Plant-pathogen interactions within the esca disease complex

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Abstract

This thesis presents an overview of the esca complex, a predominant grapevine trunk disease worldwide. Esca is one of the main causes of reduction of yield, plant decline and death of grapevines in most viticultural areas of the world. In recent years, research on esca has led to considerable progress in understanding the etiology, epidemiology and physiology of the disease and revealed its great complexity.

This work covers the symptomatology in the trunk, leaves and berries, of each syndrome belonging to the esca complex and the characteristics of the different fungal species involved in the decline of the vine.

Due to the effects of climate change, increasing attention is being paid to the impact that abiotic factors have on the development of plant diseases and on the mechanisms that can alter the relationship between pathogens and plants. Abiotic stresses, like drought or rainfall, can influence the tolerance or susceptibility of the plant to pathogens with consequences on plant-pathogen interactions, sometimes leading to a greater tendency to show symptoms, especially foliar ones. For this reason, this thesis compiles the available knowledge on the impact of host-pathogen-environment in the progress of the esca disease complex. To date, there is no certainty about the effects that abiotic factors have on plant-pathogen interaction but measurable evidences from several studies emphasize how they play a fundamental role in the definition of vine/pathogen interaction.

Grapevines have developed a plethora of defence mechanisms to face pathogen attempts to attack and infect, which are activated as soon as they can detect their presence. Based on the available evidence we evaluated the ability of the plant to respond to biotic stresses through the production, locally or systematically, of defensive compounds derived from secondary metabolism, in particular phenolic compounds. Phytoalexins are the most prominent compounds that accumulate at the site of infection, reaching toxic concentrations for pathogens. Despite this, the role of phenolic compounds, including those that are synthesized constitutively, in the protection of plant against pathogens is not yet completely clear. The knowledge of plant-pathogen interaction can have useful implications in the determination of field infections and contribute to the development of new control strategies. In addition, if the plant's innate defensive abilities could be stimulated, the use of fungicides could be reduced, along with the toxic effects for the environment, for man and for the plant.

Keywords

Grapevine trunk disease – Stress - Secondary metabolites - Phenolic compounds- Phytoalexins

Resumo

Esta tese apresenta uma visão geral do complexo Esca, um grupo de doenças do lenho da videira predominante em todo o mundo. A Esca é, atualmete, uma das principais causas de redução de produção, declínio de plantas e morte de videiras da maioria das áreas vitivinícolas à escala global. Nos últimos anos, a pesquisa sobre Esca tem levado a um progresso considerável na compreensão da etiologia, epidemiologia e fisiologia destas doenças e revelou a sua enorme complexidade. Este trabalho abrange a sintomatologia no tronco, folhas e uvas de cada síndrome pertencente ao complexo Esca e as características das diferentes espécies fúngicas envolvidas no declínio da videira.

Até ao momento, devido aos efeitos das mudanças climáticas, cada vez mais atenção tem sido dedicada ao impacto que os fatores abióticos têm no desenvolvimento das doenças das plantas e aos mecanismos que podem alterar a relação entre agentes patogénicos e plantas. Os stresses abióticos, como a seca ou a precipitação em excesso, podem influenciar a tolerância ou a susceptibilidade da planta aos microrganismos patogénicos, com consequências nas interacções planta-agente patogénico, conduzindo frequentemente a uma maior tendência para expressar sintomas, especialmente os foliares. Por esta razão, esta tese compila o conhecimento disponível sobre o impacto do hospedeiro-agente patogénico-ambiente no progresso do complexo de doenças da Esca. Até ao momento, não há certeza sobre os efeitos que os fatores abióticos têm na interação videira/agente patogénico, mas vários estudos apresentam evidências mensuráveis que enfatizam como eles desempenham um papel fundamental na definição dessa interação.

As videiras desenvolveram uma multiplicidade de mecanismos de defesa para enfrentar tentativas de ataque ou infecções por agentes patogénicos, que são ativadas assim que é detectada a sua presença. Com base no conhecimento disponível, avaliámos a capacidade da planta em responder aos stresses bióticos através da produção, local ou sistémica, de compostos defensivos derivados do metabolismo secundário, em particular compostos fenólicos. As fitoalexinas (compostos fenólicos no caso da videira) são dos compostos mais intensamente produzidos que se acumulam em torno do local da infeção, atingindo concentrações tóxicas para os agentes patogénicos. Apesar disso, o papel dos compostos fenólicos, incluindo dos que são sintetizados constitutivamente, na proteção de plantas contra agentes patogénicos ainda não se encontra completamente elucidada. O conhecimento da interação videira/agente patogénico pode ter implicações úteis na determinação de infecções de campo e contribuir para o desenvolvimento de novas estratégias de controle. Além disso, se as capacidades defensivas inatas da planta puderem ser estimuladas, o uso de fungicidas poderia ser reduzido, juntamente com os efeitos tóxicos para o ambiente, para o homem e para a planta.

Palavras - chave

Doença do lenho da videira - Stresse - Compostos fenólicos - Fitoalexinas

Resumo alargado

O complexo esca é parte do conjunto de doenças do lenho da videira, ou seja, das doenças causadas por agentes patogénicos fúngicos que colonizam e vivem dentro dos órgãos lenhosos da planta, causando em geral infeções vasculares que levam à necrose, descoloração e deterioração da madeira. De entre as doenças do lenho da videira, a esca é certamente considerada como o grupo de doenças mais importante, pela sua propagação em todas as áreas vitícolas do mundo e pela sua complexidade. O que torna o grupo de doenças da esca um problema muito complexo é a presença simultânea de diferentes agentes patogénicos, que causam diferentes síndromes baseadas na sua interação com a planta e com o meio ambiente, sendo que os diferentes mecanismos que envolvem respostas fisiológicas e estruturais nem sempre são previsíveis. Em geral, a planta é capaz de implementar mecanismos de defesa ou de resposta para tentar reparar e superar qualquer stresse. Contudo, neste caso, parece que as respostas não são eficazes no combate ao desenvolvimento e propagação dos agentes patogénicos. Outro fator que torna a doença muito complexa é a influência de fatores abióticos e antropogénicos durante o desenvolvimento da doença. Alguns dos fungos associados com as várias síndromes foram isolados de plantas sintomáticas e assintomáticas, apoiando assim a ideia de que estes mesmos fungos podem fazer parte do microbioma da planta saudável e que são fungos endofíticos, agentes patogénicos que colonizam o hospedeiro causando infecções que permanecem latentes, alternativamente, que mudam o seu comportamento, tornando-se invasivos assim que a planta sofre os efeitos de um stresse abiótico. O que muito contribuiu para a propagação da esca em todas as zonas vitícolas do mundo foi a falta de métodos e de estratégias de controlo e mitigação, que dêem uma proteção adequada à planta, tanto no viveiro como em condiçoes de campo. Isto é também agravado pelo facto de não existirem procedimentos de diagnóstico que não envolvam métodos destrutivos. O único método não destrutivo de deteção da esca é a observação de sintomas externos, que permanece um meio não confiável considerando a variabilidade anual da sua manifestação. O objetivo desta tese foi dar uma visão geral da etiologia e epidemiologia da esca, especificamente de todas as síndromes associadas ao complexo, dos agentes patogénicos associados a cada síndrome e de quaisquer estratégias de controlo e atenuação aplicáveis tanto no viveiro como em condiçoes de campo. Dentro da interação do hospedeiro-agentes patogénicos, foi importante definir se e como os fatores abióticos influenciam o desenvolvimento da doença e como a planta interage com os agentes patogénicos e, portanto, da capacidade de resposta que possui durante um estado infeccioso.

Os principais agentes patogénicos associados à esca são *Phaeomoniella chlamydospora* e *Phaeoacremonium minimum* que fazem parte dos Ascmicetos, e *Fomitiporia mediterranea*, que faz parte dos Basidiomicetos. Em geral, a esca é devida à sucessão ou sobreposição de

duas doenças principais, ou seja, a traqueomicose e, portanto, a colonização do xilema, e a podridão branca. A traqueomicose é causada por Ascomicetos e a podridão branca por Basidiomicetos, neste caso específico por *F. mediterranea*. Em 2009, Surico propôs uma nova classificação para o complexo de doenças da esca e para as síndromes associadas ao complexo, definindo os seguintes parâmetros de classificação: idade da planta, sintomas e agentes patogénicos. A estes parâmetros-base foram acrescentados a susceptibilidade do porta-enxerto e a cultivar, bem como a influência de fatores abióticos.

A videira encontra-se, como todas as outras culturas em condições de campo, exposta a um grande número de stresses abióticos, especialmente num período histórico como este, em que os efeitos/ impactos das mudanças climáticas são cada vez mais evidentes e frequentes. Assim, a comunidade científica tem questionado se e de que modo os fatores abióticos afetam o desenvolvimento da esca e os mecanismos de interação videira-agentes patogénicos. Chegou-se à conclusão de que, para ter uma visão completa e ampla da expressão da esca, é necessário avaliar três fatores principais, definidos exatamente como o triângulo da doença: a susceptibilidade da planta aos agentes patogénicos, a presença de agentes potencialmente virulentos e um ambiente favorável ao desenvolvimento da doença. Os stresses abióticos, dependendo da sua intensidade e da predisposição da planta, podem agravar muito os efeitos da interação planta-agentes patogénicos. Em geral, quando a planta é exposta a um stresse abiótico não muito intenso, é capaz de se adaptar ao stresse e de reparar os danos sofridos muito rapidamente. A exposição simultânea a stresses abióticos e bióticos leva à implementação de estratégias de defesa diferentes daquelas implementadas quando o stresse é ‘percebido’ como único. A planta tem então que ativar mecanismos que podem interagir uns com os outros. Os fatores que influenciam a interação hospedeiro-agentes patogénicos são, não só, stresses abióticos, mas também fatores relacionados com o genótipo da planta. O stresse hídrico e o ataque por agentes patogénicos são certamente uma das interações entre stresses que têm sido mais estudadas. Em condições de campo, existem duas maneiras principais de interação: os agentes patogénicos podem se comportar como agentes oportunistas, aproveitando a fraqueza da planta que está sujeita a um stresse hídrico, ou o stresse hídrico pode ocorrer após um ataque patogénico. O stresse hídrico pode aumentar a susceptibilidade da planta a agentes patogénicos, pelo que induz uma menor produção fotossintética, a que corresponde a uma menor capacidade da planta em produzir compostos defensivos, ou leva a um menor crescimento da planta, sem comprometer o desenvolvimento e o crescimento dos agentes patogénicos.

Como parte da interação planta-agentes patogénicos, é importante avaliar a resposta defensiva da planta durante o stresse biótico. Para se defender de ataques patogénicos, a planta desenvolveu mecanismos de defesa a fim de tentar neutralizar o desenvolvimento dos agentes patogénicos. Assim, uma vez que a planta reconhece que está num estado infeccioso, ativa respostas que podem ocorrer na forma de barreiras físicas e na forma de compostos químicos. Além das proteínas PR (do Inglês *Pathogenesis-Related*), os principais compostos químicos que a videira utiliza nestes casos vêm do metabolismo secundário e são compostos fenólicos (as fitoalexinas da videira). É possível dividir estes compostos fenólicos em dois grupos: os pré-formados ou constitutivos (i.e. os que são sintetizados independentemente de estar a ocorrer uma tentativa de infeção) correspondendo àqueles que são normalmente produzidos durante o crescimento e desenvolvimento saudável da planta, e os induzidos, portanto, sintetizados *de novo* durante uma situação de stresse. Estes compostos têm ação antifúngica e a sua concentração aumenta em torno do local onde a (tentativa de) infeção está a ocorrer/ocorreu. A videira responde à infeção com a acumulação de fitoalexinas, compostos pertencentes à família de estilbenóides e com a estrutura base do *trans*-resveratrol. Estes estilbenóides são sintetizados *de novo* quando a planta está sob ataque dos agentes patogénicos e, por isso, eles são compostos fenólicos induzidos e são ativados na presença de eliciadores. Entre os mais abundantes temos as viniferinas e o *trans*-resveratrol. Esses compostos influenciam o crescimento de fungos, mas seu poder de limitar esse crescimento depende da concentração em que são sintetizados e da tolerância dos agentes patogénicos. Analisamos, portanto, as variações desses compostos, tanto nos tecidos lenhosos como nas folhas.

Até à data, pouca evidência existe que possa esclarecer as variações nos compostos fenólicos. O que parece acontecer é que essas variações são respostas espontâneas das plantas na presença dos agentes patogénicos. Em conclusão, embora não haja certezas sobre a influência dos fatores abióticos durante o desenvolvimento da esca, parece que eles desempenham um papel fundamental que precisa ser esclarecido. Quanto à resposta defensiva nos tecidos lenhosos, não há evidências suficientes para esclarecer o papel dos fenóis, mas é provável que seja também uma resposta espontânea da planta. Poder avaliar as variações dos fenóis nas folhas poderia ser um ponto-chave para desenvolver novas estratégias de controlo sem recorrer a métodos destrutivos e, em especial, antes de a planta se encontrar num estado muito grave de infeção e, acima de tudo, permitir uma redução da utilização de fungicidas, um ponto importante neste período histórico em que devem ser encontrados métodos alternativos e ambientalmente sustentáveis, a fim de reduzir o impacto dos efeitos das alterações climáticas.

INDEX

List of figures

transcriptional activators and repressors, gene expression and physiological responses (Bostock *et al.,* 2014).

Figure 3.2 Effect of water stress on leaf water potential of infected plants with *Phaeomoniella chlamydospora* and uninfected plants of cv Cabernet Sauvignon (Edwards *et al.,* 2007b).

Figure 3.1 Effect of water stress on stomatal conductance of infected plants with *Phaeomoniella chlamydospora* and uninfected plants of cv Cabernet Sauvignon (Edwards *et al.,* 2007b).

Figure 4.1 Chemical structures of stilbene phytoalexins. 1 and 6, trans- and cis-piceid; 2 and 7, trans- and cis-resveratroloside; 3 and 8, trans- and cis-astringin, 4, trans-pterostilbene; 5, trans-resveratrol. Glc: glucosyl (C6H11O5; Jeandet *et al.,* 2002).

Figure 4.2 Total phenolic compounds content within the methanolic extract from grapevine wood tissue from healthy and infected plants (cvs Touriga Nacional, Chardonnay, Aragonez). Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF; Martin et al., 2009).

Figure 4.3 *trans*-Resveratrol content in healthy and infected plants, from cvs Touriga Nacional, Chardonnay, Aragonez). Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF; Martin *et al.,* 2009).

Figure 4.4 Ɛ-Viniferin content in healthy and infected plants, from cvs Touriga Nacional, Chardonnay, Aragonez. Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF; Martin *et al.,* 2009).

Figure 4.5 Total phenolic production in diseased (dl), apparently healthy (ahl) and healthy (hl) Vitis vinifera cv Alvarinho leaves (Lima *et al.,* 2017).

General abbreviations

BCA Biological Control Agent

BCO Blad-Containing Oligomer

BWS Brown Wood Streaking

cv Cultivar

e. g. *Exampli Gratia*

GLSD Grapevine Leaf Stripe Disease

GTDs Grapevine Trunks Diseases

HWT Hot Water Treatment

i.e. *Id Est*

JA Jasmonic Acid

PR Pathogenesis-related Proteins.

PAL Phenylalanine Ammonium-lyase

ST Stilbene Synthase

ROS Reactive Oxygen Species

CHAPTER I

GENERAL INTRODUCTION

1.1 Grapevine trunk diseases

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops in the world, due to its worldwide diffusion, economic profitability (Vivier and Pretorius, 2002), connection to the territory of cultivation and popular tradition. Nowadays, the global area dedicated to the cultivation of vines is approximately 7.5 million hectares (OIV, 2018).

The introduction of vines in non-native environments, the use of monoculture and clonal reproduction, are all factors that have contributed to the spread of new diseases and have complicated the approach to pathogen and pest control. In the Compendium of Grape Diseases, Disorders and Pests, Second Edition (Wilcox *et al.,* 2015), a list is tentatively provided for all significant vine diseases caused by fungi, bacteria, virus, phytoplasmas, nematodes and insects, for a total of 65 diseases including Grapevine Trunk Diseases (GTDs). Grapevine trunk diseases comprise a group of diseases probably present since the beginning of vine cultivation. They are associated to fungal pathogens that colonize and live in the perennial organs, causing vascular infections which lead to wood necrosis, wood discoloration, and wood decay (Mugnai *et al.,* 1999; Bertsch *et al.*, 2013). Symptoms related to GTDs have been reported in several Latin and Greek works and later, in the Middle Ages, in the *Opus Ruralium Commodorum* by Pietro de' Crescenzi (Mugnai *et al.,* 1999). More reliable sources describing the symptoms of GTDs are found in French scientific literature starting from the late nineteenth century (Surico, 2009).

Since the last century, due to the high incidence and severity of GTDs in all viticultural areas of the world, there has been a progressive development in the comprehension of their etiology and epidemiology. In fact, a study conducted by the International Organisation of Vine and Wine (OIV), showed that, depending on the cultivar, GTDs prevalence is between 8 and 19% in Italy, 10% in Spain and 13% in France (OIV, 2018). GTDs cause serious economic losses to the vine-wine industry, for a total of 1.13 billion euros per year approximately, as it reduces the duration of plant life and increases the related costs for the replacement of dead vines (Hofstetter *et al.*, 2012). In Portugal, GTDs are also widely spread in all vine growing regions (e.g. Alentejo, Douro, Dão; Rego *et al.,* 2005). No cultivars are known to be resistant to GTDs (Bertsch *et al.*, 2013), however, varying degrees of susceptibility have been demonstrated. In fact, varieties such as cvs Cabernet Sauvignon, Sangiovese, Trebbiano Toscano, Sauvignon blanc or Temperanillo (sin.Tinta Roriz or Aragonez) have a greater susceptibility to develop both internal and external symptoms, when compared to cvs Carignan, Merlot, Montepulciano and Pinot Noir (Almeida, 2007; Quaglia *et al.,* 2009). These observations are based on the presence of visible symptoms. In fact, infections develop initially in the wood and it is generally accepted that they appear externally only after several years from the time of infection (Bertsch *et al.*, 2013; Fontaine *et al.*, 2016). For this reason, wood pathogens have been spreading nearly unrestrictedly, with asymptomatic but infected vines contributing to such spread (Mugnai, *et al.,* 1999).

The increasing incidence of GTDs in the last two decades may be linked to different factors:

- changes in cultural practices, such as the introduction of mechanical pruning (Graniti *et al.*, 2000);
- the lack of effective containment strategies (Mondello *et al.*, 2018), such as the efficient protection of pruning wounds;
- the ban on sodium arsenite, extremely toxic for human health and the environment. Sodium arsenite was the only treatment proven efficient in reducing the appearance of esca foliar symptoms (Mondello *et al.,* 2018). Five years after the end of sodium arsenite application, mortality was reported to have increased annually up to 5% in the plots (Fontaine *et al.*, 2016);
- the prohibition of the use of Carbendazim and Benomyl as control agents of *Eutypa* dieback and *Botryosphaeria* dieback (Mondello *et al.,* 2018);
- absence of non-destructive diagnostic procedures (Rubio and Garzón, 2011);
- infected plant material originating in nurseries (Rubio and Garzón, 2011);
- the presence of several pathogens that cause different symptoms depending on the type of interaction with the plant and the environment, and different mechanisms involving structural and physiological responses not always predictable (Mondello *et al.,* 2018).

Considering all these factors, it is impossible to trace the disease to a simple scheme of “cause-effect” (Mugnai *et al.,* 1999; Mondello *et al.*, 2018).

Causal agents. Recent reviews include in the GTDs cluster five main diseases, based on similarities in their etiology and epidemiology: Botryosphaeria dieback, Eutypa dieback, the esca disease complex, Phomopsis dieback and Black foot, with the first three being considered the most important (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016). Several studies have shown the large number of fungal genera and species associated with these diseases, especially within Ascomycota and Basidiomycota, whose presence may also depend on climatic and geographical factors (Fischer, 2006; Mostert *et al.*, 2006 Van Niekerk *et al.,* 2011a).

1) In the case of Botryosphaeria dieback, many members of the Botryosphaeriaceae family have been linked with this syndrome, such as *Diplodia seriata* De Not*.,* *Neofusicoccum parvum* (Pennycook & Samuels), *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.), *Botryosphaeria dothidea* (Moug. ex Fr.) and *Lasiodiplodia theobromae* (Ú’rbez-Torres, 2011; Bertsch *et al.,* 2013).

2) *Eutypa lata* (Rappaz) is an Ascomycota of the Diatrypaceae family; it has been identified as the main causal agent of Eutypa dieback (Carter, 1988).
3) *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai), *Phaeoacremonium minimum* (Tul. & C. Tul.) and *Fomitiporia mediterranea* (M. Fisch) are the main causal agents of one or more of the syndromes associated with the esca complex. These species are certainly the most frequent, but in spite of this, several other pathogenic fungi have been isolated from esca symptomatic plants (Mugnai *et al.,* 1999; Surico, 2009).

Infection, symptoms and control strategies. The scientific community has reached a confident understanding on how the pathogens can infect grapevine:

- Infection occurs directly in the field. Fungal conidia and/or mycelium reach fresh pruning cuts or mechanical wounds, through air, water droplets, arthropods or human intervention (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016).
- There is an indirect contamination in the nursery due to the presence of pathogens inside the propagation material (rootstock and/or scion) and/or through the equipment used during the grafting process and storage of the cuttings (e.g. grafting machinery, hydration tanks and cold rooms; Gramaje *et al.*, 2018). Clearly, the presence of asymptomatic but infected plants in nurseries is a potential source of inoculum that contributes to the spread of the disease in the field (Mondello *et al.,* 2018).

One of the most common manifestation of GTDs, is the vascular discoloration, mainly in the form of brown streaking of xylem vessels and wood necrosis (Fig. 1.1; Bertsch *et al.,* 2013), which compromise to some extent, the normal operation of the plant hydraulic system. Other symptoms of advanced infections are dieback, reduced vigour, lower yield and berry quality and an earlier death (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016). It may be difficult to observe the external symptoms on leaves and berries, since they may appear after several years from the moment of the infection and discontinuously. When symptoms became visible the disease could be at an advanced stage (Calzarano and Di Marco, 2007), giving few chances to viticulturists to reduce disease impact in the vineyard. The appearance of symptoms on the leaves and in the wood remains to be fully understood. However, they are believed to be related to the presence of secondary metabolites produced by fungi (e.g. toxins) and/or by-products of the degradation of the wood (Andolfi *et al.,* 2011).

To date, control of GTDs is a big challenge for vine growers and scientists, since effective strategies are few and do not always provide adequate protection. Therefore, the most common cultural practices for the containment of these syndromes are preventive and curative

(Mondello *et al.,* 2018). It is important to ensure that the propagation material is healthy, improving the sanitation of tools and treating rooted cuttings with techniques such as the hot water treatment (Gramaje *et al.,* 2018), to avoid the uncontrolled spread of the diseases to newly planted vineyards. In the field, control is mainly based on agricultural practices, such as the application of products on pruning wounds (Sosnowski *et al.,* 2013), that aims to keep the number of infected plants in the vineyard low by decreasing the likelihood of an infection from occurring (Mondello *et al.,* 2018).

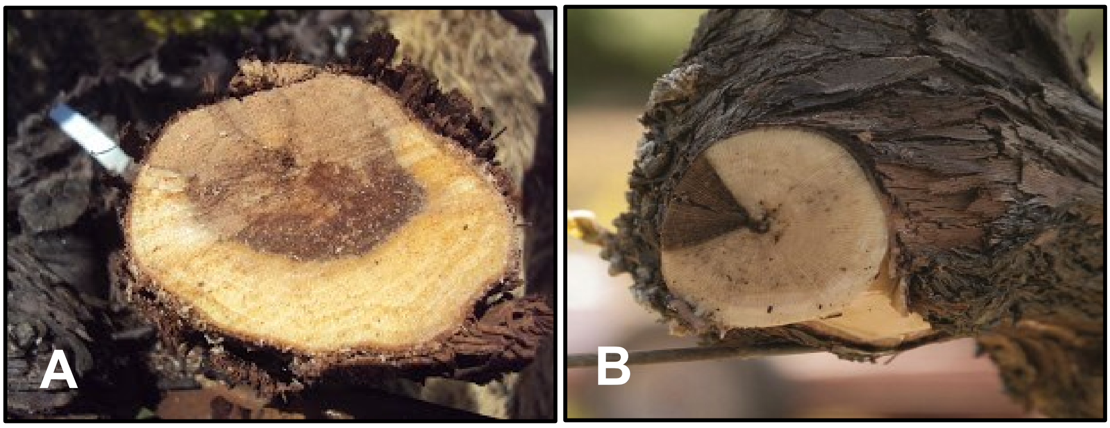


Figure 1.1 Grapevine trunk affected by wood pathogens. A- Central necrosis and vascular discoloration associated with *Botryosphaeria dieback*; B- Sectorial “wedge shaped” necrosis associated with *Eutypa lata* infection. Sources: A- http://grapepathology.blogspot.com/p/trunk-diseas-projects_6.html B- www.lodigrowers.com/labratory-testing-for-grapevine-diseases/ (Retrieved on 06-05-2020).

1.2 Aim of the thesis

Although GTDs have been well known since the end of the 19th century, their significance and impact on plant health have only been recognized recently. Among grapevine trunk diseases (GTDs), esca syndrome is one of the most complex, because it is characterized by the simultaneous presence of several fungi that cause different symptoms depending on the type of interaction with the plant and the environment, and different mechanisms involving structural and physiological responses not always predictable. Its severity is also linked to (i) the lack of effective control strategies; (ii) the absence of non-destructive diagnostic procedures; (iii) the influence of abiotic factors; (iv) the lack of effective plant defence mechanisms against pathogens, especially when cultivars are very susceptible to infection.

In the second chapter of this thesis, we will provide a general overview of the etiology of the five syndromes associated with the esca complex, the pathogens associated with the disease and the control and mitigation strategies used in the field and in the nursery.

The third chapter provides information on the relationship between abiotic factors and the esca pathogen complex, especially considering the evolution of climate change.

In the fourth chapter, we will also address the plant-pathogen relationship from the point of view of the plant ability to activate natural defence mechanisms in response to infections by fungal pathogens, with a particular focus on phenolic compounds produced in wood and leaves. Finally, in the last chapter, we will draw general conclusions on plant-pathogen interactions.

CHAPTER II

THE ESCA DISEASE COMPLEX

2.1 Syndromes involved in the esca complex of diseases

The etymology of the word *"Esca"* comes from Latin “*Fomes”*, which means *“tinder”.* Fomes is also the name of a genus of basidiomycetes used to produce an easily flammable and slow-burning material. The wood deteriorated by fungi, including that from grapevine, was then used to keep fires without flame (Mugnai *et al.,* 1999).

In 2009, Surico proposed a new classification for esca and associated syndromes, establishing a division into five main syndromes (Fig. 2.1). These were identified according to the age of the plant, symptomatology in wood or other organs, the causal agents and the presence of abiotic stress (Surico, 2009). To this list, it can also be added the susceptibility of cultivar and rootstock (Eskalen *et al.*, 2007a).

The five syndromes are:

1. Brown wood streaking of rooted cuttings (BWS);
2. Petri disease;
3. Grapevine leaf stripe disease (GLSD);
4. White rot;
5. Esca proper.

Figure 2.1 The five syndromes belonging to the esca disease complex. Symptoms in different plant organs depending on the vine age and fungal infection (Mondello *et al.,* 2018).

2.1.1 Causal agents

The esca complex is a fungal disease that, in contrast to others such as downy mildew and powdery mildew, is due to the colonization of the perennial organs by several fungal species. Three of these species are the main causes of the disease: the Ascomycetes *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* and the Basidiomycete *Fomitiporia mediterranea* (Mondello *et al*., 2018). The esca disease complex is due to the overlap or succession of two main diseases: tracheomycosis, namely the colonization of xylem vessel by fungal pathogens, and wood decay. The fungi belonging to the genera *Phaeomoniella* and *Phaeoacremonium* are the causal agents of tracheomycosis, while *Fomitiporia mediterranea* is the causal agent of white rot (Surico, 2001).

Pa. chlamydospora and Pm. minimum are two Hyphomycetes, that colonize the xylem vessels and, under favourable water and vegetative conditions, colonize the wood and compromise the translocation of water and nutrients from the roots to the vegetation. Tracheomycosis leads to: loss of leaf turgor, desiccation of the aerial part of the plant and browning of the vascular tissue. During colonization, fungi produce toxins such as scytalone and isosclerone (Fig. 2.2; Andolfi *et al.,* 2011) that, perhaps, they reach the vegetation, could confer the typical external symptoms on leaves and on bunches (Michelon *et al.,* 2007). Both fungi are frequently isolated from plants with visible external symptoms, as in the case of GLSD (Gramaje *et al.,* 2018), and from plants that do not show external symptoms but contain internal ones, as in the case of BWS and Petri disease (Michelon *et al.,* 2007).

Fomitiporia mediterranea belongs to the Basidiomycetes, fungi that produce spores inside a structure called basidium. This pathogen colonizes the wood of the branches and trunk, especially the one near the pith. This pathogen, through the production of enzymes that decompose lignin, causes wood degradation, leading to the formation of a white-yellowish spongy and friable mass that takes the name of white rot. However, white rot formation is often preceded by a first infection by *Pa. chlamydospora* and/or *Pm. minimum* (Michelon *et al.,* 2007).

The dispersion of spores can occur through the rainwater that, in its fall, carries the spores in suspension in the air or transports others flowing along the plant or on the ground. This happens especially, in case of heavy rains falling on the ground that cause the transfer of spores on the vegetation (Ferri, 1985). Another important means of dispersion is the wind, which carries the spores until they land on the host (Michelon *et al.,* 2007). The pruning wounds are certainly one of the main routes of penetration for the three main fungi responsible for esca complex. Studies by Larignon and Dubos (2001b) have shown that *Pa. chlamydospora* spores are present in the air throughout the year, including winter. The fruit bodies of *Fomitiporia mediterranea,* are formed almost exclusively on very old vines, therefore it is probable that the inoculum comes from external sources, such as near old vineyards or fruiting bodies formed by different hosts (Fischer, 2002).

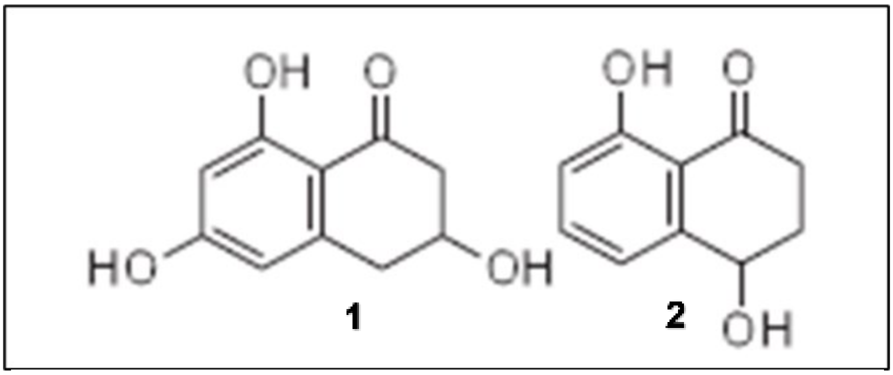


Figure 2.2 The main pentaketides isolated from *Phaeoacremonium aleophilum* and *Phaemoniella chlamydospora*: scytalone (1) and isosclerone (2; Bertsch *et al.,* 2013).

2.1.2 Brown wood streaking of rooted cuttings

The brown wood streaking of rooted cuttings (BWS) is known to affect primarily grapevine rooted cuttings. Pathogens are likely to reach the nursery through infected asymptomatic propagation material (rootstock and/or scion) and/or can be found in tools or equipment used during the grafting process and storage of the cuttings, such as grafting machinery, hydration tanks and cold rooms (Gramaje and Armengol, 2011). The two fungi associated with BWS are *Pa. chlamydospora,* that colonizes xylem vessels and xylem fibers (Pouzoulet *et al.,* 2017), and *Pm .minimum* that can colonize bark, phloem, xylem fibres and vessels, rays, metaxylem and protoxylem (Pierron *et al.*, 2015). The presence of symptoms starts frequently from the graft union and may go upwards (more often) or downwards, sometimes infecting the whole plant (Surico *et al.*, 2008). The internal symptoms most frequently observed are: (i) dark-brown streaking, when the wood is cut in longitudinal sections (Fig. 2.3 A) and (ii) dark-brown dots, when the wood is observed in a cross section (Fig. 2.3 B). Affected plants do not show external symptoms on leaves and berries (Surico *et al.*, 2008).

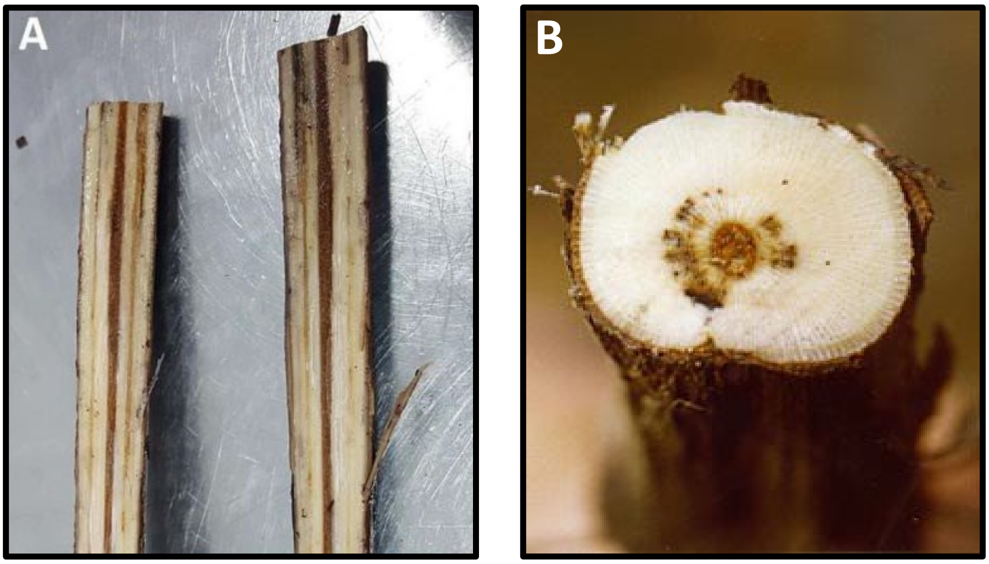


Figure 2.3 Dark-brown streaking in a longitudinal section (A) and dark-brown dots in a cross section (B) of grapevine wood (Mugnai *et al.,* 1999; Del Frari, 2018).

2.1.3 Petri disease

This disease was first observed by Lionello Petri on young vines, ranging in age from two to seven years old (Eskalen *et al.,* 2007; Surico *et al.,* 2008). Petri disease may occur as an advanced stage of brown wood streaking in rooted cuttings, or infections may originate in the field (Surico *et al*., 2008), and the causal agents associated with this disease are *Pa. chlamydospora* and/or *Pm. minimum* (Mostert *et al*., 2006; Bertsch *et al*., 2013). Petri disease was initially referred to as 'black goo' or gummosis. In fact, from the vessels affected by brown streaking, a liquid exudate of rubbery consistency and black colour, composed by pectin and polymerized phenols, can emerge. The role of this exudate and its composition, are not yet fully understood, but it is produced by the plant as a response to pathogen infection and therefore it is a means of defence to prevent its spread (Mostert *et al.,* 2006). There are two types of symptoms in plants affected by this syndrome: internal and external. Internal symptoms are black dots around the pith, a darker central pith (Fig. 2.4), and brown/dark streaking on longitudinal sections of infected wood (Surico *et al*., 2008). External symptoms are an interruption of growth, leaf chlorosis, yield losses and a decline in vigour of individual vines (Surico *et al*., 2008).

Figure 2.4 Black dots around the pith and a darker central pith in grapevine stems affected by Petri disease (Gramaje and Armengol, 2011).

2.1.4 Grapevine leaf stripe disease

Grapevine leaf stripe disease (GLSD) is also associated with an infection by *Pa. chlamydospora* and/or *Pm. minimum.* Affected vines present internal symptoms, such as brown streaking, black spots and wood necrosis (Surico *et al.,* 2008; Calzarano *et al.,* 2016), and external symptoms, such as the typical 'tiger stripes' pattern on leaves (Fig. 2.5). Symptomatic leaves present interveinal chlorotic areas, reddening followed by necrosis in red varieties, and only the main veins remain green (Surico *et al.,* 2008). The symptoms on the leaves usually occur in late spring/summer, after several years from the time of colonization by pathogens and discontinuously (Sparapano *et al.,* 2001). Another external symptom that manifests occasionally, and whose triggers are still in part elusive, is the "black measles" on berry surfaces that occurs as a result of epidermal and hypodermic necrosis (Mugnai *et al.,* 1999). The causes involving a sudden disappearance of symptoms from plants that in the previous season were externally symptomatic are not clear at all. Studies have shown that soil (Lecomte *et al.,* 2011), nutrients (Calzarano *et al.,* 2014), fungicides (Di Marco *et al.,* 2011) and environmental factors (Bruez *et al.,* 2013) could play an important role in the development of symptoms. Other authors, on the other hand, highlight the role of fungal metabolites (Andolfi *et al.,* 2011) and wood degradation products (Tabacchi *et al.,* 2000). These products could be transported via sap flow from infected woody tissues to leaves and berries, triggering a host defence response that leads to symptoms appearance (Andolfi *et al.,* 2011; Bertsch *et al.,* 2013). In addition, no variety is considered fully resistant to GLSD and the severity of external symptoms may also vary depending on the sensitivity of the host (Murolo and Romanazzi, 2014). Despite these studies, the complete mechanism of development of foliar symptoms remains to be clarified.

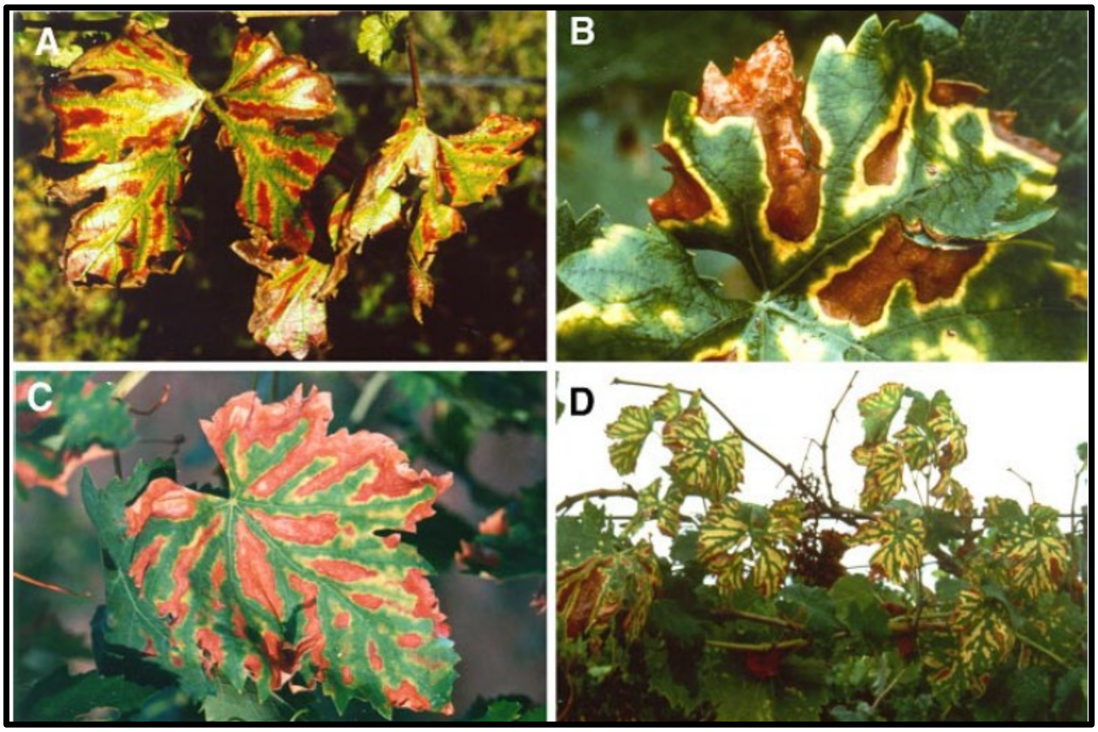


Figure 2.5 Tiger stripes pattern on leaf of grapevine cv. Cabernet Sauvignon affected by GLSD (A). Dead tissues appear dark brown to red-brown, depending on the cultivar (B and C). Symptoms often extend to the interveinal areas, leaving a narrow strip of unaffected tissue among the veins (D; Mugnai *et al.,* 1999).

2.1.5 White rot

The main causal agent of this syndrome is *Fomitiporia mediterranea (*Surico, 2009), a wood rotting Basidiomycete that can also act as a primary pathogen (Sparapano *et al*., 2001). Several Basidiomycetes genera have been linked to wood decay of white rot affected plants, despite in lower frequency, such as members of the genera *Fomitiporella, Phellinus, Inonotus, Inocutis, Stereum, Pletorus* and *Trametes* (Bertsch *et al.,* 2013; Cloete *et al.,* 2015). The typical symptom caused by these fungi is white rot (Fig 2.6), where the wood is transformed into a “spongy, friable, soft and whitish-yellow mass”, delimited by a dark line, separating rotten and healthy wood, when observed in cross sections (Surico *et al.,* 2008). Decay usually begins near a pruning cut and spreads out within the wood upwards and downwards. Another symptom that may manifest when the infection is at an advanced stage is the appearance of a crack in the trunk, which may occur when the internal rot reaches the wood surface (Surico *et al*., 2008).

Figure 2.6 Cross-section showing a central white rot surrounded by black spots and sectorial necrosis (Gramaje *et al.,* 2018).

2.1.6 Esca proper

The term esca proper is used to identify plants in which the presence of *Pa. chlamydospora* and/or *Pm. minimum* is simultaneous to that of *F. mediterranea*, and where all their related symptoms are present. The development of esca proper may follow two different paths, chronic and acute.

The chronic form includes symptoms inside the trunk, on the canes, on the leaves and sometimes on the berries and occurs after blooming, during the summer or in the first part of the autumn (Mugnai *et al.,* 1999). The acute form manifests as a sudden event, called apoplexy

(Fig. 2.7), which occurs in the middle of summer with a sudden withering of all the organs of the vine. The leaves begin changing colour until they reach a complete withering in a few days. Apoplexy is favoured by environmental factors, such as low water availability and heat (Mugnai *et al.,* 1999).

Figure 2.7 A grapevine plant showing apoplexy caused by esca disease (Mugnai *et al.,* 1999).

2.2 Control and mitigation of esca complex associated syndromes

Despite the considerable efforts made by the scientific community to identify effective control strategies against GTDs (Mondello *et al.,* 2018), to date, knowledge and information on disease management measures are still limited (Gramaje *et al.,* 2018). This is due to two main obstacles:

- The first obstacle is the difficulty in determining, with certainty, the pathogens causing the disease (Mondello *et al.,* 2018);
- The second obstacle is the complex management of GTDs at all stages of the plant's life: from the production of new propagation material in the nursery to the cultivation of vines in the field (Mondello *et al.,* 2018).

A complete eradication of all sick vines is certainly impossible and requires economic efforts certainly not sustainable, so in general control is focused on prevention/mitigation and post-infective measures (Úrbez-Torres, 2011). The use of an integrated management strategy, that includes physical, chemical and biological control in association with cultural practices, has demonstrated to be the most effective available strategy for reducing trunk pathogen infection (Halleen and Fourie, 2016).

2.2.1 In nurseries

Several studies revealed that grapevine propagation material is very prone to infection by *Pa. chlamydospora*, and *Pm. minimum* due to wounds made during the propagation process and also because the tools used may be contaminated with spores or mycelia. They spread rapidly, leading to the development of numerous latent infections, especially the appearance of Petri disease typical of young vines. Pathogens are likely to arrive from nursery to field through infected propagation material but with no visible symptoms (rootstock and/or scion) and/or through equipment used during the grafting process and storage of the cuttings such as grafting machinery, hydration tanks and cold rooms (Gramaje and Armengol, 2011). Currently, in Europe there are no satisfactory control measures in nurseries, so the integration of sanitation practices, chemical and biological control or special treatments such as hot water treatment (HWT) or ozonation, are needed to improve the quality of protection from pathogens. In fact, these have been shown to be the best approaches for improving the phytosanitary quality of the new plant material (Gramaje and Armengol, 2011; Gramaje *et al.,* 2018).

Some studies focused on the application of biological control agents, like some species belonging to the genus *Trichoderma* (Pertot *et al.,* 2016), which showed positive effects regarding the increase in callusing, the growth of the roots and, above all, avoided the colonization by wood pathogens in some parts of the plant (Di Marco and Osti, 2007). Other studies have focused on the use of fungicides on the lower end of the graft point (Rego *et al.,* 2009), which may act as a protection against pathogen attack. To avoid contamination of the propagation material in the field, it is advisable to adopt trellis systems instead of creeping and it is recommended to avoid flood irrigation (Gramaje and Armengol, 2011).

One of the techniques that showed the best results against wood pathogens is HWT. Currently, this treatment is used to fight *flavescence dorée* and *bois noir* and its efficacy against wood pathogens, including those associated with tracheomycosis, was also recently demonstrated in a study by Bruez *et al.* (2017a). Usually, it is performed at 53 °C for 30 min or at 50 °C for 45 min, according to the pathogens to fight (Gramaje and Armengol, 2011). For example, in the case of *Pa. chlamydospora*, the pathogen presence decreased 78% when vines were treated at 50 °C for 30 min. Unfortunately, HWT can also have some drawbacks, such as the delayed callus formation and rooting of cuttings (Larignon *et al.,* 2009) especially in cvs Pinot noir, Chardonnay and Merlot (Crocker *et al.,*1999). The HWT is a reasonable method to control the spread of GTD in nurseries, but it must be performed carefully, depending on the cultivar treated, because it can interfere with the vitality of the plant (Bleach *et al.,* 2013). It is important to emphasize that even if the plant has been submitted to HWT in the nursery, it remains susceptible to infection by esca pathogens under field conditions (Bruez *et al.,* 2017a).

2.2.2 Agronomical practices adopted in the vineyard

The research carried out in the last years on the pathogen biology have contributed to provide useful information about the periods of the year in which the plants are more susceptible to esca infections in the field, allowing to develop more effective management and control strategies (Gramaje *et al.,* 2018). Surico *et al*. (2008) suggest the following operations:

- Quickly eliminate dead or severely compromised plants;
- Remove from the field and burn pruning residuals or dead vines to reduce pathogen inoculum sources;
- Cover pruning wounds with chemicals or with mastics which promote wound healing;
- Check the vineyard at the end of the summer season (late August-September) when all the symptomatic plants have appeared and mark them. Perform winter pruning of infected plants separately from healthy plants to avoid any source of inoculum;
- Minimize mechanical operation in the vineyards, such as those involved in pruning, suckering and harvesting.

In recent years, research gave us other recommendations such as:

- The selection of variety and rootstock must be based on the degree of sensitivity to trunk diseases (Gramaje *et al.,* 2010b);
- The choice of a training and pruning system that causes smaller wounds able to cicatrize faster, giving less opportunity for esca-associated fungi to penetrate the vines (Lecomte *et al.,* 2017);
- Vines should be pruned during periods when the presence of the pathogen spores is less prevalent (Gramaje *et al.,* 2018).

Concerning this last recommendation, further research is needed to better understand which is the best period for pruning, considering that spore release periods may vary according to

the specific pathogen and geographical location (Gramaje *et al.,* 2018). Studies conducted in France by Larignon and Dubos (2000) have shown that *Pm. minimum* spores are present in the field only during the vegetative season, especially from the beginning of March to the first days of April and, more frequently, from the middle of May to the middle of June; the spores of *Pa. chlamydospora* have been found throughout the year, including winter. On the contrary, in California, spores of *Pa. clamydospora* are mostly dispersed from October-November to April and only occasionally during other months (Michelon *et al.,* 2007)
Other control strategies will be grouped into preventive or post-infection measures (Mondello *et al.,* 2018).

2.2.2.1 Preventive practices adopted in vineyards

Some preventive approaches are:

1. The Guyot-Poussard and the sap flux-respect pruning. The use of this pruning method is steadily increasing. This method avoids the interruption/disconnection of the sap flow inside the vessels, as pruning wounds are limited to the upper part of vine permanent structure, and allow a bipolar growth of two arms with production of shoots only at the end of each arm (Fig. 2.8; Mondello *et al.,* 2018). There are two systems used: "mixed" Guyot-Poussard with two spurs in a long cane, and "double" Guyot-Poussard with two spurs and two long canes (Mondello *et al.,* 2018). To date, the efficiency of this pruning system in the containment of esca is not yet completely clear, due to the lack of adequate experimental evidence. It still seems to have an influence on the development of symptoms and plant death (Lecomte *et al.,* 2017). Moreover, finding a training system that can have a positive impact on the disease, would be the most sustainable way to fight pathogens.
2. Double pruning. This technique is used both to accelerate pruning times but also to support the control of GTDs (Mondello *et al.,* 2018). The double pruning consists of two parts: a first mechanical winter pruning, when canes are trimmed at 30 to 40 cm above spurs position, followed by a second manual pruning, before bud break, during spring. In this way, colonization of pathogens can be restricted, as it occurs in the wounds of the first winter pruning, and it is subsequently eliminated with pruning before bud break (Elena and Luque, 2016). The newly inflicted pruning wounds from spring are also entry ports for the pathogens but occur at a time when the plant defences are becoming active. This last hypothesis remains to be experimentally demonstrated. This system also has positive effects on yield management and ripeness control (Palliotti *et al.,* 2017), but it is still little used due to high costs.

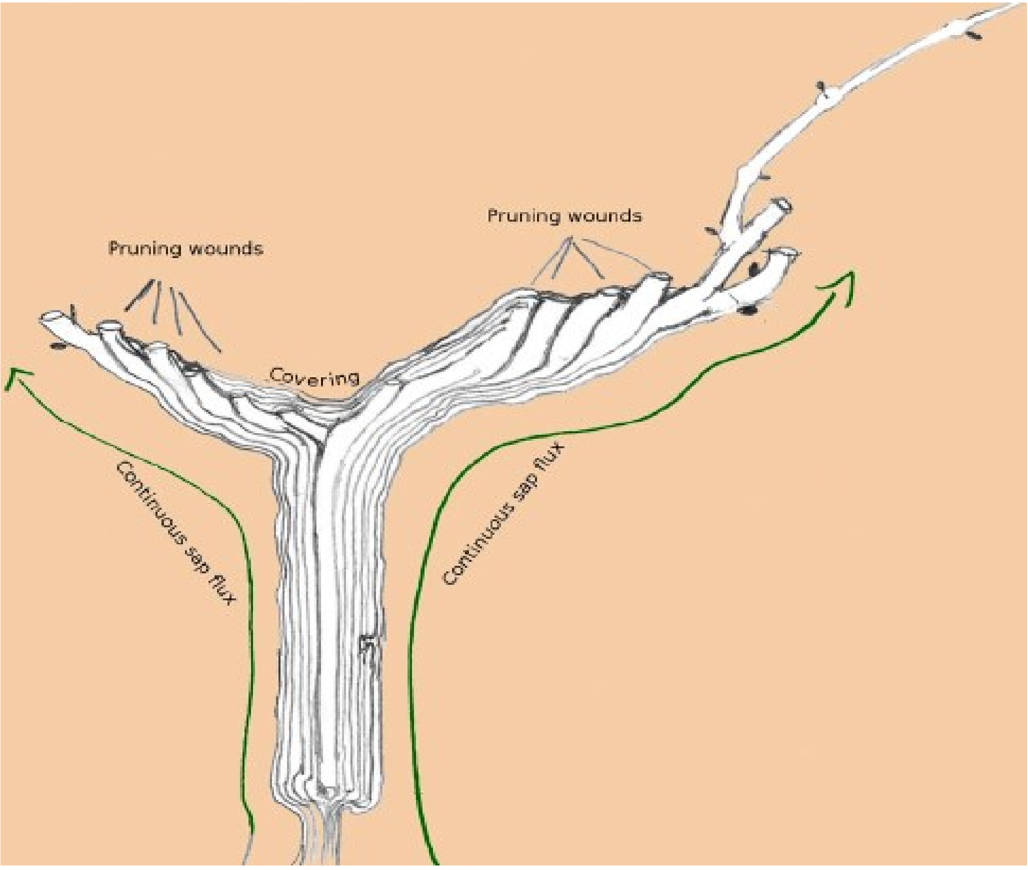


Figure 2.8 The Guyot-Poussard pruning system detection of the upper position of dead wood following pruning and respect of sap flux in the lower part (Mondello *et al.,* 2018).

3. Wound protection. It is widely known that pruning wounds are the most common entry point for esca-associated fungal pathogens (Van Niekerk *et al.,* 2011a), and for this reason pruning wound protection is essential to reduce infections. It was demonstrated that the use of fungicides for the protection of wounds has positive effects on plants such as vines that have creeping behavior (Mondello *et al.,* 2018). However, the protective action has short duration, which usually varies from 4 to 16 weeks depending on the pruning period (Van Niekerk *et al.,* 2011a). Since year 2000, more than 90 active ingredients have been tested against esca-associated fungal pathogens and other GTD fungi, but only few gave positive results under field conditions. Many chemical fungicides have also various drawbacks in terms of toxicity and efficacy, and concerns have been raised about both their environmental impact and the potential health risks associated with their use (Castillo *et al*., 2012). In Europe, different chemicals, physical and biological products are applied to wounds (Boulisset *et al.,* 2017) with the aim of protecting cuts from pathogen attacks over a long period of time (Mondello *et al.,* 2018). Some fungicides can be applied as paintbrush treatments (cyproconazole +iodocarb,

boric acid) while other chemicals can be applied as spray treatments (thiophanate-methyl, pyraclostrobin; Rolshausen *et al.,* 2010).

One of the products tested both *in vitro* and *in vivo* against *Pa. chlamydospora* is chitosan. This product resulted effective in reducing the mycelial growth of this fungus and allowed a reduction in wood colonization when compared to untreated plants, if used to protect pruning wounds (Nascimento *et al.,* 2007). Despite this, the protection of wounds by fungicides has limitations mainly due to: (i) the active ingredients, that may be effective *in vitro* but not effective *in vivo*, (ii) the delivery methods, that are not practical for vine-growers. On top of that, their success depends on several other factors, such as the method and the number of applications in the field, the persistence of the product and the species of fungi fought (Bertsch *et al*., 2013). Moreover, due to the rising issue of resistance to fungicides alternative strategies to control fungal pathogens are needed (Fisher *et al*., 2018).

4. Biological control agents. The problems encountered in the use of chemically active ingredients to control and prevent the spread of esca pathogens led researchers to test biological control agents (BCA). BCAs are believed to be useful for antagonizing wood pathogens, as they are able to colonize woody tissues (e.g. on pruning wounds), competing for the same niche, increasing the chance of protection from new infections (Mondello *et al*., 2018). Some agents have a positive impact on the plant physiology, as they contribute to the development of roots (Pertot *et al.,* 2016), and therefore to plant growth (Fourie and Halleen, 2006) and resistance against biotic or abiotic stresses (Berg, 2009). However, the effectiveness of treatments can be compromised due to the time it takes to colonize the host plant, so during this period pruning wounds remain susceptible to pathogen infection (John *et al.,* 2008).

Nerva *et al*. (2019) suggested a different approach in the field of BCA, consisting in the use by mycoviruses of grapevine trunk pathogens to control their population in the host plant. Thanks to their research, 39 new viral genomes have been discovered, and they could possibly be exploited, in the future, as biological control agents.

Recently, a new polypeptide oligomer named Blad-containing oligomer (BCO), a multifunctional product extracted from the cotyledons of *Lupinus albus* L. between days 4 and 12 after the onset of germination, was tested with positive results against some fungal diseases such as gray mold and powdery mildew (Monteiro *et al*., 2015). BCO was also tested in *vitro* against *Pa. chlamydospora* and *Pm .minimum* showing ability to inhibit fungal growth (Del Frari *et al*., 2018).

2.2.2.2 Post-infection practices adopted in vineyards

In the post-infection phase, particular importance is dedicated to the use of antifungal compounds or microorganisms, such as:

1. *Trichoderma*-inoculated wood dowels inserted into trunks. This treatment involves the inoculation of *Trichoderma* on wooden dowels. During spring, sick vines are drilled at the base of the trunk and on the arms, and inside these holes the dowels are inserted (Mondello *et al.,* 2018). This method had positive effects in Spain, in what concerns to the antagonistic role played by *Trichoderma* against pathogens. In fact, this fungus is able to colonize woody tissue, and compete for the same niche with esca-associated pathogens. In this way, the chances of protection are increased, both because *Trichoderma* exerts an antagonistic action and because its presence could stimulate a defensive response from the plant (Mondello *et al*., 2018).
2. Endotherapy: This technique consists in the injection of substances with antifungal potential inside the trunk of esca-affected vines. Del Frari (2018) tested several chemical compounds (elemental silver, fosetyl-Al, glutaraldehyde, hydrogen peroxide and Blad-containing oligomer) both *in vitro* and *in planta*, against *Pa. chlamydospora* and *Pm. minimum.* All treatments were effective *in vitro*, but *in planta* only hydrogen peroxide gave positive results against *Pa. chlamydospora* and glutaraldehyde against both fungi. This technique, was based exclusively on the observation of the appearance of external symptoms to assess the effectiveness of treatment. The pathogen may be able to colonize the tissues again as soon as the concentration of active ingredients has decreased. In addition, in adult plants, where dead xylem is large and wood is dense, it may be difficult for the active ingredients to become in contact with the fungal mycelia (Del Frari, 2018).
3. Remedial surgery. It is an innovative, but invasive, technique that aims to heal infected plant by eliminating symptomatic woody tissues. The most common remedial surgery methods used in Europe are cordon or spurs removal, trunk renewal, trunk surgery and re-grafting (Mondello et al., 2018). This technique has the advantage of allowing the plant a rapid development of the parts that were previously removed, avoiding an unbalanced growth in comparison with the healthy parts and thus ensuring a homogeneous quantitative and qualitative level of production. It is certainly a very expensive system, highly time consuming. This is the reason why it is only used when its application allows advantages that justify the economic burden (Úrbez-Torres, 2011).

CHAPTER III

THE TRIANGLE OF DISEASE

3.1 Grapevine, esca disease and environmental factors

To date, due to the effects of climate change, among others, increasing attention is being paid to the impact that abiotic factors have on the development of plant diseases and on the mechanisms that can alter the relationship between pathogens and plants (Eastburn *et al.,* 2011). In fact, the vine, as well as all other fruit crops, under field conditions, is exposed to a large number of biotic and abiotic stresses that may occur simultaneously (Pandey *et al.,* 2015). According to Agrios (2005) the expression of a disease is due to three main factors, defined as "the triangle of disease". They are: (i) the susceptibility of the plant to the pathogen, (ii) the presence of a potentially virulent pathogen and (iii) an environment suitable for the development of the disease. Therefore, only considering these three factors it is possible to have a broad and complete view of a pathosystem. Abiotic stresses can aggravate the effects of host-plant interactions, depending on their intensity and plant predisposition. Abiotic stress is defined as a change that occurs suddenly in the environment and that needs to be repaired since it causes imbalances in the plant organism. Yarwood (1959) defined the predisposition as "the tendency of non-genetic factors, acting prior to infection, to affect plant susceptibility to the disease", implicitly suggesting that stress can act on the plant in order to predispose it to susceptibility or tolerance/resistance (Bostock *et al.,* 2014).

Environmental conditions associated with climate change, such as rising temperatures or decreasing rainy events, may influence the incidence of a disease and exacerbate the severity of symptoms, although plants, in general, are able to adapt to environmental challenges and changing conditions by regulating physiological and biochemical mechanisms. In addition, changing abiotic conditions can also have effects on pathogens, as their survival is linked to environmental factors such as temperature or humidity (Eastburn *et al.,* 2011). The exposure of the plant to simultaneous biotic and abiotic stress results in the implementation of different adaptation strategies, which are sometimes different and in conflict, when compared to those of an individual stress. The plant activates response-defence mechanisms, through the expression of various genes and the production of enzymes and hormones with different biological functions, according to the type of environmental *stimulus*. In general, biotic and abiotic stress signalling networks of plants consist of several pathways that can interact with each other. In fact, the simultaneous presence of the two types of stress leads to some common physiological and molecular processes (Pandey *et al.,* 2015).

In relation to the increasing incidence of GTDs over the last thirty years, the scientific community has tried to find out how environmental factors affect the development of esca fungi, the syndromes belonging to the complex and the relative appearance of symptoms. In light of this understanding, abiotic stresses can influence the tolerance or susceptibility of the plant to pathogens. This could have consequences on plant-pathogen interactions, making more complicated to separate and clearly recognise diseases caused by wood fungi and the appearance of symptoms due to the presence of them.

3.2 Abiotic factors

In general, the abiotic stresses to which the plant is subjected are water stress, high or low temperatures, light quantity, nutrient deficiency or excess, soil salinity and the effects of fungicides. Depending on their intensity, these stresses can damage plants reversibly or irreversibly, especially when they occur in combination with pathogen attacks. Stresses can be distinguished into plastic or elastic. Plastic ones lead to irreversible changes in plant physiology and sometimes even fatal events. Elastic ones lead instead to reversible events as soon as the stress is compensated. All these stresses can cause damage to the cells of the plant, since it has to respond to combined stresses quickly. Among these damages we have membrane disruption, disturbances to ion channels, perturbation of osmo-sensors and the production of reactive oxygen species (ROS; Bostock *et al.,* 2014; Fig. 3.1).

An experiment by Dubos (2002), in France, showed that the apoplectic form of esca occurred after heavy rain during the summer. The progress of the appearance of symptoms also seems to be linked to the increase in temperature in the period from June to September, probably because high temperatures favor the activity of fungi in the woody tissues (Lecomte *et al.,* 2011). Soil characteristic (texture, availability of water and nutrients) also seems, in some cases, to play in favor of esca fungi. In fact, in a study by Guerin-Dubrana *et al.,* (2005), soils with a high percentage of clay and with a high availability of water and nitrogen, favored the development of fungi in the wood.

The main abiotic factors that will be further discussed are drought, temperature, rain, nutritional status alteration and the effects of fungicides.

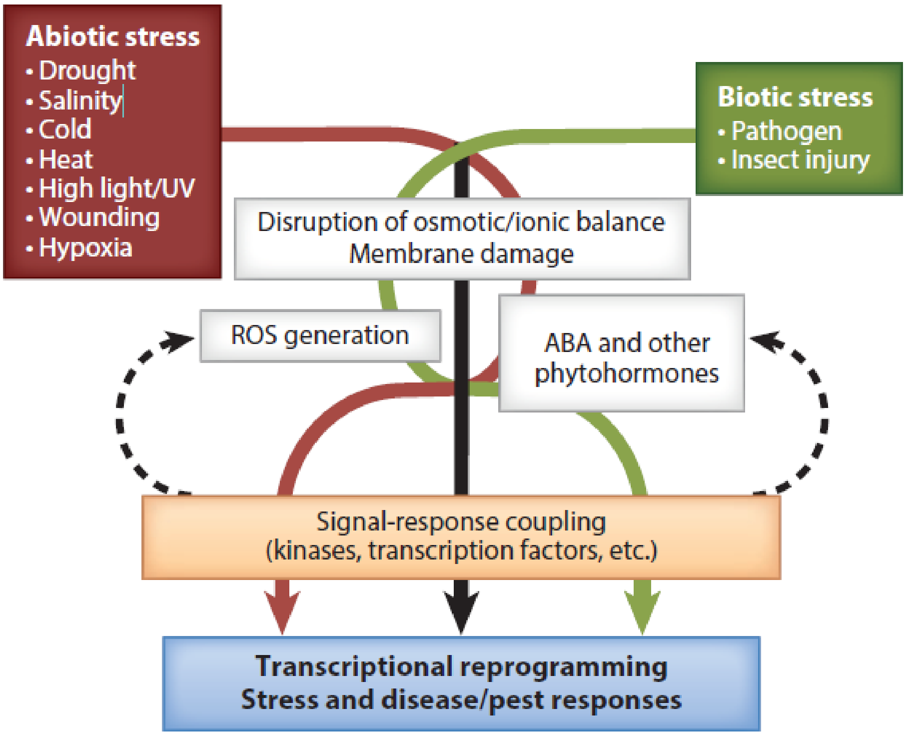


Figure 3.1 General sequence of responses to biotic and abiotic stress, with shared and transversal responses. Factors involved in the response include: kinases, phosphates, transcriptional activators and repressor, gene expression and physiological responses (Bostock *et al.,* 2014).

3.2.1 Drought

One of the most studied combined stress interactions, to which the plant is subjected, is certainly water stress-pathogen attack. Boyer (1995) proposed two mechanisms to explain how water stress increases the susceptibility of plants to pathogens: (i) reduced photosynthate production, induced by drought, reduces the plant capacity to produce defensive compounds; (ii) plant growth is reduced without reducing the pathogen capacity to reproduce. Unlike other stresses occurring simultaneously, the manifestation of combined stresses due to drought and pathogen attack, develops as water stress becomes more severe. Under field conditions, this stress can occur in two different ways: (i) the attack of the pathogen, which behaves as an opportunistic agent, taking advantage of plant weakness, can occur at the same time as drought stress; (ii) or water stress may occur after the pathogen has been established within the host. The vine may be tolerant to these stresses, as it can adapt to the two simultaneous stresses, but it may also be susceptible in case it is not able to activate tolerance mechanisms to the specific situation. Other effects due to these stresses are: (i) the increase of photorespiration processes, resulting in carbon loss and a higher probability of fatal events; (ii) down-regulation of photosynthesis, nutrient assimilation and cellular homeostasis. In particular, a lower photosynthetic capacity reduces the plant ability to activate secondary metabolism and therefore reduces the ability to produce defensive compounds. In addition, a limited photosynthesis affects the growth of the plant organs, thus facilitating, the progress of the pathogen inside the vessels and eventually the appearance of symptoms (Fischer and Peighami Ashnaei, 2019). A period of water stress could therefore exacerbate the disease due to the presence of esca fungi (Ferreira *et al.,* 1999). It has been shown that the plant is more susceptible to pathogen attacks and more likely to show symptoms in the wood of young grapevines, when it is in a state of water stress and, on the contrary, it is more resistant when water levels in the soil are high or when grapevines tolerate water stress (Fischer and Peighami Ashnaei, 2019). In general in all plants, a period of water stress occurring during a state of infection can have much more drastic effects than a period of drought that occurs in the absence of pathogens. In fact, in the case of combined stress, the presence of the pathogen can increase the probability that a water stress event will turn into a deadly event, with damage that irreversibly affects xylem and phloem vessels and leading to the withering of leaves (Oliva *et al.,* 2014).

Experiments conducted under greenhouse conditions, by Edwards *et al.,* (2007b), showed that the presence of *Pa. chlamydospora* interfered with the regulation of water reserves, especially under severe water stress conditions. Through measurements of physiological responses, they documented that the water potential levels in the leaves of infected vines were lower than those of healthy vines (Fig. 3.2), and the stomatal conductance in the infected leaves was higher than in non-symptomatic leaves (Fig. 3.3). This highlights that the presence of pathogens negatively affects the movement of water from the roots to the leaves. Assuming as a general rule that a lower water potential in the leaf corresponds to a more accentuated water-stress, it is believed that the pathogen induces a higher transpiration of water than the amount actually possessed by the plant (Edwards *et al.,* 2007b). The increase of transpiration, faced to the lack of water, also favors phenomena such as xylem embolism (Pouzoulet *et al.,* 2014).

Xylem is also affected, like any organ of the plant, by biotic and abiotic stresses. In fact, it can reduce or even lose its function of water carrier if it is subjected to cavitation-embolism phenomena due to drought, or due to gels and tyloses produced by the plant in the case of pathogen attacks. A period of moderate water stress could lead to a reduction in the diameter of xylem vessels, compromising water translocation (Lovisolo and Schubert, 1998). In addition, discontinuous appearances of foliar symptoms may be related to changes in the diameter of xylem or the formation of new vessels under different water regimes (Pouzoulet *et al.,* 2014). What can contribute to drought adaptation is certainly the choice of an adequate rootstock. In fact, a rootstock tolerant to moderate water deficits allows adequate absorption of water from the soil while keeping stomatic conductivity low (Alsina *et al.,* 2007, 2011).

Therefore, efficient water use can help to control, to some extent, the negative effects of esca complex.

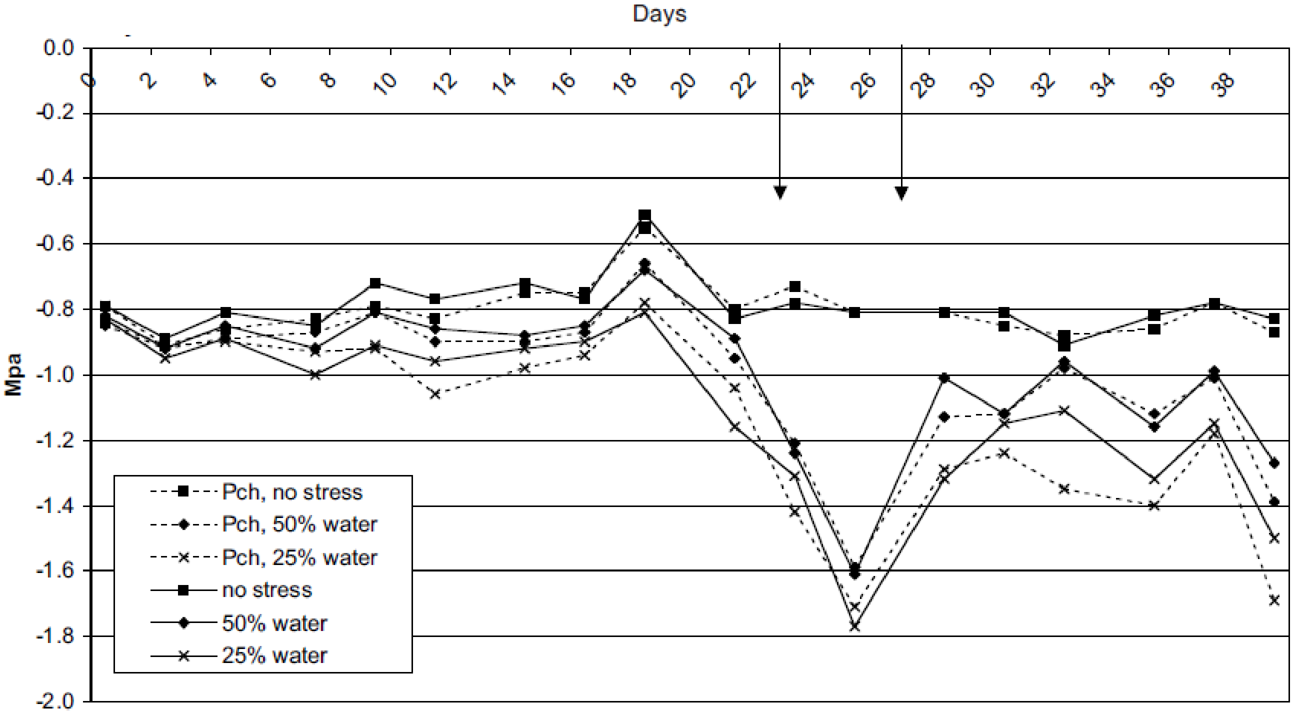


Figure 3.2 Effect of water stress on leaf water potential of infected plants with *Phaeomoniella chlamydospora* and uninfected plants of cv Cabernet Sauvignon. The first vertical arrow represents the start day of the water stress and the second vertical arrow represent the end of the water stress period (Edwards *et al.,* 2007b).

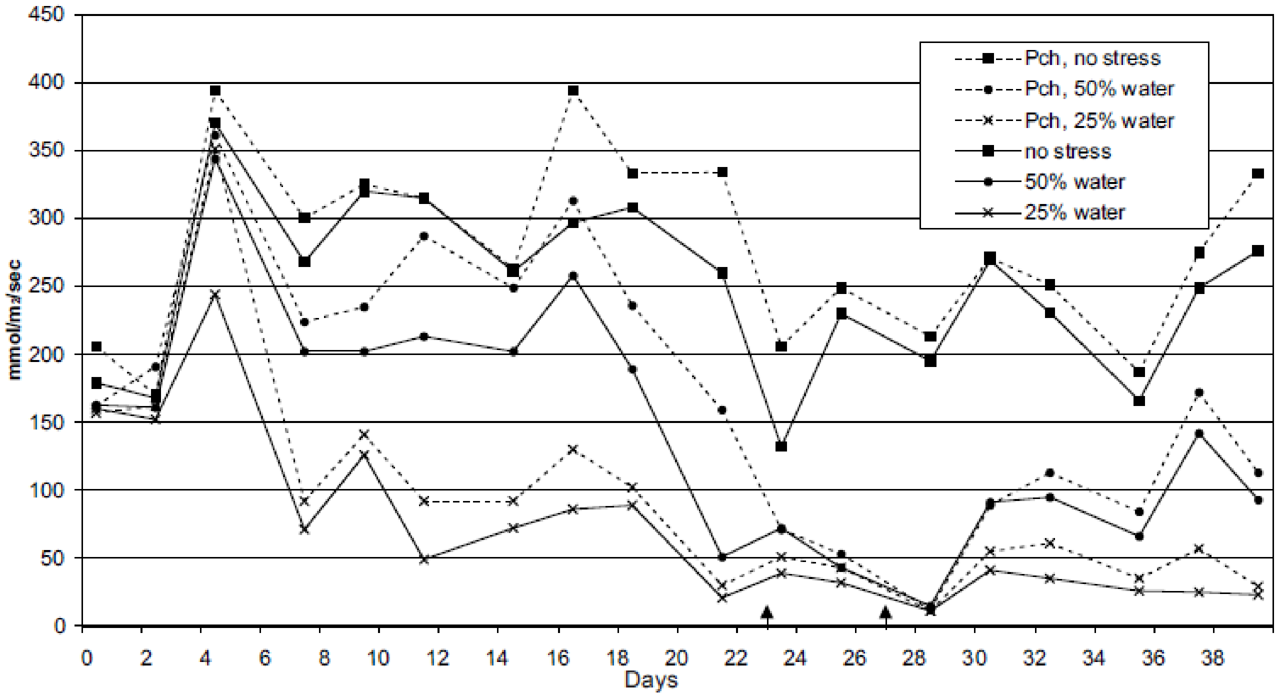


Figure 3.3 Effect of water stress on stomatal conductance of infected plants with *Phaeomoniella chlamydospora* and uninfected plants of cv Cabernet Sauvignon (Edwards *et al.,* 2007b).

3.2.2 Rainfall and temperature

It is now clear that the irregular nature of esca external symptoms is, at least in part, linked to environmental factors. Among these environmental factors, rain and temperature play a key role, especially during summer, when the plants have the greatest tendency to show foliar symptoms.

In 2000, Surico and collaborators demonstrated that there is a correlation between cool and rainy summers and the expression of foliar symptoms of chronic esca, while hot summers with water deficiency in the soil favoured the expression of the acute form. These results were also confirmed by Marchi *et al.* (2006) and Calzarano *et al.* (2018). These authors, in addition to reiterating that the cool and rainy summers allowed to reduce the amount of "hidden esca" (infected plants which remain externally asymptomatic throughout a growing season), showed how the appearance of foliar symptoms was evident in those parts of the vineyard where there was an accumulation of water due to the steep slope of the hill. Therefore, an increased flow of water from roots to the leaves, which corresponds to increased transpiration, probably allows better translocation of toxins produced by fungi. Experiments on potted vines also confirmed some of these hypotheses. In fact, regularly irrigated plants showed external symptoms earlier than plants under water stress (Surico *et al.,* 2010). However, the role of water in the expression of external symptoms is not yet completely clear.

As for thermal stress, considering its duration and intensity, it is likely that it affects the plant resistance to the pathogen. In fact, the non-acclimatization of the plant could lead to the suppression of defensive responses, increasing susceptibility to the pathogen (Pandey *et al.,* 2015). However to date, there is not enough consistent evidence to be able to define in a precise way the effect of temperature on foliar symptoms.

To date some studies have certainly confirmed the role of abiotic stresses in the expression of esca symptoms, demonstrating the complex interaction between symptoms and environmental factors as previously highlighted by Mugnai and co-workers in 1999.

3.2.3 Nutritional status of the plant

The nutritional status of vines is another factor that may influence the expression and severity of esca disease. In an experiment conducted for three years by Calzarano *et al.* (2009), the nutritional status of vines was assessed in relation to the expression of foliar symptoms. In the first two years, when the vineyard was fertilised with nitrogen, phosphorus and potassium (N-P_2O_5-K_2O) and the precipitation was regular, the appearance of symptoms was higher than in the third year when the precipitation was lower and the vineyard was not fertilised. The leaves of the symptomatic vines had lower levels of nitrogen, magnesium and potassium than the leaves of healthy vines. These elements are involved in photosynthetic processes, and since photosystem II showed imbalances even before symptoms manifested, it is possible that the plant moved these elements away from leaves located on the affected shoots. Indeed, the berries of vines with foliar symptoms benefited from this translocation, since they possessed high amounts of nitrogen, magnesium and potassium in respect to berries of healthy vines, but they were less ripe at the time of harvesting. However, higher levels of nitrogen, within the berries, could be attributed to the increase in aminoacidic substances such as proline, produced in greater quantities when the plant is under stress. In addition, there was an increase in the amount of iron in symptomatic leaves in comparison with asymptomatic. This increase is probably due to the increased activity of enzyme complexes, in response to several types of stress, of which iron is a cofactor (Calzarano *et al.,* 2009). Overall, a greater amount of nutrients available to the plant in combination with a greater amount of water in the soil, can contribute to the expression of the external symptoms, so the nutritional status plays an important role in the development of the disease. Pathogens may be able to feed on nutrients destined for the vine, especially carbohydrates. In fact, a high amount of carbohydrates in woody organs, where the pathogen is established, could affect the growth of fungi (Calzarano *et al.,* 2009). However, if nutrients and water are scarce, photosynthesis slows down and carbohydrates are moved to the berries to allow adequate maturation, thus limiting the reserves in the trunk and shoots, namely the preferred niches of esca-associated pathogens. Indeed, during the third year of the Calzarano *et al.* experiment (2009), no fertilizer was applied, helping to maintain the percentage of diseased vines low, without compromising yield. In addition, due to the poor nutritional regime, there was also a faster lignification of organs, which may have helped to limit the spread of fungi inside the vessels (Calzarano *et al.,* 2009)

However, it seems that when nutrient availability is high, the plant defense response may weaken, allowing esca fungi to express greater virulence and spread faster, with a higher probability of expression of external symptoms due to an easier release of phytotoxins (Evidente *et al.,* 2000).

However, studies on the influence of the nutritional status of the plant are still scarce, therefore it is necessary to further deepen that can clarify the role of nutrients in plant-pathogen interaction.

3.2.4 Fungicides

Since the casual discovery of the Bordeaux mixture ($CuSO_4$+Ca $(OH)_2$), the first chemical with antifungal action capable to prevent downy mildew infections (Morton and Staub, 2008), fungicides have been the most widely used products to control pathogenic fungi in crop plants. They play a significant role in disease management especially when the plant defense response to pathogen attacks, is not sufficient to limit their spread (Thind, 2017). However, the massive use of fungicide (about 80% of the total treatments carried out in the vineyard) for crop protection has negative consequences, because in the long term leads to the accumulation of residues in the environment and in the food chain (Petit *et al*., 2008). This may also cause the development of resistance in fungi (Garcìa *et al*., 2003), phytotoxic effects in grapevines (Dias, 2012) and toxic effect in humans (Komárek *et al*., 2010). Finally, fungicides have negative effects on non-target organisms (La Torre *et al*., 2018), including beneficial insects (Thomson *et al*., 2000).

In consideration of pruning wound protection, some contact fungicides inhibit fungal spores germination and, therefore, fungal mycelium development in/on plant tissues. They can be applied before an infection takes place, in order to protect the plant from fungal invasion, or used to fight an established infection, with curative action (Dias, 2012).Therefore, contact fungicides can be used only as preventive action because, as once an infection is established, their fungicidal activity is lost. This class of fungicides is relatively inexpensive and the likelihood of fungal resistances development is low (Dias, 2012). In the class of contact fungicides there are:

- Inorganic compounds. They are available as salts, inorganic acids (boric acid), elemental (sulfur) and copper-based products. In this category, the most used is copper (Cu), a heavy metal and a fundamental microelement for plants. However, excessive doses and long term application may induce phytotoxicity (Dias, 2012), due to the accumulation in the soil, leading to reduction in organic matter, fertility (Juang *et al*., 2012), and groundwater pollution (Nóvoa-Muñoz *et al*., 2007).
- Organic compounds. The massive use of chemicals in agriculture, led researchers to study new natural active ingredients, in form of simple molecules, oligomers or complex mixture. Most of these are defined as "environmental friendly" as they do not seem to have toxic effects towards plants, human and beneficial insects, and also have an easily biodegradable structure (Mondello *et al*., 2018). On the other hand, they have a strong sensitivity once in contact with UV radiation, which is why their use in agriculture is still limited.

In the vineyard, several pesticide treatments are carried out because of the susceptibility of grapevine to a range of diseases, especially those caused by fungal pathogens. Unfortunately, fungicides can also have consequences on grapevine physiology (Petit *et al.,* 2008). In some cases, fungicide phytotoxicity may be related to the formation of products that result from the degradation of the fungicide itself, once penetration into host cells and integration into the plant cellular metabolism have taken place (Garcia *et al.,* 2003). Some examples of the negative consequences of the use of fungicides are:

- Decrease of pollen germination (Pavlik and Jandurova, 2000);
- Reduction of growth and soluble carbohydrate content (Romeu-Moreno and Mas, 1999);
- Negative effects on the photosynthetic apparatus (Petit *et al.,* 2008);
- Alteration of nitrogen and/or carbon metabolism (Saladin *et al.,* 2003) fundamental elements for the plant;
- Chlorophyll fluorescence alteration (Xia *et al.,* 2006);
- Changes in the stomatal conductance and intercellular CO_2 concentration (Xia *et al.,* 2006).

One of the most commonly used contact fungicides, even in organic viticulture, is copper, in forms of copper sulphate or oxychloride. The use of this product in Mediterranean viticulture is frequent. In fact, copper levels in the soil of many wine regions are really high, ranging from 100 to 1500 mg k^{-1} (Llorens *et al.,* 2000). Copper causes damage to plant organelles, as it inhibits electron transport, leads to degradation of the chloroplast inner structure and pigment content (Ouzounidou 1993). The type of damage caused by the fungicide could also be related to the time of treatment. For example, the use of copper during flowering could lead to the loss of fertility of inflorescences, or even, a treatment with adverse climatic conditions could cause the burning of the leaves (Yruela, 2005) and stunts on the root growth (Juang *et al*., 2012). Finally, the affected organs may also vary, in fact from an investigation by Bruez *et al*. (2017b) copper could trigger phytotoxic effects inside the wood, leading to changes in the plant physiology, morphology and biochemistry. Fungicides can affect directly the secondary metabolism of the plant. In fact, they may act by eliciting the biochemical defense or reduce their synthesis.

To date, no studies have been done on how fungicides can affect the metabolism of wood phenolic compounds and on the interaction host-pathogen, when applied as pruning wound protectors. Therefore, it would be interesting to deepen the research on the effects of fungicides and on the related stress that they involve during the interactions between plant and pathogen.

3.3 Biotic factors

The effect of pathogens infection has an important impact on the physiology of the plant, especially concerning the carbohydrates metabolism and the responses that the vines exhibit as defence to pathogens attack (Fontaine *et al.,* 2015). For example, fungal colonization in woody tissues leads to the impoverishment of starch reserves, while in the leaves the alteration of carbon metabolism is due to the reduction of the photosynthetic capacity (Fontaine *et al.,* 2015). This leads to a reduced growth of the root system and buds and therefore to a decrease in development and loss in vigor of the plant (Bertsch *et al.,* 2013; Fontaine *et al.,* 2015). Sometimes, a week before the appearance of foliar symptoms, the flow of lymph inside the vessels can decrease (Bertsch *et al.,* 2013). In fact, during the warmer periods and in the presence of large quantities of grapes during ripening, the flow of the lymph can be two times lower than normal, the flow of water inside the xylem vessels decreases, as well as the transpiration of the leaves and the stomatal conductance (Ouadi *et al.,* 2017). The foliar symptoms are related to the stomatal closure and to an alteration of the photosynthetic activity, in fact there is a decrease in the assimilation of CO_2, a decrease in transpiration, a higher concentration of intercellular CO_2, a decrease in the efficiency of fluorescence and photosystem II and a decrease in the total amount of chlorophyll (Magnin-Robert *et al.,* 2011). In the leaves of symptomatic plants, there is a high presence of *trans*-resveratrol, which demonstrates the production of phytoalexins in response to toxins produced by fungi or the translocation of wood degradation compounds (Calzarano *et al*., 2016). Finally, grapevine leaf stripe disease also affects the normal ripeness of berries. This leads to a decrease in sugars content in the berries, a proportional increase of total acidity and an increment of assimilable nitrogen in must, compromising the future organoleptic and sensory qualities of wines (Lorrain *et al.,* 2012).

3.4 Genotype

Several studies have highlighted the existence of a possible correlation between the genotype of *V. vinifera* and tolerance to different biotic and abiotic stimuli. Morulo and Romanazzi (2014) argue that the complexity of a disease such as esca is not only due to the multitude of pathogens that cause infection and the interactions between them, but also to the possible influence exerted by rootstock and cultivars.

The use of grafting is now the most used agronomic practice for the propagation of grapevines. The species used as rootstocks are of American origin and have been chosen mainly for their high resistance to phylloxera (*Viteus vitifoliae*), unlike the Euro-Asiatic grapevine. The most widely used species are *Vitis berlandieri, Vitis riparia* and *Vitis rupestris*. This practice therefore makes it possible to combine the genetic heritage of several species of vine. Rootstocks play a key role in managing plant responses to water stress. During a period of drought, stress adaptation is mediated by chemical (Alsina *et al.,* 2011), hormonal (in particular through the production of abscissic acid) and hydraulic signals (embolism and/or cavitation; Soar *et al.,* 2006). Rootstock predisposition to acclimatization, at times when water availability is limited, may affect the expression of the disease. As for esca, a greater sensitivity to pathogens is attributed to the cultivars grafted on *V.rupestris* than to those grafted on *V.riparia* (Marchi 2001). In 2001, Marchi and co-workers discovered that the vines grafted on 1103P (*V. berlandieri x V. rupestris*), known to be drought-resistant, showed a higher and unexpected incidence of apoplectic strokes, when compared to vines grafted on K5BB (*V. berlandieri x V. riparia*), less resistant to water stress. Andreini *et al*. (2014), who reported that 60% of apoplectic events had occurred on 1103P vines and 30% on K5BB vines, confirmed these results. Moreover, in a research by Morulo and Romanazzi (2014), the vines grafted onto SO4 (*V. berlandieri x V. riparia*) rootstock, which adapts with difficulty to water stress, showed a greater sensitivity to esca when compared to those grafted on 1103P.

Through the comparative transcriptome analysis of the cultivar Pinot Noir, Berdeja et al. (2014) have demonstrated the presence of differences depending on the type of rootstock, in the response of genes involved in the metabolism of jasmonic acid (JA). Jasmonic acid is a phytohormone used by the plant as a elicitor, an agent capable of inducing the production of secondary metabolites involved in the defense of the plant. For example, JA is synthesized after pruning cuts as a response to pathogen attacks (Berdeja *et al.,* 2014) Therefore, it could contribute in the regulation of defence mechanisms of plants against esca pathogens

Grafted vines also show a higher incidence of Petri disease than those that grow on their own roots. The lower incidence is likely due to the decrease in the risk of contamination by fungal spores during nursery grafting processes (Andreini *et al.,* 2013).

Esca incidence may also be related to the different susceptibility of cultivars. In general, no cultivars belonging to the botanical subgenus *Euvitis*, possess complete immunity against vascular fungal diseases (Andreini *et al.,* 2013); rather there are degrees of susceptibility ranging from highly susceptible to tolerant (Murolo and Romanazzi, 2014). One of the cultivars defined as highly susceptible is cv Cabernet Sauvignon, since the percentage of vines showing external symptoms, in different climatic areas, is among the highest (Andreini *et al.,* 2014). In a study conducted by Borgo *et al.* (2008), it was found that cv Sauvignon blanc has a greater predisposition towards infections caused by esca fungi than other white grape varieties such as cvs Pinot bianco or Chardonnay. A higher tolerance of some cultivars could be linked to a higher availability of secondary metabolites that allow the plant to implement more functional defense mechanisms (Morulo and Romanazzi, 2014). Another factor that could affect the greater or lesser susceptibility of the plant in relation to the variety is the size of xylem vessels. Occlusion of xylem vessels limits the movement of fungi inside the plant. In a study by Pouzoulet *et al.* (2017), it emerged that the movement of *Pa. chlamydospora* inside the plant is favored by the presence of larger diameter of xylem vessels, as in the case of the cv Thompson seedless, when compared to cultivars such as Merlot that have vessels of lower diameter.

The selection of an adequate rootstock, and the right combination with *V. vinifera* variety, could increase the tolerance of the plant to periods of stress and consequently improve its tolerance to symptoms development. Certainly, the susceptibility of grapevine to esca depends on environmental and genotypic factors (Marchi, 2001), but further studies are still needed to explore this filed of research.

3.5 Age

The age of the plant is a critical factor in the development of the Esca complex, in fact, it can play a decisive role in regulating responses to biotic and abiotic stresses and in the manifestation of tolerance or sensitivity to stressors (Pandey et al., 2015). The incidence of GLSD, white rot and esca proper in adult plants is higher than in young ones. In fact, in adult plants over ten years old, the appearance of external symptoms is more common than in young plants (Mugnai *et al,* 1999).

This could be related to:

- Levels of tannins in the plant
- Number of pruning events to which the plant has been subjected
- Slow development of the disease
- Cumulative multi-pathogen infections

Tannin levels increase from 12-13 years to 25-30, which is when the appearance of internal and external symptoms is maximum. To date however, there are not more recent data on correlation between tannins and age. In terms of time, young plants have suffered fewer pruning cuts than adult plants and have therefore been subjected to fewer cycles of infection. In addition, it is still unclear how long it will take for leaf symptoms and wood discoloration to appear after infection (Mugnai *et al.,* 1999).

CHAPTER IV

PHENOLIC DEFENSES IN *Vitis Vinifera*

4.1 Plant defence responses

Plants are subject to attacks by several pathogens, including fungi. To defend themselves from pathogens, plants have developed many defence mechanisms, including preformed structures (e.g flavonols and hydroxycinnamic acids) and secondary metabolites, to hinder and try to stop pathogen attacks. Plant defence lines start when it recognizes the infection, it proceeds locally and sometimes become systemic along the entire plant (Kumar et al., 2020). Responses are activated very quickly and they may manifest in the form of (i) physical barriers, with cell wall reinforcement and/or (ii) in the form of chemical compounds, through the synthesis of antifungal or antimicrobial molecules. For example, through the production of tyloses inside the vessels, the plant physically hinders the passage of the pathogens (Michelon *et al.,* 2007), or the plant can produce chemical compounds, such as reactive oxygen species, phenolic compounds, or pathogenesis-related proteins (Kulbat, 2016), which have a toxic effect on the pathogens.

As part of plant-pathogen interaction, it is important to examine the defence of grapevine during a fungal attack. One of the main responses of the plant is the localized accumulation of antifungal compounds which inhibit the growth of fungi. For this reason, we believe it is necessary to study the molecular interactions between grapevine and fungi associated with esca complex syndromes, in order to understand the biology of pathogenesis and to determine potential targets to control the disease. In fact, this would be a fundamental step in controlling the spread of infection within the plant, especially considering that, to date, there are no effective mitigation strategies. Therefore, the precise understanding of how the plant responds to the pathogen could be useful for a potential exploitation of its innate defenses in case of infection.

4.2 Phenolic compounds

Most of the chemical compounds that the plant uses to defend itself against biotic stress are the result of secondary metabolism. Secondary metabolites were, long time ago, defined as "errors" of primary metabolism and therefore considered of little importance for plant growth (Bennett and Wallsgrove, 1994). Now, we are aware that these compounds play numerous fundamental roles, not only in the growth of the plant, but also in the proliferation, pigmentation, attraction of pollinators, UV-screening, allelopathy, defence signalling, and protection against attacks of pathogens and herbivores (Kumar *et al.,* 2020). Indeed, to date, the antifungal and antimicrobial activity of phenolic compounds, the most widespread class of secondary metabolites in plants, in plant defence, is widely known.

Phenolic compounds, also referred to as non-enzymatic antioxidants, are structures formed by an aromatic ring (C6) bound directly by one or more hydroxyl (-OH) groups and other substitutes such as methoxyl or carboxyl groups, which determines their polar character and dissolution capacity in water (Kulbat, 2016). They mainly accumulate in the central vacuoles of guard and epidermal cells or sub-epidermal cells in the leaves (Kumar *et al.,* 2020), but they also accumulate in the woody tissues of the plants. Depending on the complexity of the carbon chain and the number and type of substituents, phenolic compounds are divided into simple and complex phenols (i.e. those with multiple aromatic rings). All these compounds are synthesized in the shikimic acid pathway from the amino acid phenylalanine. The reaction is catalyzed by the enzyme Phenylalanine ammonium-lyase (PAL) and the starting molecule for the synthesis of phenolic compounds is cinnamic acid (Kulbat, 2016). It is possible to divide phenols into two functional groups: (i) preformed phenols, those that are normally produced by the plant during its growth; (ii) induced phenols, synthesized *“ex novo”* during pathogenic attacks or other types of stress (Goufo *et al.,* 2020). Phenolic compounds, such as lignin and related polyphenols, phenylpropanoids (phenolics and phytoalexins) are among the most important secondary metabolites in plants, representing constitutive and induced secondary metabolites. In grapevine, phenols are constitutive compounds of the lignified organs (roots, canes, seeds, stems) and of the green organs such as leaves and berries. Considering the base chemical structure phenols are divided in: non-flavonoid structure that include (i) simple phenolics such as phenolic acids (hydroxybenzoic and hydroxycinnamic acids), (ii) coumarins and stilbenic compounds; stilbenes are referred to as phytoalexins, and they are defined as an induced defence mechanism. The second group of phenols include flavonoids such as flavonols, anthocyanins, proanthocyanidins, flavones, flavanonols) usually present as preformed compounds in the tissues (Goufo *et al.,* 2020). The main phenolic compounds produced by plants will be briefly described below.

4.2.1 Simple phenols

Phenolic compounds that have at most a hydroxyl group attached to the aromatic ring belong to this category. Despite the simple phenols, are synthesized by the plant even under normal conditions, they play an important role in the defence mechanisms against possible attacks of pathogens. The rapid accumulation of phenols around the site of infection is the manifestation of plant resistance to pathogen attack. The antifungal activity of these molecules interferes with the cellular metabolism, damaging the cellular structure and interrupting the DNA chain thus leading to the death of the cells. When the level of these compounds in the tissues is high, enzymes such as polyphenol oxidases and peroxidases are activated which catalyse reactions of phenols quinones that are toxic molecules to pathogens (Kumar *et al.,* 2020).

4.2.2 Phenolic acids

Phenolic acids in their structure have at least one carboxylic group bound to the aromatic ring. Depending on the length of the carbon chain, they are divided into hydroxybenzoic acids (C6-C1) such as salicylic and gallic acid, or hydroxycinnamic acids (C6-C3) such as ferulic acid (precursor of lignin). These compounds have a fundamental role in the chemical and physical defence of the plant in case of infections, but also in enzymatic activities and during photosynthesis. Salicylic acid (2-hydroxybenzoic acid) is a plant growth regulator hormone that acts as communication molecule that activate responses at the site of infection and in other tissues to hinder the spread of pathogens. In addition, the synthesis of cinnamic and benzoic acids contributes to the strengthening of cell walls (Kumar *et al.,* 2020).

4.2.3 Flavonoids

The structure of these compounds, which derives from simple phenols, consist of a skeleton of 15 carbon atoms (C6-C3-C6), with two aromatic rings linked by a heterocyclic pyran ring (Kumar *et al.,* 2020). Flavonoids differ in the oxidation state of the central heterocycle (C), in the position of the B ring and in the number and type of substituents in the B ring; the main specific properties as antioxidants mainly depend in the combination of these traits (Kulbat, 2016). They are divided intointo flavones, flavonols, flavanones, flavanonols, flavanols, anthocyanins, and chalcones (Kumar *et al.,* 2020). In grapevine, flavonoids are present as aglycones, glycosides, and methylated derivatives (these last can exist as aglycones and glycosides). They can contribute to the development of fruit aromas and colours, but also participate in the mechanisms of defence of the plant tissue against biotic and abiotic stress. Flavonoids are mostly found in the nucleus, the vacuole, cell wall, cell membrane, and the cytoplasm. Their antifungal activity is expressed through the breaking of cell walls, through the inhibition of certain enzymes and the induction of cell death (Kumar *et al.,* 2020).

4.2.4 Coumarins

Another category of phenolic compounds that follow the shikimic acid pathway and are produced by the plant in a state of infection are coumarins. Coumaric acid is produced by hydroxylation of cinnamic acid or deamination of tyrosine by the ammonia-lyase enzyme (Kulbat, 2016). Coumarins have a very high antifungal action against pathogens, and their action is expressed through alterations in the thickening mitochondria and cell death (Kumar *et al.,* 2020).

4.2.5 Lignin

Lignin, one of the most abundant natural polymers, has a C6-C3 chain and consists of three basic phenolic molecules. Its presence in the cell wall gives to the cell rigidity and hydrophobic properties, and it has a defensive function, as it acts as a physical barrier against pathogens. Its biosynthesis takes place in three stages: biosynthesis of monolignols, translocation from cytoplasm to the cell wall, and polymerization of monolignols (Kumar *et al.,* 2020).

4.2.6 Phenolic compounds in the vegetative organs of grapevine

The levels and composition of phenolic compounds vary considerably depending on the organ of the plant. For example, although the most abundant compound is the flavonol quercetin-3-O-galactoside, flavan-3-ols (catechin, epicatechin and procyanidin B1) constitute the majority of compounds in the stems, hydroxybenzoic acids (gallic acid), hydroxyinnamic acid, anthocyanins and stilbenes (Goufo *et al.,* 2020).

The leaves contain 132 phenolic compounds in total, among the most abundant compounds are quercetin-3-O-glucuronide, quercetin-3-O-glucoside, and quercetin-3-O-galactoside. Other abundant compounds are hydroxycinnamic acid esters with tartaric acid (caftaric acid and coutaric acid), stilbenes, flavan-3-ol and hydroxybenzoic acids. Coumarins, flavones, anthocyanins and flavanons are found in smaller quantities (Goufo *et al.,* 2020).

As for canes, there are a few studies on flavonols, flavones, flavanons, anthocyanins and cumarins. Among the most abundant, there are stilbenes (*trans*-resveratrol (+)-*trans*-Ɛ-viniferin), flavan-3-ols (catechin, procyanidin B1 and epicatechin), hydroxycinnamic acids (sinapicic acid and ferulic acid), and hydroxybenzoic acids (gallic acid) (Goufo *et al.,* 2020).

In the case of trunk and cordons, there are few studies on the phenolic composition. In general, the most abundant compounds are: (+)-*trans*-Ɛ-viniferin, (+)-*cis*-Ɛ-viniferin and α-viniferin, and *trans*-resveratrol.

4.3 Grapevine main defence responses

Grapevine responds to fungal infections mainly with the accumulation of two compounds: (i) phytoalexins (ii) and pathogenesis-related proteins (PR).

Phytoalexins from the *Vitaceae,* are protective compounds belonging to the stilbene family, with the structure of trans-resveratrol (3,5,4-trihydroxystilbene; Fig. 4.1). These compounds have biological activity against a large amount of pathogens, although their toxicity is less than that of synthetic fungicides (Jeandet *et al.,* 2002). Phytoalexins, with function similar to an antibiotic, are synthesized *"de novo"* in plants that are under attack by microorganisms (bacteria, fungi, viruses) or insects, and under abiotic stress conditions (Kulbat, 2016). Phytoalexins are activated in the presence of elicitors, sometimes present as constituent elements of the plant or pathogen. The biosynthesis of phytoalexins involves three basic pathways of metabolism: acetatemevalonate, acetate-malonate and shikimate pathways. Grapevine stilbenes behave like phytoalexins when the plant is under pathogenic attack and among the most abundant we find *trans*-resveratrol and viniferins (mainly ε-viniferin and α-viniferin; Bavaresco *et al.,* 2001). These compounds increase around the site of the infection reaching toxic concentrations for pathogens. They negatively affect the sporulation, germination and growth of fungal hyphae. However, their power in limiting these processes depends on their concentration and on the tolerance of the pathogen to their presence, as the pathogen could inactivate them by demethylation or hydroxylation of aromatic rings, making these compounds more water-soluble and less stable so more susceptible to oxidation (Kulbat, 2016).

During the 1980s, the concept of pathogenesis-related proteins (PR) was introduced by Antoniw *et al.* (1980b). To this group of compounds belong all the proteins encoded by the plant exclusively as a response to an infection. Abiotic and biotic stresses were not considered as inductors of protein synthesis, despite some physiological situations and not necessarily infectious condition, such as chlorosis or necrosis, often induced their synthesis. According to Van Loon (1999), to this group of compounds belonged all proteins expressed at the time of infection. Therefore, according to this definition, proteins present in healthy tissues but which are not induced by an infectious state are not considered PR. In addition, the term PR-like proteins was introduced in order to include in the group also those basic proteins induced in a controlled way during the development of the plant. In fact, some of these basic proteins such as chitinases and glucanases, can still be expressed in response to an infection. Van Loon (2006) then introduced the general term 'inducible defence-related proteins' including proteins that are not detectable in healthy tissues and whose induction occurs after pathogenic infection, excluding many proteins present in healthy tissues but which are induced by infections. Now, PR proteins are considered as defence agents which may act in a preventive manner or may limit the infection once it has occurred. Despite this, it appears that their contribution is maximum when they are already present in the tissues or are induced in healthy but close to infected tissues. They are divided according to the similarities in their sequence of amino acids, their mechanisms of action or their enzymatic and biological activities. The target structures of PR proteins are the outer part of the cell wall, the plasma membrane and finally the intracellular parts. As for the grapevine, PR proteins induction occurs in healthy berries during veraison, as a gene expression or as an induced mechanism, and the quantities produced seem to depend on cultivars, growing region, climate and agronomic practices (Ferreira *et al.,* 2007). As demonstrated by Lambert *et al.* (2013), PR proteins participate in plant defence mechanisms during a pathogenic attack of esca-associated fungi, such as *Pa. clamydospora*. These authors studied variations of phenolic compounds and PR proteins in two cultivars, Merlot and Cabernet Sauvignon, distinguishable from each other for their susceptibility to manifesting external symptoms. They found that the most significant accumulation occurs in cultivars that have a greater predisposition to pathogen tolerance and therefore manage to activate defence mechanisms faster and more strongly, as in the case of cv Merlot. Even though PR proteins are certainly of great interest in the plant pathogen interaction, we decided to focus the following sections of this chapter on the variation of phenolic compounds in wood and in leaves.

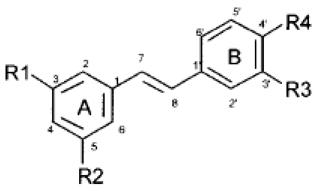
4.4 Phenolic compounds and the Esca complex of diseases

Investigation conducted in 1995 first revealed presence of phenolic compounds in wood deteriorated by fungi. In this study, the authors highlighted the increase of stilbenes in wood affected by white rot fungi (Schultz *et al.,* 1995). Therefore, as the trunk diseases are caused by fungi that lead to the deterioration of the woody parts, phenolic compounds play a fundamental role, in the defence response against pathogens. Moreover, they could contribute to the development of new control strategies, applied in the field by the vine growers, and certainly provide a "sustainable" defence mean from the environmental point of view.

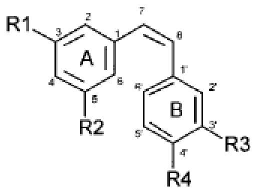
A typical reaction of woody tissues affected by esca-associated fungi is the formation of tyloses and gummosis (a mixture of polysaccharides) and the production of large quantities of phenols that accumulate in certain areas in order to hinder the passage of pathogens within the xylem (Goufo *et al.,* 2019). The plant defensive response, however, seems to be independent from external manifestation of the disease. In fact, the accumulation of flavonoids, in particular those with a high degree of polymerization, and stilbenes, especially *trans*-resveratrol and *trans*-vitisin B, occurred both in the trunks of symptomatic and asymptomatic plants (Rusjan *et al.,* 2017).

The leaves, symptomatic and asymptomatic, also respond through the production and accumulation of stilbenes in cellular tissues. Their increase is linked to the increase in the regulation of the enzyme phenylalanine ammonia-lysis (PAL) and stilbene synthase (St), which deal with the biosynthesis of phenolic compounds (Goufo *et al.,* 2019). From a study by Martin *et al.* (2009), it seems that the increase and decrease in the synthesis of secondary metabolites also depends on cultivation conditions. In fact, for the Tempranillo cultivar, grown in dry and warm conditions and with visible external symptoms, the levels of flavonoids, proanthocyanidins and hydroxycinnamic acids have decreased. On the contrary, for the same cultivar with different environmental conditions, namely warm but humid temperatures, levels of hydroxycinnamic acids increased and flavonoids decreased (Martin *et al.,* 2009).

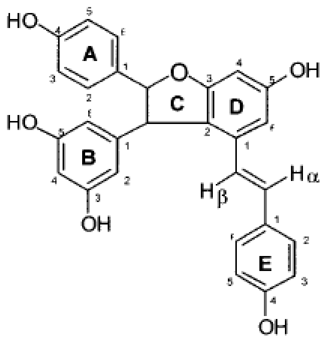
The response of the plant to the presence of esca fungi, may initially be locally induced, leading to improvements in the defence of the attacked organ, and subsequently could occur in a systemic way, as an improvement of the defences in the distant organs or not attacked. This givs the plant the ability to implement a broad-spectrum of defensive responses (Goufo *et al.,* 2019). What is certain is that there are different mechanisms of response of phenolic compounds to esca, attributable to the complexity of the disease and to different abilities of cultivars to defend themselves.



	R1	R2	R3	R4
1	GlcO	OH	H	OH
2	OH	OH	H	GlcO
3	GlcO	OH	OH	OH
4	MeO	MeO	H	OH
5	OH	OH	H	OH



	R1	R2	R3	R4
6	GlcO	OH	H	OH
7	OH	OH	H	GlcO
8	GlcO	OH	OH	OH



Trans-ε-viniferin

Figure 4.1 Chemical structures of stilbene phytoalexins. 1 and 6, trans- and cis-piceid; 2 and 7, trans- and cis-resveratroloside; 3 and 8, trans- and cis-astringin, 4, trans-pterostilbene; 5, trans-resveratrol. Glc: glucosyl ($C_6H_{11}O_5$; Jeandet *et al.,* 2002).

4.4.1 Variation in phenolic compounds in grapevine wood

Alterations in phenolic compounds profile have been studied both in vines colonized by esca pathogens and in asymptomatic ones. To date, however, there is still little evidence that can make clear the variation of secondary metabolites in the wood of vines affected by esca. To date, the most significant variations in phenolic composition are those of stilbenes. These variations are defined as spontaneous responses of the host plant to pathogenic infections.

In an experiment conducted by Amalfitano *et al.* (2000) the amount of resveratrol and dimer *trans*-Ɛ-viniferin found in the wood of infected vines was higher than that of healthy vines. However, these compounds were present, in smaller concentrations, in tissues not yet colonized by fungi. The presence of these compounds in healthy tissues was later confirmed in another study, in 2011, by Amalfitano himself. In fact, these metabolites are constitutive elements of the woody tissues, and therefore classifiable as phytoanticipins, which can increase as a result of stress (Amalfitano *et al.,* 2011). However, the quantitative difference in phenol accumulation that occurred in the Amalfitano *et al.* (2000) study, suggested that the increase in concentration was related to the defensive response of wood cells, as they showed stilbene concentration in esca colonized vines. In addition, several fungal agents were isolated from the infected wood, including *Pa. chlamydospora* and *Pm. minimum*, but also *F. mediterranea, S. hirsutum* and *E. lata*. Considering, the multitude of isolated fungi, it was not clear who, among these agents, had triggered the defensive response of the plant (Amalfitano *et al.,* 2000). From the qualitative analysis of Amalfitano, in 2011, it turned out that, in terms of chromatographic profiles, the presence of stilbenes was similar between plants affected by BWS and those used as controls. On the other hand, quantitative analysis revealed a concentration of phenolic compounds, especially of Ɛ-viniferins followed by resveratrol, higher in infected plants. This increase could be attributable to the oxidative response of the plant that accumulates ROS, which lead to radically mediated reactions between polyphenols (involving in particular the probable precursor ε-viniferin) and the embolism that occurs in xylem colonized by fungi. According to these authors, *Pa. chlamydospora* and *Pm. minimum* may induce phenol accumulation but they are not able to deactivate these compounds, thus within specific concentration they are also able to tolerate the presence of resveratrol and viniferin (Amalfitano *et al.,* 2011).

Martin and co-workers (2009) have evaluated the effects of *Pa. chlamydospora* infection on the variation of phenolic compounds in wood, following three key points:

- inoculation of two different strains (PH9, PH13);
- inoculation at two different areas (i) one at the base of the primary shoot (between the first and second node) and (ii) one at approximately half way in the trunk;

- comparison of variations in three different cultivars (cvs Chardonnay, Touriga Nacional and two clones of Aragonez).

Among the cultivars analysed, variability in phenolic content was found, which may be linked to the different degree of susceptibility to the disease expressed by the plant. In addition, significant differences in phenol production were not only related to the cultivar but also to the site of infection and the strain used during inoculum (Fig. 4.2). The differences found on the site of infection may be due to many factors, including the pathogen ability to colonize different types of tissues and the different chemical characteristics of the colonized tissue. Overall, *trans*-resveratrol and its oligomers were among the most common compounds found in tissues. One of the most accumulated compounds during the infectious state was ε-viniferin, mainly in the cv Turiga Nacional, regardless the strain used. In the two infected clones of cv Aragonez, the lowest content of phenolic compounds was found and this cultivar showed the highest degree of susceptibility to the disease. In cv Chardonnay, however, the *trans*-resveratrol was the most abundant compound and its concentration was significantly influenced by the presence of the fungus within the tissues. In fact, the increase of this compound was closely related to the infected tissue and only occasionally it was observed in healthy tissues. As already mentioned by Gubler *et al.* (2004), *Phaeomoniella chlamydospora* is able to counteract the synthesis of phenolic compounds, and this was also confirmed by Martin *et al.* (2009). In fact, the fungus developed even in presence of resveratrol or other metabolites produced by the plant, as it may have the ability to enzymatically convert phenolic compounds into lower toxicity compounds. Overall, there was a significant increase in phenols in colonized tissues, although two main pathways were observed. In fact, when there was an increase in phenolic content in response to infection in affected tissues, there was also an increase in healthy tissues (Fig. 4.3 and Fig. 4.4; Martin *et al.,* 2009). On the contrary, a decrease in phenols in infected tissues, sometimes corresponded to a decrease of phenols content even in uncolonised ones (Fig. 4.2 and Fig. 4.4; Martin *et al.,* 2009). These mechanisms responsabile for this accumulation are still unclear. It also remains to clarify if this accumulation is caused by increased localised synthesis of resveratrol or traslocation from adjacent sites. However, from the results of this study, it can be concluded that *Pa. chlamydospora* induced an upregulation of plant defense response, resveratrol-mediated.

In a study by Rusjan *et al.* (2017), an increase in the concentration of hydroxybenzoic acids, in particular gallic acid, was also detected in the rootsock infected with *Pa. chlamydospora.* However, according to Lambert *et al*. (2010), gallic acid has no inhibitory effects on the development of fungi, and it could lead to an effect defined by Mugnai *et al.,* (1999) "paradox", in fact, it could improve the growth of fungi inside the vessels. Another possible explanation of the increase of gallic acid concentration after *Pa. chlamydospora* attack is based on the fact that gallic acid is one of the most important intermediate of the biosynthesis of lignin whose capability in limiting the fungi development have already been described.

According to literature, it can be concluded that, there is a mechanism of resistance induced by the plant in the woody tissues of the vines that are under stress caused by esca fungi, expressed with localized and systemic response that allow to hinder the growth of fungi. The mechanisms underlying to the plant/pathogen interaction should be understood in detail as they can become a key step in controlling fungal infections.

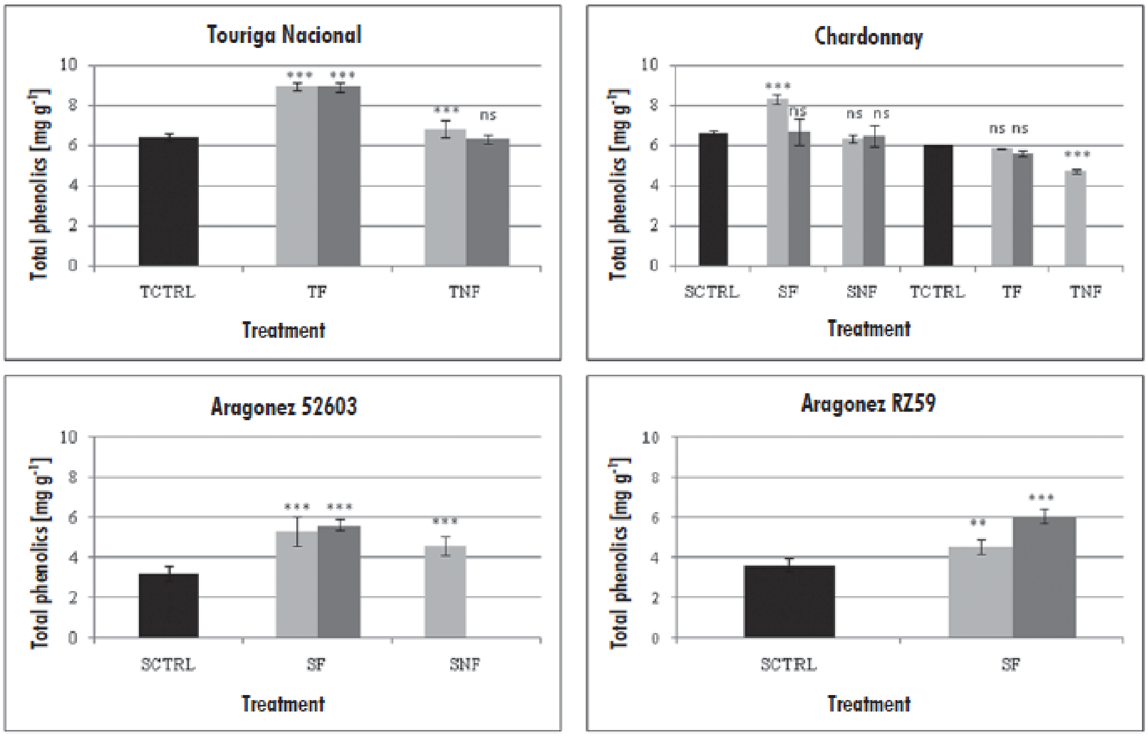


Figure 4.2 Total phenolic compound content (methanolic extract) from grapevine wood tissue of healthy and infected plants (cvs Touriga Nacional, Chardonnay, Aragonez). Different tissue types are represented for infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF) (Martin *et al.,* 2009).

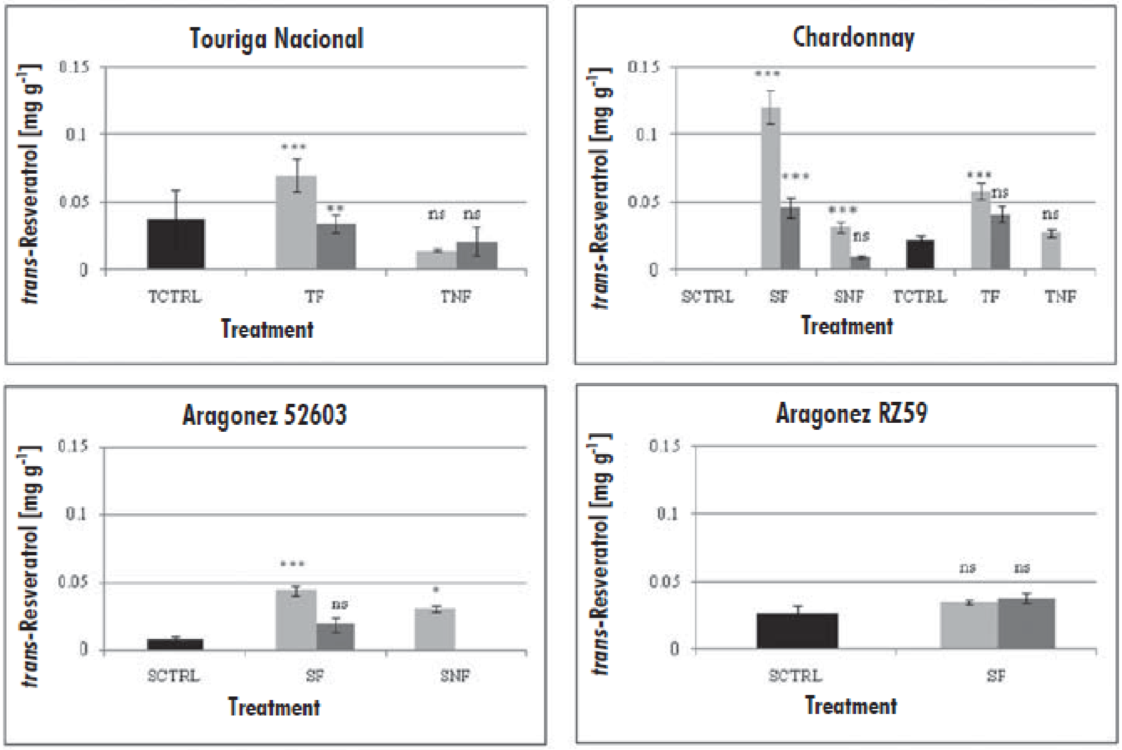


Figure 4.3 *trans*-resveratrol content in healthy and infected plants from cvs Touriga Nacional, Chardonnay, Aragonez. Different tissue types are represented for infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF; Martin *et al.,* 2009).

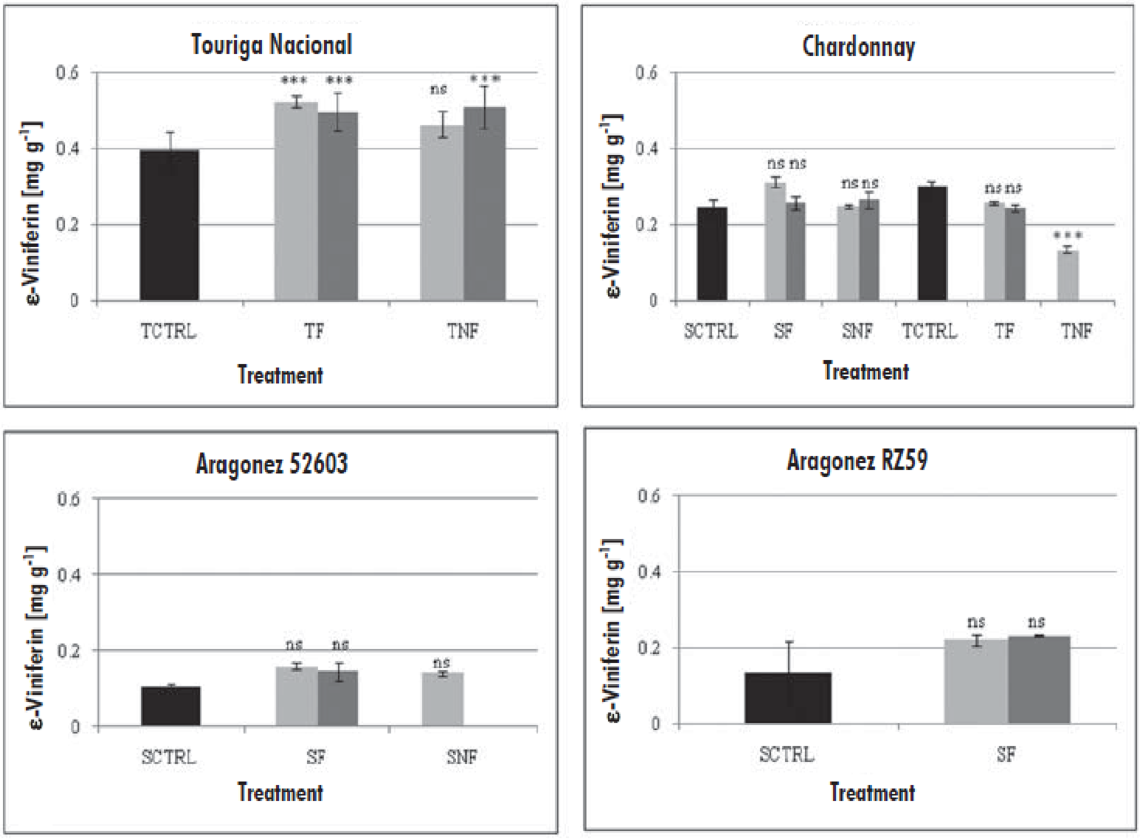


Figure 4.4 Ɛ-viniferin content in healthy and infected plants, from cvs Touriga Nacional, Chardonnay, Aragonez. Different tissue types are represented for infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF; Martin *et al.,* 2009).

4.4.2 Variations in phenolic compounds in grapevine leaves

To date, there are no non-destructive methods to determine and evaluate the presence, in the tissues of the plant, of pathogens associated with esca. Therefore, the only available means is the observation of foliar symptoms, which, however, remains an unreliable way considering the annual variability of symptoms appearance. Based on the manifestation of symptoms, cultivars have been divided into resistant or susceptible. Lambert *et al.* (2013), demonstrated, in this regard, the possible presence of two different interactions between plant and pathogen. One of them is defined as "incompatible" and the other "compatible". The difference between these two pathways is due to the response of the plant: in the incompatible relation, the responses of the plant are activated faster when compared to the compatible interaction. The cv Merlot, according to Lambert *et al*. (2013), follows the path of incompatible path interaction, that allows the plant to activate faster a strong defensive response. In fact, in the symptomatic leaves of cv Merlot, the levels of accumulation of phenolic compounds and stilbenes were higher than in the cv Cabernet Sauvignon, which on the other hand, seems to follow the compatible path of interaction.

In a study by Lima *et al.* (2017), healthy leaves (taken from non-infected cordons), diseased leaves (leaves showing symptoms) and apparently healthy leaves (leaves showing no symptoms but taken from infected cords) were examined. The purpose of their research was to assess the differences in the production of phenolic compounds, due to the presence of esca, in order to predict the presence of an infection before the appearance of visible symptoms on the leaves. By coupling HPLC and principal components analysis, the authors managed to have a classification of phenolic profile that allowed to separate clearly, the diseased leaves from the healthy ones and to place in an intermediate level the apparently healthy ones. The results of their study show that the highest levels of phenols have been found in symptomatic leaves, but, the presence of high levels even in apparently healthy leaves has shown that physiological changes occur already before the appearance of symptoms (Fig. 4.5). Among the most abundant compounds in diseased and apparently healthy leaves, there are flavonoids (quercetin-3-O-glucoside, quercetin-3-O-galactoside, kaempferol-3-glucoside and myricetin) and hydroxycinnamoyl tartaric acids (*trans*-caffeoyltartaric acid and *trans*-coumaroyl-tartaric acid; Lima *et al.,* 2017).

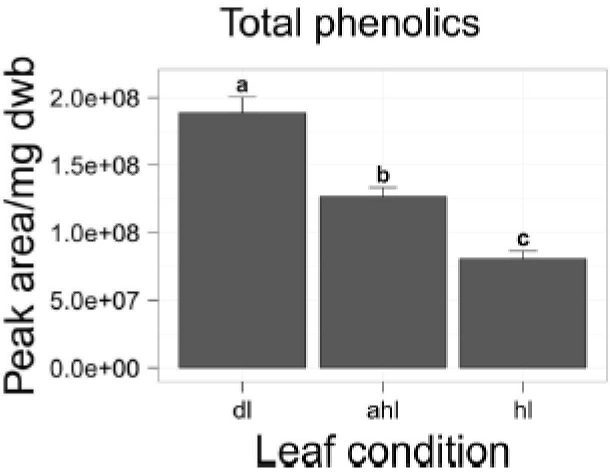


Figure 4.5 Total phenolic production in diseased (dl), apparently healthy (ahl) and healthy (hl) *Vitis vinifera* cv.Alvarinho leaves (Lima *et al.,* 2017).

Martin *et al.* (2019) evaluated the changes in the phenolic profiles of the symptomatic leaves of cv Tempranillo, taking into account the different areas of Spain where this variety is cultivated, based on climatic characteristics and water availability. The vineyards examined were three: vineyard 1 grown in warm climate with dry and warm summer, vineyard 2 with a warm climate but humid summer, and vineyard 3 with a warm climate but with dry and hot summer. Phenolic compounds identified in this study were 23 in total, the predominant ones being flavonols (flavonoids) and hydroxycinnamyc acids (non-flavonoids). In vineyard 1, the differences in variation of phenolic compounds found were mainly related to quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, kaempferol 3-*O*- glucoside and catechin gallate. In vineyard 2, above all, caffeoyltartaric, guaicol glucoside-derivative and guaiacol glucoside were found. In vineyard 3, on the other hand, where the plants were under water stress, in addition to the infection state, there were no significant variations of phenolic compounds between the leaves with symptoms and asymptomatic. Martín *et al.* (2019) demonstrated that the appearance of foliar symptoms led to a decrease in the levels of proanthocyanidins, and hydroxycinnamic acids in the leaves of vine grown under a dry and warm temperature. For the same cultivar grown under a hot and humid temperature, hydroxycinnamic acids levels increased in symptomatic leaves whereas flavonoid levels decreased. In this way, they have shown that the variation of phenolic compounds in the leaves is attributable not only to the state of infection in which the plant is, but also to environmental factors.

Trans-resveratrol levels were analysed by Calzarano *et al*. (2013), on symptomatic leaves (with different severity of symptoms), on asymptomatic leaves (taken from diseased plants) and on the healthy leaves, in different phenological phases of the plant annual growth. The levels of *trans*-resveratrol at pre-bunch closure and pre-harvest were significantly higher in symptomatic and asymptomatic (from diseased plants) leaves than in healthy leaves, and this variation corresponded to an increase in the severity of foliar symptoms. On the contrary, at veraison, levels were drastically reduced. The high levels of trans-resveratrol at pre-bunch closure and pre-harvest, and the decrease in levels during veraison (which corresponds precisely to the time when symptoms usually occur more frequently), have shown that the plant reacts through the production of phenolic compounds, but in a diversified way (probably based on the physiological state of the moment). In addition, the correlation between the increase in *trans*-resveratrol and the severity of symptoms, may be related to the effect that fungal toxins have on the activation of the plant defensive responses. In fact, the production of phenols could occur after the symptoms appeared on the leaves. The stilbenes are produced in response to stress and reach high concentrations in the symptomatic leaves, but only after the appearance of the symptoms. Therefore, the absence of symptoms on the leaves of diseased vines is not attributable to a higher production of resveratrol or other phenolic compounds (Calzarano *et al.,* 2013).

Goufo *et al*. (2019) have hypothesized that esca pathogens induce the production of phenolic compounds as part of defense responses both locally and systemically, once their toxins or compounds derived from wood degradation, are moved into the leaves. A substantial increase in phenols was observed on the leaves affected by GLSD at the time of symptoms appearance, thus confirming the assumptions of Calzarano, in 2013. However, in this case, the increased severity of the symptoms coincided with a decrease in phenols, which may be related to the increase in chlorotic and necrotic areas. In fact, when these zones increase in the leaf, photosynthetic activity is reduced, and therefore increase the levels of reactive oxygen species that compromise the activity of the primary and secondary metabolism. This would mean that, although the leaves are able to respond initially to the infection with the accumulation of phenols, when the symptoms become more serious the plant is no longer able to support the production of secondary metabolites (Goufo *et al.,* 2019).

These studies, therefore, lead to believe that phenolic compounds are part of the defensive response of the plant, but in the specific case of the esca complex, they manifest only after the appearance of symptoms and not before, therefore the action of stilbenes may not be enough to counteract in limiting the development of symptoms in the leaf.

CHAPTER V

CONCLUSION

5.1 Final considerations

Over the past few decades the incidence of grapevine trunk diseases has increased considerably. The losses in yield and in quality represent emerging problems among European winegrowers, and these losses could be exacerbated by climate change.

In the scientific community, there is good overall knowledge of the symptomatology in trunk, leaves and berries and also of the characteristics of the fungi associated with esca disease. Deepening our understanding over the impact of esca complex on plant physiology and secondary metabolism could be a key step for further understanding the mechanisms that lead to the disease development and to the appearance of symptoms. Moreover, knowledge acquired in this field could help to develop new control strategies advantageous in respect to those today available, such as the use of fungicides.

During the past two decades, there has been an important debate over the influence of environmental factors in fungal development in vineyards and in the expression of external symptoms, because viticulture is facing important environmental challenges. The reason of this debate is due to the fact that, the plant response to infections may be affected by environmental parameters, and genotype factors, such as rootstock or variety and age of the plant. Excessive water stress in combination with esca disease is one of the most important threats to viticulture. Water stress leads to a reduction in the production of the photosynthate and to a lower growth of the plant and in the presence of *Pa. chlamydospora* in the xylem, the movement of water from roots to leaves is profoundly altered. On the other hand, the excessive presence of water in the soil leads to a greater translocation, from xylem to leaves, of wood degradation compounds and/or of phytotoxins produced by fungi, which could contribute to the appearance of foliar symptoms. To date, there is no certainty about the effects that abiotic factors have on plant-pathogen interaction, but they certainly play a fundamental role that still needs to be clarified.

Regarding host–pathogen interactions, the response of grapevines affected by esca diseases is characterized by a perturbation of primary metabolism with an induction of defence reactions involving secondary metabolism, in particular the production of phenolic compounds in wood and in leaves. To date there are still little evidences that can explain the variation of secondary metabolites in the wood of vines affected by esca. In general, the most significant variations in phenolic composition are those of stilbenes and these variations are defined as spontaneous responses of the plant to pathogenic infections. However, evidences show that pathogens can survive even in the presence of these compounds.

The new synthesis of phenols in the leaves is surely not related to the physical presence of the pathogen, but probably to the presence of toxins translocated by xylem. In most analysed studies, significant variations in phenolic compounds occurred before and/or at the appearance of symptoms and, on the contrary, decreased when symptoms worsened. The action of stilbenes may not be enough to counteract the infection of the esca pathogens. Surely, however, being able to evaluate the variations of phenols in the leaves could be an alternative method to know if the plant is infected, before the appearance of the symptoms and without resorting to destructive and sometimes unreliable methods.

In conclusion, studying the interaction plant-environment-pathogen could have useful implications to counter the development of infections in the field and to be able to develop new control strategies. Furthermore, if the defensive capacities of the plant could be enhanced, the use of fungicides may be reduced, along with their toxic effects to the environment, to human and to the plant. This could be important especially in this historic period, being necessary to find alternative methods to limit pathogen damages in viticulture, in order to reduce the increasingly serious effects of climate change.

REFERENCES

Agrios G.N., 2005. *Plant Pathology*. 5th ed. Elsevier Academic Press, USA and UK, 952 pp.

Almeida F., 2007. Technical note 2 - Grapevine wood diseases - Eutypa dieback and esca. ADVID Technical Notes. 2, 1-14.

Alsina M.M., de Herralde F., Aranda X., Save R., Biel C., 2007. Water relations and vulnerability to embolism are not related: Experiments with eight grapevine cultivars. *Vitis* 46, 1-6.

Alsina M.M., Smart D.R., Bauerle T., de Herralde F., Biel C., Save R., 2011. Seasonal changes of whole root system conductance by a drought-tolerant grape root system. *Journal of Experimental Botany* 62, 99-109.

Amalfitano, C., Evidente, A., Surico, G., Tegli, S., Bertelli, E., Mugnai, L., 2000. Phenols and stilbene polyphenols in the wood of esca-diseased grapevines. *Phytopathologia Mediterranea* 39, 178-183.

Amalfitano, C., Agrelli, D., Arrigo, A., Mugnai, L., Surico, G., Evidente, A., 2011. Stilbene polyphenols in the brown red wood of *Vitis vinifera* cv. Sangiovese affected by "esca proper". *Phytopathologia Mediterranea* 50, S224-S235.

Andolfi A., Mugnai L., Luque J., Surico G., Cimmino A., Evidente A., 2011. Phytotoxins produced by fungi associated with grapevine trunk diseases. *Toxins* 3, 1569-1605.

Andreini L., Guarino L., Viti R., Scalabrelli G., 2013. Evaluation of the effect of esca disease on bud break in *Vitis vinifera* L.: Possible relationship between cultivars and rootstocks. *Vitis* 52, 33–40.

Andreini L., Cardelli R., Bartolini S., Scalabrelli G., Viti R., 2014. Esca symptoms appearance in *Vitis vinifera* L.: Influence of climate, pedo-climatic conditions and rootstock/cultivar combination. *Vitis* 53, 33-38.

Antoniw, J.F., Ritter, C.E., Pierpoint, W.S., Van Loon, L.C., 1980. Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV *Journal of General Virology* 47, 79–87.

Bavaresco, L., Fregoni, C., 2001. Physiological role and molecular aspects of grapevine stilbenic compounds. *In Molecular Biology & Biotechnology of the Grapevine*, Chapter 5, 1st ed.; Roubelakis-Angelakis, K.A., Ed.; Kluwer Academic Publishers: Berlin, Germany; pp. 153–182.

Bennett, R.N., Wallsgrove R.M., 1994. Secondary metabolites in plant defence mechanisms. Tansley Review No. 72 Biochemistry & Physiology Department, AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts ALS 2JQ, UK. *New Phytologist*. 127, 617-633.

Berdeja M., Hilbert G., Dai Z., Lafontaine M., Stoll M., Delrot S., 2014. Effects of water stress and rootstock genotype on Pinot Noir berry composition. *Australian Journal of Grape and Wine Research* 20, 409‐421.

Berg G., 2009. Plant-microbe interaction promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology* 84, 11-18.

Bertsch C., M. Ramírez-Suero, M. Magnin-Robert, P. Larignon, J. Chong, E. Abou-Mansour, A. Spagnolo, Clément C., Fontaine F., 2013. Grapevine trunk diseases: complex and still poorly understood. *Plant Pathology* 62, 243–265.

Bleach C., Jones E., Ridgway H., Jaspers M., 2013. Hot water treatment to reduce incidence of black foot pathogens in young grapevines grown in cool climates. *Phytopathologia Mediterranea* 52, 347-358.

Bostock R.M., Pye M.F., Roubtsova T.V., 2014. Predisposition in plant disease: Exploiting the nexus in abiotic and biotic stress perception and response. *Annual Review of Phytopathology* 52, 517-549.

Boulisset F., Henneguez A., Letousey P., Kempf R., Profizi C., 2017. *Trichoderma atroviride* I-1237 as a biocontrol solution to manage grapevine trunk diseases; ESQUIVE® WP field approaches. *Phytopathologia Mediterranea* 56, 568 (abstract).

Boyer, J.S.,1995. Biochemical and biophysical aspects of water deficits and the predisposition to disease. *Annual Review of Phytopathology* 33, 251-274.

Bruez E., Lecomte P., Grosman J., Doublet B., Bertsch C., Fontaine F., Ugaglia A, Teissedre P.L., Da Costa J.P., Guerin-Dubrana L., Rey P., 2013. Overview of grapevine trunk diseases in France in the 2000s. *Phytopathologia Mediterranea*. 5,: 262-275.

Bruez E., Larignon P., Compant S., Rey P., 2017a. Investigating the durable effect of the hot water treatment used in nurseries on pathogenic fungi inhabiting grapevine wood and involved in grapevine trunk diseases. *Crop Protection* 100, 203-210.

Bruez E., Anatole-Monier L., Vallance J., Bussière S., Denaix L., Rey P., Thiéry D., 2017b. High concentration of copper in vineyard soil favours the development of wood necrosis caused by the Botryosphaeria dieback pathogen *Neofusicoccum parvum*. *Phytopathologia Mediterranea* 56, 513-588.

Calzarano, F. Di Marco S., 2007. Wood discoloration and decay in grapevines with esca proper and their relationship with foliar symptoms. *Phytopathologia Mediterranea* 46, 96-101.

Calzarano F., Amalfitano C., Seghetti L., Cozzolino V., 2009. Nutritional status of vines affected with esca proper. *Phytopathologia Mediterranea* 48, 20–31.

Calzarano F., D'Agostino V., Osti F., Di Marco S., 2013. Levels of *trans*-resveratrol in leaves of vines affected with "esca". *In*: Protection and Production in Viticulture, IOBC/WPRS Bulletin Vol. 85, 129–136.

Calzarano F., Di Marco S., D'Agostino V., Schiff S., Mugnai L., 2014. Grapevine leaf stripe disease symptoms (esca complex) are reduced by a nutrients and seaweed mixture. *Phytopathologia Mediterranea* 53, 543-558.

Calzarano F., D'Agostino V., Pepe A., Osti F., Della Pelle F., De Rosso M., Flamini R., Di Marco S., 2016. Patterns of phytoalexins in the grapevine leaf stripe disease (esca complex)/grapevine pathosystem. *Phytopathologia Mediterranea* 55, 410-426.

Calzarano F., Osti F., Baranek M., Di Marco S., 2018. Rainfall and temperature influence expression of foliar symptoms of grapevine leaf stripe disease (esca complex) in vineyards. *Phytopathologia Mediterranea* 57: 488-505.

Castillo F., Hernández D., Gallegos G., Rodríguez R., Aguilar C.N., 2012. Antifungal properties of bioactive compounds from plants.

Cloete M., Mostert L., Fischer M., Halleen F., 2015. Pathogenicity of South African Hymenochaetales taxa isolated from esca-infected grapevines. *Phytopathologia Mediterranea* 54, 368-79.

Crocker J., Waite H., Fletcher G., 1999. Development of effective, efficient and reliable hot water treatments. Grape and wine research and development corporation project. *Phytopathologia Mediterranea* 46, 5-17.

Del Frari G., Costa J., Oliveira H., Ferreira R.B, 2018. Endotherapy of infected grapevine cuttings for the *control of Phaeomoniella chlamydospora and Phaeoacremonium minimum*. Phytopathologia Mediterranea 57, 439-448.

Di Marco S., Osti F., 2007. Applications of *Trichoderma* to prevent *Phaeomoniella chlamydospora* infections in organic nurseries. *Phytopathologia Mediterranea* 46, 73–83.

Di Marco S., Osti F., Mugnai L., 2011. First studies on the potential of a copper formulation for the control of leaf stripe disease within esca complex in grapevine. *Phytopathologia Mediterranea* 50, 300-309.

Dias M.C., 2012. Phytotoxicity: An overview of the physiological responses of plants exposed to fungicides. *Journal of Botany* 12, 1-4.

Dubos, B., 2002: Le syndrome de l'Esca. *In* Editions Féret, Maladies Cryptogamiques de la Vigne, 2nd ed., Bordeaux, pp. 127-136.

Eastburn D.M., McElrone A.J., Bilgin D.D., 2011. Influence of atmospheric and climatic change on plantpathogen interactions. *Plant Pathology* 60 54–69.

Edwards J., Salib S., Thomson F., Pascoe I.G., 2007. The impact of *Phaeomoniella chlamydospora* infection on the grapevines physiological response to water stress, part 2: Cabernet Sauvignon and Chardonnay. *Phytopathologia Mediterranea* 46, 38–49.

Elena G., Luque J., 2016. Seasonal susceptibility of grapevine pruning wounds and cane colonization in Catalonia, Spain following artificial infection with *Diplodia seriata* and *Phaeomoniella chlamydospora*. *Plant Diseases* 100, 1651-1659.

Eskalen A., Rooney-Latham S., Gubler W.D., 2007. Identifying effective management strategies for esca and Petri disease. *Phytopathologia Mediterranea* 46, 125-126.

Evidente A., Sparapano L., Andolfi A., Bruno G., 2000. Two naphthalenone pentaketides from liquid cultures of *Phaeoacremonium aleophilum*, a fungus associated with esca of grapevine. *Phytopathologia Mediterranea* 39, 162-168.

Ferreira J.H.S., Van Wyk P.S., Calitz F.J., 1999. Slow dieback of grapevine in South Africa: Stress-related predisposition of young vines for infection by *Phaeoacremonium chlamydosporum*. *South African Journal of Enology and Viticulture* 20, 43–46.

Ferreira R.B., Monteiro S., Freitas R., Santos C. N., Chen Z., Batista L. M., Duarte J., Borges A., Teixeira A.R., 2007. The role of plant defence proteins in fungal pathogenesis. *Molecular Plant Pathology* 8, 677–700

Ferri F., 1985. I funghi micologia, isolamento, coltivazione. Bologna: Edagricole, pp. 38-42.

Fischer M., 2002. A new wood-decaying basidiomycete species associated with esca of grapevine: *Fomitiporia mediterranea* (Hymenochaetales). *Mycological Progress*, 315–24.

Fischer, M., 2006. Biodiversity and geographic distribution of basidiomycetes causing esca-associated white rot in grapevine: A worldwide perspective. *Phytopathologia Mediterranea* 45, 30-42.

Fisher M.C., Hawkins N.J., Sanglard D., Gurr S.J., 2018. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science* 360, 739-742.

Fischer M., Peighami A.S., 2019. Grapevine, esca complex, and environment: The disease triangle. *Phytopathologia Mediterranea* 58, 17-37.

Fontaine F., Pinto C., Vallet J., Clément C., Gomes A.C., Spagnolo A., 2016. The effects of grapevine trunk diseases (GTDs) on vine physiology. *European Journal of Plant Pathology* 144, 707-772.

Fourie P. Halleen F., 2006. Chemical and biological protection of grapevine propagation material from trunk disease pathogens. *European Journal of Plant Pathology* 116, 255-265.

Fregoni C., Bavaresco L., 2000. Physiological role and molecular aspects of grapevine stilbenic compounds. *Molecular Biology and Biotecnology of the Grapevine* 153-182.

Garcìa P.C, Rivero R.M., Ruiz J.M., Romero L., 2003. The role of fungicides in the physiology of higher plants: implications for defense responses. *Botanical Review* 69, 162-172.

Goufo, P., Marques, A. C., Cortez, I. 2019. Exhibition of local but not systemic induced phenolic defenses in *Vitis vinifera* L. affected by Brown Wood Streaking, Grapevine Leaf Stripe, and Apoplexy (Esca Complex). *Plants* 8, 412. doi: 10.3390/plants8100412.

Goufo, P., Singh, R. K., Cortez, I. 2020. A reference list of phenolic compounds (including stilbenes) in Grapevine (*Vitis vinifera* L.) roots, woods, canes, stems, and leaves, *Antioxidants*, 9, 398.

Gramaje D., García-Jiménez J., Armengol J., 2010. Field evaluation of grapevine rootstocks inoculated with fungi associated with Petri disease and esca. *American Journal of Enology and Viticulture* 61, 512-520.

Gramaje D., Armengol J., 2011. Fungal trunk pathogens in the grapevine propagation process: Potential inoculum sources, detection, identification, and management strategies. *Plant Disease* 95, 1040-1055.

Gramaje D., Úrbez-Torres J.R., Sosnowski M.R., 2018. Managing grapevine trunk diseases with respect to etiology and epidemiology: Current strategies and future prospects. *Plant Disease* 102, 12-39.

Graniti A., Surico G., Mugnai L., 2000. Esca of grapevine: A disease complex or a complex of diseases? *Phytopathologia Mediterranea* 39, 16-20.

Gubler W.D., Baumgartner K., Browne G.T., Eskalen A., Rooney Latham S., Petit E., Bayramian L.A., 2004. Root diseases of grapevines in California and their control. *Australasian Plant Pathology* 33,157–165.

Guérin-Dubrana, L., Destrac-Irvine, A., Goutouly, J.-P., Letouze, A., Gaudillère, J.-P., 2005. Relationship between incidence of Esca and black dead arm foliar symptom expression in the vineyard, ecophysiological indicators and cultural practices. *Phytopathologya Mediterranea* 44, 110.

Gullino M.L., Leroux P., Smith C.M, 2000. Uses and challenges of novel compounds for plant disease control. *Crop Protection* 19, 1-11.

Hofstetter V., Buyck B., Croll D., Viret O., Couloux A., Gindro K., 2012. What if esca disease of grapevine were not a fungal disease. *Fungal Diversity* 54, 51-67.

Jeandet, P., Douillet-Breuil, A.C., Bessis, R., Debord, S., Sbaghi, M., Adrian, M., 2002. Phytoalexins from the Vitaceae: Biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *Journal of Agricultural* 50, 2731-2741.

Juang K.W., Lee Y., Lai H.Y., Wang C.H., Chen B.C., 2012. Copper accumulation, translocation, and toxic effects in grapevine cuttings. *Environmental Science and Pollution Research* 19, 1315-1322.

Komárek M., Čadková E., Chrastný V., Bordas F., Bolinger J.C., 2010. Contamination of vineyards soils with fungicides: A review of environmental and toxicological aspects. *Environmental International* 36, 138-151.

Kulbat K., 2016. The role of phenolic compounds in plant resistance. *Biotechnology and Food Sciences* 80, 97-108.

Kumar, S. 2020. Role of phenolic compounds in plant-defensive mechanisms, *In* Lone R., Shuab R., Kamili A.N., *Plant Phenolics in Sustainable Agriculture*, 517–532.

La Torre A., Lovino V., Caradonia F., 2018. Copper in plant protection: Current situation and prospects. *Phytopathologia Mediterranea* 57, 201-236.

Lambert C., Bisson J., Waffo-Teguo P., Papastamoulis Y., Richard T., Corio-Costet M. F., Merillon J. M., Cluzet S., 2012. Phenolics and their antifungal role in grapevine wood decay: Focus on the *Botryosphaeriaceae* family. *Journal of Agricultural* 60, 11859-11868.

Lambert C., Khiook I.L.K., Lucas S., Télef-Micouleau N., Mérillon J.-M., Cluzet S., 2013. A faster and a stronger defense response: One of the key elements in grapevine explaining its lower level of susceptibility to Esca? *Phytopathology* 103, 1028–1034.

Larignon P., Dubos B., 2001. Il punto sul ciclo biologico dei funghi coinvolti nella sindrome dell’esca. *Informatore Fitopatologico* 5, 27-31.

Larignon P., Fontaine F., Farine S., Clément, C., 2009. Esca et Black Dead Arm: Deux acteurs majeurs des maladies du bois chez la Vigne. *Comptes Rendus Biologies* 332, 765-783.

Lecomte P., Darrieutort G., Laveau C., Blancard G., Louvet G., Goutouly J.P., Rey P., Guerin-Dubrana L., 2011. Impact of biotic and abiotic factors on the development of esca decline disease. *Integrated Protection and Production in Viticulture* 67, 171-80.

Lecomte P., Diarra B., Carbonneau A., Rey P., Chevrier C., 2017., Esca of grapevine and training practices in France: Results of a 10-year survey. *Phytopathologia Mediterranea* 57, 472-487.

Lima R.M.M., Machado F.A., Gubler D.W., 2017. Metabolomic study of chardonnay grapevines double stressed with esca-associated fungi and drought. *Phytopathology* 107, 669–680.

Llorens N., L. Arola, C. Blade´, Mas A., 2000. Effects of copper exposure upon nitrogen metabolism in tissue cultured Vitis vinifera. *Plant Science* 160, 159–163.

Lorrain B., Ky I., Pasquier G., Jourdes M., Dubrana L.G., Geny L., Rey P., Donèche B., Teissedre P., 2012. Effect of esca disease on the phenolic and sensory attributes of Cabernet Sauvignon grapes, musts and wines. *Austalian Journal of Grapevine and Wine Research* 18, 64-72.

Lovisolo C., Schubert A., 1998. Effects of water stress on vessel size and xylem hydraulic conductivity in *Vitis vinifera* L. *Journal of Experimental Botany* 49, 693–700.

Magnin-Robert M., Letousey P., Spagnolo A., Rabenoelina F., Jacquens L.,Mercier L., Clément C., Fontaine F., 2011. Leaf strip of esca induces alteration of photosynthesis and defence reactions in presymptomatic leaves. *Functional Plant Biology* 38, 856–866.

Marchi G., 2001. Susceptibility to esca of various grapevine (*Vitis vinifera*) cultivars grafted on different rootstocks in a vineyard in the province of Siena (Italy). *Phytopathologia Mediterranea* 40, 27-36.

Marchi G., Peduto F., Mugnai L., Di Marco S., Calzarano F., Surico G., 2006. Some observations on the relationship of manifest and hidden esca to rainfall. *Phytopathologia Mediterranea* 4, 117–126.

Martin N., Vesentini D., Rego C., Monteiro S., Oliveira H., Ferreira R B., 2009. Phaeomoniella chlamydospora infection induces changes in phenolic compounds content in *Vitis vinifera*. *Phytopathologya Mediterranea* 48, 101–116.

Martín L., Fontaine F., Javier Castaño F., Songy A., Roda R., Vallet J., Ferrer-Gallego R., 2019. Specific profile of Tempranillo grapevines related to Esca-leaf symptoms and climate conditions. *Plant Physiology and Biochemistry* 135, 575–587.

Michelon L., Pellegrini C., Pertot I., 2007. Il mal dell’esca della vite. Istituto Agrario di San Michele all’Adige, *SafeCrop Centre*.

Mondello V., Songy A., Battiston E., Pinto C., Coppin C., Trotel-Aziz P., Clément C., Mugnai L., Fontaine F., 2018. Grapevine trunk diseases: A review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Disease* 102, 189-217.

Monteiro S., Carreira A., Freitas R., Pinheiro A.M., Ferreira R.B, 2015. A nontoxic polypeptide oligomer with a fungicide potency under agricultural conditions which is equal or greater than that of their chemical counterparts. *PLoS ONE* 10, 1-23.

Mostert L., Halleen F., Fourie P., Crous P.W., 2006. A review of *Phaeoacremonium* species involved in Petri disease and esca of grapevines. *Phytopathologia Mediterranea* 45, 12-29.

Mugnai L., A. Graniti and Surico G., 1999. Esca (black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Disease* 83, 404–18.

Murolo S., Romanazzi G., 2014. Effects of grapevine cultivar, rootstock and clone on esca disease. *Australasian Plant Pathology* 43, 215–21.

Nascimento T., Rego C., Oliveira H., 2007. Potential use of chitosan in the control of grapevine trunk diseases. *Phytopathologia Mediterranea* 46, 218-224.

Nerva L., Turina M., Zanzotto A., Gardiman M., Gaiotti F., Gambino G., Chitarra W., 2019. Isolation, molecular characterization and virome analysis of culturable wood fungal endophytes in esca symptomatic and asymptomatic grapevine plants. *Environmental Microbiology* 21, 2886-2904.

Nóvoa-Muñoz J.C., Queijeiro J.M.G., Blanco-Ward D., Álvarez-Olleros C., Martínez-Cortizas A., García- Rodeja E., 2007. Total copper content and its distribution in acid vineyards soils developed from granitic rocks. *Science of the Total Environment* 378, 23-27.

OIV: International Organization of vine and Wine 2018. “OIV Statistical Report on World Vitiviniculture”. 4 pp. Available from: http://www.oiv.int/public/medias/6371/oiv-statistical-report-on-world-vitiviniculture-2018.pdf.

Oliva J., Stenlid J., Martinez-Vilatta J., 2014. The effect of fungal pathogens on the water and carbon economy of trees: Implications for drought-induced mortality. *New Phytologist* 203, 1028–1035.

Ouadi, L., Bruez, E., Bastien, S., Domec, J., Rey, P., 2017. Highlights on ecophysiological changes in esca-diseased grapevines in comparison to healthy plants. [abstract]. *In*: 10th International Workshop on Grapevine Trunk Diseases, Reims. 67.

Ouzounidou G., R. Lannoye, Karataglis S., 1993. Photoacoustic measurements of photosynthetic activities in intact leaves under copper stress, *Plant Science* 89, 221–226.

Palliotti A., Frioni T., Tombesi S., Sabbatini P., Cruz-Castillo J.G, Lanari V., Silvestroni O., Gatti M., Poni S., 2017. Double-pruning grapevines as a management tool to delay berry ripening and control yield. *American Journal of Enology and Viticulture* 68, 412-421.

Pandey P., Ramegowda V., Senthil-Kumar M., 2015. Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. *Frontiers in Plant Science* 6, 723.

Pavlı́k M., Jandurová O.M., 2000. Fungicides cytotoxicity expressed in male gametophyte development in Brassica campestris after in vitro application of converted field doses. *Environmental and Experimental Botany* 44, 49-58.

Pertot I., Prodorutti D., Colombini A., Pasini L., 2016. *Trichoderma atroviride* SC1 prevent *Phaeomoniella Chlamydospora* and *Phaeoacremonium aleophilum* infection of grapevine plants during the grafting process in nurseries. *BioControl* 61, 257-267.

Petit A.N., Fontaine F., Clement C., Vaillant-Gaveau N., 2008. Photosynthesis limitations of grapevine after treatment with the fungicide fludioxonil. *Journal of Agricultural and Food Chemistry* 56, 6761-6767.

Pierron R., M. Gorfer, H. Berger, A. Jacques, A. Sessitsch, J. Strauss and Compant S., 2015. Deciphering the niches of colonisation of *Vitis Vinifera* L. by the esca-associated fungus *Phaeoacremonium aleophilum* using a gfp marked strain and cutting systems. *PLoS ONE* 10, 1–22.

Pouzoulet J., Pivovaroff A.L., Santiago L.S., Rolshausen P.E., 2014. Can vessel dimension explain tolerance towards fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. *Frontiers in Plant Science* 5, 253.

Pouzoulet J., Scudiero E., Schiavon M., Rolshausen P.E., 2017. Xylem vessel diameter affects the compartmentalization of the vascular pathogen *Phaeomoniella chlamydospora* in grapevine. *Frontiers in Plant Science* 8, 1-13.

Quaglia M., Covarelli L., Zazzerini A., 2009. Epidemiological survey on esca disease in Umbria, central Italy. *Phytopathologia Mediterranea* 48, 84-91.

Rego C., Nascimento T., Cabral A., Oliveira H., 2005. Fungi associated with young vine decline in Portugal: results of nine years surveys. *Integrated Protection in Viticulture* 29, 121-126.

Rego C., Nascimento T., Cabral A., Silva M.J., Oliveira H., 2009. Control of grapevine wood fungi in commercial nurseries. *Phytopatologia Mediterranea* 48, 128-135.

Rolshausen P.E., Úrbez-Torrez J.P., Rooney-Latham S., 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. *American Journal of Enology and Viticulture* 61, 113-119.

Romeu-Moreno A., Mas A., 1999. Effects of copper exposure in tissue cultured *Vitis vinifera*. *Journal of Agricultural and Food Chemistry* 47, 519-522.

Rubio J.J., Garzón E., 2011. Las enfermedades de madera de vid como amenaza del sector vitícola. *Revista Winetech* 11, 18-21.

Rusjan, D., Persic, M., Likar, M., Biniari, K., Mikulic-Petkovsek, M., 2017. Phenolic responses to esca-associated fungi in differently decayed grapevine woods from different trunk parts of ‘Cabernet Sauvignon’. *Journal of Agricultural* 65, 6615–6624.

Soar C.J., Dry P.R., Loveys B.R., 2006. Scion photosynthesis and leaf gas exchange in *Vitis vinifera* L. cv. Shiraz: Mediation of rootstock effects via xylem sap ABA. *Australian Journal of Agricultural Research* 12, 82-96.

Sosnowski M.R., Loschiavo A.P., Wicks T.J., Scott E.S., 2013. Evaluating treatments and spray application for the protection of grapevine pruning wounds from infection by *Eutypa lata*. *Plant Disease* 97, 599-604.

Sparapano L., Bruno G., Graniti A., 2001. Three-year observation of grapevines cross-inoculated with esca-associated fungi. *Phytopathologia Mediterranea* 40, 376-386.

Schultz, T. P.; Harms, W. B.; Fisher, T. H.; McMurtrey, K. D.; Minn, J.; Nicholas, D. D., 1995. Durability of angiosperm heartwood: The importance of extractives. *Holzforschung* 49, 29-34.

Surico G., 2001. Towards commonly agreed answers to some basic questions on esca. *Phytopathologia Mediterranea* 40, 487-491.

Surico G., Mugnai L., Marchi G., 2008. The Esca Disease Complex. In Integrated Management of Diseases Caused by Fungi, Phytoplasma and Bacteria. *Phytopathologia Mediterranea* 3, 119-136.

Surico G., 2009. Towards a redefinition of the diseases within the esca complex of grapevine. *Phytopathologia Mediterranea* 48, 5–10.

Tabacchi R., Fkyerat A., Poliart C., Dubin G.M., 2000. Phytotoxins from fungi of esca of grapevine. *Phytopathologia Mediterranea* 39, 156-161.

Thind T. S, 2017. Role of Fungicides in Crop Health Management: Prospects and challenges. *Developments in Fungal Biology and Applied Mycology* 433-447

Thomson L.J., Glenn D.C., Hoffmann A.A., 2000. Effects of sulfur on *Trichogramma* egg parasitoids in vineyards: Measuring toxic effects and establishing release windows. *Australian Journal of Experimental Agriculture* 40, 165-171.

Úrbez-Torres J.R., 2011. The status of *Botryosphaeriaceae* species infecting grapevines. *Phytopathologia Mediterranea* 50, 5-45.

Van Loon L.C**.,** 1999. Occurrence and properties of plant pathogenesis related proteins. *In* Pathogenesis-Related Proteins in Plants (Datta, S.K. and Muthukrishnan, S., eds) 1–19.

Van Loon L.C., Rep M., Pieterse C.M., 2006. Significance of inducible defense-related proteins in infected plants. *Phytopathology* 44, 135–162.

Van Niekerk J., Halleen F., Fourie P.H., 2011. Temporal susceptibility of grapevine pruning wounds to trunk pathogen infection in South African grapevines. *Phytopathologia Mediterranea* 50 139-150.

Vivier M.A., Pretorius I.S., 2002. Genetically tailored grapevines for the wine industry. *Trends in Biotechnology* 20, 472-478.

Wilcox W.F., Gubler W.D., Uyemoto J.K., 2015. Compendium of grape diseases, disorders, and pests. Edited by and Jerry K. Uyemoto Wayne F. Wilcox, Walter D. Gubler. Second Edi. Vol. 2. Ed. *The American Phytopathological Society*.

Xia X.J., Huang Y.Y., Wang L., 2006. Pesticides induced depression of photosynthesis was alleviated by 24-epibrassinolide pretreatment in *Cucumis sativus* L. *Pesticide Biochemistry and Physiology* 86, 42-48.

Yarwood CE., 1959. Predisposition. *Plant Pathology*, ed. JG Horsfall, AE Dimond, 521–62.

Yurela I., 2005. Copper in plants. *Brazilian Journal of Plant Physiology* 17.