and Dubos, 1997; Crous and Gams, 2000; Bruno and Sparapano, 2007; Bertsch et al., 2009). The general symptoms are expressed at the foliar level by discolouration and drying and at the wood level through sectorial necrosis with the presence of brown streaking or cankers. (Mugnai et al., 1999). In addition, characteristic symptoms of grapevines affected by these diseases are sunken necrotic root lesions with a reduction in root biomass and root hairs (Rego et al., 2000). As a result, esca is detrimental to the resilience of the wine-growing heritage with symptom (Bertsch et al., 2009). Since the esca pathogens are mainly soilborne (Agustí-Brisach and Armengol, 2013; Berlanas et al., 2017), a helpful role in plant health management could be played by rhizosphere microorganisms, such as mycorrhizal fungi.

Mycorrhizal symbiosis relies on an ancient evolutionary process dating back to early land plants and it was the key factor when these ones conquered the land. Arbuscular mycorrhizal fungi (AMF) are the most common symbiotic fungi, associated with many different plant species and completely dependent on their host's carbon (Smith and Read, 2008). In return the plant receives additional nutrients, improved water relations (Marschner and Dell, 1994; Smith and Read, 2008; Ivanov

et al., 2019). Furthermore, to this nutritional function, AMF can enhance plant tolerance to both biotic and abiotic stresses. Several mechanisms whereby AMF could cause pathogen protection are known, including changes in root architecture, improvement of nutrient status, activation of plant defense mechanisms, or competition for infection sites (Azcón-Aguilar, 1996; Pozo and Azcón-Aguilar, 2007; Porcel et al., 2011; Augé et al., 2015; Fiorilli et al., 2018). This multifunctional ability of the partner fungi has led to the development of mycorrhizal inoculants as biofertilizers in agriculture. Mycorrhizal inoculation has been applied for decades to promote a better plant growth for various crop plants (Osonubi et al., 1995; Carretero et al., 2009). On the other hand, several studies point out the higher efficiency of native AMF inoculum, starting from native soils as an alternative to commercial ones (Affokpon et al., 2011; Wehner et al., 2011; Berruti et al., 2016; Ceustermans et al., 2019). However, all these studies concerning the biocontrol action were carried out by AMF, singular or in community, species selected and re-inoculated. A different role could be played by the non-target indigenous mycorrhiza, naturally present in the soil. At this regard, there are not enough information about the relationship of mycorrhizal native species, already established in the plant, and the plant diseases. Concerning the grapevine plants, they are remarkably dependent on mycorrhizae, since this plant has low-density roots and few root hairs. In the vineyard, the AMF communities, are mainly belong to the *Glomerales* group. They are highly influenced by the soil characteristics but also to a smaller extent by the host plant development stage (Schreiner and Mihara, 2009; Balestrini et al., 2010). In this work we have investigated the indigenous AMF community in both, asymptomatic and esca symptomatic grapevine. The total quantification of AMF was performed using the non-vital

Trypan blue (TB) staining, a suitable fluorochrome for staining total AMF spores, fungal hyphae, arbuscules and vesicles, whose specificity for mycorrhizal fungi was demonstrated (Kumar et al., 2008).

In addition, an innovative molecular approach using droplet digital PCR (ddPCR) (Pinheiro et al., 2012) was setup to investigate the absolute quantification of *Rhizophagus irregularis* (syn. *Glomus intraradices*) and *Funneliformis mosseae* (syn. *Glomus mosseae*) species identified in the vineyard soils. The ddPCR is based on the amplification of single target DNA molecules in many separate droplets (water-in-oil emulsion) resulting in a positive-negative call for every droplet and greater amenability to multiplexed detection of target molecules (Whale et al., 2012). These factors offer the advantage of direct and independent quantification of DNA without standard curves giving more precise and reproducible data versus Real time quantitative (q)PCR especially in the presence of sample contaminants. These features make this technology particularly suitable for the analysis of roots and soil samples DNA, contaminated by high content of humic acid and root exudates, that result in PCR inhibition (Racki et al., 2014). It demonstrates high sensitivity and precision for low-copy-number target nucleic acids (Hayden et al., 2013). Thereby, ddPCR technology can be used for extremely low-target quantitation from contaminated samples. These characteristics have made this technology suitable for AMF species investigations in the roots.

3.2 Materials and Methods

3.2.1 Experimental trials

The goal of this works was to quantify, in roots tissue of esca symptomatic and asymptomatic grapevine

plants, the whole indigenous AMF colonization microscopically, using total non-vital staining. In addition, using the molecular ddPCR technology, the abundance of two indigenous AMF species, *R. irregularis* and *F. mosseae*, was quantified.

The experiment was performed in 2017 year from January to July and was carried out in the three-commercial vineyards, located in Ancona district, Marche region, in central-eastern Italy. In detail:

Vyn_1 - The vineyard, located in Castelplanio (AN), 43°30'10''N - 13°05'42''E, at 272 metres above sea level (m a.s.l.) was planted in 2004. The grape cultivar planted was 'Verdicchio' on Kober 5BB (*Vitis berlandieri* × *V. riparia*) rootstock. The soil has a medium texture tending to melted, with a scarce amount of organic substance. The morphology of the land is hilly, and the exposure of the vineyard is south / west. The roots of the selected vine plants were collected in January.

Vyn_2 - The vineyard, located in San Biagio of Osimo (AN), site at 43°31'16''N – 13°28'50''E at 188 metres above sea level (m a.s.l.), was planted in 2002. The grape cultivar was 'Verdicchio' grafted on Kober 5BB (*Vitis berlandieri* × *V. riparia*) rootstock. The soil has a medium texture tending to clayey, fresh, fertile, deep and mainly grassy. The roots from the selected grapevine plants were collected in May.

Vyn_3 - The vineyard, located in Casenuove of Osimo (AN), site at 43°29'04''N – 13°26'10''E, at 115 m a.s.l., was planted in 2002. The grape cultivar planted was 'Chardonnay', grafted on Kober 5BB (*Vitis berlandieri* × *V. riparia*) rootstock. The vineyard is exposed to the south / east. The soil has a medium texture, with a high organic substance. Usually the soil is ploughed both in the row and along the rows, with elimination of the infesting flora. The roots from the selected grapevine plants were taken in July.

Vineyards were not irrigated, the fertilizers were distributed in winter, and an additional green pruning was applied in spring and summer, as are the normal practices for the area. In all vineyards, an integrated pest management program was applied to control the main fungal diseases (downy mildew, powdery mildew, gray mold) and pests (moths).

3.2.2 Samples collected

In each vineyard, several sites were randomly identified, four for the vineyards *Vyn_1* and *Vyn_2*, and six for vineyard *Vyn_3*. From each site, one symptomatic plant and one nearby asymptomatic plant were selected for the study. A total of 28 plants were analyzed: 14 esca symptomatic and 14 asymptomatic plants. From each plant the roots were collected at a depth of about 20 cm. A portion of the terminal root system from each plant was randomly collected and washed with water. A total of 8-10 g from each plant were collected, half of these were conserved on acetic acid:ethanol (50:50) for total non-vital staining, and half at -20°C for DNA extraction.

3.2.3 Estimation of total indigenous AMF in esca symptomatic and asymptomatic grapevine roots

For assessing the total AMF symbiosis, from each plants, 50 section 1 cm-long pieces were randomly chosen and analyzed by non-vital staining with TB (Phillips and Hayman, 1970). With this aim, after cleaning the root samples in 10% KOH for 20 min at 80°C, the roots were stained with 0.05% (w/v) TB in lactoglycerol (lactic acid:glycerol:H_2O, 1:1:1, v/v/v) for 20 min at 80°C. Stained roots were observed with a light microscope, Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan). The intensity of root cortex colonization by AM fungus was determined as described by Trouvelot et al. (1986).

The MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) was used to calculate the %F, %M and %A parameters. The %F correspond to frequency of mycorrhiza in the root system, %M, correspond to the colonization intensity of cortical cells occupied by AMF structures, and %A, correspond to the estimation of abundance of arbuscules in the root system.

3.2.4 *Rhizophagus irregularis* and *Funneliformis mosseae* absolute quantification with ddPCR

3.2.4.1 DNA extraction

Total DNA from roots samples was extracted by modified CTAB (cetyl trimethyl ammonium bromide) procedure (Doyle and Doyle, 1990) modified by Landi et al. (2019). For each sample, two subsamples were obtained. From each subsample 2 g of pooled roots were ground in liquid nitrogen, then 200 mg of pulverized materials were collected and put in 2 ml microcentrifuge tube. One ml of extraction buffer (CTAB 3%, 100 mM Tris-HCl, pH8.0, 20 mM EDTA, 1.4M NaCl, 2% [w/v] soluble PVP-40) was added at each tube. The samples were incubated at 68°C for 30 min. After centrifugation for 5 min at 8,000g, the supernatants were transferred to new tubes with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 10,000g for 8 min. This last step was repeated for two times. The total DNA was precipitated with isopropanol 0.6% for 10 min at room temperature. The samples were then centrifuged at 14,000g for 20 min, washed with 70% ethanol, dried, and re-suspended in 50 µL of double-distilled water. DNA purity and quantity were measured with BioPhotometer plus (Eppendorf Inc., Westbury, NY, USA).

3.2.4.2 Primer selections and validation

For the mycorrhizal analysis specific primers were selected related to 28S rRNA and large subunit (LSU) ribosomal RNA genes according to *R. irregularis*, (National Center for Biotechnology Information, NCBI cod. HF968988.1) and *F. mosseae* (NCBI cod. FN377865.1) respectively using Primer3web version 4.1.0. (http://bioinfo.ut.ee/primer3-0.4.0/). The primers named RI/f (5'-GGCGTTATTGTCGCACCTAT), and RI/r (5'-CCTTGGTTTTTCAAGGGTCA), that amplify a 248 bp PCR fragment, were developed for *R. irregularis* species and primers named FM/f (5'-CCTATGGATCCCCCTTTTGT) and FM/r (5'-AGATGCTGCAGAAGGCAAAT), that amplify a 190 bp PCR fragment, were developed for *F. mosseae* species.

Before using the primers in ddPCR technology, their ability to detect the species present in the vineyard soils was validated according Real Time qPCR protocol followed by amplicon sequencing analysis. A serial dilution from 100 ng to 1 ng of total DNA was obtained from a pool of DNA from roots of asymptomatic and esca symptomatic plants. As a negative control, the DNA from grapevine leaf tissue was analyzed. The qPCR was carried out in a total volume of 12 µl, which in addition to the DNA template described above, contained 6 µL SsoFast EvaGreen supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 1 µl of the designed primers (1 µM each). The reactions were subjected to the following conditions that were selected as optimal: initial denaturation step for 3 min at 98°C, followed by 40 cycles of 15 s denaturation at 98°C, and 40 s annealing–elongation at 60°C. The final step included the melting curve analyses (0.5°C step increments; 10 s hold before each acquisition), which were analyzed from 70°C to 95°C.

The sequence analysis PCR amplicons obtained from RI/f, -r and FM V/f -r primers on qPCR of

representative symptomatic and asymptomatic roots were performed. The fragments were sequenced by Genewiz (Hope End, Takeley, UK) and subjected to bioinformatic analysis. Sequence similarity searches were performed using Blast analysis in NCBI. Phylogenetic trees were constructed using the molecular evolutionary genetics analysis (MEGA) program, version 5.2 (Tamura et al. 2011), according to the neighbor-joining method (Saitou and Nei 1987). The sequences have been deposited with NCBI GenBank.

3.2.4.3 ddPCR assay

The ddPCR assay was performed using QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA). To identify the lowest limit of detection (LoD), a pool of DNA from symptomatic and asymptomatic plant roots ranging from 100 ng to 1 ng, previous analyzed in qPCR, was tested. At the same way, DNA from grapevine leaf tissue was analyzed as negative control. Finally, the analysis was performed for each subsample using in 25 µl including 1× QX200 ddPCR EvaGreen supermix (Bio-Rad), 10 µM each primers and 10 ng of DNA for each subsample. The Mastermix and sample DNA were thoroughly mixed and transferred to a DG8 Cartridge for a QX100™/ QX200 Droplet Generator (Bio-rad). Next, Droplet Generation Oil (Bio-Rad) was added to the cartridge which was placed into the QX200 Droplet Generator™ (Bio-Rad). After droplet generation, the mix were carefully transferred to a ddPCR™ 96-well PCR plate (Bio-Rad) after which the plate was sealed at 180°C using PX1™ PCR plate sealer (Bio-Rad). The amplification was performed in the thermal cycler, ICycler (Bio-Rad), with a ramp rate of 2°C/s with the following protocol: 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s and 58°C for one min. The

enzyme was deactivated at 4°C for 5 min followed by 90°C for 5 min. Droplets were read in a QX200 Droplet reader (Bio-Rad) after which the ddPCR data were analyzed using Quantasoft Version 1.6.6. The script analyzed the data of the signals exported from the QuantaSoft software, with its automatic threshold defined or with a selected, manually defined, threshold applied. This incorporates the calculation of the basic parameters of the ddPCR (e.g. concentration, mean amplitudes of positive and negative droplets); the mean copies per partition and the total volume of the partitions measured, as defined by the digital MIQE guidelines (Huggett et al., 2013). Two positive droplets were enough to determine a sample as positive, and only the reactions with more than 10,000 accepted droplets were used for analysis.

3.2.5 Statistical analysis

The difference among AMF symbiosis in the esca symptomatic plants and asymptomatic plants according to the vineyards were analyzed. The arcsin square-root transformation was used to normalize the percentage ratios data prior to statistical analysis. Data from AM fungi colonization (*Vyn*_1 and *Vyn*_2, n=4; *Vyn*_3, n=6) were subjected to one-way ANOVA for mean comparisons, standard deviation (SD) and significant differences calculated according to Duncan's Multiple Range Test, $P < 0.05$. Data sets were also tested for correlations using the Pearson's coefficient (r) $P < 0.05$. Regarding the ddPCR test, the DNA from each sub-sample was analyzed two times in four independent experiments (n=2). The means and SD were calculated.

3.3 Results

3.3.1 Estimation of total indigenous AMF in esca symptomatic and asymptomatic grapevine roots

Indigenous AMF colonization was detected in all root samples analyzed from both, esca symptomatic and asymptomatic plants with frequency (%F) of 100%. However, different quantity of mycorrhizal infection was observed according to esca symptomatic and asymptomatic plants. Regarding the roots from esca symptomatic plants, non-vital staining revealed for each vineyard an average colonization ranged from 28.2% (*Vyn_1*) to 47.4% (*Vyn_2*), while the presence of arbuscules ranged from 13.1% (*Vyn_1*) to 21.6% (*Vyn_2*). At the same way in the asymptomatic plants average colonization ranged from 24.9% (*Vyn_1*) to 40.5%, (*Vyn_2*), while the presence of arbuscules ranged from 12.1% (*Vyn_1*) to 15.8% (*Vyn_2*). The *Vyn_3* showed intermediate values compared to the others vineyards (**Figure 1**).

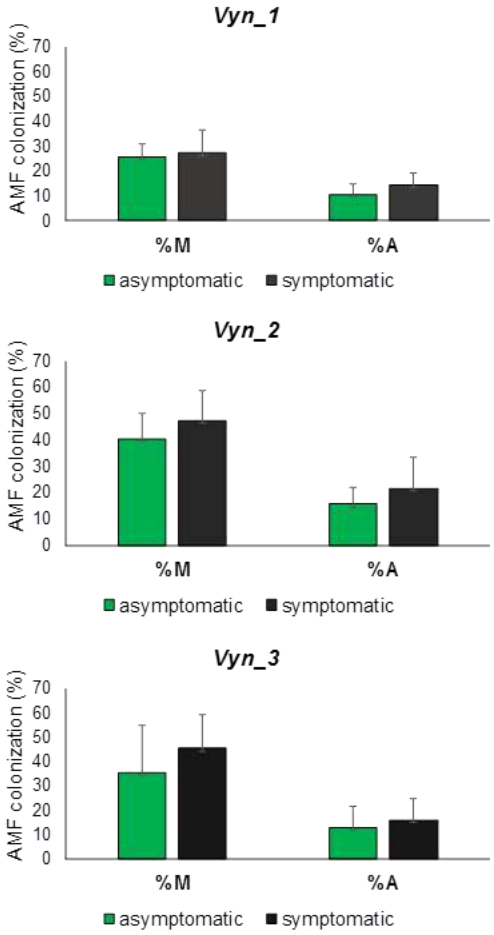

Figure 1 – AMF colonization (%M) and arbuscular abundance (%A) detected by non-vital staining of roots of esca symptomatic and asymptomatic grapevine plants collected in three different vineyards (*Vyn_1*, *Vyn_2* and *Vyn_3*). For each trait, means followed by at least one common letter are not significantly different, according to Duncan's Multiple Range Test ($P < 0.05$). The data were normalized according to the arcsine square root.

Regarding to each of the 14 sites identified in the vineyards, all the esca symptomatic plants showed higher mycorrhizal infection when compared with nearby asymptomatic plants (**Figure 2**).

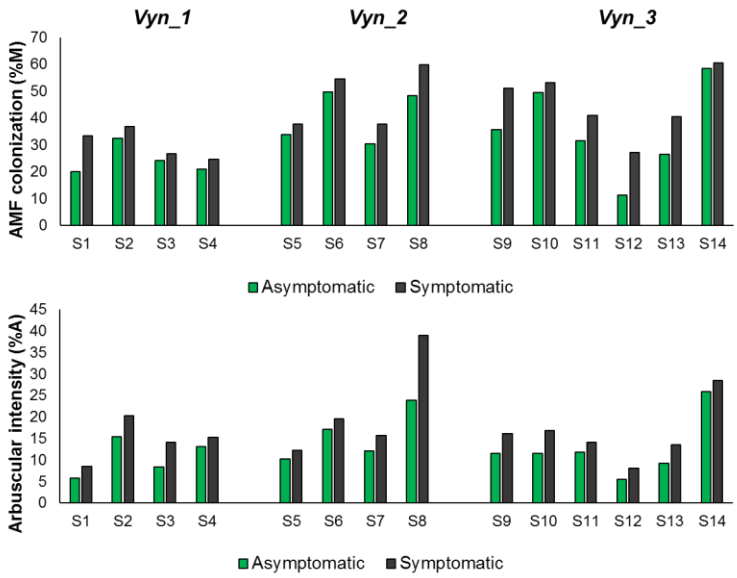

Figure 2 – AMF colonization (%M) and arbuscular abundance (%A) detected by non-vital staining from each asymptomatic and contiguous esca symptomatic plants from each site (S) individuate in the vineyards (*Vyn_1*, *Vyn_2* and *Vyn_3*).

Correlation coefficient among esca symptomatic and asymptomatic plants of both, AMF colonization and arbuscular shown high positive correlation (**Figure 3**).

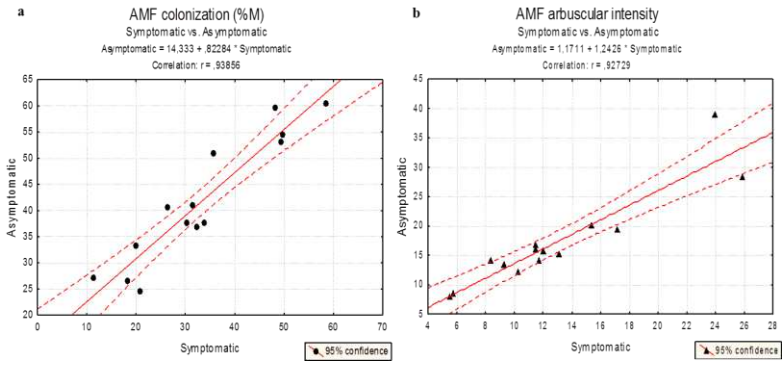

Figure 3 – Correlation related to AMF colonization (a) and Arbuscular abundance (b) detected by non-vital staining in asymptomatic and contiguous esca symptomatic plants. Pearson's coefficient (r) $P < 0.05$.

3.3.2 Primer selections and validation

The qPCR analysis shown a singular amplicon of 81.5°C melting temperatures (TM), according to primers RI/f-r, specific for *R. irregularis*, while the primers FM/f-r, amplify for a singular amplicon of 80.5°C TM, specific for *F. mosseae* (**Figure 4a**). For qPCR, pool DNA 10-fold serial dilution from 100 ng to 1 ng, both the primers gave reaction efficiencies (between 104% and 113%) and Quantification Cycle (Cq) ranging from 25.5 to 35 cycles for *R. irregularis* and from 26.8 to 36 for *F. mosseae* (**Figure 4b**). The ddPCR data generated from the identical reaction mixtures revealed the separation between the negative and positive droplets (**Figure 4c**). Sequence analysis of the PCR amplicons indicated as isolates S1_V1 (NCBI accession number MK513942) and S5_V3 (MK513943) were homologous to > 98% with the reference sequences related to 28S rRNA gene of *R. irregularis,* NCBI cod. HF968988.1, and FJ235574.1 (**Figure 4d**). Sequence analysis of the PCR amplicons indicated as isolates S4_V1 (MK513941) and S10_V3

(MK513943) were homologous to 99% with the reference sequences related to LSU gene of *F. mosseae*, NCBI cod. FN377862.2 and FN377865.1 (**Figure 4e**).

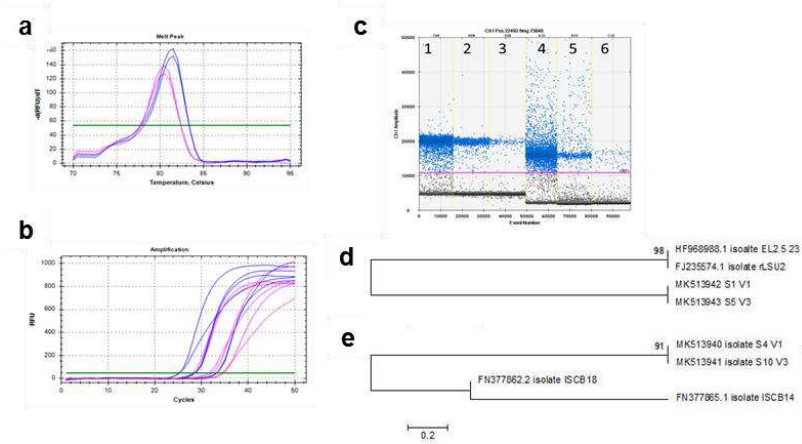

Figure 4 – Assessment of primers in qPCR and ddPCR platforms. **a**) Melt curve obtained from qPCR related to primers specific for *Funneliformis mosseae* species (pink lines) and *Rhizophagus irregularis* species (blue line). **b**) Traces obtained from qPCR amplification of DNA from esca symptomatic and asymptomatic pool sample 10-fold serial dilution ranging from 100 to 1 ng of DNA. (*F. mosseae* pink lines, *R. irregularis* blue lines). **c**) ddPCR obtained from amplification of DNA from esca symptomatic and asymptomatic pool sample 10-fold serial dilution ranging from 100 to 1 ng of DNA (lines 1,2,3 *F. mosseae*; lines 1,2,3 *R. irregularis*). **d**) Phylogenetic tree according to 28S gene of the of isolates S1_V1(NCBI accession number, MK513942) and S5_V3 (MK513943) homologous to *R. irregularis* species (No HF968988.1 and No FJ235574.1 from NCBI). **e**) Phylogenetic tree according LSU gene of isolates S4_V1 (MK513940) and S10_V3 (MK51394) homologous to *F. mosseae* species (No FN377862.2 and FN37865.1 from NCBI).

3.3.3 Rhizophagus irregularis and *Funneliformis mosseae* absolute quantification with ddPCR

The amount of the droplets was > of 15,000 of 72% of reaction and included on 15,000 to 10,000 in the 28% of samples. The analysis performed showed the presence of both mycorrhizal species in all roots analyzed, no amplification was observed in the DNA from leaf tissue used as negative control (data not showed). Related to vineyard sites, a great variability was observed in both, esca symptomatic and asymptomatic plants (**Figure 5**).

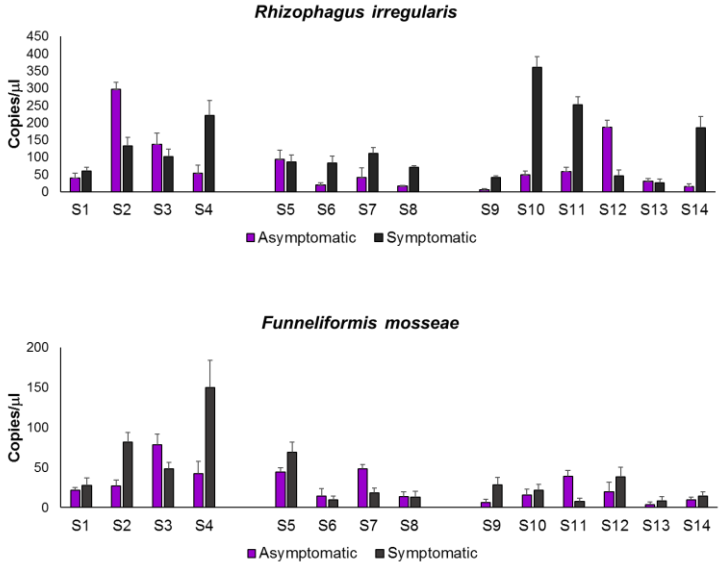

Figure 5 – Amount results of *R. irregularis* and *F. mosseae* quantified by ddPCR from each asymptomatic and contiguous esca symptomatic plants from each site (S) individuate in the vineyards (*Vyn_1*, *Vyn_2* and *Vyn_3*). The standard deviation was shown.

Concerning the *Vyn_1*, in esca symptomatic plants, the abundance of *R. irregularis* ranged from 59 copies/µl

to 221 copies/µl, while in asymptomatic plants from 40 copies/µl to 296 copies/µl were detected. About *F. mosseae*, in the roots from esca symptomatic plants the quantity ranged from 27 copies/µl to 150 copies/µl while in the roots from asymptomatic plants the amount ranged from 21 copies/µl to 78 copies/µl (**Figure 5**).

In the *Vyn_2* the abundance of *R. irregularis* ranged from 71 copies/µl to 111 copies/µl in esca symptomatic plants, and from 16 copies/µl to 94 copies/µl in asymptomatic plants. From 9 copies/µl to 69 copies/µl of *F. mosseae* were detected in the esca symptomatic plants, and from 13 copies/µl to 48 copies/µl in asymptomatic plants (**Figure 5**).

In the *Vyn_3*, the *R. irregularis* ranged from 25 copies/µl to 360 copies/µl in the esca symptomatic plants while from 5 copies/µl to 187 copies/µl in the asymptomatic plants. About *F. mosseae*, in esca symptomatic plants the abundance ranged from 8 copies/µl to 69 copies/µl, while in asymptomatic plants ranged from 3 copies/µl to 48 copies/µl (**Figure 5**).

Correlation coefficient among AM species abundance in esca symptomatic and asymptomatic plants was detected in each site, and no constant relationship was observed (**Figure 6**).

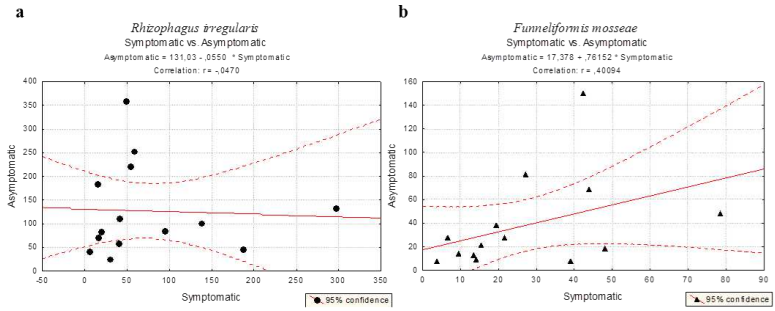

Figure 6 – Correlation related to amount of *R. irregularis* (**a**) and *F. mosseae* (**b**) quantified by ddPCR detected in each asymptomatic and contiguous esca symptomatic plants.

3.4 Discussion

Management of esca must be integrated with an interdisciplinary approach (Gramaje et al., 2018). Since the esca pathogens are often existing in soils before planting (Agustí-Brisach and Armengol, 2013; Berlanas et al., 2017), the study of rhizosphere microorganisms as the mycorrhizal fungi could be provide important information to better understand the dynamics of esca. In this work, we analyzed the interaction among the indigenous mycorrhizal symbiosis in both asymptomatic and esca symptomatic grapevines. The non-vital TB staining the total AMF symbiosis allowed us a comprehensive approach to determine the roots mycorrhizal status. Staining and low magnifications microscopic method not only provided reliable data on the degree of root colonization, but also allowed to visualize the presence and distributions of key features such as arbuscules, which are the morphological criteria that define AMF associations (Vierheilig et al., 2005). Our study shown greater presence of AMF colonization related to both, mycelium and arbuscular colonization in roots from esca symptomatic plants than asymptomatic plants. This was

observed according to the average value of AMF status in each vineyard. However, no significant differences were detected due to great variability of AMF colonization observed among the vineyard plants. These suggest the probable influence of both, soil conditions and rhizosphere on AMF symbiosis establishment variability (Goh et al., 2013). The AMF may express different symbiotic lifestyles, depending on environmental factors and stress conditions (Johnson and Graham, 2013; Nogales et al., 2019). Our data show, related to each of the 14 sites analyzed, a higher AMF colonization in esca symptomatic plants than the nearby asymptomatic ones. Our work underlines that the different cultivars analyzed as well as the seasonal variations do not affect the relationship among the AMF indigenous infection and the esca. In addition, *R. irregularis* and *F. mosseae*, previous identified among the most representative species in the vineyards (Mathimaran et al., 2005; Trouvelot et al., 2015), were quantified using the ddPCR technology. The protocols setup in this study allowed us to assess the mycorrhizae amount of these two species. Both *R. irregularis* and *F. mosseae* were detected in all plants analyzed. However, differently from the non-vital staining, no significant correlation between amount of AMF species and the esca symptoms was observed. This highlights that the highest amount of AMF symbiosis observed in symptomatic plants is not associated with a specific attraction for individual AMF species, but involves the whole mycorrhizal community and their rhizosphere relationship. Overall results suggest a greater quantity of these species in symptomatic plants than in the asymptomatic ones, and this supports the results evidenced by non-vital staining.

Some recent work focused on trunk disease-affected vines highlighted the importance of microbial community influence on esca (Alaimo et al., 2018). These

authors reported that in esca-affected vines the whole fungal community structure, included beneficial and deleterious microorganisms, undergoes a considerable change in comparison with healthy plants. In our samples the presence of *P. chlamydospora*, a pathogen involved in esca, was detected only in some root samples from symptomatic plants (data not shown). This emphasizes the complexity of the microbial community and plant root relationships.

The beneficial effects of mycorrhizae against the pathogens were demonstrated in several plant-mycorrhizae-pathogen interactions involving both woody perennial crops and annual crops (Wehner et. al. 2011; Cameron et al., 2013; de Souza et al., 2016; Berdeni et al., 2018). In particular, studies comparing different fungal species highlighted that the degree of protection is highly dependent on the AMF involved (Kobra et al., 2009). Concerning grapevine trunk diseases, the inoculation of the roots with the *R. irregularis* has reduced both, the number of root lesions and the severity of diseases caused by pathogens (Petit and Gubler, 2006). Many studies pointed out a higher protector effect of *F. mosseae* in comparison to other AMF species (Pozo et al., 2002; Utkhede, 2006; Ozgonen and Erkilic, 2007). Our study carried out according these two species underlines again that a different relationship could be established by native AMF species compared to exogenous species.

The indigenous AMF composition is a pool of species not foreign to the rhizosphere, compared to that established applying exogenous mycorrhizal inoculum (Wehner et al., 2010). About indigenous mycorrhiza species, the host plant and other rhizosphere organisms recognize certain organisms as their own and do not activate defense mechanisms. These characteristics make native mycorrhizal symbiosis useful as a bioindicator of environmental stresses (Smith and Read, 2008). On the

other hand, is known that both plant mutualists and pathogens share common molecular and cellular mechanisms for colonizing their hosts (Corradi and Bonfante, 2012; Zamioudis and Pieterse, 2012; Zhang et al., 2019). The difference could be linked to modality in which the defense mechanism is activated in the plant host among beneficial or pathogen organisms.

The relationship established by the plant with indigenous mycorrhizal species naturally present in the soil, requires a continuous exchange of signals between the host's roots and mycorrhizae (Smith and Read, 2008; Ivanov et al., 2019). Then this suggest that the pathogens establishment, changes the relationship between plant and all mycorrhizal symbiosis. Esca disease induces important changes in the root system, including a reduction in root biomass and root hairs. This condition could further increase the need of the plant to establish mycorrhizal symbiosis useful for its nutritional needs.

Other aspects may influence the different amount of AMF symbiosis observed in the roots. Among these an altered pattern of root exudation could affect the plant-mycorrhizae-pathogen relationship. The root exudates are key factors in shaping soil microbial communities (Badri and Vivanco, 2009), and the changes in exudation into the mycorrhizosphere may result in alteration of the microbial communities, including possible antagonistic organisms. In esca symptomatic plants, vascular necrosis or black streaking with black gummy exudates occurs. It has been hypothesized that the metabolites first act in the place in the wood where they are produced, and that subsequently some of these metabolites also act at a distance from the place of production (Andolfi et al., 2011). Others works suggest that changes in the root exudates from mycorrhizal plants are partially involved in the susceptibility of these plants to soil microorganisms. Therefore, while the root exudates of mycorrhizal strawberry plants have reduced

the sporulation of *Phytophthora fragariae* (Norman and Hooker, 2000), the root exudates of mycorrhizal potato plants have increased the hatching of the nematodes (Ryan and Jones, 2004), or in mycorrhizal tomato plants was reported to increase the germination of *Fusarium oxysporum* conidia (Singh et al., 2010). It is conceivable that pathogens take advantage of this symbiosis program to gain access to the host plant's resources (Rey and Schornack, 2013).

This work can contribute to better understand the interactions between indigenous mycorrhiza and esca disease, and it underlines the role that mycorrhizae can have as bioindicators of the soil changes taking place in the rhizosphere.

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CONCLUDING REMARKS

The understanding of the mechanisms that trigger the interaction between the host plant and a complex disease as esca, requires an inclusive study that involves the characteristics of the host and the context in which it grows. In this thesis, the size of xylem vessels, in a broad number of grape cultivars, having national and international viticultural relevance, whose different susceptibility to esca disease is known, was analyzed. We have shown that there is a difference among cultivars regarding the size of xylem vessels, in which develop causal agents of esca, thus spreading the disease.

Previous studies, carried out not only on grapevine, had shown that a higher vessel diameter size increase susceptibility to vascular diseases. Our data related to the disease incidence did not show this for the individual cultivars. There are many environmental aspects associated with the appearance of symptoms, that are not always closely related to the presence of the pathogen, in particular when there are different causal agents of GTDs that interact each other. However, the difference between red-berried and white-berried cultivars is intriguing. The latter have on average a larger vessel size than red-berried. This result, considering that recent investigations have highlighted a higher susceptibility of white-berried cultivars to the esca pathogens, represents an important information, especially because this research has involved a considerable number of grape cultivars.

In the same way, the study carried out on natural mycorrhizal state of plants affected by esca strengthen the knowledge on how the diseased plant interacts with beneficial microorganisms, modifying their concentration. The analysis of the symbiosis grapevine roots-AMF showed wide difference, according to the vineyards considered, although the symptomatic plants showed a

higher level of total mycorrhization than the asymptomatic plants. The presence of disease in grapevine interacts and modifies the relationship of the plant with the microorganisms of the rhizosphere. Furthermore, the results on interactions between plants and their rhizosphere microbial communities are an important point to consider for development of strategies for the sustainable management of grapevine esca disease. For this reason, it becomes increasingly important the need to better understand the plant defense mechanisms and to identify less susceptible genotypes within existing germplasms, as key factors for sustainable agriculture.

The investigation carried out in this study encourages to foster the studies on the relationship between anatomical characteristics of grape cultivars and esca, since it can provide useful information for grapevine trunk disease management. Moreover, it is evident that, in the identification of agronomic practices aimed to identify the most suitable control strategies against esca disease, the study of the plant-rhizosphere interaction can play an important role.

PUBBLICATIONS

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