PHD THESIS

SPATIAL DISTRIBUTION OF GRAPEVINE ESCA DISEASE IN VINEYARDS OF THE MARCHE REGION, AND STUDY OF FUNGAL ENDOPHYTIC COMMUNITIES

PhD XVII CYCLE

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A blue fly, if it clings to the tail of a thoroughbred horse, can travel ten thousand miles, and the green ivy that twines around the tall pine can grow to a thousand feet.

Nichiren, Rissho Ankoku Ron

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Chapter I: INTRODUCTION

GRAPEVINE TRUNK DISEASES

Grapevine (*Vitis vinifera* L.) is one of the oldest perennial crops and one of the most important fruit species worldwide (**Alfonso et al. 2012**, **Álvarez-Pérez et al. 2017**), both because of its cultivation throughout the world and because of its high commercial value (**Vivier & Pretorius 2002**). The species is cultivated in the world of approximately 7.5 million ha (**OIV 2016**) and it comprises more than 5 thousand cultivars for the production of wine, grape juice and other foods (**Ali et al. 2010**).

Several fungal pathogens have been reported for grapevine (**Alfonso et al. 2012**) and, among all grapevine diseases worldwide, Grapevine Trunk Diseases (GTDs) are considered the most destructive of the past three decades (**Van Niekerk et al. 2011**, **COST 2013**). GTDs cause a significant reduction of yields as well as in grape quality (**Surico et al. 2000**, **Almeida 2007**, **Lorrain et al. 2012**), dramatically shortening vineyard longevity and compromising their sustainability (**Rolshausen et al. 2010, Álvarez-Pérez et al. 2017, Moisy et al. 2017**). Hofstetter et al. (**2012**) estimated that the economic cost worldwide for the replacement of GTD-linked dead grapevines was higher than 1.5 billion dollars per year, whereas Vasquez et al. (**2007**) calculated that in Californian table grape production the losses due to esca, a very common GTD, were between 532 and 1970 United States dollars per ha. These grapevine diseases are considered, therefore, a major threat to the wine and grape industry all over the world (**Siebert 2001**, **Gubler et al. 2005**, **Álvarez-Pérez et al. 2017**), one of the most relevant challenges for the viticulture and a major concern in all wine producing countries (**Bertsch et al. 2009**, **2013**).

GTDs are considered as the result of infection by multiple fungal pathogens which simultaneously or sequentially colonize the host tissue (**Hiscox et al. 2015**), entering through pruning wounds and/or the root system (**Álvarez-Pérez et al. 2017**). Although different strategies have recently been developed to protect pruning wounds using antifungal compounds (natural or synthetic) or biocontrol agents, no tools are yet available for controlling soil pathogens that infect plants through their root system (**Álvarez-Pérez et al. 2017**).

The presence of a GTD pathogen in the vine does not necessarily result in the immediate appearance of disease symptoms (**Kovács et al. 2017**). Incidence of grapevine trunk diseases is influenced by soil, topology and vineyard age (**Kovács et al. 2017**).

The frequency of the GTDs around the world over the last decades increased due to different factors; in particular the extremely toxic sodium arsenite (the only treatment which seemed to reduce the esca foliar symptoms) have been banned in 2003 (**Sass & Colangelo 2006**).

The presence of GTDs increased since 2003 (the year of sodium arsenate ban) from 1.8% to 10.5% in 2007 in Spain (**Rubio et al. 2011**). A decreasing of wine production was reported in France by 13% in 2014 (**OIV 2016**). A 2.7% annual increasing of appearance of foliar symptoms was assessed in Austrian vineyards (**Reisenzein et al. 2000**). An incidence of esca disease that was estimated above 50% in some Italian vineyards (**Surico et al. 2006**) and the replacement of 1% to 5% plants in the new established vineyards occurred in California, because of young vine decline (**Eskalen et al. 2007**).

The issue became worse because of a broad establishment of new vineyards globally, that was accompanied by a dramatic increasing of “Petri disease”, associated with growth reduction and browning of vascular tissues in young plants (1 to 9 years old) (**Hofstetter et al. 2012**). Moreover, infections are brought to vineyards from the nurseries planting material (**Rubio et al. 2011**).

GTDs include different impacting fungal diseases (Tab. 1), the most relevant of which are esca,

Phomopsis dieback, Eutypa dieback, Petri disease, black foot and Botryosphaeria dieback, that impact the perennial organs of the plant leading to premature decline and dieback in some cases (**Bertsch et al. 2013**, **Álvarez-Pérez et al. 2017**).

Table 1: Main Grapevine Trunk Diseases and causing agents.

GRAPEVINE TRUNK DISEASES	PATHOGENS
Esca diseases	
Esca, grapevine leaf stripe diseases	*Phaeomoniella chlamydospora* (syn. *Phaeoacremonium chlamydosporum*), *Phaeoacremonium minimum* (syn. *P. aleophilum*), *Phaeoacremonium* spp. (**Petri 1912**, **Graniti et al. 2000**), *Fomitiporia mediterranea* and/or other basidiomycetes, *Inocutis* spp., *Inonotus* spp., *Fomitiporella* spp., *Phellinus* spp., *Stereum* spp. (**Mugnai et al. 1999**, **Graniti et al. 2000**, **Fischer 2002**, **Sánchez-Torres et al. 2008**, **Surico et al. 2008**, **Cloete et al. 2015**)
Petri disease	*Phaeomoniella chlamydospora*, *Phaeoacremonium minimum, Phaeoacremonium* spp., *Cadophora luteo-olivacea*, *Pleurostoma richardsiae* (**Petri 1912**, **Graniti et al. 2000**, **Halleen et al. 2007**, **Agustí-Brisach et al. 2013**)
Phomopsis dieback - Phomopsis cane and leaf spot	*Phomopsis viticola* (sexual stage: *Diaporthe ampelina*) (**Bertsch et al. 2013**, **Fischer et al. 2016**)
Botryosphaeria dieback - 'bot' canker - black dead arm	*Botryosphaeria obtusa* (anamorph: *Diplodia seriata*), *Diplodia mutila*, *Lasiodiplodia theobromae* (teleomorph: *Botryosphaeria rhodina*) (**Úrbez-Torres et al. 2008**, **Djoukeng et al. 2009**, **Úrbez-Torres 2011**, **Fischer et al. 2016**, **Paolinelli-Alfonso et al. 2016**)
Eutypa dieback	*Eutypa lata* (**Rolshausen et al. 2006**)
Black foot	*Cylindrocarpon* spp., *Ilyonectria* spp., *Dactylonectria* spp., *Campylocarpon* spp., *Cylindrocladiella* spp., *Neonectria* spp. (**Fourie & Halleen 2004**, **Agustí-Brisach & Armengol 2013**, **Úrbez-Torres et al. 2014**)

The plant infection in vineyard can occur through root system and pruning wounds, both in adult and in young grapevines (**Álvarez-Pérez et al. 2017**). Pathogens belonging to *Dactylonectria* or *Ilyonectria* genera (**Halleen et al. 2006**) have the root system as a primary mode of colonization, whereas fungi like *Botryosphaeriaceae* family and *Eutypa lata* mainly infect the plant through pruning wounds that are produced (**Rolshausen et al. 2010**, **Van Niekerk et al. 2011**, **Luque et al. 2014**). Other pathogens like *Phaeoacremonium minimum* (formerly *P. aleophilum*) or *Phaeomoniella chlamydospora* can use both pathways to penetrate the plant (**Gramaje & Armengol 2011**, **Bertsch et al. 2013**, **Gramaje et al. 2015**).

It is well known that planting material (grafted plants) produced in nurseries is frequently infected with fungal pathogens, especially those involved in black foot and Petri diseases (**Álvarez-Pérez et al. 2017**). In addition, it has been widely reported that grafts can be infected at different stages of the propagation process that takes place in the nurseries (**Gramaje & Armengol 2011**, **Agustí-Brisach et al. 2013**).

COMPLEXITY OF ESCA

The disease currently known as esca is a multiple fungal syndrome, considered as a complex disease (**Mugnai et al. 1999**). The most commonly associated fungi are *P. chlamydospora*, *P. minimum* and *Fomitiporia mediterranea* (**Bertsch et al. 2013**). The main fungal agents involved in the first step of the disease are *P. chlamydospora* and/or species of *Phaeoacremonium* (expecially *P. minimum*) being the most prevalent and virulent and considered as pioneer fungi (**Larignon & Dubos 1997**, **Gramaje et al. 2018**); they colonize the living wood (fig. 1) so that it is prepared to further colonization by those basidiomycete fungi which are responsible for the typical decay associated with esca, belonging to the genera *Fomitiporia*, *Inocutis*, *Inonotus*, *Fomitiporella*, *Phellinus* and *Stereum* (**Cloete et al. 2015**). In particular, *Phaeoacremonium* spp. have been reported as major contributors to grapevine mortality (**Scheck et al. 1998**, **Rego et al. 2000**).

In the past, esca was generally related to old vineyards, whereas nowadays it shows recurrence in young grapevines (less than 10 years old) and it has emerged as a significant problem in newly established vineyards (**Pollastro et al. 2000**, **Halleen et al. 2003**, **Gimenez-Jaime et al. 2006**, **Romanazzi et al. 2009**). Symptomatic vines have been recorded even in 2- to 3-year-old vineyards, and from such plants *P. chlamydospora* and *P. aleophilum* are consistently isolated (**Mugnai et al. 1999**, **Romanazzi et al. 2009**). A histochemical study of necrotic wood tissue of vines infected with *P. chlamydospora* revealed that the fungus spreads through the xylematic area (**Troccoli et al. 2001**, **Valtaud et al. 2009**).
All species of *Vitis* and varieties of *V. vinifera* are susceptible to esca (**Mugnai et al. 1999**), but the syndromes produced by the causal agents and their severity depends on various factors (**Feliciano et al. 2004**, **Eskalen et al. 2007**, **Surico et al. 2008**). Amongst grapevine genotypes for instance, differences in degree of susceptibility have been assessed. Some cultivars commonly show a higher susceptibility to esca disease (**Christen et al. 2007**, **Andreini et al. 2013**, **2014**), such as Cabernet Sauvignon, Sangiovese, Trebbiano Toscano, Thompson Seedless, Sauvignon blanc, Mourvèdre, Ugni blanc, Cinsault, Trousseau or Temperanillo (sin. Tinta Roriz or Aragonez). They seem to be more prone to express both foliar and wood esca internal symptoms in comparison to Merlot, Pinot Noir, Carignan, Roussane, Montepulciano (**Almeida 2007**, **Christen et al. 2007**, **Quaglia et al. 2009**, **Lorrain et al. 2012**). The different susceptibility to esca among cultivars also appears to be affected by the rootstock combination (**Marchi 2001**). Age in which the plant becomes infected (**Chiarappa 1997**, **Mugnai et al. 1996a**, **Mugnai et al. 1999**, **Feliciano et al. 2004**, **Eskalen et al. 2007**), concurrence of abiotic stresses (**Surico et al. 2008**) and pedoclimatic factors such as rainfall, air temperature, vineyard slope, soil type, and sun exposure are also related with the esca incidence (**Graniti et al. 2000**, **Landi et al. 2012**, **Lecomte et al. 2012**, **Andreini et al. 2014**). It has been observed for instance that cool rainy summers favour the development of the "chronic" form of esca, whereas high temperatures associated with drought promote the "acute" form of the disease (**Surico et al. 2000**). Recently, Van Niekerk et al. (**2011**) showed that incidence and symptom profiles of the pathogens varied greatly between different climatic areas. This indicates that rainfall and temperature influence not only the distribution of pathogens but also their symptomatology (**Andreini et al. 2014**).

ESCA SYNDROMES

The esca disease complex is associated to two main syndromes: i) esca and grapevine leaf stripe disease, and ii) Petri disease, as reported.

i) Esca and grapevine leaf stripe disease

The development and evolution of esca disease may have two ways: chronic and acute. In the "chronic esca", grapevine leaf stripe disease, leaf symptoms are highly variable ranging from drying, dropping, reddening, yellowing (**Lecomte et al. 2012**).

The most characteristic foliar symptoms correspond to small chlorotic spots between the veins and along the margins (**Haleem et al. 2013**). These spots may later coalesce and become necrotic, giving the leaves the main and typical leaf symptom of esca: the "tiger-stripe" pattern (**Haleem et al. 2013**), that is to say chlorotic areas and reddening of leaves resulting in necrotic stripes with only a narrow green stripe along the midrib (**Surico et al. 2008**). Sparapano et al. (**2001**) demonstrated that the first leaf symptoms occur years after infection by wood pathogens. Leaf symptoms normally appear in late spring/early summer (**Surico et al. 2008**).

On the berries, particularly on white varieties, "black measles" - characteristic dark brown spots - may occur on the skin (**Mugnai et al. 1999**, **Surico et al. 2008**, **Lima et al. 2017**). Berries with heavy spotting often show skin cracks and become prey to soft rotting fungi or bacteria (**Mugnai et al. 1999**). Shoots arising from infected wood show stunting and tip dieback (**Haleem et al. 2013**); the most prominent internal symptom is the presence of brown to black spots or streaks in the xylem vessels of the woody cylinder (**Scheck et al. 1998**, **Mugnai et al. 1999**, **Edwards et al. 2001**, **Gubler et al. 2004**).

Apoplexy, also known as "acute" or "severe" form of esca, is characterized by the sudden wilting, followed by the shrivelling and drying of the berries and leaf drop. The healthy-looking leaves dry up in a few days (**Mugnai et al. 1999**). Normally, this event happens in late spring/early summer, when a rainfall event is followed by dry and hot weather, which may lead to an imbalance between foliar transpiration and root absorption (**Mugnai et al. 1999**, **Surico et al. 2006**, **2008**). After this event, the vine can recover growth in the current season or the following one, but, more often, the event kills the plant within a few days (**Bertsch et al. 2013**). The most accepted theory is that apoplexy is caused mainly by a dysfunction of the conducting xylem caused by white rot and tracheomycosis (**Surico et al. 2008**).

Cross section of esca affected trunks reveal a variety of internal wood symptoms, such as brown or black wood-streaking and to a pink, reddish or brown discoloration of the woody portions of grapevine that still look apparently healthy grapevines (**Graniti et al. 2000**). In older vines, the wood may develop a white to yellow soft rot (**Fischer et al. 2002**).

The etiology of esca disease has been a matter of discussion among scientists over the last 20 years. The main hypothesis is that young vines infected with the pioneer fungi *P. chlamydospora* and *Phaeoacremonium*, can later develop esca symptoms following further colonization by several basidiomycetous species belonging to the genera *Fomitiporia*, *Inocutis* spp., *Inonotus* spp., *Fomitiporella* spp., *Phellinus* spp., and *Stereum* spp. (**Sánchez-Torres et al. 2008**, **Cloete et al. 2015**).

ii) Petri disease

It was first reported by the pathologist Lionello Petri (**1912**), and is due to the infection by *P. chlamydospora* and/or some species of *Phaeoacremonium*, mostly *P. minimum* (**Mostert et al. 2006**, **Bertsch et al. 2013, Gramaje et al. 2015**). It may also be related to *Cadophora luteo-olivacea* and *Pleurostoma richardsiae* (**Agustí-Brisach et al. 2013**). Together with black foot, Petri disease is the main disease affecting young vineyards, even from 1 to 7 years old, and it is then recognized as amongst the prevalent causes of young grapevine decline (YGD) (**Eskalen et al. 2007**, **Álvarez-Pérez et al. 2017**).

Internal symptoms can be observed in trunk and cordons (**Mostert et al. 2006**); affected plants show a darkened central pith, black dots circumscribing the pith or sparse over the cross section surface, seen as brown to black streaking in longitudinal sections. The black dots correspond to

blockage of the xylem vessels, showing a dark, gummy substance called “black goo”, containing polymerized phenolic compounds inside these vessels as a response by the host against the fungus growing in and around the xylem vessels (**Mugnai et al. 1999**, **Mostert et al. 2006**, **Surico et al. 2008**). Externally, the plant shows an interruption of growth, yield losses and a decline in vigour leading, in some cases, to the death of the vine (**Surico et al. 2008**, **Bertsch et al. 2013**).

The main difficulty on facing esca, as well as most GTDs, are perhaps the externally asymptomatic nature characteristic of its steps. A peculiar characteristic of esca is indeed the discontinuous expression of chronic symptoms in diseased plants from year to year, generally followed by death from apoplexy (**Mugnai et al. 1996b**, **Surico et al. 2000**, **Calzarano et al. 2001**, **Christen et al. 2007**, **Andreini et al. 2014**). As a consequence of the discontinuous expression of symptoms it is not possible to know when a plant becomes infected. Despite progress in recent years on epidemiology and control of esca, several aspects of the disease still are unclear.

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Chapter II: AIM OF THE STUDY

The research focused on spatial distribution of grapevine esca, and the ecological significance and role of fungal endophyte communities in symptomatic and asymptomatic (healthy) plants. Concerning to the first aspect, different commercial vineyards in Marche region (Italy) have been examined to study the spread of the symptomatic plants, to understand dissemination patterns that provide indications both on directionality and on the evolving of the infection over time. The research included visual assessment of the level of the severity of symptoms for each single plant, an activity of computation of the collected data and a processing phase on statistical software for the creation of graphic maps. The elaborated maps were more easily to interpret and to compare among different years, to reconstruct the development of the disease in the space and in the time. Another aspect that was investigated in this research was the estimation of fungal endophytic communities both in roots of healthy plants and in roots of vines showing typical symptoms of esca in three different commercial vineyards in Marche region (Italy). The main aim was to establish how the population structure of the fungal endophytes change according to the presence of esca pathogens, and if the cultivable endophytic fungi interfere with the pathogens related to esca. The study of variability of fungal communities in asymptomatic and symptomatic vines allowed to mark a first step towards further studies for biological control.

Chapter III: EPIDEMIOLOGY AND SPATIAL DISTRIBUTION OF ESCA DISEASE IN COMMERCIAL VINEYARDS IN MARCHE REGION (ITALY)

ABSTRACT

Esca is a multiple fungal syndrome which can cause serious economic damages in different cultivation areas. The aim of this study was to describe the epidemiology of esca in three commercial vineyards located in Marche region (Italy).

The investigated vineyards were referred to as “OS1”, “OS2” and “CAST” in this study, and the collected data show that esca is a common disease in them all, with the disease incidence which ranged from 10% to 40% in 2017 and from 16 up to 50% in 2018. In both years a consistent percentage of cut, uprooted and dead vines because of esca was recorded.

The severity of symptoms varied significantly from sporadic leaves that showed "tiger-stripes" up to the apoplectic stroke, represented by an entire dry up of the plant. Longitudinal cracks of trunk and presence of typical basidiocarps of *Fomitiporia mediterranea*, an agent of esca, have also been found. Maps describing severity and spread of the disease within the vineyards were elaborated.

Data of severity of symptoms (z) were defined with respect to number of rows (x) and position within the rows (y), allowing to reproduce a spatial distribution of infected plants in the vineyard using SYSTAT v.13 software (Systat Software Inc.). Contours were followed using the method of Lodwick & Whittle (**1970**) combined with linear interpolation. The data processing made possible to verify in 2017 and 2018 that i) none of the vineyards showed a gradient of infection among the rows; ii) there was a west-east gradient of infection in the vineyard OS1, and in the vineyard OS2 a greater concentration of disease in the center than the edges was observed; iii) a significant correlation was recorded between the esca disease incidence and different combination of rootstocks (420A, SO4, 41B, 110R) in vineyard OS1.

The obtained result represents a further set of information for a better understanding of the epidemiology of esca, still particularly damaging and difficult to control.

INTRODUCTION

Vitis vinifera (L.) is known to host a wide range of pathogens respect to any woody agricultural plant (**Martelli 1997**). Among them, fungal pathogens are of significant importance since *V. vinifera* is susceptible to 29 fungal diseases (**Wilcox et al. 2015**), including esca disease, which is currently considered one of the most detrimental (**Bertsch et al. 2013**).

Changes in cultural techniques, prohibition of sodium arsenite treatment most of all in Europe allowed to esca to become an emergent disease.

Nowadays, esca is widespread in all vine-growing regions of Italy (**Pollastro et al. 2000**, **Serra et al. 2000**, **Sidoti et al. 2000**, **Surico et al. 2000**, **Romanazzi et al. 2006**, **Michelon et al. 2007**), and of the World, including California, USA (**Scheck et al. 1998**), Portugal (**Rego et al. 2000**), France (**Larignon & Dubos 1997**), Spain (**Gimenez et al. 2006**), Australia (**Edwards & Pascoe 2004**), Greece (**Rumbos & Rumbou 2001**), New Zealand (**Ridgway et al. 2002**) and South Africa (**Halleen et al. 2003**). It affects the old vineyards, and more and more often now, the young vineyards.

The sanitary situation of Italian vineyards relative to esca is not dissimilar from that of the rest of the World. In some regions of central and southern Italy where epidemiological studies have been carried out, such as Tuscany, Marche, Abruzzi, Apulia, and Sicily, esca incidence has reached 60%

to 80% in some old vineyards (**Pollastro et al. 2000**, **Sidoti et al. 2000**, **Surico et al. 2000**, **Romanazzi et al. 2006**, **Calzarano & Di Marco 2007**), while it was overall lower in northern Italy, in Trentino Alto Adige and Veneto (**Michelon et al. 2007**, **Borgo et al. 2008**), although with a different behavior of the cultivars.
The wide range of foliar symptoms appears to be due to interactions among the microorganisms involved, cultivar susceptibility, rootstock, age of the vines, and pedoclimatic conditions (**Graniti et al. 2000**, **Landi et al. 2012**, **Lecomte et al. 2012**, **Petit et al. 2006**, **Valtaud et al. 2011**). In particular, the main fungal agents involved in esca disease are *Phaeomoniella chlamydospora* (Pch), *Phaeoacremonium minimum* (Pmi), and *Fomitiporia mediterranea* (Fomed) which seems to be responsible for a chronic form of the disease characterized by leaf discolorations (tiger-stripes), brown-red wood necrosis, dark streaks, and spongy wood (**Mugnai et al. 1999**, **Fischer 2002**). Foliar symptoms may not occur in the same plant every year (**Mugnai et al. 1996**, **Calzarano et al. 2001**, **Andreini et al. 2014**). The acute or apoplectic form manifest itself in a sudden wilting of the entire plant or of one arm or several shoots (**Dubin et al. 2000**, **Gramaje et al. 2018**). Leaf symptoms include now scorching, dropping and shriveling (**Gramaje et al. 2018**), and it is also frequently observed the drying of grape clusters (**Mugnai et al. 1999**).
To solve some open questions about the epidemiology and spreading of esca disease, the spatial distribution may give an essential contribution. The spatial distribution of a specific disease is defined as how the pathogen spreads in space (**Carstens 2014**). Spatial statistics and spatial mathematical modeling have been used to describe the distribution and spread of esca in vineyard (**Li et al. 2017**). Several spatial statistical analyses, based on annual data from different vineyards in Europe, revealed that the random distribution of esca prevails in most vineyard situations (**Cortesi et al. 2000**, , **Surico et al. 2000Edwards et al. 2001**, **Redondo et al. 2001**, **Sofia et al. 2006**), suggesting that the spread of esca within the vineyard is mainly airborne, via external and/or internal sources of inoculum, rather than due to contaminated pruning tools along rows of vines (**Surico et al. 2000**). Aggregated patterns of symptoms have been anyhow observed in certain vineyard scenarios. Edwards **et al.** (**2001**) found for instance foliar symptoms of esca both randomly distributed and clustered, whereas the symptoms of esca seemed to have a tendency toward aggregation - especially in young vineyards (**Pollastro et al. 2009**). This situation leads to wonder whether there are specific patterns that are related with environmental and/or management factors.
Three studies started to investigate the esca spread over time through spatial modeling. Stefanini et al. (**2000**), after proposing a parametric statistical model to estimate the probability of symptoms expression, observed a slight increasing in the probability of symptoms outbreak when infected vines were present along the same row in one Italian vineyard. Zanzotto et al. (**2013**), using Bayesian spatio-temporal methods, found a higher probability of esca expression over time and greater spread along rows, rather than among adjacent rows, whereas the results of Li et al. (**2017**), obtained through JC statistics adapted for binary data (**Moran 1948**), suggested a limited potential for secondary local spread from neighboring symptomatic grapevines.

Aim of this research was to monitor three different commercial vineyards located in Marche region in Italy and analyzing temporal and spatial distribution of esca symptomatic plants to increase knowledge regarding the epidemiology. Spatial analyses using recorded data should help us to explain the changing over time of esca symptoms, to evaluate the spatial relationship between previously and newly symptomatic vines, and to improve future management strategies to apply in the commercial vineyards.

MATERIALS AND METHODS

Study areas

During late August 2017 and 2018, in Marche region two visual inspections were carried out in three commercial vineyards. Each vineyard was defined with a reference abbreviation (OS1, OS2, and CAST) and the main characteristics of vineyards were reported and listed (tab. 1; fig. 1).

Vineyard OS1

The vineyard located in Montegallo (Osimo, Ancona) was coded as "OS1". With a position at around 463 m above sea level, it measures about 2 ha, whereas the portion taken into consideration for the visual surveys was about 1.3 ha, mainly cultivated with cv Verdicchio. All vines were planted in 1994, and grafted on four different rootstocks (420A, SO4, 41B, and 110R) with Guyot as training system (tab. 2, fig. 1). The vineyard is in a hilly position, with an exposition south/east. The soil is medium mixture tending to clayey (clay loam soil), fresh, fertile and deep. The vineyard is essentially covered by natural grass between the rows, mainly during the warm season, and a mechanical weeding is carried out within the rows. A mechanical long-type-pruning is carried out on the vines. The management of green (summer pruning) consists of suckering, removing of side-growths, binding and topping of shoots, defoliation, and thinning of bunches. The control of fungal diseases (downy mildew, powdery mildew and gray mold) is carried out according to the organic regulation. Immediately after pruning, a commercial formulation (Remedier, Isagro), based on *Trichoderma* was applied to prevent esca disease.

Vineyard OS2

The vineyard coded as "OS2" is owned by the Azienda Vitivinicola Moncaro (tab. 1; fig. 1), is located in Casenuove (Osimo, Ancona) at about 44 m a.s.l., and the considered area was 0.95 ha, grown with cv Chardonnay. Soil in OS2 is medium-textured, with good amount of organic substance (loam - sandy loam soil). The vineyard is exposed to the south/south east. The vineyard was planted in 1994, and grafted onto Kober 5BB rootstock. The planting size allowed about 2200 plants per hectare (about 102 plants per row). The training system is single curtain. Grapevine diseases rely on integrated pest management.

Vineyard CAST

The CAST vineyard, in Castelplanio, owned by the Azienda Vitivinicola Moncaro, has a total area of around 0.95 ha, with medium-textured and relatively loose soil and low amount of organic substance (tab. 1; fig. 1) (loamy sand soil). The ground morphology is hilly, with exposure south/west. The vineyard was planted in 2004, with about 3485 plants per hectare. The farm techniques are mostly mechanically managed, like the winter pruning, which is carried out during the winter mainly through mowing bar pruning machines. The summer (or green) pruning is made up by suckering and removing of side-growths of shoots, binding and topping of shoots, leaf stripping. The soil processing along the row is managed in alternate rows: especially in emergency period, this makes possible the intervention regardless of weather conditions, precisely thanks to the grass layer along the row which makes walkable the ground. The disease management is carried out according integrated control methods.

Visual inspection and disease assessment

The visual survey at the end of August 2017 and 2018 were carried out in OS1, OS2 and CAST vineyards to verify the presence of leaf symptoms related to esca (tiger-stripes) and the spreading in the framework of two years and in the space. Symptomatic plants were identified in the field and reported on a bi-dimensional map.

Moreover, the disease severity was estimated for each plant, according to an empirical scale

consisting of five disease severity classes: 0 = symptomless/healthy plant; 1 = plant showing 1-10 leaves with symptoms; 2 = plant showing up to 50% of symptomatic leaves; 3 = plant with more than 50% of canopy with symptoms; 4 = apopletic stroke.
We recorded also the vines which died in the previous years. The collected data and the adoption of the empirical scale made possible to calculate, for each vineyard, incidence (D) severity (S), and disease intensity (I) (or McKinney Index).

The incidence (D) expresses the percentage of infected vines on the total of examined vines. This parameter was calculated with the following formula (1)

$$D = n \times 100/N \qquad (1)$$

with n = number of symptomatic plants and N = total number of observed plants.

Disease Severity (S) was calculated with the formula (2)

$$S = \Sigma(c \times f)/n \qquad (2)$$

with c = value of severity class, f = frequency in the severity class and n = number of symptomatic vines. The plants which were recorded as died and absent were considered as previously affected by esca disease.

The McKinney's index (I) (**McKinney 1923**) includes information on both disease incidence and disease severity; this parameter, expressed as the weighted average of the disease with a percentage of the maximum possible level, was calculated with the formula (3)

$$I = \Sigma(c \times f) \times 100/N \times v \qquad (3)$$

with c = value of severity class, f = frequency in the severity class, N = total number of plants observed and v = highest value of severity class.

Analysis of the spatial distribution of esca disease
The occurrence of disease gradients within the vineyards was studied. For this purpose, to analyze the spatial distribution of the symptomatic plants in the vineyards, the position of each plant was identified by referring to the row number (X) and the position within the row (Y). This made it possible to create two-dimensional maps reproducing the spatial distribution of the infected plants in the vineyard, using the SYSTAT software (Systat Software, Inc., San Jose, CA), to spatially monitor the epidemiology.
The same software allowed to create a mosaic maps of the vineyard according to the method proposed by Lodwick & Whittle (**1970**), in addition to take in account the position of the plants (X, Y) also consider the disease severity (Z), combined with linear interpolation. The plot automatically determines the number of contours to draw, so that the surface is delineated and the contour labels can be characterized by different colors.
To verify the presence of inter-row disease gradient within the vineyards, the percentage of symptomatic plants in the different rows was calculated. Then for evaluating the intra-row disease gradient, each vineyard was divided in different plots, and in each plot the percentage of symptomatic plants were calculated. For the vineyard OS1 the plot was designed according to the different location of rootstock (420A, SO4, 41B, 110R) onto which cv Verdicchio was grafted. To determine if there was an inter-row or intra-row disease gradient, the regression curve was calculated. The found regression curves represent the percentage of symptomatic plants in

relation to the distance from the edge of the vineyard.

RESULTS

Visual assessment

According with the collected data, esca is a common disease in the three investigated vineyards. In both years a consistent percentage of cut, uprooted and dead vines for esca was recorded (fig. 2). Considering the three parameters (diffusion, severity and McKinney Index), in the three vineyards investigated, we can say that there is a recrudescence of the disease from 2017 to 2018 (fig. 2, tab. 3).

During the visual inspections both in 2017 and 2018, the main symptoms recorded was the chlorotic and necrotic spots on leaves ("tiger-stripes"). The stripes wedged on leaves between one veining and the other, first with yellow color then gradually necrotic, with a more vivid color on the edge both on the cv Verdicchio at vineyards CAST and OS1 and on cv Chardonnay at vineyard OS2 (fig. 3A-B).

During the inspection, several cases of apoplexy were found. It usually occurs during the summer months, with a rapid progress of the disease that leads to a sudden death of the plant. Before falling, the leaves remained on the plant for a long period, while the ripening of bunches stopped (fig. 3C). New shoots growing at the base of the rootstock appeared generally asymptomatic (fig. 3D), or showed mild symptoms of esca (disease severity class 1).

Further symptoms related to esca disease were found in both years of visual assessment on the woody part. In particular, on the cv Chardonnay at vineyard OS2, different plants were reported as showing symptoms of "mal dello spacco" (**Surico et al. 2008**). The plants showed longitudinal cracking and woody tissues, symptoms mainly determined by *F. mediterranea*, a fungal agent of white rot wood decay, able to degrade the wood making it friable and spongy (fig. 3E). On some vine were found the typical fruiting bodies of the fungus (fig. 3F). They appear and are mainly evident in the most humid periods, producing basidiospores that when released can reach nearby other hosts. Due to the presence of both dead plants and apoplectic plants, subsequently cut, it was possible to find a high absence of plants along the rows, especially in the vineyards cultivated with Verdicchio and Chardonnay at vineyards OS1 and OS2, respectively (fig. 3G).

In the vineyard CAST, especially during the inspections carried out in 2017, it was possible to observe many plants previously undergone (during the winter period) to a pruning, required for changing the training system (fig. 3H-I). The plants showed evident cuts without any protection, and their surface showed an emission of blackish mucilaginous substances usually associated with the reaction of the plant to infections of esca agents.

Spatial distribution of esca disease

The data collected in 2017 and 2018 were elaborated allowing to obtain two-dimensional maps of presence of symptoms (fig. 4), severity (fig. 5), analysis of the spatial distribution of infected plants, gradient of inter-row and intra-row infection respect to the edge of the vineyards. Between the inspections, a slight progression of the disease with a slight accentuation of the severity of the symptoms has been highlighted.

The number of grapevines in each vineyard changed over time. This factor is mainly related to both death of plants and to planting of new vines (mostly to replace the dead ones).

The results for each year will be shown and commented on below.

Vineyard OS1

In OS1, the survey carried out in 2017 regarded a total of 5412 plants. During the visual inspection 1529 (28.8%) plants were recorded as symptomatic or dead, with 410 of them showing leaf symptoms and 1119 plants which died in previous years, presumably due to esca (fig. 4). The disease severity recorded in 2017 was 2.01, most symptomatic plants were in class 1 (44.4%), followed by class 3 (26.6%), class 2 (19.7%) and 4 (9.3%) (fig. 5).

The McKinney's index, that includes information on both disease incidence and disease severity, was equal to 24.47% (tab. 3).

Concerning to 2018, the survey regarded a total of 5405 plants, with 1788 of them (33.98%) recorded as symptomatic or dead. 534 plants showed esca leaf symptoms indeed, and 1254 died in previous years due to esca (fig. 4). There was an increment of disease severity at 2.35, most of symptomatic vines were ranged in class 3 (26.50%), with several in class 1 (44.30%), followed by class 2 (20%) and 4 (9.20%) (fig. 5). The McKinney's index for 2018 corresponded to 29.00% (tab. 3).

Furthermore, there was no statistically significant disease gradient between rows (fig. 6A), whereas a progressive reduction in the percentage of symptomatic plants from west to east was recorded, which can be summarized with the following binomial regression curves for 2017 ($y = -0.3769x^2 + 0.161x + 34.691$; $R^2 = 0.9938$) and 2018 ($y = -0.9447x^2 + 4.8886x + 30.764$; $R^2 = 0.9737$) (fig. 6B).

Analysing the data of esca disease incidence and spatial distribution according the four different rootstocks, a highly significant correlation was described. We found then a significant difference of symptoms between the rootstock types, and the highest incidence was recorded on vines grafted on rootstock 420A (31.65 in 2017, 35.52 in 2018), followed by 41B (26.92 in 2017, 30.13 in 2018), S04 (23.27 in 2017, 26.75 in 2018) and 110R (16.15 in 2017, 18.84 in 2018). The result is summarized with the following binomial regression curves for 2017 ($y = -0.5996x^2 - 2.0184x + 34.04$; $R^2 = 0.9919$) and 2018 ($y = -0.6277x^2 - 2.2035x + 38.028$; $R^2 = 0.9854$) (fig. 7A-B).

The recorded disease severity varied between rootstocks: in 2017 we found a disease severity of 2.16 on vines grafted on rootstock 420A, 1.96 for rootstock 41B, 1.63 on S04 and 1.41 on plants grafted on rootstock 110R. The following year we found a similar trend, with 420A (2.39) followed by 41B (2.33) and S04 (2.17), with a swift increasing of 110R (2.64, from 1.41 in the previous year).

A clear decreasing of McKinney's index values amongst the rootstocks was also observed in both years (tab. 2).

Vineyard OS2

In 2017, OS2 consists of 2613 plants, of which 1039 (40%) were identified during the visual inspection as symptomatic or dead because of esca. Amongst them, 196 showed ~~typical~~ symptoms of esca disease, whereas 843 had died in previous years, presumably due to esca disease (fig. 4). The disease severity recorded in 2017 was 2.03, and most of symptomatic plants were in class 1 (40.3%), followed by class 2 (28.6%), class 3 (20%) and 4 (11.7%) (fig. 5). In 2017 a McKinney's index value of 36.07% was calculated (tab. 3).

In 2018, 477 vines out of 2615 showed esca leaf symptoms, and 872 as dead or cut/uprooted for esca (fig. 4). The disease severity recorded in this vineyard was 2.52, in particular 30% range in class 3, 27% in class 2 and 21% in class 1 and 4 (fig. 5). The McKinney's index in 2018 was 44.84% (tab. 3).

Furthermore, for both years there was no infection gradient between the rows with respect to the edge of the vineyard (fig. 8A), while a gradient of intra-row infection was found, which can be summarized with the following binomial regression curves in 2017 ($y = -5.9777x^2 + 36.939x – 5.597$; $R^2 = 0.9025$) and 2018 ($y = -3.5594x^2 + 22.71x + 22.078$; $R^2 = 0.9395$) (fig. 8B).

Vineyard CAST

In vineyard CAST, 3485 plants were monitored during the inspection in 2017, and 335 vines were reported as showing symptoms ascribable to esca, with a percentage of infection of 9.6% (fig. 4). The disease severity was 1.54, most of the symptomatic plants were in class 1 (65%), followed by class 2 (17%) and 3 (16.7%). Occasionally, apoplectic plants class 4 (1%) were found (fig. 5). The corresponding McKinney's index in 2017 in CAST vineyard was 8.32% (tab. 3).

During 2018, 3324 plants were recorded, among which 545 showed symptoms ascribable to esca (fig. 4). The disease severity recorded in 2018 was higher (2.52), in particular 42% of plants were ranged in class 3, 26% in class 2, and 18% and 14% respectively in class 1 and 4 (fig. 5). The value of McKinney's index in 2018 was 14.14% (tab. 3).

No gradient of esca disease was found between rows during both of years (tab. 9A). The intra-row disease gradient, on the other hand, was rather significant and can be summarized with the following binomial regression curves for 2017 ($y = -0.098x^2 + 2.3838x + 12.546$; $R^2 = 0.74721$), which shows us how the disease it progresses from west to east within the inter-row, and 2018 ($y = 0{,}4009x^2 - 5.8913x + 48.635$; $R^2 = 0.9231$) (fig. 9B).

DISCUSSION

Nowadays, esca appears as one of main diseases of vine internationally, causing an extensive economic damage in vineyards all over the world (Mugnai et al. 1999, Bertsch et al. 2013). At national level, the importance of vine in Italy as an economic resource and the recurrence of esca in recent years has prompted researchers to intensify research about this complex phytosanitary problem. The data collected during the survey period (August 2017 and 2018) showed that esca is a common disease in the studied vineyards of Marche region. In the two years, an increase of the incidence of plants with esca symptoms was noted in accordance with Zanzotto et al. (**2013**). The cumulative number of plants showing foliar symptoms of esca increased through the years as well, with symptoms of the affected vines generally more severe over time, confirming the graduation and the irreversibility of the disease within the vineyards. The recorded percentages confirm what already showed in wide study conducted previously in Marche region (**Romanazzi et al. 2009**, **Murolo & Romanazzi 2014**), but also those recorded in monitored vineyards of Apulia and Tuscany (**Pollastro et al. 2000**, **Surico et al. 2000**).

In both years of surveys, the most recurrent symptoms recorded in the three vineyards were tiger-stripes, representing the chronic form of esca. As reported by literatures, at least a part of external symptoms is caused by phytotoxical fungal metabolites (**Bruno & Sparapano 2007**). Generally, most of the plants that externally show such symptoms, internally (wood level) may show blackish streaks due to the colonization of tracheomycotic fungi *P. chlamydospora* (*Pch*) and *Phaeoacremonium* (*Pmi*) (**Mugnai et al. 1997**, **Sparapano et al. 2000**, **Eskalen et al. 2007, Romanazzi et al. 2009**). Such characteristic symptoms seem to derive from a plant, that produces phytoalexins and polysaccharides in relation to the production of pectinolytic enzymes and toxins synthesized by pathogens.

Only in the vineyard OS2, longitudinal cracking of the trunk was also observed, as a structural alteration of wood generally associated with infections of *F. mediterranea*, whose fruiting bodies are formed mainly in rainy and humid periods. In Europe, this fungus on vine determines white rot wood decay, making friable and spongy wood (**Mugnai et al. 1999**, **Fischer 2002**).

Different cases of apoplexy have been found in the vineyards, i.e. plants that suddenly wither and dry (**Surico et al. 2008**). The causes of apoplexy are not yet fully known, although it has generally been observed in the vicinity of summer rains followed by hot windy days in plants whit a particularly compromised host system (**Surico et al. 2008**). At the resuming of the vegetative growth, the following year, plants affected by apoplexy can sometimes resume vegetating by emitting suckers on the side of the stem underneath the altered wood (**Geoffrion 1982**). The data collected during the present study provide us a picture of the health status with respect to two vegetative seasons. To calculate the real incidence of esca disease, without neglecting any infected plants, it is necessary to extend the observation period considering at least 3 to 5 years (**Romanazzi et al. 2009**). Leaf symptoms of esca are indeed clearly visible during the summer, and can occur unevenly since influenced by factors which are external to the plant-pathogen complex (rains and temperatures), which can vary from year to year (**Surico et al. 2000**).

In the studied vineyards, the record of the disease was useful to elaborate two-dimensional maps that identify spread and severity of esca in 2017 and 2018. Maps of severity easy to interpret, revealed a recrudescence of esca disease: not only the number of symptomatic vines but also the severity increased from 2017 to 2018. In the literature, the incidence and symptom profile of trunk disease can greatly vary between regions and according rainfall and temperature (**Van Niekerk et al. 2011**), which seems to influence also the pathogen distributions (**Magarey & Carter 1986**, **Merrin et al. 1995**, **Úrbez-Torres et al. 2006**, **Pitt et al. 2010**).

Besides the climatic factors, the symptoms seem to depend on various sensitivity amongst different vine variety, vessel size (**Tramontini et al. 2014**, **Pouzoulet et al. 2014**), on the characteristics of vineyards and agronomic input such fertilization (**Calzarano et al. 2009**), on the breeding system, on the influence that the type of rootstock can exerted and on stress conditions (excess soil moisture) (**Surico et al. 2000**, **Marchi 2001**, **Corti et al. 2004**, **Surico et al. 2004**, **Murolo & Romanazzi 2014**).

Particularly significant is the situation recorded in the Castelplanio vineyard, where there is a retraining of the growth system, with evident wounds that can easily be colonized by pathogenic fungi, as previously proved by several works (**Eskalen et al. 2007**, **Petzoldt et al. 1983**, **Rooney Latham et al. 2005**, **Úrbez-Torres & Gubler 2008**, **Lecomte et al. 2012**). The vine is exposed to the infection risk during the pruning period due to the high number of wounds, which remain particularly receptive for several weeks (**Eskalen et al. 2007**, **Úrbez-Torres & Gubler 2008**). Tests about protection of fresh pruning wounds which were carried out using biological compounds based on *Trichoderma* did not allow a lasting and significant protection against the agents of esca disease (**Di Marco 2010**, **Frisullo et al. 2010**).

Analyzing the position of the vines with respect to the distance from the edge of the vineyard, a statistically significant disease gradient was not found between the rows for none of the examined vineyards. Statistically significant instead the intra-row infection gradient for the vineyards, even if represented by different regressions curves in both years. In the vineyard OS1, a high correlation was recorded between the disease incidence and the different rootstock used. It has long been known that some species of *Vitis* contain characters conferring resistance to numerous disorders of biotic origin. According to Marsais (**1923**) grapevines grafted on *V. rupestris*, richer in tannins than *V. riparia*, are more frequently affected by esca. Rootstock have been reported to modify the water status and gas exchange under field conditions with *V. vinifera* varieties (**Koundouras et al. 2008**), and indirectly they can affect the plant reactions. The rootstock has been reported to affect the efficiency of water transport to the shoots via conductivity constraints imposed by the

anatomy of the xylem vessels (**de Herralde et al. 2006**), which can be modified and/or occupied by the esca disease agents. This could be at the bases of the different incidence of esca disease recorded in previous studies carried out on different varieties grafted on different rootstocks both in commercial vineyards under natural conditions (**Marchi et al. 2006**; **Murolo & Romanazzi 2014**) and under artificial inoculation (**Gramaje et al. 2010**, **Landi et al. 2012**).

We recorded a progressive reduction of the percentage of symptomatic plants from west to east in OS1 (2017-2018) and CAST (2018), whereas in CAST (2017) a progressive increase in the same direction was recorded. In OS2, instead, a wider incidence of symptomatic plants was found in the central part and a reduction towards both edges.

In all three vineyards the distribution of symptomatic plants was random, as similarly described in several annual surveys that have been published on previous works in Europe (**Cortesi et al. 2000**, **Edwards et al. 2001**, **Redondo et al. 2001**, **Sofia et al. 2006**, **Li et al. 2017**). These data are in accordance to the fact that the spread of esca in the vineyard is mainly due to air flows, through internal and external sources of inoculation rather than through contamination of pruning tools along the row (**Surico 2000**). A particularly interesting recent study by Li et al. (**2017**) highlights that esca disease does not generally spread among neighboring plants, but in a random manner. Random distribution can be explained by the spread of pathogens associated with esca. Romanazzi et al. (**2009**) in Marche region found *P. chlamydospora* and *P. minimum* on symptomatic plants, which are potentially spread by rain and insects out (**Edwards et al. 2001**, **Mostert et al. 2005**). Moyo et al. (**2014**) have shown that even arthropods can be potential vectors of esca, and be particularly efficient in spreading over short and long distances. Another fungus previously found on symptomatic plants in the studied vineyards is *F. mediterranea* (**Romanazzi et al. 2009**), considered as a fungus able to spread through the air flows.

The assessment we presented, as well as those of previous studies of spatial modeling and distribution of esca (**Stefanini et al. 2000**, **Zanzotto et al. 2013**, **Li et al. 2017**), might be improved by considering weather and soil parameters, i.e. relationship between availability of water, nutrients and structure of the soil. The present study on the monitoring of spatial distribution of esca in three different vineyards in Marche region, enriched with data on the etiology, and considering environmental parameters are essential knowledges to plan management strategies to mitigate esca disease.

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Tables

Table 1 - Main characteristics of OS1, OS2 and CAST vineyards.

Site characteristics	Vineyards		
	OS 1	**OS 2**	**CAST**
Location	Osimo	Osimo	Castelplanio
Province	Ancona	Ancona	Ancona
Ownership	Azienda Agraria Cantina Badialetti	Moncaro winery	Moncaro winery
Vineyard area (ha)	1.3	0.95	0.95
N. vines (2017/2018)	5412/5405	2613/2615	3485/3324
Latitude	43°31'09''N	43°29'81''N	43°50'30''N
Longitude	13°28'47''E	13°26'12.83''E	13°09'50.3''E
Altitude (m)	463	44	334
Cultivar	Verdicchio	Chardonnay	Verdicchio
Rootstock	420A (*V. berlandieri* × *V. riparia*) S04 (*V. berlandieri* × *V. riparia*) 41B (*V. vinifera* × *V. berlandieri*) 110R (*V. berlandieri* × *V. rupestris*)	Kober 5BB (*V. berlandieri* × *V. riparia*)	Kober 5BB (*V. berlandieri* × *V. riparia*)
Plant spacing (m)	3.0 by 1.5	2.5 by 1.5	2.5 by 1.5
Trellis system	Single curtain	Single curtain	Single curtain
Year of planting	1994	1997	2004
Soil management	Tillage and chemical	Chemical	Chemical
Fertilization	Mineral	Mineral	Mineral
Control of main fungal disease	Biological control	Guided and integrated control	Guided and integrated control

Table 2 - McKinney's index values on rootstocks in 2017 and 2018, OS1 vineyard.

Rootstock	2017	2018
420A	27.18%	31.95%
S05	22.52%	25.88%
41B	19.11%	23.41%
110R	13.55%	17.07%

Table 3 - McKinney's index values in OS1, OS2 and CAST vineyards (2017 and 2018).

Vineyard	2017	2018
OS1	24.47%	29.00%
OS2	36.07%	44.84%
CAST	8.32%	14.14%

Figures

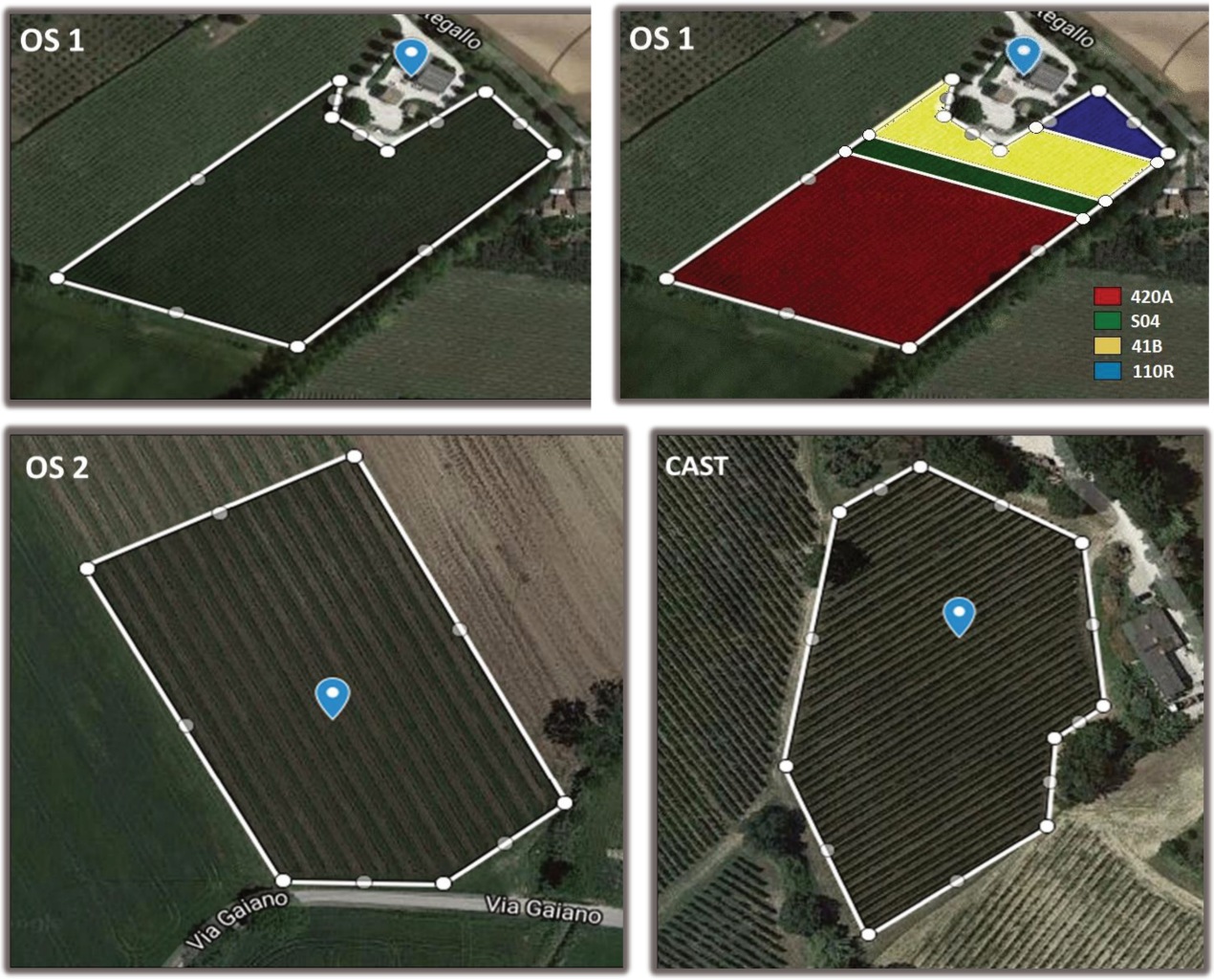


Figure 1 - Maps of OS1, OS2, and CAST vineyards and distribution of rootstocks in OS1 vineyard (Google Earth Pro 7.3.0.3830, images 2017, modified).

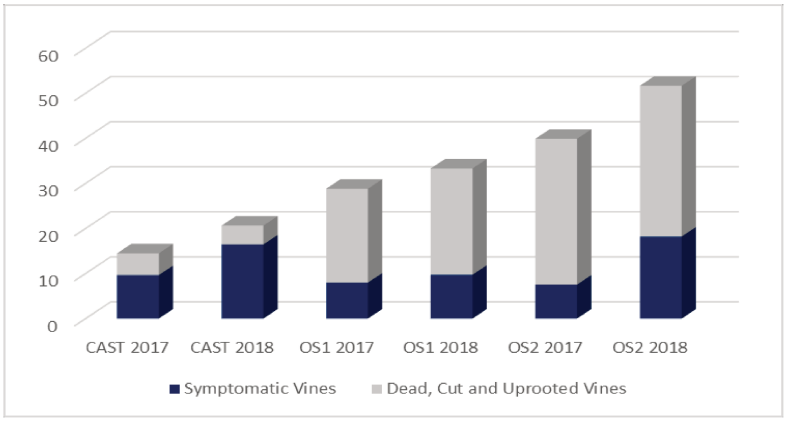


Figure 2 - Incidence of esca in 2017-2018.

Figure 3 - Esca symptoms in the studied vineyards: tiger-stripes on adult leaves in Chardonnay (A) and Verdicchio (B); apoplectic stroke (C); with asymptomatic shoots at the base of the rootstock (D); white rot on adult Chardonnay vine (E) and carpophores of *Fomitiporia mediterranea* (F); absence of vines along the row in Osimo (G) (OS2 - Chardonnay); conversion cuts on Verdicchio vines (H-I).

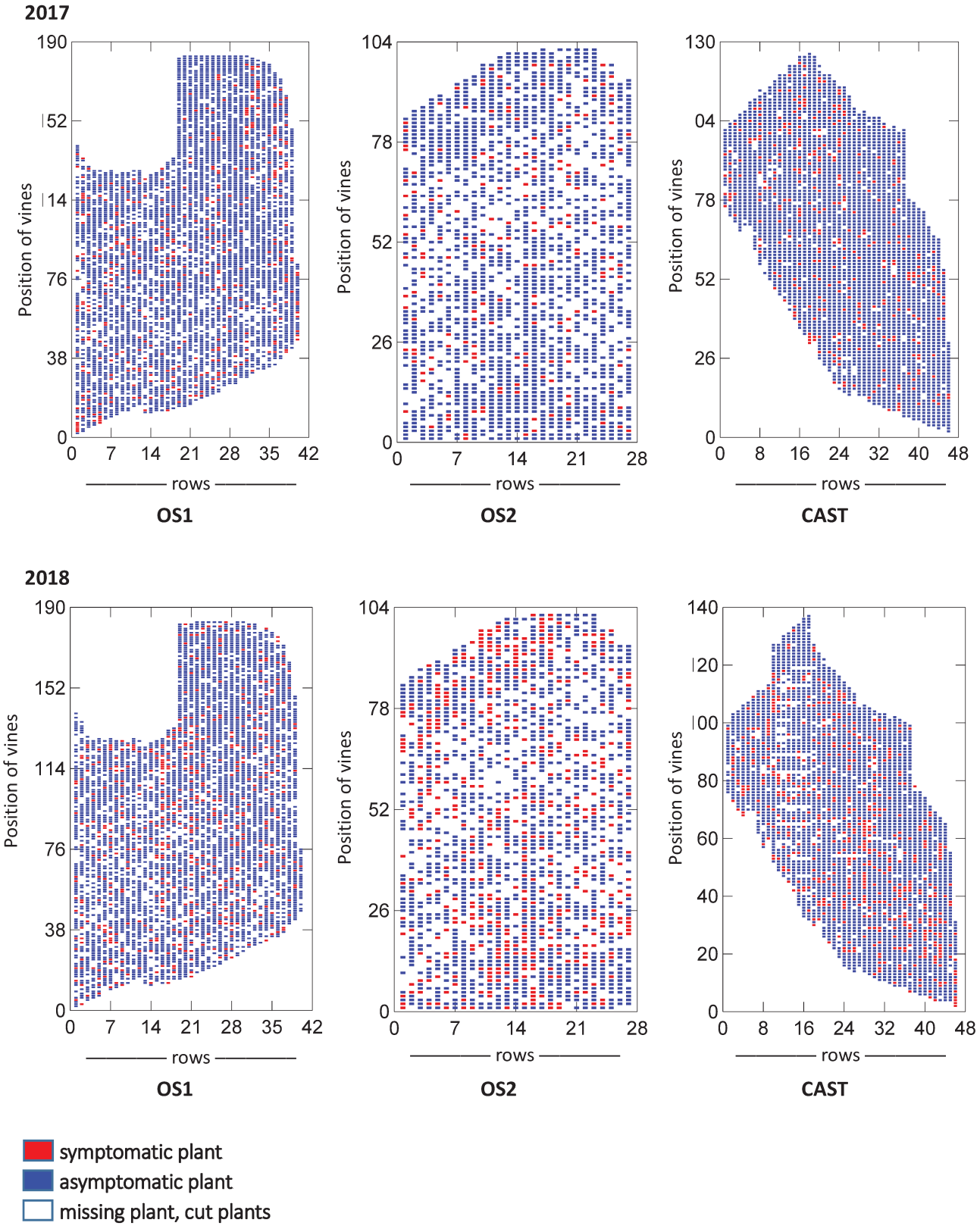


Figure 4 - Presence of esca disease (leaf tiger- stripes) in OS1, OS2 and CAST vineyards, according to the data collected in 2017 and 2018.

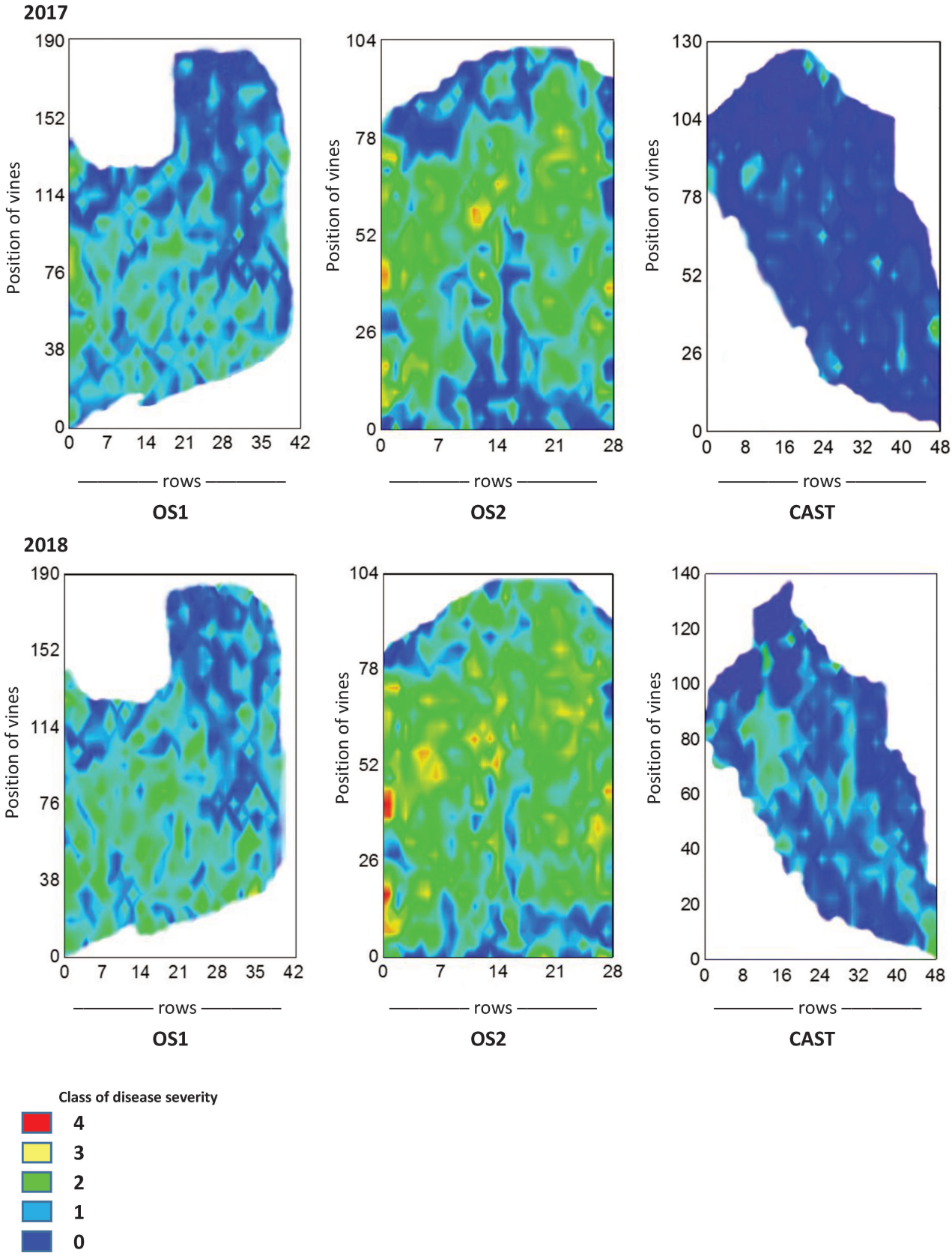


Figure 5 - Disease severity in OS1, OS2 and CAST vineyards, according to the data collected in 2017 and 2018.

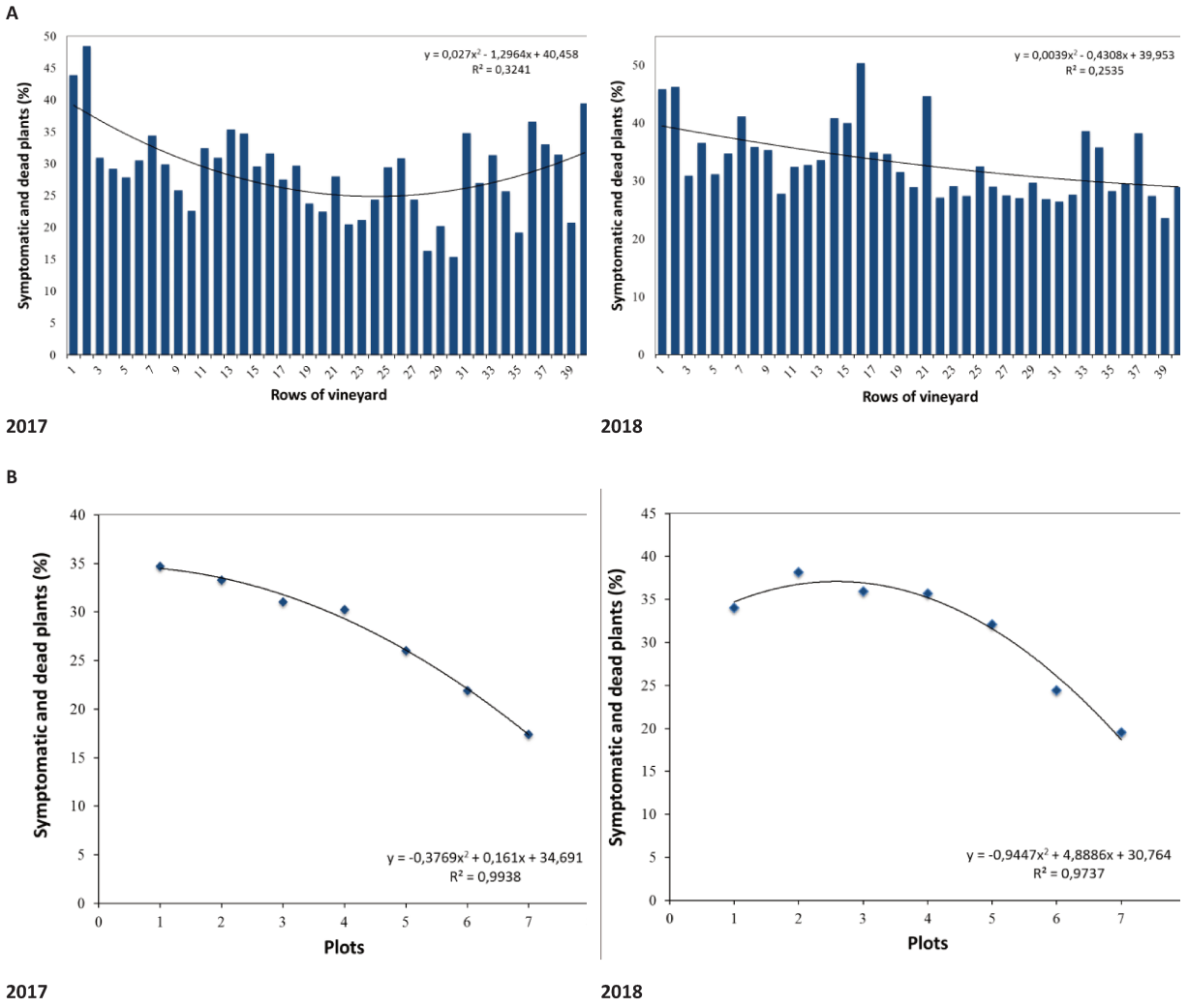
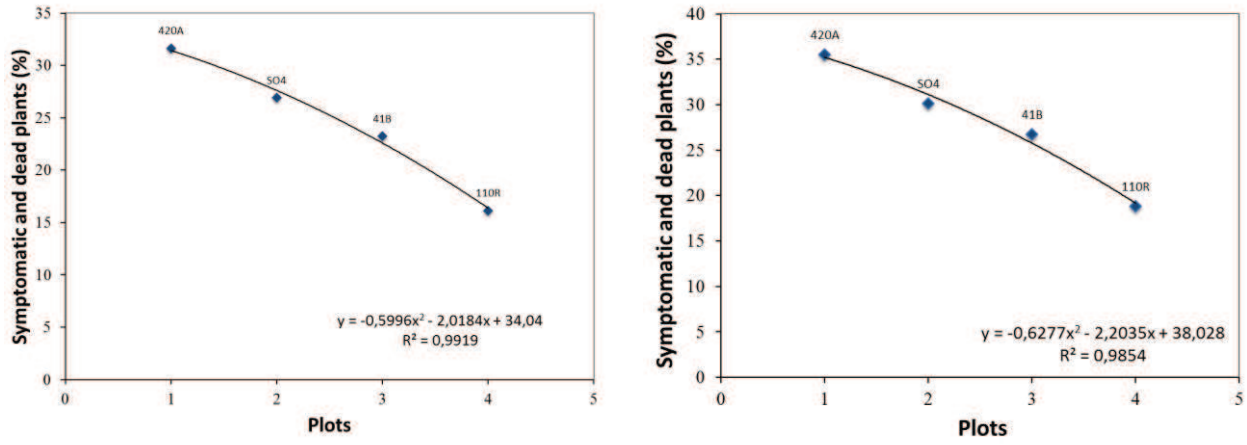


Figure 6 - Inter-row (A) and intra-row (B) disease gradient, respect to the edge of the vineyard OS1.

(A) Inter-row disease gradient within the vineyard represents the percentage of symptomatic plants in the each rows.

(B) The intra-row disease gradient was evaluated, considering the percentage of symptomatic vines in each plot. The vineyard OS1 was divided in 7 plots.
The regression curves represent the percentage of symptomatic plants in relation to the distance from the edge of the vineyard.



2017 **2018**

Figure 7 - Intra-row infection gradient according to rootstocks (420A, SO4, 41B, 110R), respect to the edge of the vineyard OS1.

The intra-row disease gradient was evaluated, considering the percentage of symptomatic vines in each plot. The vineyard OS1 was divided in 4 plots, according the position of the different rootstocks on which cv Verdicchio was grafting. The regression curves represent the percentage of symptomatic plants in relation to the distance from the edge of the vineyard.

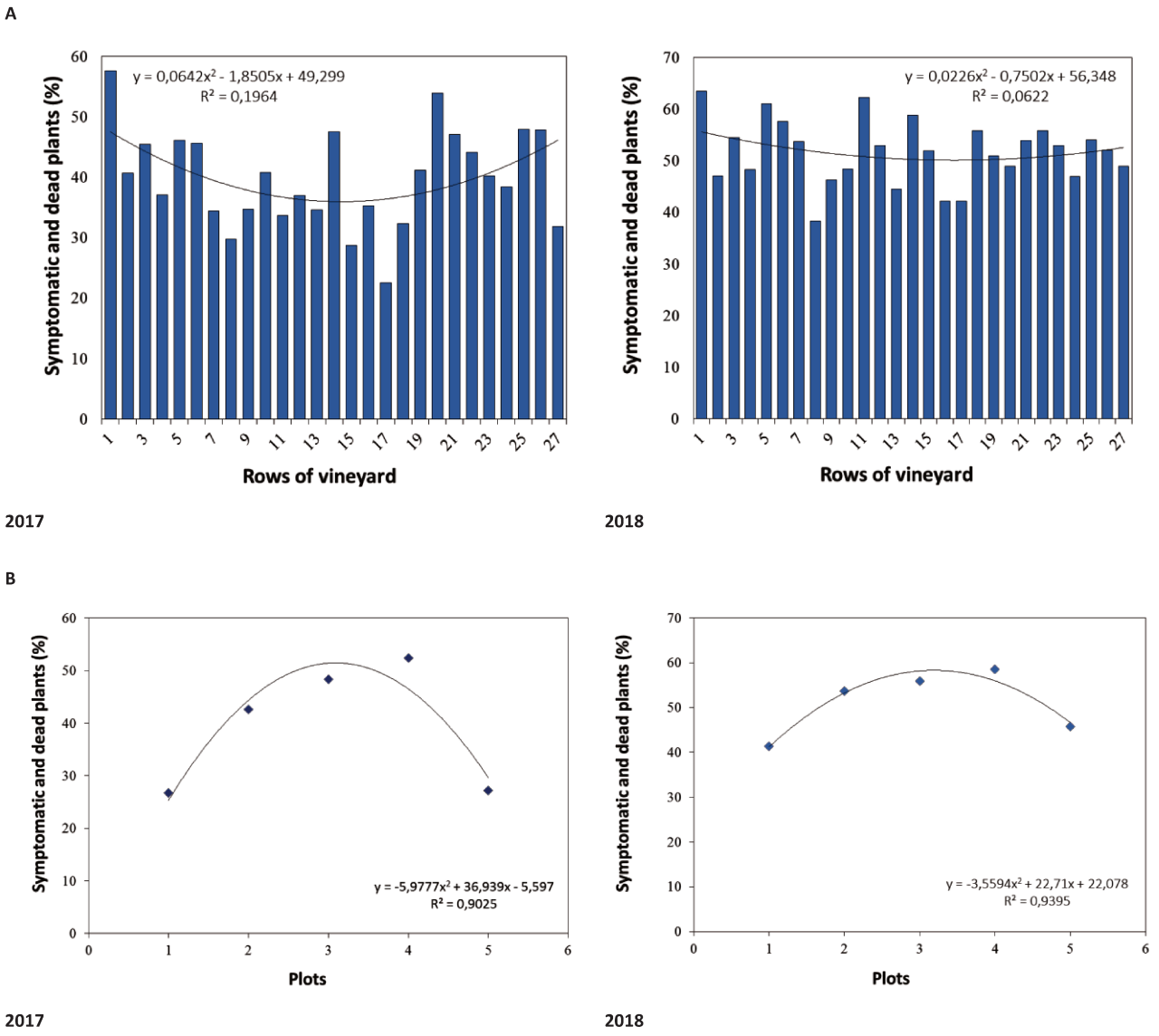


Figure 8 - Inter-row (A) and intra-row (B) infection gradient, respect to the edge of the vineyard OS2.

(A) Inter-row disease gradient within the vineyard represents the percentage of symptomatic plants in the each rows.

(B) The intra-row disease gradient was evaluated, considering the percentage of symptomatic vines in each plot. The vineyard OS2 was divided in 5 plots.
The regression curves represent the percentage of symptomatic plants in relation to the distance from the edge of the vineyard.

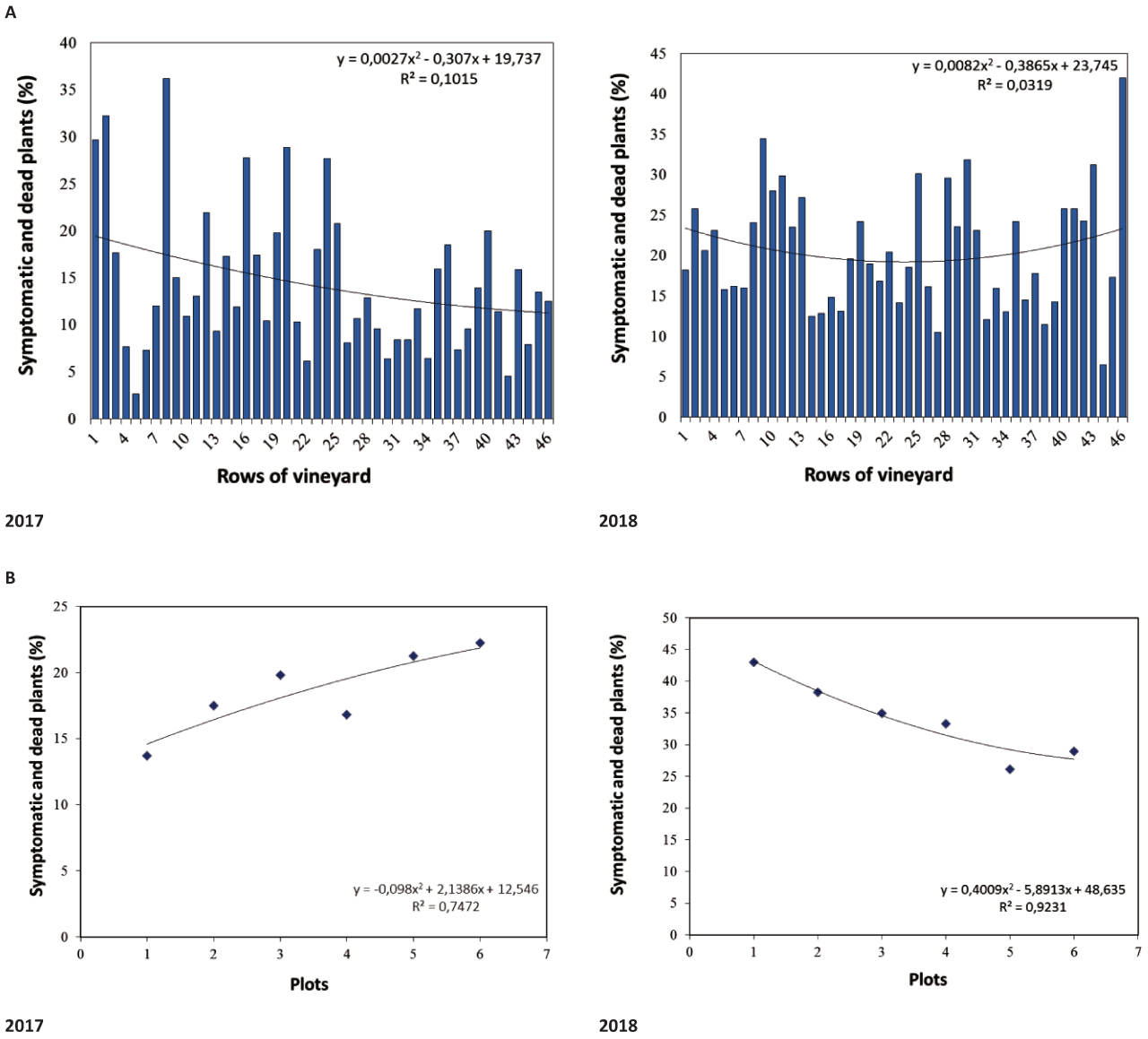


Figure 9 - Inter-row (A) and intra-row (B) infection gradient, respect to the edge of the vineyard CAST.

(A) Inter-row disease gradient within the vineyard represents the percentage of symptomatic plants in the each rows.

(B) The intra-row disease gradient was evaluated, considering the percentage of symptomatic vines in each plot. The vineyard CAST was divided in 6 plots.
The regression curves represent the percentage of symptomatic plants in relation to the distance from the edge of the vineyard.

Chapter IV: ENDOPHYTIC FUNGAL COMMUNITIES IN GRAPEVINE ROOTS

ABSTRACT

The aim of this research was to detect differences in fungal endophytic populations (about species and quantity) in grapevines in relation with: i) presence of esca symptoms, ii) sampling location, and iii) cultivar. The sampling was performed during summer 2017 in three commercial vineyards OS1, OS2, CAST located in Marche region (Italy), selecting 14 symptomatic plants and 14 symptomless. The survey included roots sampling from two most representative cultivars in the region: Verdicchio and Chardonnay. Portions of disinfected roots were placed in growth in Petri dishes with Potato Dextrose Agar, and each single grown fungus was isolated. Fungal isolates were grouped according to morphological characteristics, and species were identified. The work allowed to isolate *in vitro* 34 fungal taxonomic groups. In particular, *Phaeoacremonium* sp., *Fomitiporia mediterranea*, and *Phaeomoniella chlamydospora* were mainly isolated from symptomatic plants. The molecular detection confirmed the classical mycological analysis, and revealed that these pathogens were sporadically present also in asymptomatic vines. Whereas, *Dactylonectria* spp. was isolated from both asymptomatic and symptomatic vines. These results were confirmed also in a pivotal study carried out a pool of symptomatic and asymptomatic plants using Next Generation Sequencing of ITS amplicon. A QIIME analysis allowed to assess the abundance of each fungal OTU in the samples, to compare the composition of taxa between symptomatic and asymptomatic sampled vines and to investigate the structure of the fungal communities. Considering the main endophytic species, we isolated *Fusarium* spp., *Clonostachys rosea*, *Trichoderma* spp., *Penicillium* spp. and *Alternaria* spp. both from asymptomatic and from grapevine showing leaf tiger stripes. Some of the species found in the present study have been reported as being suitable bio-control agents against grapevine pathogens. However, it was not possible to postulate that non-pathogenic fungi were always more numerous in asymptomatic grapevines than in esca-foliar symptomatic ones. There is a microbial balance between the potentially plant-beneficial and plant-pathogenic fungi. When this equilibrium is broken the plants become diseases and show the symptoms.
Further study will be done to improve the knowledge about the fragile equilibrium and how the mycoflora is shaped, using innovative technology (such as Next Generation Sequencing) and considering not only the endophytic fungal communities but also the whole microbiome.

INTRODUCTION

The disease currently known as esca is a very impacting multiple fungal syndrome affecting grapevine (**Gramaje et al. 2018**). Often chronic, esca reduces grape harvest and perturbs sensory attributes of grape, must and wine (**Calzarano et al. 2004**, **Lorrain et al. 2012**) and can occur in a slow evolving form that is recognizable by visible foliar symptoms (chronic esca) or an apoplectic form (acute esca) that kills the plants within a few days (**Mugnai et al. 1999**, **Bertsch et al. 2013**). The disease is characterized by an etiological plurality that is related to different fungi (**Mugnai et al. 1999**). As described by several authors, the main fungal pathogens involved in the first step of esca are the hyphomycetes *Phaeomoniella chlamydospora* and/or species of *Phaeoacremonium* (mainly *P. minimum*), species then considered as pioneer fungi (**Gramaje et al. 2018**), the basidiomycete *Fomitiporia mediterranea* and ascomycetes *Botryosphaeriaceae*. *P. chlamydospora*, *P. minimum* and *F. mediterranea* are responsible for brown-red wood necrosis, dark streaks, and

spongy wood, respectively (**Mugnai et al. 1999**). Further colonization of wood are fulfilled by several other basidiomycetous species belonging to the genera *Inocutis*, *Inonotus*, *Fomitiporella*, *Phellinus* and *Stereum* (**Cloete et al. 2015**).

The rhizosphere (**Hiltner 1904**) is identified as a portion of soil where microorganism-mediated processes are under the influence of the root system. Functions of the rhizosphere are of central importance for plant nutrition, health and quality (**Berg & Smalla 2009**), and plant-microorganism interactions in the rhizosphere are important for carbon sequestration, ecosystem functioning and nutrient cycling in natural ecosystems as well as in agricultural and forest systems (**Singh et al. 2004**). This soil area is colonized by a number of fungal communities including endophytes and mycorrhizal fungi, and it is known as fungal pathogens can have the root system as a primary mode of colonization (**Halleen et al. 2006**, **Álvarez-Pérez et al. 2017**). Together with pruning wounds, the root system is actually one of the pathways that pathogens like *P. minimum* and *P. chlamydospora* can use to penetrate the plant (**Gramaje & Armengol 2011; Bertsch et al. 2013**). A reduction of the root system has been noticed on plants grown *in vitro* (**Rooney & Gubler 2001**) and in greenhouses (**Khan et al. 2000**), infected by *Phaeacremonium*. The reduced growth of canes and shoots is also reported (**Wagschal et al. 2008**), and these alterations lead to a decreasing of the plant carbohydrate reserves during the winter rest, as a consequence of the decrease of total leaf photosynthetic rate during the previous season, thus contributing to a significant reduction in plant vigor and development during the following year or, in worse cases, to plant death (**Petit et al. 2006**, **Bertsch et al. 2013**, **Fontaine et al. 2016**).

Together with cultivar susceptibility, age of the vines, and pedoclimatic conditions, the symptoms of esca seems to be due to interactions among the microorganisms involved (**Graniti et al. 2000**), and considering rhizosphere as a space where biological components strongly interact (**Hiltner 1904**, **Dessaux et al. 2009**) and that strong interactions occur between soils and plant roots, or plant roots and microorganisms, between plants themselves and microorganisms themselves (**Dessaux et al. 2009**), it appears evident the value of a survey on fungal endophytes from roots as approach for new knowledges about grapevine esca. The structure of fungal endophytic communities is normally a dynamic system, where changes in composition of plant-associated microbial communities have often been associated with plant physiology, age, health, geography, environmental factors, and differs also among the various tissues and organs into the same host plant (**Arnold et al. 2003**, **Saikkonen 2007**, **González & Tello 2011**, **Moricca et al. 2012**). Because of different kinds of stress, or during senescence of the host, the endophytic fungi can also become pathogenic and cause disease symptoms in the plant (**Kogel et al. 2006**, **Rodriguez & Redman 2008**) or alternatively the fungus is excluded due to plant defense responses (**Kogel et al 2006**, **Schulz 2006**, **Rodriguez & Redman 2008**). The term “endophytic”, in the most basic sense of the word, refers in effect only to the location of the microorganism, not making distinction between endophytic and pathogen (**Wilson 1995**, **Schulz 2006**).

It is known that endophytes may interact with their hosts by producing plenty of substances that provide protection and ultimately survival value to the plant (**Yu et al. 2010**), by enhancing their growth and improving their resistance to environmental stresses (**Clay 1988**, **Wolock-Madej & Clay 1991**, **Knoch et al. 1993**) or their capacity to resist attacks from herbivores or other plant pathogenic fungi (**Clay 1988**, **1990**, **Leuchtmann et al. 2000, Faeth & Fagan 2002**), sometimes inducing systemic resistance mechanisms and the expression of defense genes against the attack of certain pathogens (**Gwinn & Gavin 1992, Arnold et al. 2003**), or competing with pathogens for space and nutrients (**Arnold et al. 2003**, **Gonzalez & Tello 2011**).

Several studies have been conducted in recent years on the use of biological control agents against various diseases, especially those caused by fungi (**Alabouvette et al. 2006**), and the main of this research was the characterization of endophyte communities in grapevine, and checking the presence of difference between asymptomatic and symptomatic plants, in order to establish how

the population structure of the fungal endophytes changes according to the presence of esca pathogens, and if the cultivable endophytic fungi interfere with the pathogens related to esca.
Considering how the discontinuous expression of esca symptoms (**Surico et al. 2000**, **Romanazzi et al. 2009**, **Andreini et al. 2014**) makes normally not possible to know when a plant becomes infected, the present work brings to light ecological presences of fungal endophytes and rule of fungal endophyte communities into the root, allowing to better understand the turning of an asymptomatic grapevine into a symptomatic one.

MATERIALS AND METHODS

Sample collection and isolation of fungal endophytes

The research was carried out in three commercial vineyards OS1 (cv. Verdicchio), OS2 (cv. Chardonnay), and CAST (cv. Verdicchio), located in Marche region (Italy) whose main characteristic were mentioned in table 1 of the previous Chapter. Root samples were collected in June 2017 from 14 plants that showed leaf tiger-stripes, symptom associated to esca, and 14 asymptomatic plants, presumably healthy. Studied plants were named with code "A" ("asymptomatic") or "S" ("symptomatic"), followed by a number. We considered an equal number of symptomatic and asymptomatic plants for each surveyed vineyard: A1-A4 and S1-S4 (vineyard CAST), A5-A8 and S5-S8 (vineyard OS2), finally A9-A14 and S9-S14 (vineyard OS1).
The root samples were collected, from different parts of root system of each plant, after removing the first 20-30 cm of top soil. From each of these selected vines, portions of secondary roots (length 10-15 cm, diameter 3-5 mm) were put into sterile plastic bags with a small quantity of soil in order to preserve the humidity, and stored in a portable refrigerator to maintain vitality of sampling tissues.
In the laboratory, the root samples were separated from the soil and preliminary washed in tap water, then treated with 0.05% (v/v) Tween 20 in 50-mL tubes (Falcon) and vortexed for 10 min. A second treatment was developed and applied to the samples to obtain strict external decontamination and reach the isolation of fungal endophytes. The samples were washed in a 70% solution of ethanol for 3 min, treated in a 2% sodium hypochlorite solution for 5 min and then washed in 70% ethanol (v/v) during 30 s, with a final five-times-repeated wash in sterile distilled water for 1 min each (**Verma et al. 2007**, modified). Portions of disinfected roots were cut under laboratory hood into small pieces (2-4 mm). To test the efficacy of this treatment, random pieces of externally decontaminated roots were repeatedly rolled on PDA Petri dishes, followed by incubation for 2 weeks at 20 to 25 °C to confirm the absence of any microbial growth. Then, the small pieces of roots were placed into Petri dishes (9 cm) with PDA (Potato Dextrose Agar) for growing. From each grapevine, 50 pieces of decontaminated root were analysed, putting 5 pieces into each PDA petri dishes. The dishes were incubated for seven days at room temperature (25 ± 1 °C). Fungi were further sub-cultured, transferring the mycelia from the margin of the growing fungal colonies to individual Petri dishes (5 cm) containing fresh PDA and incubated at room temperature (25 ± 1 °C) another 7 days. The subculturing step was repeated until pure cultures were obtained.

Fungal identification by stereomicroscopy

Each single grown fungal colony was isolated as explained. Isolated colonies were grouped according to morphological characteristics, and the species were identified by morphological characterization.
Fungal identification was based at first on morphological characters (shape, form, size and growth time, border, surface, opacity, pigmentation) of the colonies and shape and size of fungal fruiting

bodies, spores and conidiophores; fungi were observed with laboratory microscope (Leica DM 2500®), and measures of their structures were performed using LAS v. 3.8 software applied on fifty units per structure.

DNA extraction, ITS region amplification and sequencing

Approximately 100 mg of mycelium was harvested from pure colonies of each taxonomic group, and ground to a fine powder in liquid nitrogen. Each powder was then suspended in 800 µl preheated 2% CTAB buffer (200 mM Tris-HCl, pH 8.0, 50 mM EDTA, 2.2 M NaCl, 2% CTAB, 0.06% sodium metabisulphite) on the basis of the DNA extraction protocol described by Doyle and Doyle (**1990**). A 1.5% agarose gel was run to determine the amounts and the integrity of the DNA.

The amplification of the internal transcribed spacer (ITS) region in the rDNA of the isolates (tab. 1) was performed using 20 µl of PCR reaction mix containing 2 µl genomic DNA (30-80 ng) of the fungal isolate, 10 µl Master Mix, 0.5 µl of ITS_1 primer (5´-TCCGTAGGTGAACCTGCGG-3´), 0.5 µl of ITS_4 primer (5´-TCCTCCGCTTATTGATATGC-3´) (**White et al. 1990)**, 0.2 mM dNTPs (Fermentas), and 2.5 U of DreamTaq DNA polymerase (Fermentas).

Amplification was carried out in a Thermal Cycler (Bio-Rad) at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 50 s, and 72 °C for 60 s with a final extension at 72 °C for 10 min. Amplified products were analysed by agarose gel electrophoresis. PCR products were purified using DNA Clean & Concentrator (Zymo Research) and sequenced in forward and reverse directions by Macrogen (The Netherlands). Sequence analysis of the ITS sequences was carried out using BioEdit Sequence Alignment Editor v. 7.2.3 (**Hall 1999**). The search for homologous sequences was done using Basic Local Alignment Search Tools (BLAST) at the National Center for Biotechnology Information (NCBI). Sequences were identified to the species level whenever possible. All fungal sequences considered were at least 98 % identical to the best hit in the NCBI database.

DNA extraction from root tissues

Total DNA was extracted from roots using the cetyl trimethyl ammonium bromide (CTAB) procedure according to Landi et al. (**2019**). For each sub-sample, 3 g of pooled roots were ground in liquid nitrogen, and 200 mg of the pulverized material was added to 2-mL microcentrifuge tubes with 1 mL extraction buffer (3% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% [w/v] soluble PVP-40), and 1% (w/v) sodium metabisulphite was added. After incubation at 68 °C for 30 min, purification with twice chloroform/isoamyl alcohol (24:1) and precipitation with 0.6% isopropanol were conducted. Finally, the DNA was dissolved in 50 µL of pure water. The DNA purity and quantity were also determined (BioPhotometer plus; Eppendorf Inc., Westbury, NY, USA) estimating the absorption ratios at 260/280 in the range of 1.6-1.8, and at 260/230 in range of 1.3-2.0.

Molecular detection of main fungal pathogens related with esca disease

Total DNA extracted from the root samples of symptomatic (S1-S14) and asymptomatic plants (A1-A14) was analyzed in order to detect the main fungal pathogens generally related with esca disease, in particular *Phaeomoniella chlamydospora* (Pch), *Phoeacremonium minimum* (Pmi), *Botryosphaeria dothidea* (Bdot). The details of amplification for each pathogen were reported in the following lines.

To detect Pch, 1 µl DNA was added to the PCR reaction mixture containing 1× PCR buffer (Promega Corporation, Madison, USA), 1.5 mM MgCl2, 200 µM of each dNTP (Promega), 0.125 U Taq DNA polymerase (Promega) and 200 nM of each primer OPA13844Df/OPA13844Dr (Invitrogen, Life Technologies, Carlsbad, CA, USA) (**Abbatecola et al. 2006**). PCR was carried out in a thermal Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA) programmed for an initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 60 s, with a final extension cycle

at 72 °C for 7 min. A second round of PCR (nested-PCR) was carried out in the presence of another Pch-specific primer pair OPA13844Ff/ OPA13844Fr (**Abbatecola et al. 2006**) using the same PCR conditions except for the annealing temperature (Ta = 60 °C) and the number of cycles (30).
Pal1N and Pal2R were used directly for the detection of *Phaeocremonium* (Pmi) according to the PCR conditions suggested by Tegli et al. (**2000**).
The total DNA was also amplified with the primer pair EBdF and EBdR, reported as specific to *Botryosfaeria dothidea* (*Bot*) according to the PCR conditions described by Ma et al. (**2003**).
For the detection of *Fomitiporia mediterranea* (*Fom*), the species-specific primers developed by Fischer (**2006**) were used.
The amplification products were separated by electrophoresis in 1.5% agarose (Molecular Biology Certified Agarose, Bio-Rad) gels in 1× Tris-acetic acid-EDTA (TAE) buffer. The PCR products in the gels were stained with Gel Red solution (Biotium), and visualised under UV light at 312 nm on a transilluminator; the images were acquired with a digital camera integrated in GelDoc (BioRad). The expected lengths of the amplified DNA fragments were estimated by comparison with a 100 bp DNA Ladder (Invitrogen).

Amplification with High-Coverage ITS Primers
Total DNA extracted from the asymptomatic (A9, A10, A11, A12, A13, A14) and symptomatic (S9, S10, S11, S12, S13, S14) root samples, collected in the vineyard OS1 was amplified in total volume 25 µl using the primer pair ITS1F_KYO2 (5' TAGAGGAAGTAAAAGTCGTAA 3') and ITS2_KYO2 (5' TTYRCTRCGTTCTTCATC 3') (**Toju et al. 2012**). PCR was conducted using under a temperature profile of 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 56 °C for 30 s, and 72°C for 20 s, followed by 72uC for 7 min. The concentration of $MgCl_2$, dNTPs, and PCR primers in the reaction buffer were 1.5 mM, 200 mM, 0.5 mM, respectively.For each sample, an aliquot of 30-50 ng of DNA was added.

The amplified samples were checked on agarose gel, then the amplicons of six asymptomatic (A9-A14) and six symptomatic (S9-S14) samples were pooled, obtaining two different samples identified as End-AS and End-S, respectively. The two pool samples were purified using Wizard® SV Gel and PCR Clean-Up System (Promega), then quantified with the Biophotometer (Eppendorf), to estimate the threshold of quantity (60 ng/µl; at least 500 ng) and quality (260⁄280 > 1.8; and at 260⁄230 in range of 1.7).

DNA sequencing and bioinformatics analysis
Fungal ITS1 amplicon pools (End-As and End-S) were sent to GENEWIZ, Inc. (South Plainfield, NJ, USA), which provided library preparations, Illumina MiSeq sequencing and data analysis. In particular,
DNA Library Preparation, clustering, and sequencing reagents were used throughout the process using NEBNext Ultra DNA Library Prep kit following the manufacturer's recommendations (Illumina, San Diego, CA, USA). End repaired adapters were ligated after adenylation of the 3'ends followed by enrichment by limited cycle PCR. DNA libraries were validated using a DNA 1000 Chip on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and were quantified using Qubit 2.0 Fluorometer and multiplexed in equal molar mass. The pooled DNA libraries were loaded on the Illumina instrument according to manufacturer's instructions. The analysis was conducted using GENEWIZ's ITS analysis pipeline, as described below.
The raw data were subjected to the following steps for the optimization:
(i) The two sequences of each read pair were merged according to overlapping sequences (Vsearch 1.9.6). The read merge is deemed to be successful only if the overlapping sequence is least 20bp long. After merging, undetermined bases (N) were removed from the resulting sequence;

(ii) Primer and adapter sequences were removed (software Cutadapt v. 1.9.1). Then the 5' and 3' bases with Q score lower than 20 were also removed. The resulting sequences with length > 200bp would pass this step of processing;
(iii) The sequences obtained were then aligned to database to identify and remove chimera sequence (Vsearch 1.9.6 and QIIME version 1.9.1). Sequences passed this filtering step are deemed as clean data ready for analysis.
Finally, the "clean nucleotide sequences" were used for the for OTU analysis and clustering and species annotation, using the software QIIME version 1.9.1.
Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (1.9.6) against the UNITE ITS database (https://unite.ut.ee/) pre-clustered at 97% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic category to all OTUs at confidence threshold of 0.8. The RDP classifier uses the UNITE ITS database which has taxonomic categories predicted to the species level. Qiime software (1.9.1) was used for analysis of community richness, community diversity and to measure sequencing depth.

RESULTS

Fungal identification by stereomicroscopy and ITS region sequence analysis

In July 2017, we collected 14 root samples from plants showing leaf tiger stripe symptoms associated with esca disease and other 14 root samples from asymptomatic plants. From the *in vitro* isolation, after different sub-culturing, we were able to distinguish 14 taxonomic group of fungi according to the morphological characteristics of colonies, and further observations by aid of microscopy (fig. 1). The DNA extraction of the representative fungal isolates, the amplification and the sequence analysis of ITS region allowed to corroborate the result of morphological characterization and define other 14 taxonomic groups. Among the 34 taxonomic groups, 28 were well identified, other 6 groups remained unknown.
Both in asymptomatic and symptomatic vines, the most recurrent endophytic fungi were *Fusarium* spp. (100%), and *Clonostachys rosea* (92.85%) followed by *Trichoderma* spp. (42.85%), *Alternaria* spp. (42.85%), *Phlebia acerina* (57.14%), and *Penicillium* spp., isolated from more than 39% of studied plants (tab. 1A). Less recurrent were *Ceratobasidium* sp. / *Rhizoctonia fragariae* and *Macrophomina phaseolina* isolated from around 28% of symptomatic and asymptomatic vines (tab. 1A).
Only from asymptomatic vines, we were able to isolate *Trichoderma harzianum* (28.57%), *Aureobasidium pullulans* (14.29%) and an unidentified specie (Unknown 09) (tab. 1A).
Fungi only isolated from root samples collected from vines showing leaf tiger stripes were *Xylaria* sp. (21.43%), and *Acremonium* sp. (35.71%).
Among the fungi related to trunk disease, we were able to isolate *Dactylonectria* spp. (89.28%) and *Phomopsis* spp. (39.28%) which however were present both in asymptomatic and symptomatic vines.
Among the fungi more strictly related to leaf tiger stipes and esca, we isolated *Phaeomoniella chlamydospora* (50%), *Fomitiporia mediterranea* (50%), and *Phaeoacremonium* (35.71%) (fig. 2), only detected in symptomatic vines.
F. mediterranea and *P. chlamydospora* were isolated from samples coming from vineyards CAST, OS1 and OS2, whereas *Phaeoacremonium* was isolated in samples S9, S10, S11, S12, and S14, all symptomatic samples collected in vineyard OS1 (tab. 1, fig. 3).
Considering the difference of main fungal taxonomic groups according to the cultivars, we recorded the prevalence of *Diaporthe/Fomopsis*, *Fusarium* spp., *Penicillium* spp., *Macrophomina*

phaseolina and Trichoderma sp. in Verdicchio samples respect to Chardonnay. Whereas *Dactylonectria* spp. were recurrent in Chardonnay samples (fig. 4).
Considering the difference of main fungal taxonomic groups according to the vineyard location, we recorded that *Phlebia acerina*, *Clonostachys rosea* and *Dactylonectria* spp. were prevalent in the vineyard OS2, *Diaporthe/Fomopsis* and *Trichoderma* sp. were more frequent in the vineyard OS1, and finally *Fusarium* spp. were more recurrent in the vineyard CAST (fig. 5).

DNA extraction from root tissues and molecular detection of fungal pathogens related with esca disease

The DNA was extracted from 28 root samples (14 symtopmatic and 14 asymptomatic vines). The method proposed by Landi et al. (**2019**) was very simple and it was able to obtain high DNA quality as reported in tab. 2.
The DNA was amplified for a preliminary screening with the primer pairs specific for *Phaeomoniella chlamydospora*, *Phoeacremonium*, *Botryosphaeria* spp., and for *Fomitiporia mediterranea*.
From the molecular detection, we recorded in some asymptomatic plants the presence of *P. minimum* (3), and *F. mediterranea* (2). All plants resulted negative for the presence of *P. chlamydospora*, whereas in 6 vines coming from the three vineyards we recorded the presence of *Botryosphaeraceae* species (tab. 3). The molecular detection, carried out on symptomatic samples, confirmed the presence of *Pch* in 12 out 14 samples, which resulted the most abundant, followed by *Bot* in 8 out of 14 and finally *Pmi* (7⁄14) and *Fom* (5⁄14) (tab. 3).

Amplification with High-Coverage ITS Primers and QIIME analysis

Total DNA extracted from the root samples, collected in the vineyard OS1, was amplified with primer pair ITS1F_KYO2 and ITS2_KYO2, described as tool able to have a high coverage for fungi. All samples were amplified and we recorded a specific band whose bands were of about 350 bp (fig. 6).

The two pooled samples, one obtained from asymptomatic plants (A9-A14) and symptomatic vines (S9-S14), were analyzed on the platform Illumina MiSeq, which was able to generate 206.978 reads with a length of about 206 bp and a Q30 (%) of about 95% for the sample End-S. On the other hand, we recorded 161.744 reads with a length of about 230 bp and a Q30 (%) of about 92% for the sample End-As.
The raw data were optimized, and after merging, removing undetermined bases, removing adapters and chimeras, we obtained “the clean data” (tab. 4) ready for the QIIME analysis.
From the QIIME analysis, all the sequences in a sample were classified to obtain information on species and genus. By classification, the sequences were grouped according to their similarity, and one group is an OTU. If we consider the two samples we obtained 241 OTU, most of them were in common (127 OTU) for both, then we obtained 65 OTU specific for End-S and 49 for End-AS (fig. 7).
The heatmap analysis (fig. 8) shows the abundance information of selected OTU as well as the similarity and difference across OTUs and samples by similarity clustering.
An OTU abundance clustering heatmap (GENEWIZ Next Generation Sequencing) were processed, considering the top 30 OTUs with the highest abundance. As indicated in fig. 7, the row name is the OTU ID, the column name is the sample information, the left side of the figure is the OTU cluster tree, and the top is the sample cluster tree. The value of each colored box is the relative abundance of each OUT after normalization. The heatmap showed differences in composition of fungal communities, through abundance information of selected OTU as well as similarity and difference across OTUs and samples by similarity clustering. OTUs 5, 6, 8, 9, 15, 19, 12, 13 were

more frequent in sample End-AS than samples End-S. In the samples End-S the OTUs 3, 4, 16, 17 were more abundant.
The QIIME analysis revealed that both for sample End-AS and End-S, more than 80% of nucleotide sequences matched with the sequences belonging to the phylum *Ascomycota*, followed by *Basidiomycota* (about 5%) and finally by *Glomeromycota* (tab. 5). A part of nucleotide sequences resulted not identified.

Considering only the nucleotide sequences well identified we could record that in the sample End-S the sequences belonging to Ascomycota, the orders more abundant respect End-AS were *Chaetothyriales*, *Phaeomoniellales*, and for Basidiomycota was *Malasseziales*. In the sample End-AS we recorded that the fungi belonging to Orders *Botryosphaeriales*, *Hypocreales* and *Agaricales* are more abundant than in the samples End-S (tab. 6).

DISCUSSION

The aim of this work was to study the composition of fungal endophytic communities both in symptomless grapevines and in grapevines showing leaf tiger stripes, in vineyards affected by esca. This could help to understand the turning of a symptomless vine into a symptomatic one and *vice versa*, being esca symptoms discontinuous in vineyards (**Surico et al. 2000**, **Andreini et al. 2014**), and to offer further perspectives for the challenge against esca disease.
The result that is based on *in vitro* fungal isolation, indicates that the most abundant fungal division was Ascomycota, followed by Basidiomycota. This distribution pattern of Ascomycota is consistent with other studies on the endophytic communities of woody tissues or shoots, and on the leaves of different plant hosts (**Bills 1996**, **Frohlich et al. 2000**, **Rungjindamai et al. 2008**), including grapevines (**Hofstetter et al. 2012**, **Pancher et al. 2012**, **Bruez et al. 2014**, **Bruez et al. 2016**). Moreover, this data corroborated what the pivotal study carried out applying NGS technology, where we found that 83% of fungal OTUs were related to *Ascomycota* and 5% were *Basidiomycota*. The low percentage of *Basidiomycota* found in our study is consistent with other works (e.g. **Bettucci & Saravay 1993**, **Sánchez et al. 2010**). Stone et al. (**2004**) indicated the low proportions that is usually reported in endophytic inventories as related to sampling bias that can facilitate the sporulating and fast-growing species, which generally belong to asexual ascomycetes, rather than basidiomycetes. In literature can be found anyhow papers suggesting instead basidiomycetes as important component of certain endophytic communities (**Crozier et al. 2006, Rungjindamai et al. 2008**, **Pinruan et al. 2010**).

It is well known that the main fungal agents involved in the first step of the disease are *P. chlamydospora* and/or species of *Phaeoacremonium* (expecially *P. minimum*), being the most prevalent and virulent and considered as pioneer fungi (**Larignon & Dubos 1997**, **Gramaje et al. 2018**). After the first colonization of *P. chlamydospora* and/or species of *Phaeoacremonium*, the grapevine wood is more receptive to further colonization by the basidiomycete fungi which are responsible for the typical decay associated with esca, belonging to the genera *Fomitiporia*, *Inocutis*, *Inonotus*, *Fomitiporella*, *Phellinus* and *Stereum* (**Cloete et al. 2015**).
In our analysis we detected pioneer fungi related to esca, such as *Phaeoacremonium* and *Phaeomoniella*, mainly in symptomatic vines. And it was possible to also isolate them together with *Fomitiporia* spp. from roots of symptomatic plants.

From the molecular detection, using as template the DNA extracted directly to the grapevine wood and specific primer for the main fungi related to esca, we detected in some asymptomatic

samples *Phaeoacremonium*, *Botryosphaeria,* and *Fomitiporia*. In particular, *Botryosphaeria* spp., esca-pathogens (**Casieri et al. 2009**, **González & Tello 2011**) was found both in asymptomatic and symptomatic vines only applying molecular detection.
This discordant result can depend on the method of detection that is applied. In particular, classical used cultural method facilitates fast-growing species respect to other fungi with a slower growth in vitro condition (**González & Tello 2011**, **Bruez et al. 2014**, **Stone et al. 2004).**
Moreover, the molecular technique applied is more sensitive and it is able to detect low titles of DNA target. The DNA extraction, proposed by Landi et al. (**2019**) and starting from roots, was easy to apply. The following amplifications with specific primers allowed the intermediate steps to be by-passed (i.e. *in vitro* cultures, subcultures and microscopy identification), thus obtaining results in a shorter time (**Abbatecola et al. 2006**).
Considering that fungal pathogens can have the root system as a primary mode of colonization (**Halleen et al. 2006**, **Álvarez-Pérez et al. 2017**) and that the root system is actually one of the pathways that pathogens like *P. minimum* and *P. chlamydospora* can use to penetrate the plant (**Bertsch et al. 2013**, **Gramaje & Armengol 2011**), we can conclude that the presence of these fungi is not necessarily associate with the onset of esca disease. It was demonstrated by Sparapano et al. (**2001**) that the first leaf symptoms occur years after infection of pathogens.
From the roots of symptomatic and asymptomatic vines, we isolated a high percentage of colonies identified as *Dactylonectria* spp. Over the last 15 years, several studies have reported the occurrence and increasing incidence of black foot disease (BFD) of grapevine in production areas around the world. *Dactylonectria* spp. were responsible for cankers, root rot and decay of woody and herbaceous plants (**Bae et al. 2004**, **Domsch et al. 2007**, **Fu et al. 2015**) and was isolated from symptomatic vines. Recently, have also been isolated from asymptomatic rootstock mother-plants, rootstock cuttings, young grafted vines, and young nursery vines (**Rumbos & Rumbou 2001**, **Halleen et al. 2003**, **2006**, **2007**, **Fourie & Halleen 2004**, **Oliveira et al. 2004**, **Dubrovsky & Fabritius 2007**, **Aroca et al. 2010**, **Cardoso et al. 2012**, **Agustí-Brisach & Armengol 2013**, **Carlucci et al. 2017**).

Considering the main endophytic species, we isolated *Fusarium* spp., *Clonostachys rosea*, *Trichoderma* spp., *Penicillium* spp. and *Alternaria* spp. both from asymptomatic and from grapevine showing leaf tiger stripes, as already reported by Casieri et al. (**2009**). Only from asymptomatic vines, we were able to isolate *Trichoderma harzianum* (*Aureobasidium pullulans* and an unidentified specie (Unknown 09).
Some of the species found in the present study have been reported as being suitable bio-control agents against grapevine pathogens. For instance, *Cl. rosea*, was reported as one of a number of promising biocontrol agents for *B. cinerea* (**Sutton et al. 1997**, **Köhl et al. 1998**, **Cota et al. 2008**, **Sutton et al. 2008**). *Trichoderma* is considered as ubiquitous genus, easily isolated from decaying wood and any forms of plant organic matter and soil, present in a broad range of habitat and at high population densities. *Trichoderma* spp. can interfere with pathogens through a wide range of mechanisms that are based on mycoparasitism, antibiotic production, competition through the rhizosphere, enzyme production, metabolism of germination stimulants, plant growth-promoting activities, and resistance to biotic and abiotic stress (**Shoresh et al. 2010**, **Mukherjee et al. 2013**).
Against grapevine trunk diseases, there are available some *Trichoderma*-based formulates (**Di Marco et al. 2004, Harvey & Hunt 2006**, **Di Marco & Osti 2007**, **Parizi et al. 2012**, **McLean et al. 2012**). Some species of *Fusarium* have also been identified as promising biocontrol (**Bakshi et al. 2001**, **Musetti et al. 2006**, **Rania et al. 2016**).
Penicillium spp., have been reported as antagonist fungi of plant pathogens, with mechanisms of action based on induction of resistance (**Hossain et al. 2007**, **Nicoletti & De Stefano 2012**), production of antibiotic compounds (**Nicoletti et al. 2004**, **Yang et al. 2008**) and mycoparasitic interactions (**Sempere & Santamarina 2008**).

Amongst all fungi isolated from grapevine roots in this study, there are some which may play an active role in balancing fungi related to esca disease and/or enhancing the host response in order to avoid an excessive growth of pathogenic fungi. However, it was not possible to postulate that non-pathogenic fungi were always more numerous in asymptomatic grapevines than in esca-foliar symptomatic ones.

As claimed by Bruez et al. (**2016**), there is a microbial balance between the potentially plant-beneficial and plant-pathogenic fungi. When this equilibrium is broken the plants become diseases and show the symptoms. Further study will be done in order to improve the knowledges about the fragile equilibrium and how the mycoflora is shaped, using innovative technology (such as Next Generation Sequencing) and considering not only the endophytic fungal communities but also the microbiome.

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Tables

Tab. 1 A - Fungal colonies isolated from roots of asymptomatic (A1-A14) and symptomatic vines (S1-S14), identified by morphological and molecular tools.

Fungal isolate	Asymptomatic vines														Symptomatic vines													
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
Phlebia acerina	+	+		+	+	+		+							+		+	+	+	+	+	+	+		+	+		
Alternaria spp.	+		+	+			+		+	+	+		+		+										+	+	+	
Diaporthe / Phomopsis		+		+						+		+		+						+	+		+	+	+			+
Fusarium spp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Clonostachys rosea	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	
Ceratobasidium sp. / Rhizoctonia fragariae	+	+	+	+		+		+								+	+					+						
Penicillium spp.		+	+							+	+	+	+			+		+							+	+	+	
Macrophomina phaseolina				+					+	+		+	+												+		+	+
Sordariomycetes				+	+			+			+	+		+													+	
Cylindrocarpon olidum					+	+	+	+											+		+	+						
Dactylonectria spp.	+	+	+	+	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	
Unknown 01									+		+			+							+				+		+	+
Trichoderma sp.			+						+	+	+	+		+						+			+	+	+	+		+
Unknown 06			+			+	+		+		+		+				+						+		+	+	+	
Xylaria / Halorosellinia											+							+				+			+	+		
Ilyonectria sp.	+								+	+	+																+	
Aspergillus niger									+		+	+									+					+		
Aspergillus sp.					+		+																+		+	+	+	
Idriella lunata	+		+	+														+										
Mortierella alpina						+									+								+		+	+		
Unknown 02						+								+							+							

Fungal isolate	Asymptomatic vines														Symptomatic vines													
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
Truncatella angustata	+			+			+										+											
Phaeoacremonium											+												+	+	+	+		+
Trichoderma harzianum		+							+		+	+																
Aureobasidium pullulans			+									+																
Phaeoacremonium minimum													+															
Unknown 03												+																
Unknown 08											+																	
Unknown 09		+									+	+																
Xylaria sp.																		+							+	+		
Acremonium sp.															+		+				+			+		+		
Phaeomoniella chlamydospora															+				+		+			+	+	+		+
Lophiostoma / Massarina																							+					
Fomitiporia mediterranea															+		+				+	+	+		+	+		

Tab. 1 B - Fungal colonies isolated from roots of asymptomatic (A1-A14) and symptomatic vines (S1-S14), according to location, cultivar and foliar symptoms

B

ISOLATE	Location			Cultivar		Presence of leaf tiger-stripe	
	CAS	OS2	OS1	VERDICCHIO	CHARDONNAY	ASYMPTOMATIC VINES	SYMPTOMATIC VINES
Phlebia acerina	+	+	+	+	+	+	+
Alternaria spp.	+	+	+	+	+	+	+
Diaporthe / Phomopsis	+	+	+	+	+	+	+
Fusarium spp.	+	+	+	+	+	+	+
Clonostachys rosea	+	+	+	+	+	+	+
Ceratobasidium sp. / Rhizoctonia fragariae	+	+		+	+	+	+
Penicillium spp.	+		+	+		+	+
Macrophomina phaseolina	+		+	+		+	+
Sordariomycetes	+	+	+	+	+	+	+
Cylindrocarpon olidum		+			+	+	+
Dactylonectria spp.	+	+	+	+	+	+	+
Unknown 01		+	+	+	+	+	+
Trichoderma sp.	+	+	+	+	+	+	+
Unknown 06	+	+	+	+	+	+	+
Xylaria / Halorosellinia	+	+	+	+	+	+	+
Ilyonectria sp.	+		+	+		+	+
Aspergillus niger		+	+	+	+	+	+
Aspergillus sp.		+	+	+	+	+	+
Idriella lunata	+			+		+	+
Mortierella alpina	+	+	+	+	+	+	+
Unknown 02		+	+	+	+	+	+
Truncatella angustata	+	+		+	+	+	+
Phaeoacremonium sp.			+	+		+	+
Trichoderma harzianum	+		+	+		+	
Aureobasidium pullulans	+		+	+		+	
Phaeoacremonium minimum			+	+		+	
Unknown 03			+	+		+	
Unknown 08			+	+		+	
Unknown 09	+		+	+		+	
Xylaria sp.	+		+	+			+
Acremonium sp.	+	+	+	+	+		+
Phaeomoniella chlamydospora	+	+	+	+	+		+
Lophiostoma / Massarina			+	+			+
Fomitiporia mediterranea	+	+	+	+	+		+

Tab. 2 - Quality and quantity of DNA, extracted from root samples collected in the three vineyards

Sample	Quantity (ng/µl)	Ratio (A260/280)	Ratio (A260/230)
P1A	210	1.78	1.55
P2A	312	1.75	1.61
A7	153	1.69	1.53
A8	189	1.72	1.63
A13	301	1.62	1.64
A14	153	1.74	1.57
P1S	525	1.62	1.61
P2S	314	1.69	1.72
S7	270	1.64	1.58
S8	186	1.66	1.32
S13	240	1.61	1.62
S14	601	1.61	1.52

Tab. 3 - Molecular detection carried out on roots collected from asymptomatic (A1-A14) and symptomatic vines (S1-S14), using specific primers.

Sample	Presence of Symptoms	Molecular detection by specific primer for			
		Pch	*Pmi*	*Bot*	*Fom*
A1	no	-	+	+	-
A2	no	-	-	-	
A3	no	-	-	-	+
A4	no	-	-	+	-
A5	no	-	-	+	-
A6	no	-	-	-	-
A7	no	-	-	-	+
A8	no	-	+	-	-
A9	no	-	-	-	-
A10	no	-	-	+	-
A11	no	-	-	-	-
A12	no	-	-	+	-
A13	no	-	+	+	-
A14	no	-	-	-	-
Total		***0***	***3***	***6***	***2***

Sample	Presence of Symptoms	Molecular detection by specific primer for			
		Pch	*Pmi*	*Bot*	*Fom*
S1	yes	+	-	+	+
S2	yes	+	+	-	+
S3	yes	+	-	+	+
S4	yes	+	-	-	+
S5	yes	+	-	+	-
S6	yes	-	+	-	-
S7	yes	+	+	-	-
S8	yes	+	-	+	-
S9	yes	+	+	-	-
S10	yes	+	-	+	-
S11	yes	+	+	-	+
S12	yes	+	-	+	-
S13	yes	-	+	+	-
S14	yes	+	+	+	-
Total		***12***	***7***	***8***	***5***

Tab. 4 - Statistical data GENEWIZ NGS data report.

Sample	PE_reads	Nochimera	AvgLen(bp)	GC(%)	Effective(%)
SM01_End_S[1]	103.489	78.305	269,14	48,11	75,67
SM02_End_AS[2]	80.872	66.823	278,78	49,46	82,63

[1] Pool of amplicons of symptomatic samples generated with primer pair ITS1F_KYO2 and ITS2_KYO2.
[2] Pool of amplicons of asymptomatic samples generated with primer pair ITS1F_KYO2 and ITS2_KYO2.

Tab. 5 - Nucleotide sequences according the *phylum* in End-S and End-AS samples after QIIME analysis.

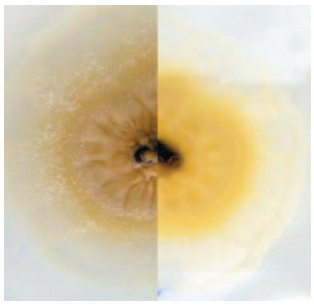
Phylum	End-S (reads)	End-AS (reads)
Ascomycota	56878	54541
Basidiomycota	3603	4441
Others	275	318
Glomeromycota	275	193
Unidentified	5596	7052
Total	***66627***	***66545***

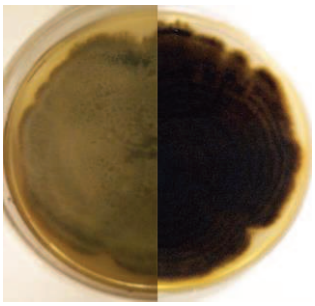
Tab. 6 - Nucleotide sequences, according to Order of in End-S and End-AS samples after QIIME analysis.

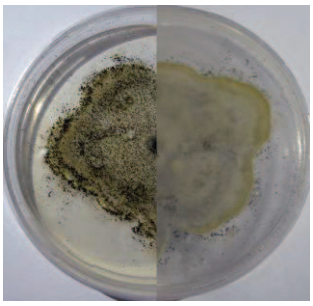
Phylum	**Order**	End-S (reads)	End-AS (reads)
Ascomycota	Botryosphaeriales	62	8512
	Capnodiales	95	12
	Pleosporales	43	76
	Chaetothyriales	5042	2161
	Eurotiales	138	200
	Phaeomoniellales	5020	0
	Hypocreales	1136	4749
Basidiomycota	Agaricales	2372	3843
	Cantharellales	100	271
	Entylomatales	14	0
	Malasseziales	1093	314
	Tremellales	4	13
Total		***15119***	***20151***

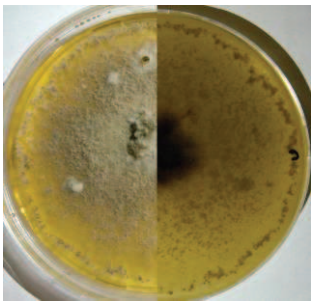
Figures

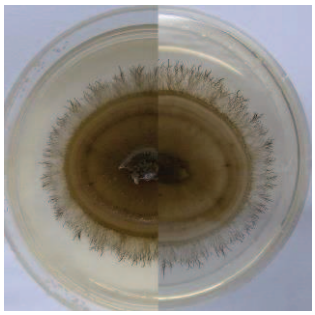
Fig. 1 - Representative fungal taxonomic groups isolated from roots of symptomatic and asymptomatic vines.

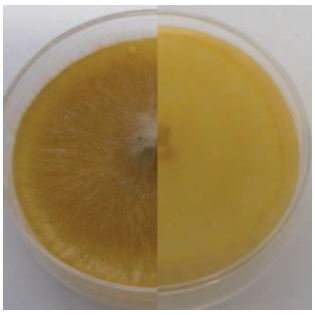

Acremonium sp.

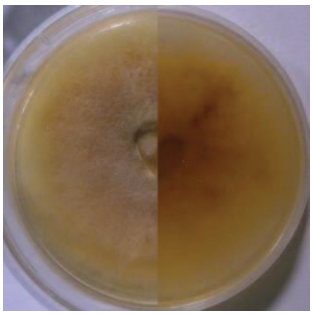

Alternaria spp.

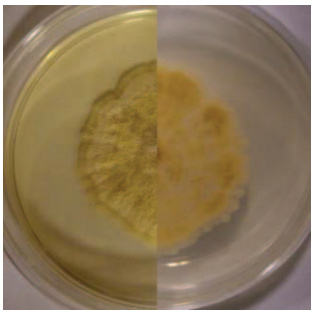

Aspergillus niger

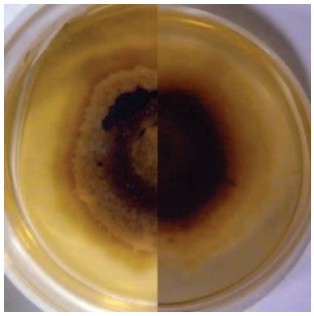

Aspergillus sp.

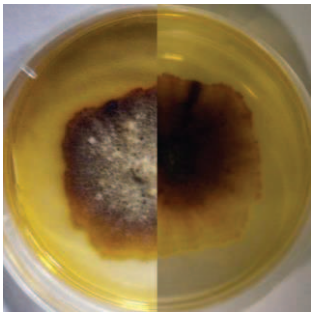

Aureobasidium pullulans

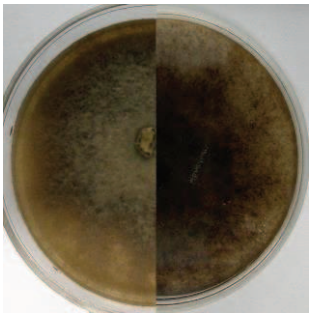

Ceratobasidium sp. / Rhizoctonia fragariae

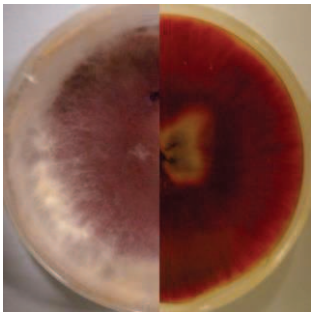

Phaeomoniella chlamydospora

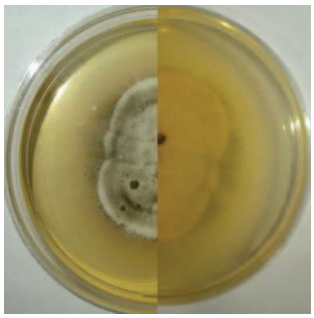

Clonostachys rosea

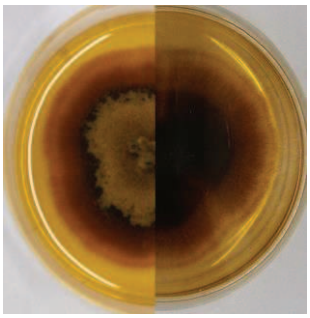

Cylindrocarpon olidum


Dactylonectria spp.


Diaporthe / Phomopsis


Fusarium spp.


Idriella lunata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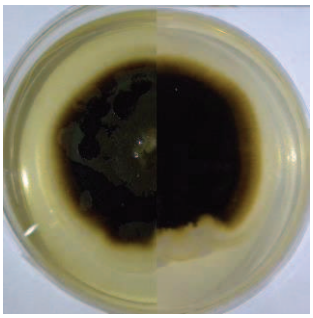
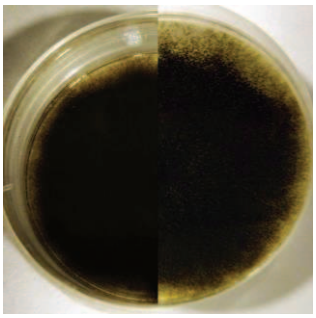
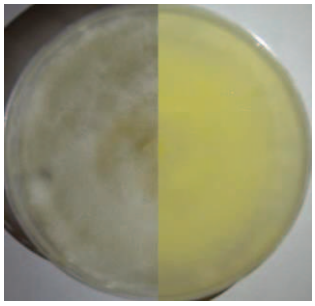
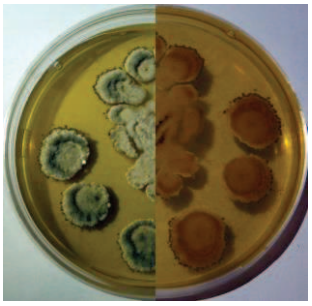
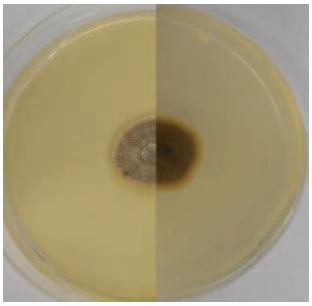
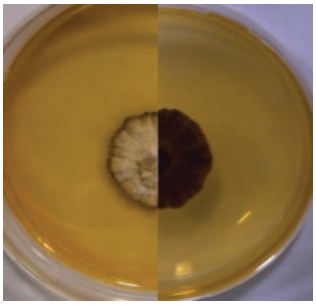

Lophiostoma / Massarina


Macrophomina phaseolina


Mortierella alpina


Penicillium spp.


Phaeoacremonium minimum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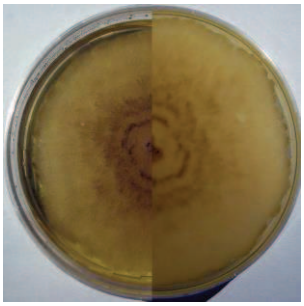
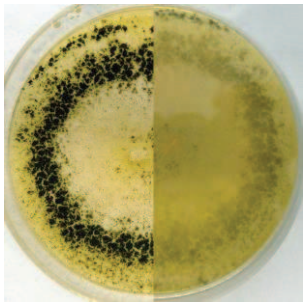
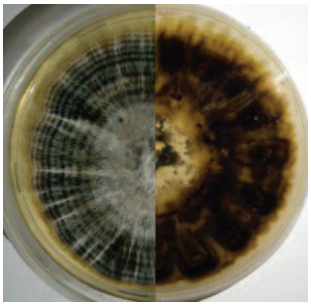
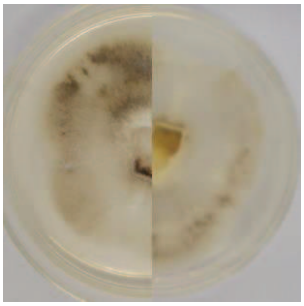
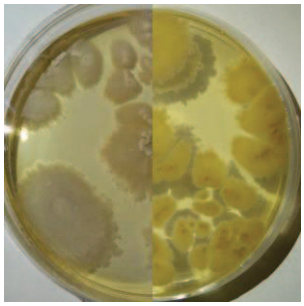

Sordariomycetes


Trichoderma harzianum


Truncatella angustata


Xylaria / Halorosellinia


Xylaria sp.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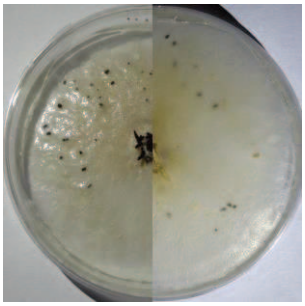
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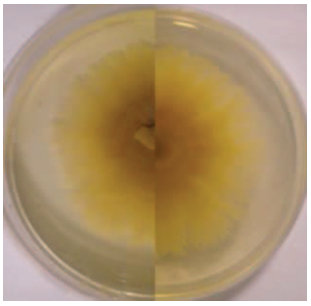
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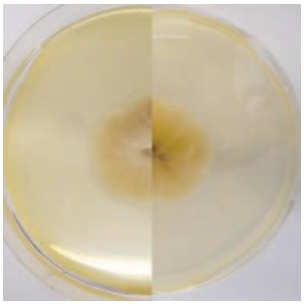
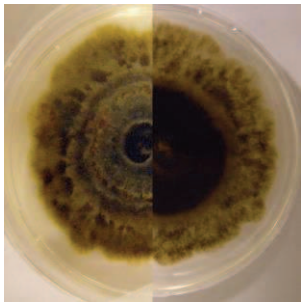
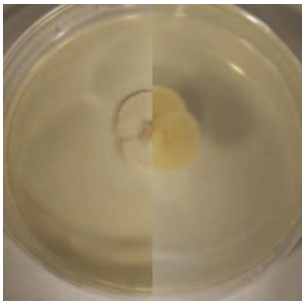

Fomitiporia mediterranea


Trichoderma sp.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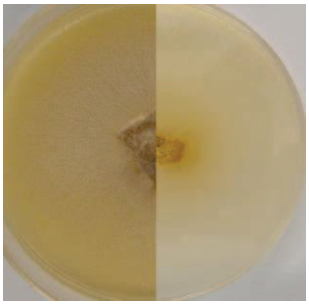
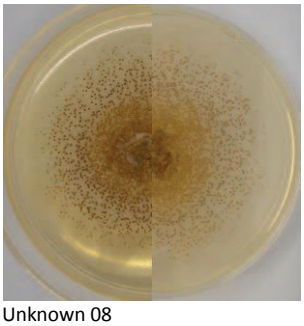

Phaeoacremonium sp.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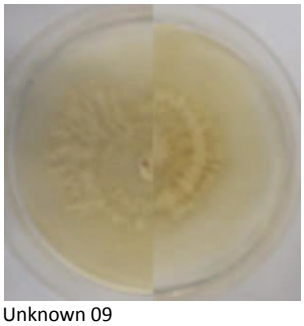
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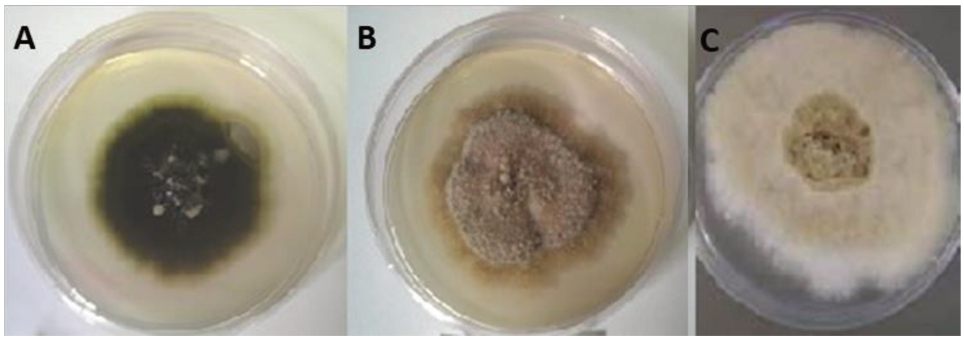

Fig. 2 - Main fungal pathogens isolated from roots of plants showing leaf tiger stripes. (A) *Phaeomoniella chlamydospora*, (B) *Phaeoacremonium minimum* and (C) *Fomitiporia mediterranea*.

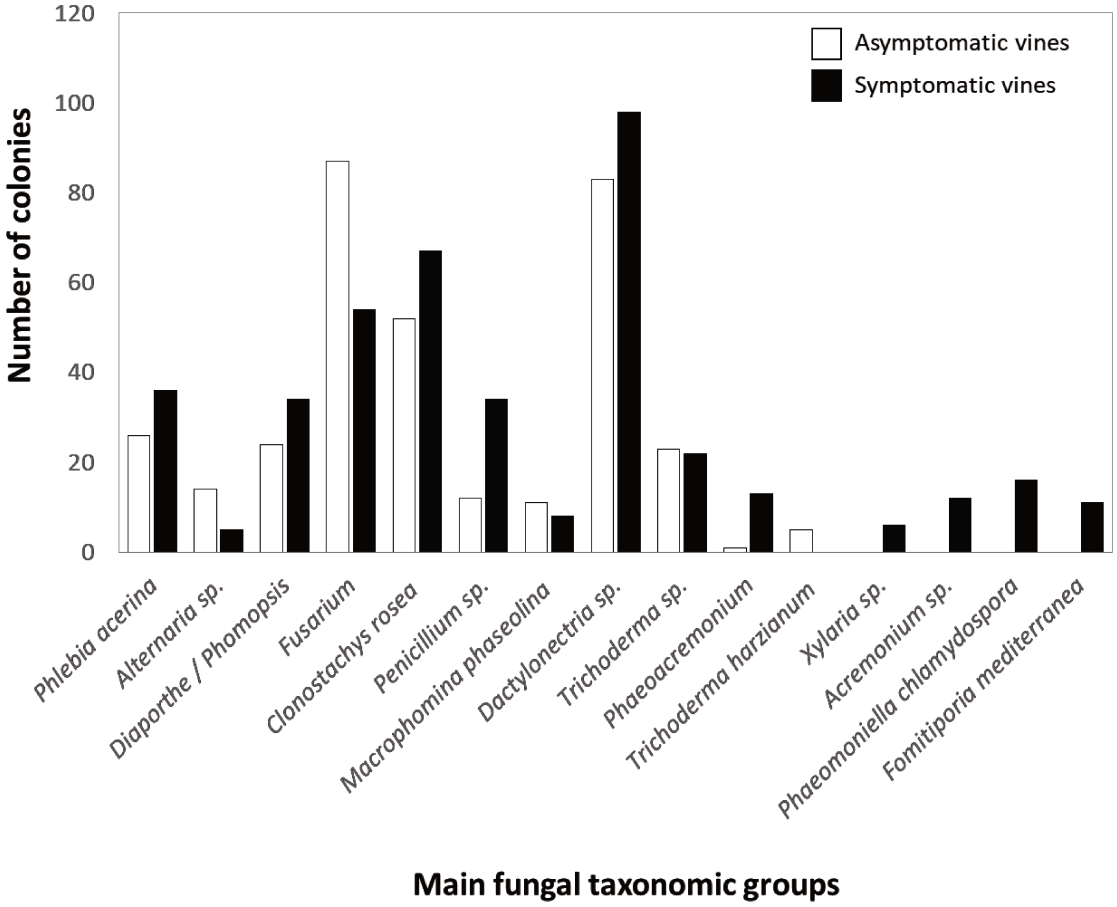

Fig. 3 - Main fungal taxonomic groups isolated from roots of symptomatic and asymptomatic vines.

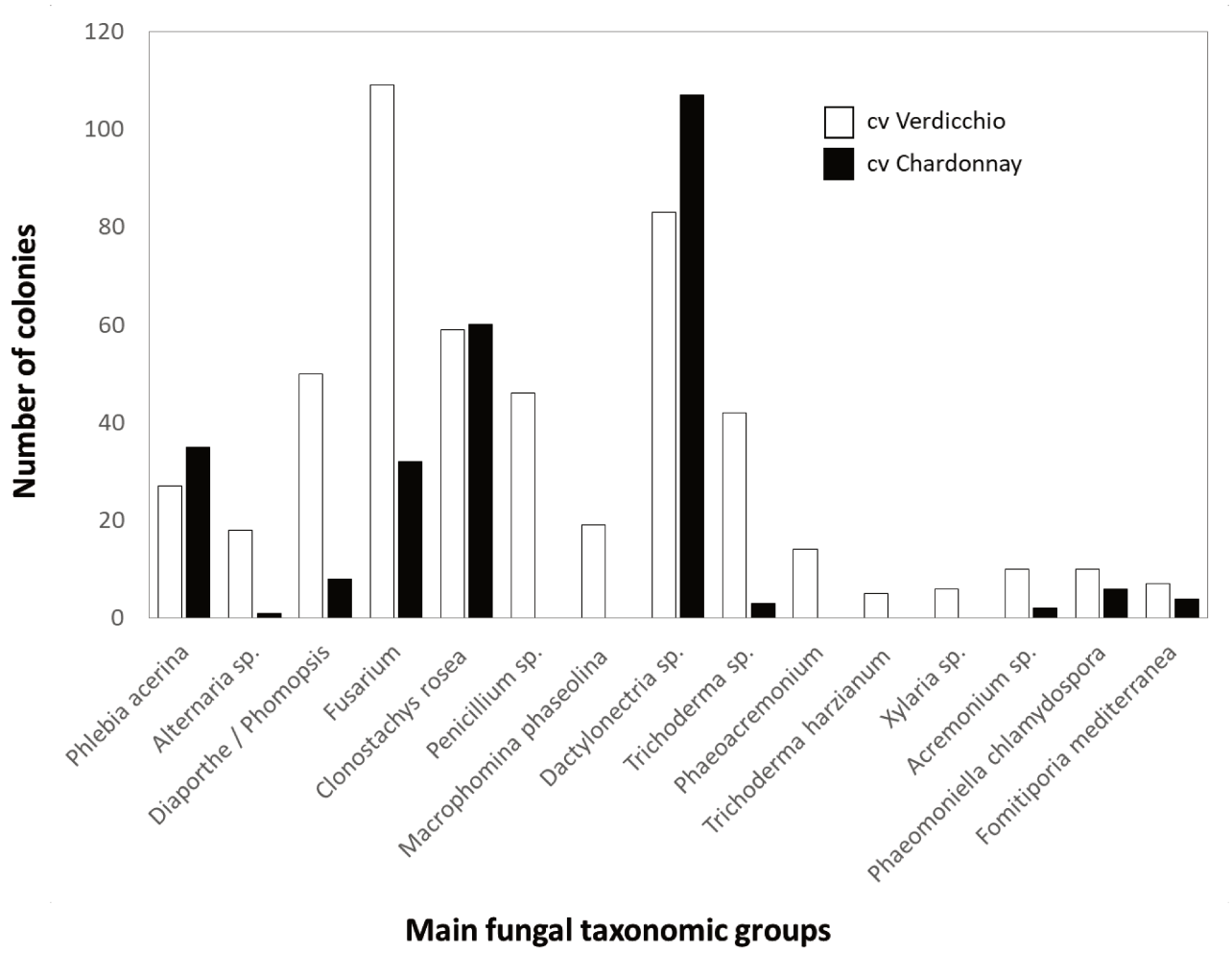

Fig. 4 - Main fungal taxonomic groups isolated from roots of grapevine cvs. Verdicchio (V) and Chardonnay (C).

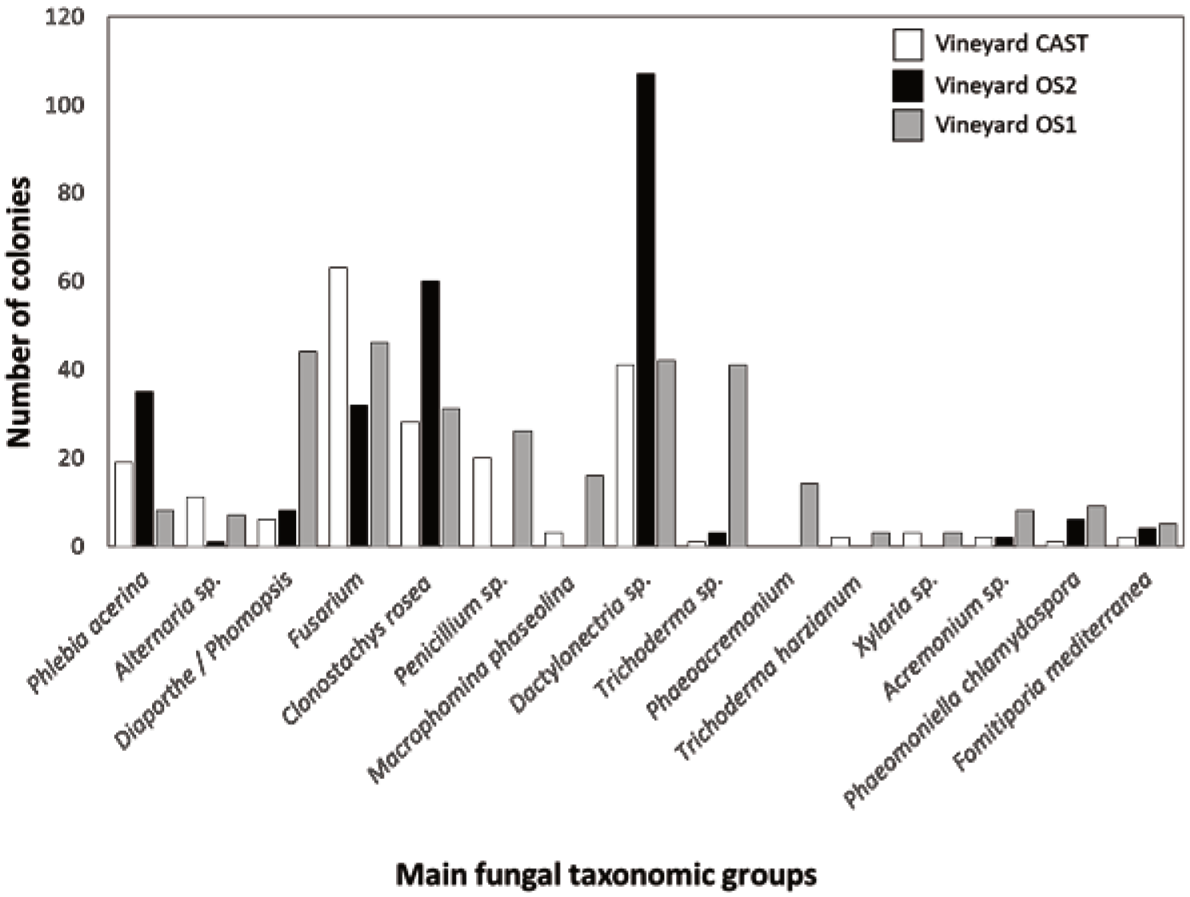

Fig. 5 - Main fungal taxonomic groups isolated from roots of grapevine collected in vineyards in Osimo (OS1, OS2) and Castelplanio (CAST).

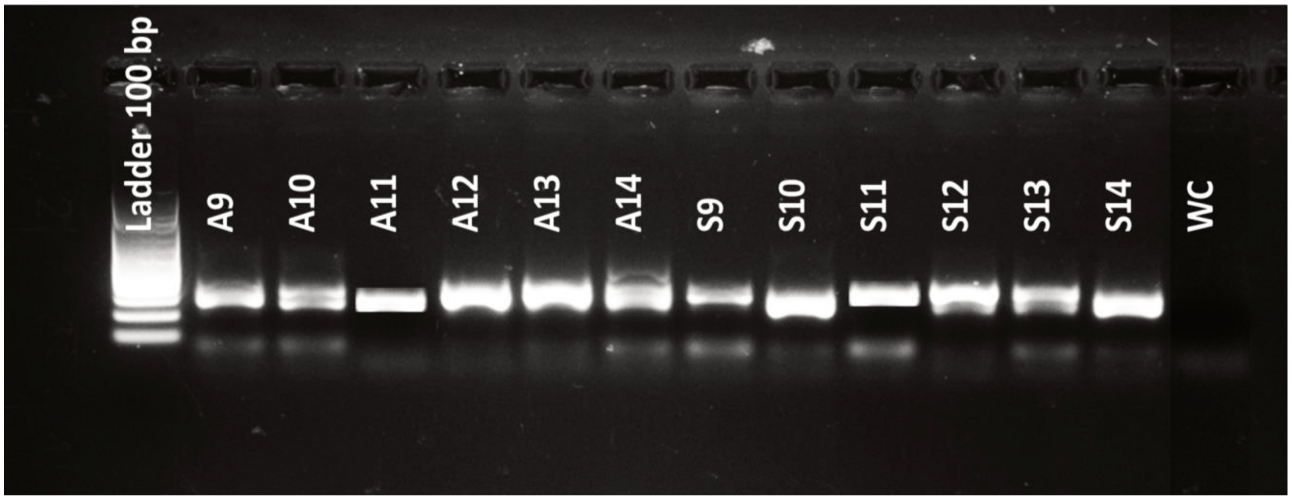

Fig. 6 - Agarose gel with specific amplicons generated after PCR with primer pair ITS1F_KYO2 and ITS2_KYO2 in asymptomatic samples (A9-A14) and symptomatic samples (S9-S14).

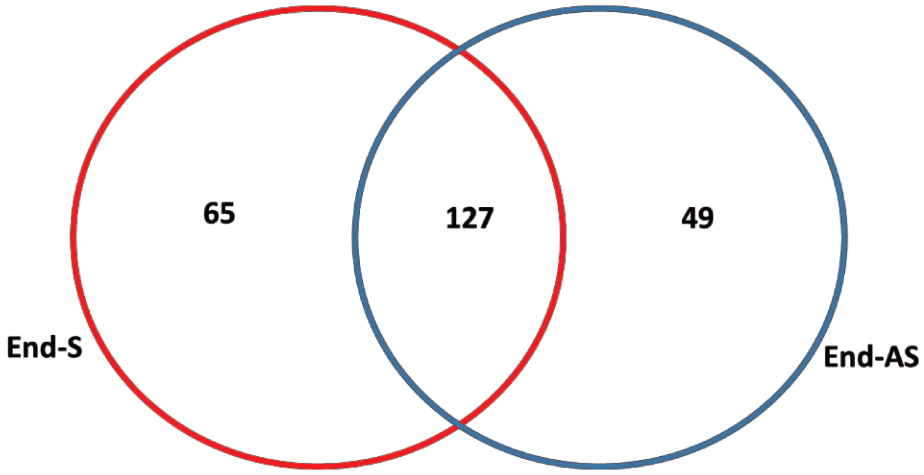

Fig. 7 - Venn Diagram which represents the different distribution of OUTs in the two analyzed samples: End-S (pool of symptomatic samples, S9-S14), End-AS (pool of asymptomatic samples, A9-A14).

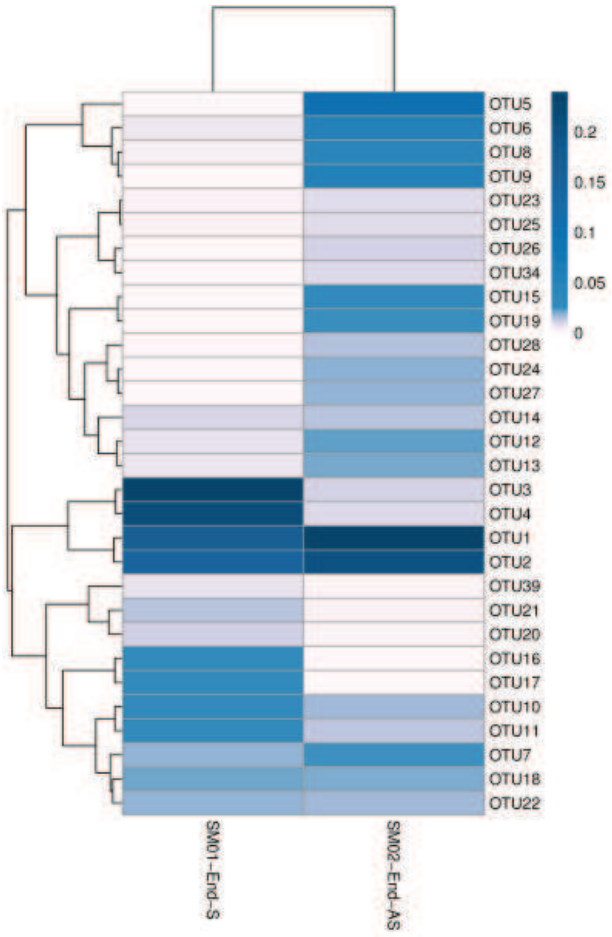

Fig. 8 - OTU abundance clustering heatmap (GENEWIZ Next Generation Sequencing), top 30 OTUs with the highest abundance, in sample End-S and End-As. The different intensity of blue colors is in relationship with the abundance of sequences.

Chapter V: CONCLUSION

The disease currently known as esca is a multiple fungal syndrome, considered as a complex disease (**Mugnai et al. 1999**). All species of *Vitis* and varieties of *V. vinifera* are susceptible to esca (**Mugnai et al. 1999**), but the syndromes produced by the causal agents and their severity depends on various factors (**Feliciano et al. 2004**, **Eskalen et al. 2007**, **Surico et al. 2008**).

The research focused on i) spatial distribution of grapevine esca, and ii) the ecological significance and role of fungal endophyte communities in symptomatic and asymptomatic (healthy) plants.

Concerning to the first aspect, three commercial vineyards in Marche region (Italy) have been examined in order to study the spread of the disease, in order to understand dissemination patterns that provide indications both on directionality and on the evolving of the infection over time. The research included visual assessment of the level of the severity of symptoms for each single plant, an activity of computation of the collected data and a processing phase on statistical software for the creation of graphic maps.
The data collected and elaborated, made possible to verify in 2017 and 2018 that i) none of the vineyards showed a gradient of infection among the rows; ii) there was a west-east gradient of infection in the vineyard OS1, and in the vineyard OS2 a greater concentration of disease in the center than the edges was observed; iii) a significant correlation was recorded between the esca disease incidence and different combination of rootstocks (420A, SO4, 41B, 110R) in vineyard OS1.

The second aspect investigated in this research was the estimation of fungal endophytic communities both in roots of healthy plants and in roots of vines showing leaf tiger-stripe symptoms of esca in three different commercial vineyards in Marche region (Italy). In particular, the main aim was to establish how the population structure of the fungal endophytes change according to the presence of esca pathogens, and if the cultivable endophytic fungi interfere with the pathogens related to esca.
From classical mycological analysis, fungal isolates were grouped according to morphological characteristics, and species were identified. The work allowed to isolate *in vitro* 34 fungal taxonomic groups. In particular, *Phaeoacremonium sp.*, *Fomitiporia mediterranea*, and *Phaeomoniella chlamydospora* were mainly isolated from symptomatic plants. The molecular detection confirmed the classical mycological analysis, and revealed that these pathogens were sporadically present also in asymptomatic vines. Wheares *Dactylonectria* spp. was isolated from both asymptomatic and symptomatic vines.
Considering the main endophytic species, we isolated *Fusarium* spp., *Clonostachys rosea*, *Trichoderma* spp., *Penicillium* spp. and *Alternaria spp*. both from asymptomatic and from grapevine showing leaf tiger stripes. Some of the species found in the present study have been reported as being suitable bio-control agents against grapevine pathogens. However, it was not possible to postulate that non-pathogenic fungi were always more numerous in asymptomatic grapevines than in esca-foliar symptomatic ones.

Further study will be done in order to improve the knowledges about the fragile equilibrium and how the mycoflora is shaped, using innovative technology (such as Next Generation Sequencing) and considering not only the endophytic fungal communities but also the microbiome.

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