Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Viticultura e Enologia

Dissertação

Identification of fungi species associated to grapevine trunk disease in Alentejo region

Angela Billar de Almeida

Orientador(es) | Maria do Rosário Fernandes Félix
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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

Presidente | João Manuel Mota Barroso (Universidade de Évora)

Vogais | Hélia Cristina Guerra Cardoso (Universidade de Évora) (Arguente)
Maria do Rosário Fernandes Félix (Universidade de Évora) (Orientador)

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ABSTRACT

Grapevine trunk diseases (GTDs) is one of the most important groups of fungal diseases affecting grapevine plants in all the major growing regions of the world, with more than 130 fungal species associated. All grapevine species are susceptible to these diseases and their complete eradication is not possible for many reasons. In addition, GTDs are influenced by the type of disease and/or pathogens involved, leading management of this diseases complex to focus primarily on disease prevention and mitigation. Focusing on finding alternatives to avoid the spread and higher incidence of the disease, the present work had the aim to identify molecularly the phytopathogenic fungi responsible for GTDs present in vineyards of Alentejo region, and to test the antagonist potential of some endophytes against those pathogens. PCR assays followed sequencing of ITS region were performed to identify fungi and among them, three GTDs fungi were identified at genera level (*Diaporthe sp., Pestalotiopsis sp*., *Neofusicocum sp.*) and six at specie level (*Hormonema viticola, Stereum armeniaccum, Phialophora fastigiata, Truncatella angustata, Cytospora acaciae, Diplodia pseudoseriata*). The most prevalent fungus verified in the samples were *Diaporthe sp*., *Neofusicocum sp.* and *Hormonema viticola* in symptomatic plants. Almost all these pathogens were also verified in asymptomatic plants, highlighting the incidence of *Hormonema viticola,* what confirms the need of early diagnosis of this diseases complex. All the endophyte used in the direct inhibition antagonism test had the capacity of inhibit the pathogen mycelia growth, showing their potential biocontrol. This study allowed a deeper knowledge of the fungi present in vineyards from Alentejo region associated to GTDs and will contribute to further studies on fungi molecular identification in order to monitor the behaviour of the disease in the vineyards.

Key-words: Grapevine trunk disease, *Vitis vinifera*, molecular identification, endophytic fungi, antagonism, ITS region

Resumo: Identificação de espécies de fungos associadas a doenças do lenho em videira na região do Alentejo

As doenças do lenho da videira são um dos grupos mais importantes de doenças fúngicas que afetam as plantas da videira em todas as principais regiões produtoras do mundo, com mais de 130 espécies de fungos associadas. Todas as espécies de videira são suscetíveis a essas doenças e sua erradicação completa não é possível por diversos motivos. Além disso, as doenças do lenho são influenciadas pelo tipo de doença e / ou agentes patogénicos envolvidos, levando a gestão deste complexo de doenças a concentrar-se principalmente na prevenção e mitigação de doenças. Com o objetivo de encontrar alternativas para evitar a disseminação e maior incidência da doença, o trabalho aqui apresentado teve como objetivo identificar molecularmente os fungos fitopatogénicos responsáveis pelas doenças do lenho presentes em vinhas da região do Alentejo, e testar o potencial antagonismo de alguns fungos endófitos contra esses agentes patogénicos. Os testes de PCR seguidos de sequenciação da região ITS foram realizados para identificar os fungos, tendo sido identificados três fungos causadores de doenças do lenho ao nível de género (*Diaporthe* sp., *Pestalotiopsis* sp., *Neofusicocum* sp.) e seis ao nível de espécie (*Hormonema viticola*, *Stereum armeniaccum*, *Phialophora fastigiata*, *Truncatella angustata*, *Cytospora acaciae*, *Diplodia pseudoseriata*). Os fungos mais prevalentes verificados nas amostras foram *Diaporthe* sp., *Neofusicocum* sp. e *Hormonema viticola* em plantas sintomáticas. A grande maioria dos agentes patogénicos foram também verificados em plantas assintomáticas, destacando a incidência de *Hormonema viticola*, o que confirma a necessidade de diagnóstico precoce desse complexo de doenças. Todos os endófitos utilizados no teste de antagonismo de inibição direta tiveram a capacidade de inibir o crescimento mecelial do patogénico, mostrando seu potencial de biocontrole. Este estudo permitiu um conhecimento mais aprofundado dos fungos presentes nas vinhas da região do Alentejo associadas às doenças do lenho, e contribuirá para mais estudos sobre a identificação molecular dos fungos, a fim de monitorizar o comportamento da doença nas vinhas.

Palavras-chave: Doença do lenho da videira, *Vitis vinifera*, identificação molecular, fungos endófiticos, antagonismo, região ITS.

NOMENCLATURE

GTDs - Grapevine Trunk Diseases

ha - hectare

sp. - species

spp. - species

BDA - black dead arm

DNA - deoxyribonucleic acid

PCR - polymerase chain reaction

qPCR – quantitative real-time PCR

FEA - Fundação Eugénio de Almeida

VRH - Vale do Rico Homem

VCM - Vinhas de Campo Maior

PDA - Potato Dextrose Agar medium

% - Percetage

gDNA - genomic DNA

CTAB - Cetyl trimethyl ammonium bromide

NaCl - sodium chloride

EDTA - Ácido Etilenodiamino Tetra Acético

ITS - internal transcribed spacer

dNTPs - Phosphate Deoxyribonucleotides

TBE - Tris-Borate-EDTA buffer

NCBI - National Centre for Biotechnology Information

BLAST - Basic Local Alignment Search Tools

Cq - quantification cycle

Bp - base pair

cm – centimetres

Nucleotide bases

A - Adenine

C - Cytosine

G - Guanine

T - Timina

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1.Introduction

1.1 Study Presentation and Relevance

Wine has evolved as part of life, culture and diet for centuries and its role has changed over time from an important source of nutrition to a cultural complement to food. The wine sector makes an invaluable economic, social, agricultural and environmental contribution to wine regions across the world.

Good wines are made from high quality grapes and to obtain this quality is indispensable to provide excellent cultivation of the grapevines, mainly considering the phytosanitary care of the vineyard. Nowadays, fungi are the principal organisms disease-causing in grapevines and Grapevine Trunk Diseases (GTDs) complex have been gaining importance through the years especially due to the inadequate pruning techniques, the lack of strategies against the fungi and the variety of fungi and symptoms involved. Considering that GTDs management is difficult, their control is mainly focused on disease prevention and mitigation. Therefore, the identification of fungi is very important to characterize and have an overview of the disease, besides contributing to the planning of fight strategies.

1.2 Objective and hypotheses

The aim of this work was to investigate, through molecular tools, the endophytes and phytopathogenic fungi associated to the Grapevine Trunk Diseases in the Alentejo region, Portugal. The main scientific questions under this study were:

1. What are the fungi endophytic population found in vineyards in Alentejo region?
2. What are the phytopathogenic fungi associated with GTDs in those vineyards?
3. What will be the influence in the growth of some identified GTDs phytopathogenic fungi by endophytes?

The fungi were isolated from three commercial vineyards in Alentejo region; from two cultivars (Alicante Bouchet and Trincadeira); from GTDs symptomatic and asymptomatic plants and from different plant organs (roots, petioles and offshoots). The following working hypotheses were tested: (i) there are differences in the abundance and diversity of GTDs phytopathogenic fungi between the grapevine cultivars in the three vineyards;(ii) there are differences between the abundance and diversity of GTDs phytopathogenic fungi between symptomatic and asymptomatic plants; iii) there are differences in the abundance and

diversity of phytopathogenic fungi in the different plant organs (roots, petioles and offshoots); and finally iv) there are interference in the GTDs phytopathogenic growth by endophytic fungi found.

1.3 Work Organization

This work is composed by the summary of the work in English and Portuguese, the general index of the work, the figure index, the table index and seven chapters.

The first chapter consists of the introduction, which is divided into three parts: the presentation and relevance of the study, the objective and hypotheses of the study and finally the organization of the work.

The second chapter is the bibliographic review, which is split in four parts. The first part describes the viticulture, its economic importance in the world and in Portugal. The second part characterizes a group of grapevine diseases named "Grapevine Trunk Diseases". The third part emphasizes the importance of Grapevine Trunk Disease's diagnosis and it describes the types of diagnosis. The fourth part indicates the use of endophytes as a control against GTDs.

The third chapter consists of the materials and methods, which describes the all procedures performed during the study.

The fourth chapter describes the results obtained in this research and it is divided in 5 parts. The first part presents the fungi identified obtained from plant material. The second part focus on GTDs fungi identified in this work. The third part present the results of the development of SYBR@ Green Primer primers for *Diaporthe sp*. and the fourth part the development of TaqMan probes specific for *Diaporthe sp.* The fifth part shows the results of the direct inhibition antagonism tests between some GTDs pathogens and some endophytic fungi.

The fifth chapter is devoted to explaining and discuss the results obtained in this research.

The sixth chapter consist of the research conclusions and future perspectives and ideas made from the present study.

The seventh chapter is the bibliographic references where is listed all the material consulted to develop and write the thesis.

2.Bibliography revision

2.1 General aspects of the vine culture

2.1.1 The viticulture and world economic aspects

Grapevine (*Vitis vinifera* L. and *Vitis* spp.), belonging to *Vitaceae* family, is one of the most economically important woody perennial fruit crops in the world (Gramaje *et al*, 2018). *Vitis* is the most studied and cultivated genus in the world with more than 100 species already described (The Plant List, 2013), and *Vitis vinifera is* the most widely grown specie for fresh table grape, juice, dried fruit, and wine. However, some other species, are also used such as *Vitis labrusca,* for fresh table grape and juice; and *Vitis rupestris*, *Vitis riparia, Vitis berlandieri* as breeding rootstock (Gramaje et al., 2018). *Vitis vinifera* was, for a long period, mainly cultivated in regions located between latitudes 30° and 50°, in both hemispheres (Terral *et al*., 2010; Gramaje *et al*, 2018); however, nowadays, it has been growing all over the world (OIV, 2019).

According to The International Organisation of Vine and Wine (OIV) (2019), the 2018 world area under grapevines was estimated in 7.4 million ha, that includes wine grapes, table grapes, drying grapes and the vineyards not yet in production (Figure 1).

The global grape production has been suffering some oscillation; however it reached the highest amount in 2018 (77.8 million ton), comparing with the past 18 years (Figure 2) (OIV, 2019).

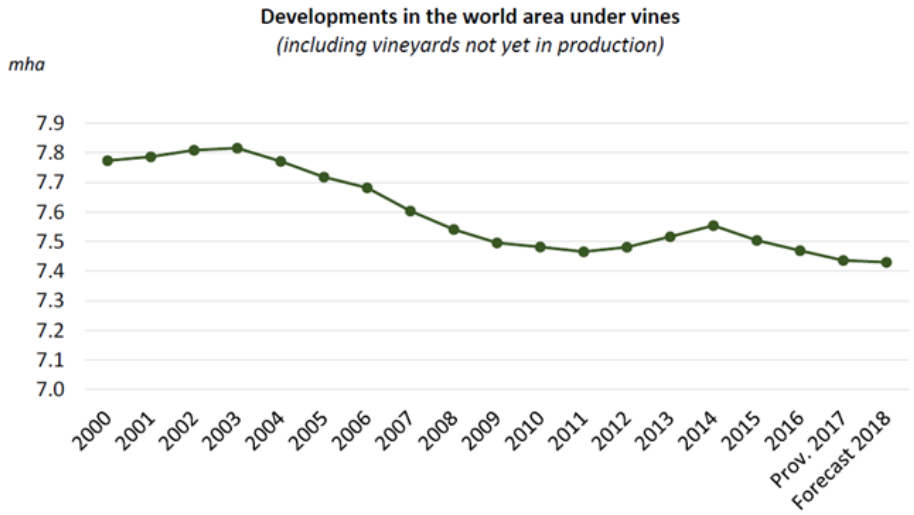

Figure 1. The development in the world area under vines (million ha) in the past 18 years (OIV, 2018).

Figure 2. Evolution of global grapes production in tonnes since 2000 (OIV, 2019).

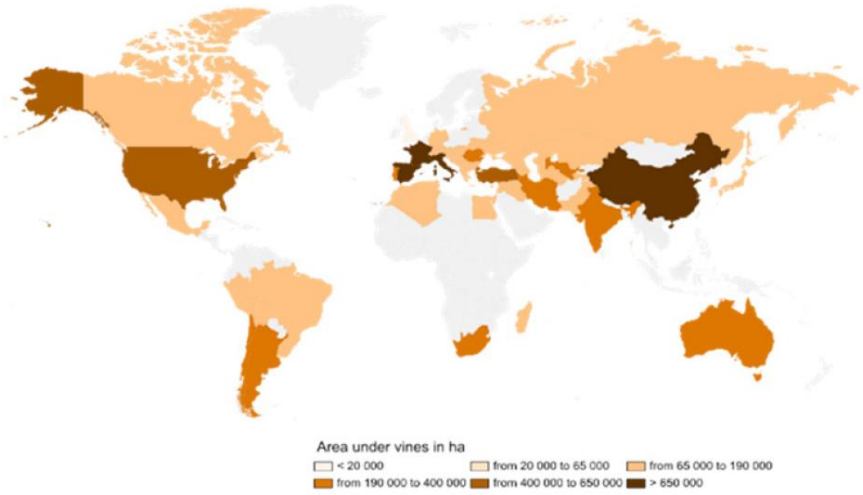

Figure 3. Grapevine areas around the globe (OIV, 2019).

The European continent leads grape production worldwide with 3.55 million ha, followed by 2.04 million ha in Asia, 0.53 million ha in South America, 0.43 million ha in North America, 0.33 million ha in Africa and 0.18 million ha in Oceania (Figure 4) (FAO 2017). According to OIV (2019), in 2018 five countries accounted for approximately 50% of the world's grape production, including Spain (13%), China (12%), France (11%), Italy (9%) and Turkey (6%).

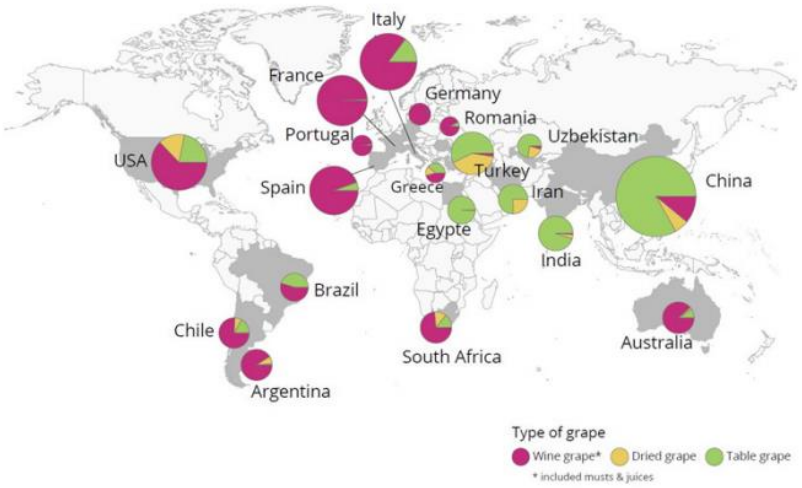

Figure 4. Distribution map of major producers by type of grape in 2018 (OIV, 2019).

million t	2014	2015	2016	2017	2018	Production. 2018 (in %)		
						Table grape	Dried grape[b]	Wine grape [c,d]
China	12.5	13.2	12.6	13.1	11.7	84.1%	5.6%	10.3%
Italy	6.9	8.2	8.4	6.9	8.6	13.5%	0.0%	86.5%
USA	7.1	6.9	7.0	6.7	6.9	16.3%	18.1%	65.6%
Spain	6.1	6.0	6.3	5.0	6.9	4.0%	0.0%	96.0%
France	6.2	6.3	6.3	5.0	6.2	0.4%	0.0%	99.6%
Turkey	4.2	3.7	4.0	4.2	3.9	56.1%	40.7%	3.2%
India	2.6	2.6	2.6	2.9	2.9	92.6%	5.9%	1.5%
Argentina	2.7	2.5	1.9	2.1	2.7	0.9%	5.5%	93.7%
Chile	2.2	2.7	2.2	2.0	2.5	26.0%	3.9%	70.2%
Iran	2.3	2.3	2.3	1.9	2.3	76.3%	23.7%	0.0%
Australia	1.8	1.9	2.0	2.2	1.9	7.1%	1.9%	90.9%
South Africa	1.9	2.0	2.0	2.0	1.8	15.8%	15.5%	68.7%
Uzbekistan	1.4	1.6	1.6	1.6	1.7	78.4%	17.8%	3.7%
Egypt	1.6	1.7	1.7	1.7	1.6	99.5%	0.0%	0.5%
Brazil	1.4	1.5	1.0	1.7	1.6	53.5%	0.0%	46.5%
Germany	1.2	1.2	1.2	1.0	1.4	0.4%	0.0%	99.6%
Romania	0.7	0.8	0.8	1.0	1.3	6.9%	0.0%	93.1%
World	74.3	76.7	75.5	73.0	77.8	36%	7%	57%

Figure 5. Major grape producers from 2014 to 2018, in million tonnes and percentage of grape production destination (table grape, dried grape, wine grape) in each country (OIV, 2019).

According to OIV (2019), in 2018, 57% of grapes production was destinated to wine, 36% to table grape production and 7% to dried grape production. China is today the major

grape producer with 11.7 million ton (84.1% table grape, 5.6% dried grape and 10.3% wine grape), followed by Italy with 8.6 million ton (13.5% of table grape and 86.5% of wine grape). Spain and France are also among the top 10 grape producer in the world, however the majority of grape production goes to the wine sector, 96% and 99.6%, respectively (Figure 5).

2.1.2 The importance of grapevine in Portugal

The hot dry summers and schists soil turn Portugal to an ideal country to grown grapes. Vertically bedded schists soil permits the roots to extend deeply into the soil for searching water and nutrients (Unwin, 1991). Viticulture was already established in Portugal in Roman times (Gonçalves, 1984), mainly in the north-west and in the basin of the Douro river, where grapevines were trained precariously on small wooden fencelike structures. England and Portugal had established close economic links since their first political alliance in the Treaty of Windsor in 1386, which led to the beginning of an interest of English traders in the lucrative Viana do Castelo wine export trade in the northwest of the country. The trade between these two countries became stronger after the European wars of the late seventeenth and early eighteenth century, when Britain paved the way for a considerable expansion of the wine trade. The declining quality of the wines and the search for better quality wines for British merchants, led the Portuguese government to establish a monopoly over the trade, and one result of this was that the Upper Douro valley became the first officially demarcated wine region in the world (Unwin, 1991).

In the twentieth century other regions were demarcated, and in 1986 new regions were created, with the objective of reinforcing the quality of Portuguese wines. According to data from the International Organization of Vine and Wine (2017), the area of vineyard planted in Portugal has been decreasing over the last 20 years, however the wine production does not follow this decrease in reason of the better agricultural practices and the better use of resources (Santos, 2018).

Although, the wine production in Portugal has been increasing (7.0 million hl in 2015, 6,8 million hl in 2017 and 6,1 million hl in 2018), the country is still among the twelve major wine producers in the world, and the wine consumption grew from 4.3 million hl in 2014 to 5,5 million hl in 2018 (OIV, 2019).

In Alentejo, the second largest wine producer region (Figure 6) (Wines of Portugal, 2015), as in all over Portugal, wine and history walk together since before the romans time (Freire and Ramos, 2019). However, it was only after the regulation of the first denomination

of region in 1988, with benefits from the European Union's financial, the entrepreneurial spirit of the region's economic players and the creation of wineries cooperatives, that the Alentejo modern oenology was capable to develop and settle (CVRA, 2013).

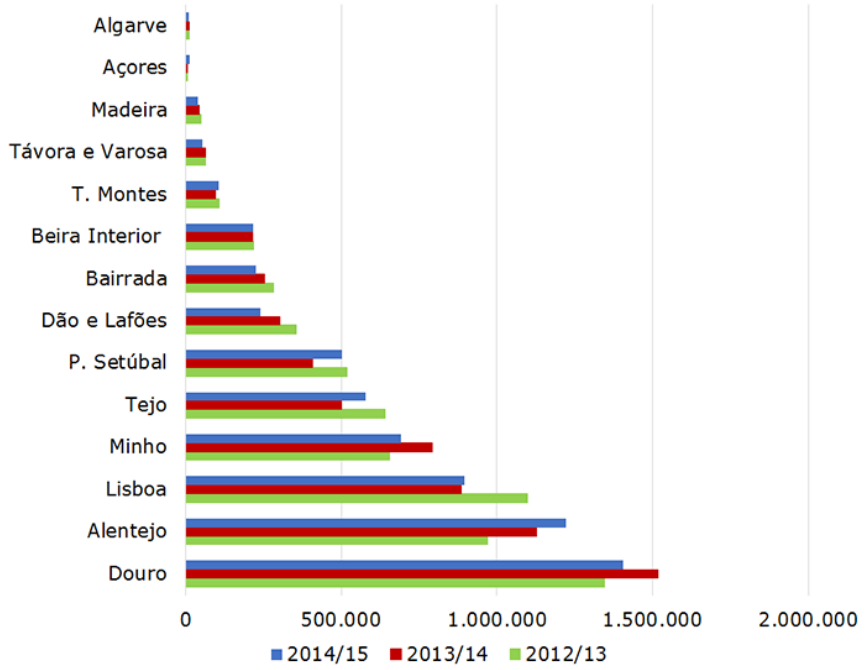

Figure 6: Total wine production in Portugal by Region (hl) from 2012 to 2015 (Wines of Portugal, 2015).

Alentejo is a plain region characterized by a variety of, not very fertilized soils. . The sunny, hot and dry weather in this region contributes for great maturation of the grapes. All these characteristics contribute for the adaptation of the grapevine, which occupied approximately 22.000 hectares in this region in 2018 with 1.07 million hl of wine production corresponding17.8% of the national production (CVRA, 2019).

2.2 Grapevine trunk diseases (GTDs)

2.2.1 General aspects

GTDs are among the most important and the most destructive fungal diseases affecting grapevines in all the major growing regions of the world (Bertsch *et al.,* 2012; Pinto *et al*, 2018; Gramaje *et al*, 2018). Despite having been known since the end of the 19th century, GTDs importance and impact on plant health have only been recognized decades ago (Bertsch *et al.,* 2012) and they have been rapidly growing concern in all wine producing countries (Fontaine *et al*., 2016). These destructive diseases cause in vineyards several damages every year (Fontaine *et al*., 2016). GTDs fungi subsequently grow, decay the wood and slowly kill the vines (Rolshausen and Kiyomoto, 2007). Replacement of dead grapevines

worldwide cost was estimated to be more than 1.5 billion dollars per year (Hofstetter *et al*., 2012).

According to some authors, GTDs have impact on grape and wine economic production reducing the productivity, quality and longevity of vineyards (Hofstetter *et al*., 2012; Bruez *et al*., 2012; Dissanayake *et. al.*, 2015; Gramaje *et al*., 2016; Kaplan *et al.,* 2016). The productivity is mainly reduced over time by the death of the spurs, canes, and/or cordons (Gramaje *et al*, 2018).

"Esca", the white rot caused by basidiomycetes, was the first GTD reported worldwide, to which several symptoms in the crown was linked. Only in 1990s this manifestation was recognized as part of a larger problem, involving a complex of different diseases known as esca complex. Later, many authors discovered different pathogens causing different wood symptoms in the grapevine (Modello *et al.*, 2017). Nowadays, the term 'Grapevine Trunk Diseases' refers to a set of complex diseases, which include esca, Petri disease, Eutypa dieback, Botryosphaeria dieback, Phomopsis dieback and black foot (Bertsch *et al.,* 2013; Chebil *et al*., 2017; Pintos *et al.*, 2018). However, these different diseases overlap in the same vine and develop at different stages of the vine, since the plants can be infected by different pathogens over the years due to the multiple infection opportunities. Therefore, the grapevines can be affected by one or more GTDs at the same time (Modello *et al.,* 2017; Gramaje *et al*, 2018)

Although GTDs are caused by different fungal pathogens (Mugnai *et al*., 1999), their life cycles and epidemiology are very similar (Berstch *et al*., 2013). To date, up to 133 fungal species belonging to 34 genera have been associated with GTDs worldwide (Gramaje *et al.*, 2018), including the following 18 genera: *Botryosphaeria*, *Diplodia*, *Lasiodiplodia*, *Fusicoccum*, *Neofusicoccum*, *Dothiorella*, *Phomopsis*, *Diaporthe*, *Eutypa*, *Eutypella*, *Diatrypella*, *Diatrype*, *Cryptovalsa*, *Cylindrocarpon*, *Phaeomoniella*, *Fomitiporia*, *Phaeoacremonium*, and *Greeneria* (Úrbez-Torres *et al*., 2013; Chebil *et al*., 2017; Pintos *et al.*, 2018).

Open wounds are the most likely way for spores of GTDs fungi infect grapevines. These wounds are commonly caused by retraining, trimming, and de-suckering (Makatini *et al*., 2014). However, annual pruning wounds are the mainly way of fungi entrance in the plant (Gramaje *et al.*, 2018). In a Mediterranean climate, trunk diseases of grapevines fungi release their spores during rainfall and infect grapevines through pruning wounds during the dormant season (Diaz and Latorre, 2013). Another way of spreading the disease is the release of

spores, from fruiting bodies produced in dead wood, in the presence of water and the dispersion them by wind (Rolshausen and Kiyomoto, 2007).

Some authors emphasize that one of the main causes of GTDs infection occur during nursery plant production processes (Larignon and Dubos, 1997; Agustí-Brisach *et al*., 2013; Gramaje *et al*., 2018), causing decreases in survival rates of grafted grapevines in nurseries and young vineyards (Gramaje *et al*., 2009; Agustí-Brisach *et al*., 2011; Gramaje and Armengol, 2011; Gramaje *et al*., 2018). The impacts caused in young vines has mainly been attributed to *Cylindrocarpon* species, Petri disease fungi, and *Botryosphaeriaceae* species that could act alone or in combination (Probst *et al*., 2012; Pintos *et al.*, 2018). Stunted growth, reduced vigour, delayed or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, bud mortality, fruit rotting, cane bleaching, failure of the graft unions, wilting and dieback are the most common external symptoms of the GTDs diseases in young grapevines, and these symptoms can be accompanied by sunken necrotic root lesions and reductions in the root biomass and root hairs (Gramaje and Armengol, 2011).

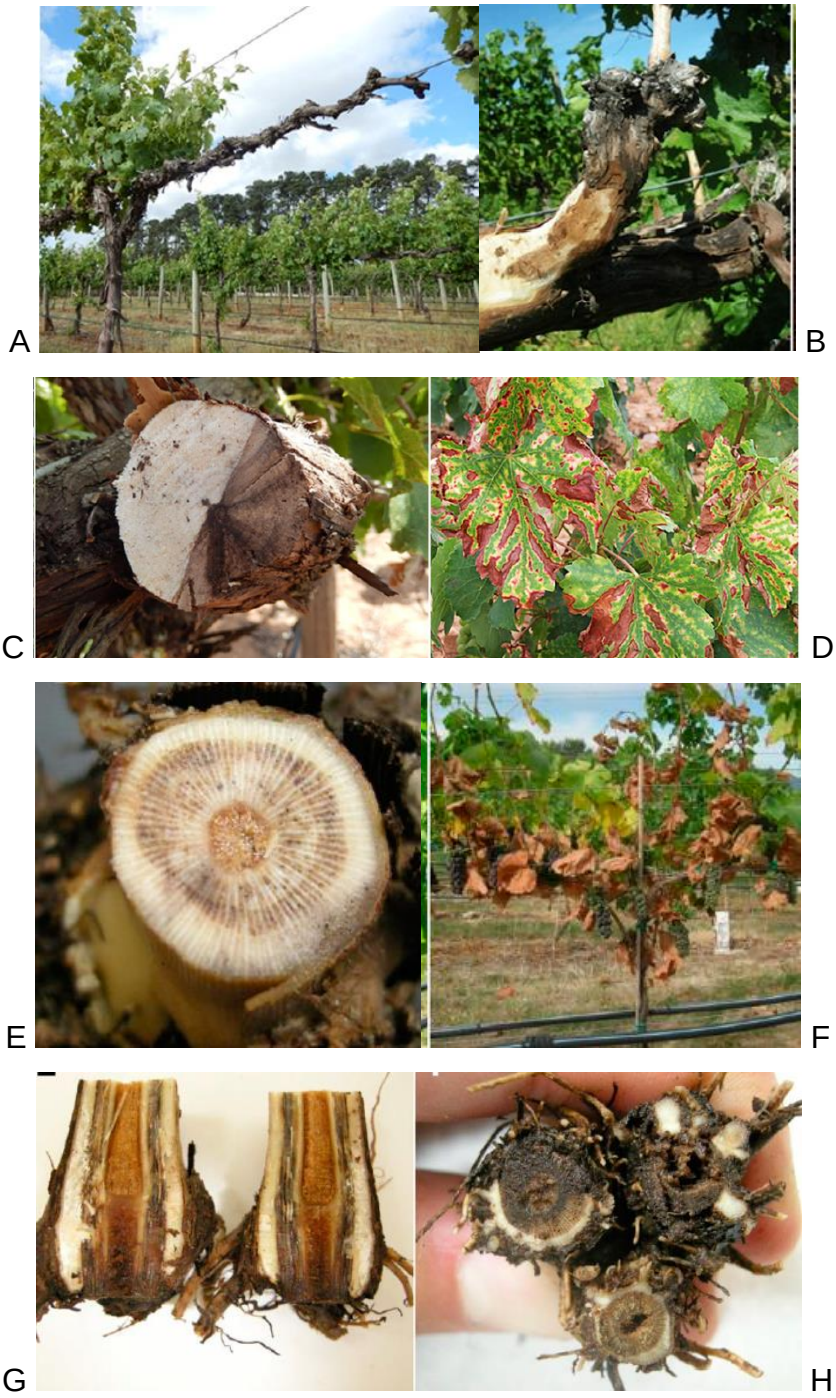

Figure 7. Some symptoms of GTDs. **A)** cordon dieback and **B)** spur dieback observed in vines affected by "Botryosphaeria dieback". **C)** cordon with wedge-shaped canker infected by "Botryosphaeria dieback". **D)** leaves symptoms characteristic of "grapevine leaf stripe disease". **E)** rootstock cross-section showing dark xylem vessels infected by "Petri disease". fungi. **F)** withered leaves and buds caused "Petri disease" or "black foot". **G)** longitudinal-section of rootstock with necrotic streaks infected by "Petri disease". **H)** rootstock wood infected by "black foot" (Gramaje *et al.* 2018).

GTDs fungi live in and colonize the wood of the perennial organs (Mugnai et al. 1999) impeding the water transport in plants by clogging the xylem vessels (Carter, 1991),

consequently decreasing the adsorption capacity of water and nutrients by the plant (Andolfi *et al*., 2011). The common effects in adults vines are: cankered wood, poor vigour vines, stunted shoots (Gramaje *et al.* 2018), delayed budburst, dead buds, dieback, stunted development, chlorosis, apoplexy (Mondello *et al*., 2017), wood necrosis, wood discoloration (Bertsch *et al*. 2012). Leaf symptoms depend on the fungi involved in the infection and can include scorching, dropping, shrivelling, chloroses, discolorations surrounding dry, necrotic tissue on the leaf blade (Gramaje *et al.* 2018). However, these effect are not detectable for years, due to the slowly growth of the pathogens into the vascular tissues (Carter, 1991) or due to the fact that GTDs fungi may live as endophytic , asymptomatically, a part of their life inside a plant and, at some point, associated with plant stress, modify their behaviour becoming pathogenic, which leads to the development and expression of the disease symptoms (Rolshausen and Kiyomoto, 2007; Hofstetter *et al*., 2012).

The long latency time of these diseases enables the visibility of aerial symptoms after several years (Carter, 1991). At the time of the appearance of leaf symptoms, the disease has already developed to an irremediable point leading rapidly to the death of a cordon or of the entire plant (Moller and Kasimatis, 1981). Due to the high incidence and severity of these diseases, which lead to low yields, the maintenance of infected plants becomes unsustainable, so the wisest measure is the removal of infected plants (Pinto, 2010).

Eutypa dieback, esca and Botryosphaeria dieback are slow perennial diseases, which symptoms usually appear on 7 years old or older grapevines (Bertsch, 2012). Their causal pathogens attack long-lasting organs compromising the plants sustainability, what can cause shorten vineyard longevity and induce vine death (Moisy, 2017*).* The long latency time and the high variability of symptom expression hampers the diagnosis of GTDs fungi in vineyards and the detection of the internal symptoms (Moller and Kasimatis, 1980). Furthermore, some symptomology overlaps among different GTDs what makes the accurate identification in the field even more difficult (Gramaje *et al*, 2018). Some grapevine trunk disease symptoms are often confused with other diseases, occasional plagues, nutritional and water deficits what mislead on management solutions (Sofia *et al*., 2013).

According to several authors, there are not any grapevine cultivated or wild known to be resistant to trunk diseases (Surico *et al*., 2006; Wagschal *et al*., 2008; Larignon *et al*., 2009). Complete eradication of the fungi is not possible due to the lack of strategies for fighting GTDs, the limitation of information on control measures, inadequate pruning practices and the necessary protection of the environment. (Bertsch *et al*, 2012). The management of GTDs is influenced by the type of the disease and/or by the pathogens involved what makes

the GTDs control even more difficult. For all the reason listed above, GTDs control is primarily focused on disease prevention and mitigation (Úrbez-Torres, 2011). Therefore, some authors believe that the most effective procedure to reduce infections by fungal trunk pathogens is the deployment of integrated management program including physical, chemical, biological, and/or other control strategies (Halleen and Fourie, 2016; Gramaje *et al*, 2018).

2.2.2 Grapevine trunk disease (GTD): around the world

From the end of the 1980's, the incidence of the Esca complex diseases increased throughout Europe (Larignon *et al*., 2008). However, the most drastic increase of these diseases in Europe happened in 2003, after the definitive banishment of the most effectively fungicides used to keep esca under control (arsenate), due to its high risks for humans and environment, which was replaced by less effective fungicides (Spinosi *et al.,* 2009). In Spain, trunk diseases grew up from 1.8% of degree affections in vineyards to 10.5% in 2007 (Rubio and Garzón, 2011).

According to a survey led by the French General Directorate of Food in 2012, close to 13% of French vineyard was affected by trunk diseases (Grosman and Doublet, 2012). In 2014, diseases known as esca, Botryosphaeria dieback and Eutypa dieback lowered potential wine production by 13% in France, according to the agriculture ministry and French Wine Institute. The agriculture ministry and French Wine Institute said more than 100,000 hectares of vineyard was lost in 2014 and between 10 to 15% of potential production was lost in 2015 last year, what costed France the equivalent of 1.14 billion euros annually in lost wine production (Fontaine *et al.*, 2016).

In Italy, it was noticed that the incidence of the disease on plants, of 15 to 18 years, fluctuated around 12 to 19% for white grapes and around 8 to 10% for the red grapes. In some regions under extreme conditions of central and southern Italy esca incidence reached 60% to 80% in some old vineyards (Romannazzi *et al*., 2009).

GTDs are widely spread all over the grape Portugal regions (Fontaine *et al.*, 2016) and, grapevine decline and mortality in vineyards often related to GTDs cause economic losses. However, grapevine growers do not often understand their causes, due to the similarity of GTDs symptoms with nutritional problems, virus, occasional plagues and water deficits (Sofia *et al*, 2013). Eutypa dieback, Petri disease, Botryosphaeria dieback are diseases present in Portugal (Gramaje et al., 2018)

In other parts of the world, GTDs have also played an important role in the grape production chain. In California (USA), a loss of at least US$260 million per year was attributed

to trunk diseases (Fontaine *et al*., 2016). In Australia, economic loss around $8.3 billion in the wine industry was linked mainly to Eutypa dieback (Ridgway *et al.*, 2014; Fontaine *et al*., 2016).

2.2.3 Main diseases associated to GTDs

2.2.3.1 Esca disease complex and grapevine leaf stripe disease (GLSD)

The esca disease name derives from the Latin for ‘tinder’ and it was used in the beginning of the 20th by grape growers in southern Italy (Surico, 2009) probably because of the presence of rotted trunk wood noted mainly in apoplectic plants, which were in fact used as tinder. The typical foliar discoloration caused by apoplexy and/or rotted trunk wood led to the use of ‘esca’, even in absence of these problems in the plant. Noting the variety of the symptoms caused by esca, many researchers have defined esca as a complex of diseases. However, the term ‘esca’ is still commonly used to refer to most of the diseases forming the complex (Bertsch *et al.*, 2012).

The esca disease complex is mainly caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*, *Fomitiporia mediterranea* (Murolo and Romanazzi, 2014) and several basidiomycetes species (Fischer, 2006), belonging to the genera *Inocutis, Inonotus, Fomitiporella, Fomitiporia, Phellinus*, *Phaeoacremonium* and *Stereum* (Cloete *et al*., 2015). Other species such as *Eutypa lata* and *Stereum armenicum* can also play roles in the esca disease complex (Bertsch *et al.*, 2012).

The aetiology of esca disease has been studying for many years and can be still a mystery, once a broad range of taxonomically unrelated fungal trunk pathogens and even endophytic bacteria have been isolated from wood tissues of esca diseased vines, which the role and interaction with the primary fungi responsible for disease symptoms is still uncertain. The main hypothesis is that young vines are first infected with the pioneer fungi *P. chlamydospora* and/or species of *Phaeoacremonium* and can later develop esca symptoms following further colonization by several basidiomycetous species (Gramaje *et al.*, 2018).

Esca disease was always associated to old vineyards. However, nowadays, the disease has been a significant problem in newly established vineyards (Murolo and Romanazzi, 2014). The disease in mature vines is associated with symptoms that can range from mild to severe and chronic (Martín *et al.*, 2012). The chronic or mild form of the disease is known as "grapevine leaf stripe disease" (GLSD), and its leaf symptoms of affected vines are highly variable (Lecomte *et al*., 2012). The most characteristic symptom of "grapevine leaf stripe disease" is the ‘tiger-stripe’ pattern in the leaves (Gubler *et al*. 2015), which present

multiple banding discolorations surrounding by dry, light or red-brown necrotic tissue, edged by narrow red or yellow blotches and superficial brown to purple spots scattered over the surface of berry epidermis (Martín *et al.*, 2012). On the epidermis of white cultivars berries is common to find superficial small reddish and dark spots, known as 'black-measles' (Mugnai et al., 1999; Gramaje *et al*, 2018).

The severe form of esca is called apoplexy and it is characterized by a sudden wilting in the leaves (Martín *et al.*, 2012), in the shoots or in the entire plant (Mugnai *et al*., 1999). Leaf symptoms include scorching, dropping, and shrivelling (Mugnai *et al*., 1999). Other symptoms can be included such as shrivelling of fruits (Martín *et al.*, 2012), drying of grape clusters (Mugnai *et al*., 1999), death of cordons or death of the plant in midsummer (Murolo and Romanazzi, 2014).

Foliar symptoms usually appear several years after the fungus infection in the plant, when the wood symptoms have already developed (Surico *et al*., 2008). Foliar symptoms of both forms of esca appear in late spring or summer, and can vary from year to year (Fischer, 2002), indicating that several factors are probably involved in their development (Bertsch *et al*, 2012). On the trunk, of both forms, the symptomatology is characterized by dark brown to black vascular streaking, pink-brown areas on the margin of necrotic tissues (Surico et al., 2006; Murolo and Romanazzi, 2014; Gramaje *et al.*, 2018) and white rot which gives the wood a silver appearance (Fischer, 2002).

2.2.3.2 Eutypa dieback

Eutypa dieback is recognized as causing serious damage in some countries, mainly in Australia, France, and California (Modello et al., 2017). Eutypa dieback, or eutypiosis, is a disease caused by 24 species in the Diatrypaceae. Other Diatrypaceous genera include Anthostoma, Cryptosphaeria, Cryptovalsa, Diatrype, Diatrypella, and Eutypella. However, the most virulent and common specie is *Eutypa lata*, which is the only species known to be responsible for the foliar symptoms (Gramaje *et al.*, 2018). This fungus has a wide host range and it is frequently found in vineyards that receive more than 250 mm of rainfall per year (Bertsch *et al*, 2012). This fungus colonizes vines, eventually causing a brown sectorial necrosis in wood as well as stunted vegetative growth. The expression of external symptoms is usually manifested several years after the infection (Moisy *et al*., 2017).

The type of wood decay that is caused by *Eutypa lata* is classified as a "soft rot" (Rudelle *et al*., 2005) because it develops inside the secondary walls forming cavities (Larignon *et al*., 2009). Plants colonized by *Eutypa dieback* present internal, necrotic and

wedge-shaped staining in the cross-section of cordons and trunk. Cordon dieback and loss of spurs are also symptoms caused by this fungus. Over the dieback progresses, external cankers can appear which characterized by flattened areas of the wood with no bark what can lead an eventual death of the plant (Kovács and Sándor, 2016; Gramaje *et at*, 2018).

Eutypa lata produces secondary metabolites in the wood, mainly acetylenic and heterocyclic compounds (Bertsch *et al*, 2012), which are toxic to the vine and are made only by this fungus (Gramaje *et at*, 2018). The foliar symptoms are caused by these toxic metabolites produced in the wood (Mahoney *et al*., 2005), which will be manifested later, 3 to 8 years after infection (Gramaje *et at*, 2018) and can vary from year to year. Vine colonized by *Eutypa dieback* can present stunted or shriveling shoots (fan leaf) with chlorotic leaves, wrinkled and ripped leaves with marginal necrosis (Fontaine *et al*, 2016). Bunches on stunted shoots ripen unevenly, are small, and in severe cases, berries shrivel and die (Gramaje *et at*, 2018).

2.2.3.3 Petri disease

In 1912, an Italian scientist called Lionel Petri completed for the first time Koch's postulates and demonstrated that the necrosis observed in the vascular system of young grapevines were caused by *Cephalosporium* and *Acremonium* spp. (Gramaje *et al*., 2018). After that, his surname was given, during the second Congress IWGTD in 2001, to name the disease associated to the process of decay of the vascular tannings in vine (Larignon, 2012).

The fungal species associated with Petri disease include: *Phaeomoniella chlamydospora*, 29 species of *Phaeoacremonium, Pleurostoma richardsiae*, and six species of *Cadophora*. Among the different *Phaeoacremonium* and *Cadophora spp*. occurring in Petri disease symptomatic vines, *Phaeoacremonium minimum* and *Cadophora luteo-olivacea* are the most prevalent (Gramaje *et al.,* 2018). Other genera of fungi that might be associated to the decline in nurseries or in young vineyards are *Acremonium* (*A. charticola* and *A. ochraceum*) and *Phialemoniopsis curvata* (= *Phialemonium curvatum*) (Perdomo *et al.,* 2013).

Petri disease affects mostly 1- to 5-year-old grapevines (Martín *et al.*, 2012; Bertsch *et al*, 2012) and is recognized when dark striations are found in the trunk cut in the longitudinal section and when dark-coloured phenolic compounds from xylem vessels exude from the vessels when cut in cross sections (Gramaje *et al.*, 2018). The external symptoms include stunted growth or the complete cessation of growth, leaf chlorosis, loss of yield and a decline in vigour. Infected plants form teloses, gums, and phenolic what became xylem vessels brown

because of the blockade what accentuate water stress and lead to insufficient water and nutrient supplies to the vegetative parts of the plant (Martín *et al.*, 2012).

Petri disease has been reported in different parts of the world where grapevine is cultivated (Gramaje *et al.*, 2018). In Portugal, this disease was first detected in the 90s, in young vines when they were planted following an intensive replanting of old vineyards (Rego *et al*., 2000).

2.2.3.4 Black foot

The symptoms of black foot were first described in the 1960s under the name of 'gangrene' (Maluta and Larignon 1991). Initially, the disease was associated with a "*Cylindrocarpon*" species in Italy in 1975. Nowadays, it is known that up to 24 species in the genera *Campylocarpon, Cylindrocladiella, Dactylonectria, Ilyonectria, Neonectria, and Thelonectria* are responsible for the symptoms associate to "black foot disease" (Gramaje *et al*, 2018).

Black-foot is an important disease of grapevines which affects grapevine propagation material, newly planted vines or established vineyards in most of the wine-producing countries worldwide (Agusti-Brisach *et al*., 2014), and it is considered the most significant grapevine phytosanitary problem in nurseries (Gramaje and Armengol, 2011). It has been reported, over the last decades, as responsible for loss of productivity, decline and death of young vines (Agusti-Brisach *et al*., 2014) and it can be recognized by black, sunken, necrotic lesions on roots, reddish brown discoloration in the base of the trunk of affected vines (Gramaje *et al*, 2018), with a reduction in root biomass and root hairs (Gramaje and Armengol, 2011). As external symptoms of black food include stunted growth, reduced vigour, retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting and dieback (Gramaje and Armengol, 2011).

According to Agusti-Brisach *et al*. (2014) nursey soil is an important source of inoculum for black-foot pathogens, once some of disease-causing pathogens species are known to be saprobes in soil. Thus, nursery soils are the major source of black-foot inoculum, causing infection of grafted vines (Chaverri *et al*., 2011). Grafted vines are frequently infected when cuttings are in contact with infected nursery soils during some practices, mainly after planting of callused cuttings or, covering grafted cuttings with soil (Halleen *et al*., 2003).

2.2.3.5 Botryosphaeria dieback

"Botryopshaeria dieback" was in the past named "black dead-arm disease" and this name was used for many years to describe similar symptoms observed in grapevines around the world. Black dead arm (BDA) was first described in 1974 in Hungary, associated with *Diplodia mutila* and later others *Botryosphaeriaceae* species, mainly *Diplodia seriata* and *Neofussicoccum parvum*, were also associated with this disease. Noting that symptoms caused by species belonging to the family *Botryosphaeriaceae* were similar, scientists proposed, in the 1990s, to rename the disease as "botryopshaeria dieback" (Gramaje *et al.*, 2018).

To date, 26 botryosphaeriaceaous taxa in the genera *Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia, Neofusicoccum, Neoscytalidium, Phaeobotryosphaeria*, and *Spencermartinsia* have been associated with Botryosphaeria dieback of grapevines (Úrbez-Torres 2011; Yang *et al.* 2017).

The first symptoms of "Botryosphaeria dieback" appear only 1 or 2 years after the infection (Úrbez-Torres *et al*. 2006) and are mainly observed in vineyards over 8 years old (Gramaje *et al*, 2018). Some symptoms caused by *Botryosphaeriaceae* in grapevines, mainly canker symptoms, are difficult to differentiate from symptoms caused by others grapevine trunk disease pathogens, such as *Eutypa lata*, *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* (Chebil *et al*., 2017). Wedge-shaped perennial cankers are the most common wood symptom caused by "Botryosphaeria dieback"; which is similar to Eutypa dieback symptom. However, plants infected by Botryosphaeria dieback generally do not exhibit leaf symptoms (Gramaje *et al.*, 2018). Sometimes only chlorosis weaknesses or some deformations of leaves are detected (Larignon, 2012).

Other very typical symptoms in the grapevine trunk caused by "Botryosphaeria dieback" are sectorial necrosis with vascular discoloration (Fontaine *et al.*, 2016) and circular to nonuniform central staining of the wood in cross-sections of affected wood (Gramaje *et al.*, 2018). The affected plants by "Botryosphaeria dieback" are characterized by branches low percentage of bud break caused by shoot dieback, bud and xylem necrosis (Larignon, 2012; Úrbez-Torres 2011).

According to Pinto et al. (2018), some surveys carried out in nurseries showed that grapevine infections by *Botryosphaeriaceae* fungi may originate from the propagation nurseries.

2.2.3.6 Phomopsis dieback

Phomopsis dieback disease was named first as Excoriose and it is primarily caused by *Phomopsis viticola* (Ascomycota, Diaporthales; syn. = *Diaporthe ampelina*) (Barba *et al*., 2017), which is known as the causal agent of Phomopsis cane and leaf spot (Phillips 2000). Although *Phomopsis viticola* has been detected worldwide wherever grapes are grown, "Phomopsis cane and leaf spot" is more severe in grape-growing regions characterized by a humid temperate climate through the growing season. "Phomopsis cane and leaf spot" symptoms can be observed on leaves as small pale green to yellow spots with necrotic centres, in canes as brown to black necrotic irregular-shaped lesions, and clusters as rachis necrosis and brown, shrivelled berries close to harvest (Úrbez-Torres and Gubler, 2013).

In a research by Baumgartner *et al*. (2013), grapevine severely affected by "Phomopsis cane and leaf spot" showed symptoms of "Phomopsis dieback" in the vineyards (). The most characteristic symptoms attributed to "Phomopsis dieback" are similar to those resembling "Botryosphaeria dieback" and include perennial cankers in the framework of the vine and lack of budbreak from infected spurs (Úrbez-Torres *et al*., 2013).

According to some authors Until 2015, seven species in the genera Diaporthe showed to be were pathogenic on grapevine wood (Baumgartner *et al*., 2013; Dissanayake et al. 2015; Úrbez-Torres *et al.*, 2013).

2.3 Grapevine trunk disease's diagnosis

2.3.1 Classical methods

Knowing that GTDs is a complex of diseases caused by fungi and complete eradication is not possible, their characterization and detection are crucial to early diagnosis (Fleurat-Lessard *et al*., 2010). The diagnosis of GTDs diseases is mainly performed by foliar observations, which is completed by the harvest of a piece of wood for detection of possible trunk symptoms that develops in the wood tissues because of the pathogen infection (Valtaud *et al.*, 2009). This method can be imprecise due to the variation of the foliar symptoms from year to year in the same branch (Fleurat-Lessard *et al.*, 2010).

The subsequent characterisation of the pathogen involves culturing and isolation of the fungus from the infected plant material and the observation of the mycelium and the conidia of the growing fungus through microscope (Fleurat-Lessard *et al.*, 2010). For Retief *et al.* (2005) these traditional analyses of the isolated and cultured fungal samples are precise and offered another advantage, as living microorganisms can be storage for further phytophathological investigations (Ward *et al*.,1990). However, symptoms can be unreliable

due to the lack of symptomatology which some plants present for a long period (Martín *et al.*, 2012). Other limitations presented by classical method are: the time-consuming due to the slow growth of the pathogen, long period needed to distinguish the species based on their morphology (Valtaud *et al*., 2009), and the destruction of part of the plant because of the necessity to remove parts of some plant organs. Conventional methods detect only active cells, which is grown on culture medium under laboratory conditions and take around 3 months for identification (Martín *et al.*, 2012).

According to Guo *et al*. (2000), isolates obtained using traditional techniques are limited due to the highly probability of some endophytes might not sporulate on the cultures and the fail in the isolation techniques. Furthermore, identification of some fungi such as *Fusicoccum* sp., based on morphological characteristics requires an experienced observer, once morphological characteristics can be similar among some species. This is the case of *Botryosphaeria* species which have similar growth and their characteristics can be strongly influenced by the substrate in which they are produced (Hartman *et al.*, 2017).

A second approach has been proposed by Mahoney *et al.* (2005), using Eutypa dieback as an example, based on detection of metabolites secreted by fungi in infected plant tissues, with also requires a time-consuming for isolation and for the analysis of the extracted products. In some cases, like esca disease, this method is not specific, once the same compounds can be secreted by others grapevine pathogens (Fleurat-Lessard *et al.,* 2010).

The diagnosis of GTDs using traditional methods is not easy due to the diversity of the fungi involved and the difficult in attributing the responsibility for a given symptomatology to a single causal agent. Therefore, accurate, rapid, and affordable disease testing is needed to overcome the disadvantages of the traditional cultivation approach (Martín *et al*., 2012) based on independent culture techniques, molecular DNA, and phylogenetic techniques (Ettenauer *et al.,* 2014; Pouzoulet *et al*., 2017).

2.3.2 Molecular methods

Molecular DNA techniques, such as polymerase chain reaction (PCR), have provided opportunities for a rapid and precise way to detect fungal plant pathogens, using DNA from culture and from plant tissues (Ma and Michailides, 2012). The DNA extraction from plant tissues and the following amplifications with specific primers skip the intermediate steps (culturing and isolation), providing results in about 1 day (Ridgway *et al*., 2002) and can detect DNA from alive and/or dead cells (Martín *et al.*, 2012).

After the production of the first thermal cycler instrument in 1987, molecular techniques revolutionated the detection of pathogens and molecular methods, essentially based on PCR, are now indispensable tools for the diagnosis diseases (Paiva-Cavalcanti *et al.*, 2010). PCR-based methods have been developed and adapted to solve problems in fungal taxonomy techniques and to identification of fungi (GUO *et al*., 2000; Ettenauer *et al*., 2014). PCR-based methods do not require a previous experience with the pathogen, it is easy to learn, provide reliable results and it can be based on amplification of specific DNA sequences and genes, randomly amplified regions, or species-specific repetitive sequences (Ma and Michailides, 2012).

2.3.2.1 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) is a molecular method based on *in vitro* DNA replication (Paiva-Cavalcanti *et al.*, 2010). This method involves *in vitro* enzymatic amplification of specific DNA sequences which use two oligonucleotide primers that hybridize to opposite strands and flank the interest region of the target DNA. The amplification of the DNA sequences by DNA methods consists in repetitive cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase, which results in exponential accumulation of a specific fragment whose termini are defined by 5' end of the primers (Atawodi *et al*., 2011).

PCR methodology initially requires the identification of part of the DNA target for the development of primers or probes that will hybridize specifically to the target sequence (Yang and Rothman, 2011). Traditional PCR primers have been successfully used for studying the epidemiology of the species associated to Petri disease and esca (Martín *et al*., 2012) and for detection or identification of either *Eutypa lata*, *Phaemoniella* spp. and *Phaeoacremonium* spp. (Gramaje *et al*, 2018).

Conventional PCR are highly sensitive and specific. However, it has some limitations including the requirement of agarose or polyacrylamide gel for electrophoresis, risk of contamination, lack of quantitative capacity, and the use of reagents such as ethidium bromide, which is harmful to the health of the operator (Paiva-Cavalcanti *et al.*, 2010).

In the past decades, conventional PCR technique has been modified to overcome limitations, expand its use and versatility (Yang and Rothman, 2004). The multiplex-PCR enables the use, in the same reaction, of several pairs of primers with simultaneous amplification for multiple target DNA sequences; allowing the amplification of more than one DNA sequence (Bahk *et al*., 2004). Nested-PCR, however, use different two pairs of primers

to amplify internal DNA sequences in a selected target. The first pair is used for an initial reaction and its products are subjected to a second amplification with another pair of primers. This technique presents increased sensitivity and specificity, but it reveals risk of contamination by the amplified product from the first reaction (Paiva-Cavalcanti *et al.*, 2010). PCR-based method is also easy to learn and can provide reliable results, even when there is no previous knowledge of the pathogen (Ma and Michailides, 2002).

2.3.2.2 Quantitative Real-time PCR (qPCR)

In the last decades, the polymerase chain reaction (PCR) technique has significantly advanced towards expanding its use and versatility by working with quantitative real-time PCR (qPCR) (Paiva-Cavalcanti *et al.*, 2010), developed by Kary Mullis (Higuchi et al., 1992). qPCR promotes an accurate quantification in real-time of the amplicon, allowing the monitoring of the reaction and quantitative ability (Paiva-Cavalcanti et al., 2010).

Researches data from the literature show that both, conventional PCR and qPCR, present interesting characteristics for the diagnosis of grapevine trunk disease. However, qPCR enables the elimination of a laborious post-amplification stage (gel electrophoresis preparation) used conventionally for the observation of the amplified product in conventional PCR (Paiva-Cavalcanti *et al.*, 2010). Other benefits of qPCR in relation to conventional PCR include reproducibility, quantitative ability, specificity and sensitivity (Martín *et al.*, 2012).

This method is based on the use of dyes or fluorescent probes that permit the monitoring of the amplified product. SYBR Green is the most widely used dye, which binds without specificity to the DNA duplexes produced during amplification. TaqMan probe is another way to generate the fluorescence which uses a probe specifically targeted to a region of the internal sequence that needs to be amplified. During amplification, the TaqMan is degraded and the reporter is realised emitting light (Mortarino *et al*., 1999), which analysis are made by a light signal detector that creates a graphic with the absorption obtained after each round of PCR, the generated signal reflects the amount of product formed (Kubista *et al*., 2006).

qPCR enable the performance of four types of tests: absolute quantification, relative quantification, high melting resolution analysis, and allelic discrimination. The results are recorded through interconnected computer graphics generated in the thermal cycler and basically, four kinds of analysis are carried out: amplification curve, dissociation curve, spectrum, and component (Paiva-Cavalcanti *et al*., 2010).

qPCR combined with SYBR Green methodology were investigated for the detection of Petri disease pathogens *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. (Overton et al., 2004) and esca (*Phaeomoniella chlamydospora*, *Botryosphaeria* spp., *Fomitiporia mediterranea* and *Phaeoacremonium aleophilum*) (Romanazzi *et al*., 2009). Edwards *et al*. (2007) concluded that TaqMan PCR were the most sensitive methods for detecting *P. chlamydospore*. Martin *et al*. (2012), also used TaqMan PCR assays to test primers and probe designed for *Phaeomoniella chlamydospora* and *Phaeomoniella aleophilum*.

2.4 Potential use of endophytes against GTDs

2.4.1 Endophytic fungi definition

Endophytic fungi are defined as microorganisms that colonize healthy internal plant tissues at some time in their life without causing any apparent disease symptoms. The endophytic community in a single plant is usually composed by numerous species of microorganism and fungi are some of them (Petrini 1991; Mostert *et al*., 2000). The number and diversity endophytic fungi of species are influenced by the environment, plant physiology, anthropogenic factors, and pathogen infections (Varanda *et al*., 2016). They may confer tolerance to environmental stresses and pathogen (Oono *et al.*, 2015). It is presumed that endophytes comprise a range variety of fungi species, with the majority belonging to the Ascomycota (Sessa *et al*., 2018).

Endophytes fungi have been gaining attention in the past decade in agriculture mostly due to their roles within plants and beneficial effects on their hosts. The role of endophytes in pathogen defence is attained through different mechanisms since the competition for the same ecological niches in terms of nutrients and space until the production of secondary metabolites that inhibit fungal growth (Gonzalez and Tello, 2011).

Although most of the time the symbiotic relationship between the endophytic fungus and the plant is mutualistic, this relationship can sometimes become parasitic, thus leading to plant disease. This flexible interaction is determined by the nutritional needs of the endophyte, the environment, as well as small differences in the fungal gene expression. When the equilibrium established in the interaction is disturbed, endophytic fungi can become pathogenic fungi and cause symptoms of disease in the plant or lead to the exclusion of the fungus by plant defence mechanisms (Schulz and Boyle, 2006). Therefore, some pathogenic fungi may live as endophytes during part of their life, which is a big challenge for plant

pathologists, especially the ones that work with grapevine due to isolation of trunk disease pathogens inside plant tissues from both symptomatic and asymptomatic plants (Varanda *et al*., 2016).

2.4.2 Antagonism as biological control

Biological agents for fungal plant pathogens control are very popular among researchers and numerous microorganisms were already identified as potential biocontrol agents (Alabouvette *et al*., 2006). Biological control using antagonistic microorganisms assumes a great importance, and it is considerate a sustainable and environmentally acceptable management method for numerous pathogens (Punja and Utkhede, 2003).

According to Landum *et al*. (2016), endophytes show great potential as biocontrol agents, whereas competing with pathogens for space or nutrient and producing compounds that inhibit the growth of other fungi. The most studied endophytic fungi belong to the genus *Trichoderma.* The efficiency of *Trichoderma* spp. as a biological control in grapevine was tested in the protection of pruning wounds or in the different stages of vine propagation in nurseries providing good results against pathogens (Perlot *et al*., 2016). Fourie et al. (2001), studied *Phaeomoniella chlamydospora* and *Phaeacremonium spp*. in rootstock cuttings and observed an incidence reduction after root drench treatments with *Trichoderma* spp. Some others fungal endophytes have shown antagonistic effects against grapevine pathogens, such as fungi belonging to the genus *Alternaria* and *Epicoccum* against *Plasmopara viticola* and *Botrytis cinerea* (Musetti et al., 2007; Polizzotto et al., 2009). Yacoub et al. (2016), studied the colonization of the root system by *Pythium oligandrum* and discovered that this fungus was effective in colonizing grapevine roots and reducing the wood necroses caused by *Phaeomoniella chlamydospore*. Other endophyte belonging to the genera *Epicoccum*, *Cladosporium* and *Alternaria* have shown reveal potential for biological control against esca-associated pathogens (Bruez *et al*., 2014; Pancher *et al*., 2012).

Antagonism activity of some endophytic have also been tested by *in vitro* analyses such as volatile compounds test (Lee *et al*, 2012; Rahmansyah and Rahmansyah, 2013; Reddy *et al*., 2014; Landum *et al*., 2016) and direct opposite method inhibition antagonism test (Royse and Ries**,** 1977**;** Demici *et al*., 2011; Sezões, 2016; Coletto *et al*., 2018). Landum *et al*. (2016), isolated several fungi from olive trees to test their antagonism activity against the growth of *Colletotrichum acutatum* and verified that *Aspergillus niger* and *Nigrospora oryzae* demonstrated the highest growth inhibition percentages, 86.3% and 66.7%, respectively, resulting from direct inhibition tests. Pimenta (2016), also used direct inhibition

tests to verify the potential antagonist of fungi present in Alentejo vines against pathogenic fungi responsible for GTDs and concluded that all the isolates from *Alternaria* sp. and *Epicoccum sp*. were able to inhibit the mycelia growth of *Cladosporium sp*. in 12 days of the test.

3. Materials and Methods

3.1 Origin of plant material

This study was carried out in September 2017, in three private vineyards located in Alentejo region (south of Portugal): Fundação Eugénio de Almeida (FEA), Vale do Rico Homem (VRH) and Vinhas de Campo Maior (VCM). Plants from two important cultivars in this region; Trincadeira and Alicante Bouchet; were randomly chosen (Table 1) and their roots, petioles and offshoots were collected for analyses.

Table 1. Number of collected plants from different vineyards and cultivars.

Location	Cultivar	Asymptomatic	Symptomatic
Vale do Rico Homem	Trincadeira	3	3
Fundação Eugénio de Almeida	Trincadeira	5	5
Vinhas de Campo Maior	Alicante Bouchet	3	3

3.2 Fungal isolation and purification

From each plant, 8 branches were cut, and secondary roots (length 10-15 cm, diameter 3-5 mm) were collected from different parts of the root system. The roots samples were placed into sterilized plastic bags along a small portion of soil in order to preserve the humidity. All the samples were transported to the laboratory and stored at 4°C until further analyses, which occurred within 48 h.

Roots were separated from the soil by washing them in running water and treated with 0.05% (v/v) Tween 20 in 50 mL tubes (Falcon). Roots, petioles and offshoots were disinfected to suppress epiphytic microorganisms and cut into 0.5 cm sections. Disinfection consisted in a succession of 3 min immersions, conducted under a sterile laminar airflow chamber, in a series of 96 % ethanol, 3 % sodium hypochlorite solution, 70 % ethanol, and ultra-pure water.

After disinfection, the pieces were dried in sterile Whatman paper, placed on Petri dishes of 90 mm diameter (four pieces per plate) containing Potato Dextrose Agar medium (PDA) and incubated, in darkness, for 1 to 2 weeks at 25ºC. The different colonies morphologically caracrterized were separated by transferring them to a small agar disk (about 5 mm^2) of the growing fungi to a fresh 60 mm diameter PDA medium. The obtained colonies were grouped and numbered according to their morphological characteristics which were based on their shape, form, size, growth time, border, surface, opacity and pigmentation and the shape and size of the fungal fruiting bodies, spores and hyphae. Shape of hyphae and spores was observed under an Olympus BX-5ed0 compound microscope (400x magnification). The pure colonies were stored in a fridge at 4°C until April 2018, when a new isolation was performed. Upon removing samples from the fridge, the dishes were kept at room temperature for a few days to let the fungi grow before their re-isolation. For the re-isolation, a small agar dish (approximately 5mm^2 with the mycelia) was cut and transfer to the new 60 mm PDA petri dish and three repetitions were considered. Fungi where kept at room temperature for 1 to 2 two weeks to grow until they cover the entire dish surface.

All the laboratorial operations for fungal isolation and re-isolation were performed on a clean and superficial disinfected bench, next to a burner flame with the aim to achieve the asepsis conditions and decrease the risk of contamination for undesirable microorganisms.

3.3 Extraction of genomic DNA (gDNA).

Once the entire dish surface was covered with the fungus, the mycelia was removed using a sterilized scalpel, transferred into a 2 ml tube and kept in a box with ice until the tube was stored in the freezer at -20ºC. This operation was also performed on a sterilized bench, next to a burner flame.

The gDNA extraction was made following the CTAB method (hexadecyltrimethylammonium bromide) described by Doyle and Doyle (1987), with some modifications. After the incubation, 100 mg of the material was placed into a 1.5 mL micro tube containing 600 µL of an extraction buffer CTAB 3%. The CTAB extraction buffer (provided with 10% CTAB, 5 M NaCl, 0.5 M EDTA pH 8.0, 1 M Tris-HCl pH 8.0, 4% PVP, 0.1% β-mercaptoethanol), was prepared just before its use. Samples were incubated at 55ºC for 90 minutes in an orbital shaker (Aerotron HT) just after the addition of the mycelium into the CTAB extraction buffer and subjected to a manual upside-down shake for 2 minutes, every 15 minutes. After the incubation, 600 µL of chloroform-isoamyl alcohol (24:1) were added to each tube. The samples were manually agitated by inversion for 10 minutes and

centrifuged (centrifugal machine Himac CT 15RE) at 12000 rpm for 10 minutes. The supernatant was transferred to a new 1.5 ml tube already with 800 µL of frozen absolute ethanol (-20°C). The tubes were manually homogenized again for few minutes and centrifuged for 20 minutes at 13000 rpm. The supernatant (liquid) was carefully discarded and the remaining "pellet" at the bottom of the tube, with the DNA, was washed with 500 µL of 70% ethanol to eliminate all the residues adhering to the DNA. The samples were centrifuged for 15 minutes at 13000 rpm and the liquid supernatant was discarded. In order to dry the "pellet" to eliminate all the ethanol, the tubes were placed into a centrifugal concentrator (LABCONCO® CentriVap® micro IR), with their lids open, for approximately 20 minutes at 55°C, until they were totally dry. To hydrate the "pellets", 20 µL of ultra-pure water were added to the tubes.

To quantify and assess gDNA purity, the absorbance was measured on a NanoDrop-2000C spectrophotometer (Thermo Scientific). Samples were kept in the freezer (-20ºC) until further analyses.

3.4 Amplification, purification and sequencing of the ITS region

The internal transcribed spacer (ITS) region of nuclear rDNA was amplified through PCR from gDNA by using ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') primers, according to White *et al*. (1990). PCR reactions consisted of 1 µL of gDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas), 1 mM of each primer, and 2.5 U of Dream-Taq DNA polymerase (Fermentas) in a total reaction volume of 50 µL. Amplification was carried out in a Thermal Cycler (Bio-Rad) following the program described in Table 2.

Table 2. Amplification program for PCR using ITS1 and ITS4 primers.

Steps	Temperature (ºC)	Time	Number of cycles
Initial denaturation	95	3 minutes	1
Denaturation	95	30 seconds	40
Annealing	55	55 seconds	
Extension	72	2 minutes	
Final extension	72	10 minutes	1

Amplified products were analysed by agarose gel electrophoresis, running 10 µL of each sample in a 1% agarose gel and 6 µL of a DNA marker (GeneRulerTM 1 kb Plus DNA

Ladder) used as a reference, performed at 80 V for approximately 1 hour and 20 minutes. Agarose gel was prepared with 0.5x Tris-Borate-EDTA buffer (TBE) (1x: 1 M Tris-HCl, 0.83 M boronic acid, 10 mM EDTA, pH 7.5). PCR products were visualized, on a Gene Flash Bio Imaging system (Syngene).

The PCR reaction products showing the expected size were purified from agarose gel using the NZYTech kit according to the manufacturer's protocol. The samples were verified and quantified using a spectrophotometer NanoDrop-2000C (Thermo Scientific) and sequenced by Macrogen Inc. (Madrid, Spain).

ITS sequence homology was explored at the "National Center for Biotechnology Information" (NCBI) database using the BLAST algorithm (Karlin and Altschul 1993) (http://www.ncbi.nlm.nih.gov/) (BLASTn), and ITS sequences analysis were based on a ClustalW Multiple alignment using BioEdit software, according to Hall (1999). All the fungi sequences that showed resemblance at least 89% were used to identify the fungus analysed.

Phylogenetic studies that were performed on the identified fungi responsible for GTDs, were based on a ClustalW Multiple alignment made in BioEdit software (Hall, 1999); the alignment was bootstrapped with 1000 replicates by the Neighbour-Joining (NJ) method using the MEGA 7 software.

3.5 Quantitative Real-time PCR (qPCR)

3.5.1. Establishment of a Diaporthe sp. SYBR®Green qPCR assay

A gene-specific primer set (Table 3) was designed in a conserved region of *Diaporthe* sp. β-actin, sequence collected from NCBI GenBank database (accession ID JN230391.1). The conserved region was identified after alignment of full-genome β-actin sequence from *Diaporthe* together with other fungi β-actin collected from NCBI GenBank database. To design the primers, it was used the Primer Express 3.0 Software (Applied Biosystems), using the default parameters of the software, and their specificity was tested *in silico* using the BLAST tool at NCBI database.

To ensure the specificity of the *Diaporthe* SYBR®Green assay against the gDNA of the fungi identified in this study (identified through isolation of the ITS region), a qPCR test was performed.

qPCRs were carried out on a 7500 Real Time PCR System (Applied Biosystems) with SYBR Green qPCR Master Mix (Nzytech). To perform a total of 18 ml reaction volume it was used 100 ng of gDNA and 560 nM of each specific primer. The quantification cycle (Cq) values were acquired for each sample and the cycling program consisted of three steps: (1)

an initial denaturation step at 95°C for 10 minutes, (2) amplification program of 40 cycles at 95°C for 15 seconds followed by annealing and extension at 60°C for 1 min and (3) an additional melting analysis of 40 min from 60 to 95°C. Three technical replicates were considered for each sample. A *Diaporthe* sp. reference isolate and no template control were included in all plates.

Table 3: qPCR oligonucleotide primers designed on β-actin region of *Diaporthe sp.*

Specie	*Accession ID*	'Primes' (5'→ 3')	Amplicon (bp)
Diaporthe sp.	JN230391.1	Fw: TTTTCGTAAGTCACCCCCGC Rv: TAGCCTTCATGGTCGTTGCA	104 bp

The fluorescence threshold was manually set above the background level. The specificity of qPCR reactions was evaluated by melting curve analysis.

3.5.2 Establishment of a Specific *Diaporthe* sp. TaqMan Assay

A set of primers and a probe were designed for detection of *Diaporthe* sp. after alignment of ITS sequences previously isolated by ITS1 and ITS4 primers. It was used the Primer Express 3.0 software (Applied Biosystems), selecting the option MGB TaqMan probes, and using the default parameters of the software (Table 4).

Table 4. qPCR oligonucleotide primers and probes designed on internal transcribed spacer (ITS) gene region of *Diaporthe sp.*

Specie	'Primers' (5'→ 3')	Probe (5'→ 3')	Amplicon (bp)
Diaporthe sp.	Fw: GTTCTGGCATCGATGAAGAACGCA Rv: CAAGCCAGGCTTGAGGGTTGAAA	CAAGCCAGGCTTGAGGGTTGAAA	154

A bioinformatic approach was performed to assure the specificity of the primers and the probe, which included a BLASTn query at NCBI and the others ITS sequences of the GTD fungi identified in this study, and also to ensure the specificity of the *Diaporthe* TaqMan assay against the gDNA of the identified fungi a qPCR test was performed.

qPCRs were performed on a 7500 Real Time PCR System (Applied Biosystems), using 100 ng of gDNA as template, 2x NZY qPCR Probe Master Mix (Nzytech), 400 nM of each primer, and 100 nM of probe (Nzytech) in a total volume of 20 µL. The quantification cycle (Cq) values were acquired for each sample with the Applied Biosystems 7500 software v2.0.6 (Applied Biosystems) and the cycling program consisted of three steps: (1) an initial denaturation step at 95°C for 10 minutes, (2) amplification program of 40 cycles at 95°C for 15 seconds followed by annealing and extension at 60°C for 1 min. and (3) an additional melting analysis of 40 min from 60 to 95°C.

The fluorescence threshold was manually set above the background level. Three technical replicates were considered for each sample. A *Diaporthe* sp. positive target control and no template controls were included in all plates.

3.6 Direct inhibition antagonism test

Fungal isolates were tested *in vitro* for their antagonistic activity using the direct opposition method described by Dennis and Webster (1971), with some modification. Briefly, a 3 mm mycelia disc from a pure culture of the potential antagonistic actively growing was placed next to the edge of a 60mm PDA dish and in the opposite side was placed a similar sized mycelium disc of a GTDs pathogen (Figure 8).

Figure 8 - Representative scheme of the direct antagonism test. GTDs pathogen fungi on the left side and the endophytic on the right side of the Petri dish.

For this study 6 endophytic fungi (potential antagonist), identified previously by the ITS region, were used for the tests: *Fusarium oxysporum, Aspergilus niger, Penicillium* sp., *Trichoderma* sp., *Clonostachys rosea and Epicocum nigrum,* and 3 GTDs pathogens:

Diaporthe sp.*, Phialophora* sp. *and Diplodia pseudoseriata*, totalizing 18 pathogen/antagonist combinations. Each combination of pathogen/antagonist was repeated 3 times and, as negative control, Petri dishes were inoculated with only the pathogen or only the endophytic fungus.

All Petri dishes were incubated at 22ºC ± 2ºC, in dark conditions. During the interaction, the radial growth towards (internal radius) the interacting fungus were measured daily by using a graduated ruler for 9 days. Measurements were made since the edge of the plug with the mycelium of the pure culture, until the end of the colony growth. The growth of the negative control fungi was also measured. The inhibition percentage was calculated using the following formula (Royse & Ries, 1977).

$$I = \left(\frac{R1 - R2}{R1}\right) \times 100$$

I: inhibition percentage
R1: the fungus colony radius in the control Petri dish
R2: the fungus colony radius in the test Petri dish

Analysis of variance (ANOVA) was conducted regarding differences among antagonistic fungi in each pathogenic fungus, using the IBM SPSS statistical package v.20. Multiple mean comparisons were made using Tukey HSD test when statistical differences were found between data sets ($p < 0.05$).

4. Results

4.1 Isolation and identification of fungal isolates obtained from plant material

In the present work, plant material was collected from symptomatic and asymptomatic grapevines of three wine-producing areas: Vale do Rico Homem (VRH), Fundação Eugénio de Almeida (FEA) and Vinhas de Campo Maior (VCM). Samples were collected from three different grapevine organs (roots, petioles and offshoots). Fungal isolates were obtained in all samples tested. The 22 collected sampled harboured 3054 endophytic fungal isolates: 912 isolates from VRH, 1315 isolates from FEA and 827 isolates from VCM (Table 5).

Table 5. Number of isolates by location, vegetal material conditions and vegetal material culture of the plant collected.

Location	Vegetal Material Conditions	Vegetal Material Culture	Number of isolates
VRH	Asymptomatic	Roots	205
		Petioles	100
		offshoots	166
		Total	**471**
	Symptomatic	roots	143
		petioles	132
		offshoots	166
		Total	**441**
FEA	Asymptomatic	roots	165
		petioles	165
		offshoots	255
		Total	**585**
	Symptomatic	roots	218
		petioles	234
		offshoots	278
		Total	**730**
VCM	Asymptomatic	roots	143
		petioles	125
		offshoots	122
		Total	**390**
	Symptomatic	roots	152
		petioles	117
		offshoots	168
		Total	**437**
TOTAL			**3054**

Fungi were then grouped according to their morphological characteristics, the shape and size of the fungal fruiting bodies, spores and hyphae. In total, 101 fungi were identified as different individuals based on their morphological characteristics (Figure 9).

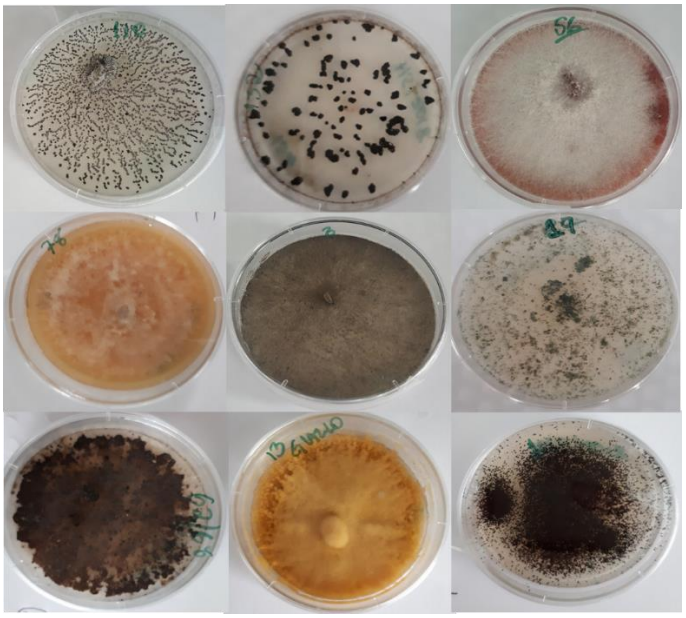

Figure 9. Examples of fungal isolates with different morphological characteristics.

For additional studies, the fungi were submitted to a molecular identification by a conventional PCR with the aim to amplify their ITS region. All isolated fungi were successfully identified, through the search for homologous sequences using BLAST at the NCBI, based on ITS region. The size of the generated PCR products ranged from 500 to 700 bp (Figure 10).

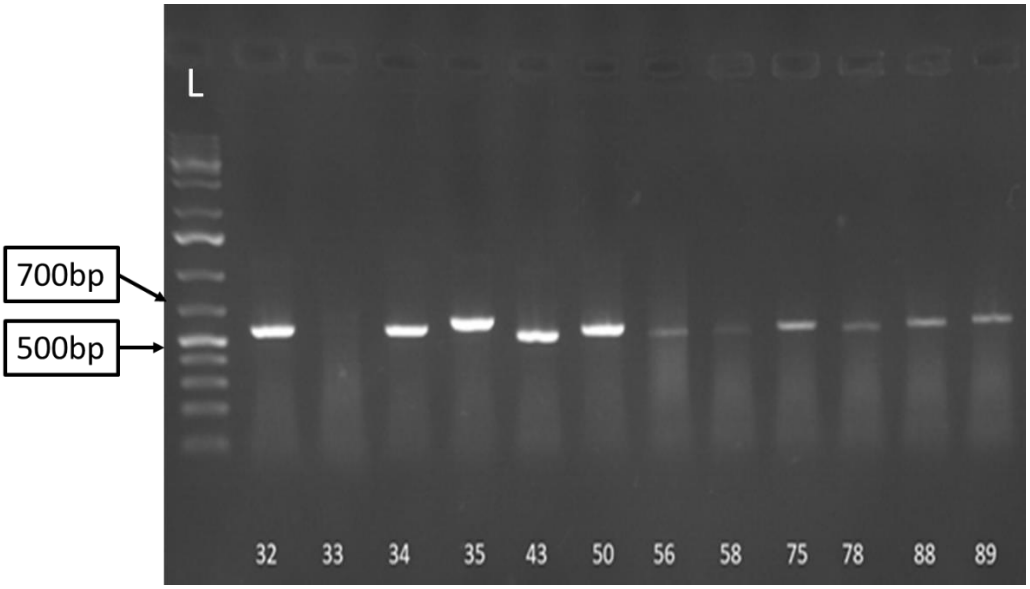

Figure 10. Agarose gel electrophoretic analysis of some of the amplified DNA products using the ITS1 and ITS4 primers. L – ladder GeneRulerTM 1 kb Plus DNA Ladder; bp – base pairs.

Each endophytic isolate was named as taxonomic group such as a species or genus. Forty different fungi were successfully identified based on ITS sequence analyses, with 65% of the identification at species level (Table 6).

Table 6. Endophytic fungi identified by their % of homology based on ITS region.

Fungus	% homology based on ITS region
Acrocalymma vagum	100,00%
Alternaria alternata	100,00%
Alternaria sp.	100,00%
Aspergillus niger	100,00%
Aspergillus niveus	89,01 %
Aspergillus terréus	98,75%
Aspergillus sp.	96,48%
Beauveria bassiana	100,00%
Bjerkandera adusta	98,65%
Botryotinia fuckeliana	99,72%
Botrytis cinérea	99,45%
Chaetomium succineum	99,71%
Clonostachys rósea	100,00%
Clonostachys sp	99,47%
Colletrotrichum sp.	99,33%
Cytospora acaciae	100,00%
Diaporthe sp.	100,00%
Diplodia pseudoseriata	97,89%
Epicoccum nigrum	100,00%
Eupenicillium sp	98,55%
Fusarium oxysporum	100,00%
Fusarium sp	100,00%
Fusarium verticillioides	97,89%
Hormonema vitícola	99,71%
Macrophomina phaseolina	99,72%
Neofusicoccum parvum	92,05%
Penicillium chrysogenum	100,00%
Penicillium glabrum	98,02%
Penicillium sp.	100,00%
Penicillium thomii	98,21%
Pestalotiopsis sp	91,09%
Phialophora fastigiata	99,50%
Phialophora sp	99,40%
Phlebia setulosa	99,27%
Phlebiopsis gigantea	100,00%
Rutstroemiaceae sp.	99,72%
Stereum hirsutum	99,71%
Talaromyces sp.	99,50%
Trichoderma sp.	96,50%
Truncatella angustata	99,08%

Among the fungi studied, 9 were considered GTDs pathogens, with 6 of them identified at specie level (*Hormonema viticola*, *Truncatella angustata*, *Stereum hirsutum*, *Phialophora fastigiata*, *Cytospora acaciae*, *Diplodia pseudoseriata*) and 3 at genera level (*Diaporthe* sp., *Pestalotiopsis* sp., *Neofusiccoccum* sp.)

To represent evolutionary relation related to the species of interest a phylogenetic tree was graphically designed. In a phylogenetic tree, species belonging to the same taxonomic group are grouped in the same cluster. In this study, the phylogenetic analysis obtained through the sequence's alignment showed a wide diversity of GTD fungi genera identified (Figure 11).

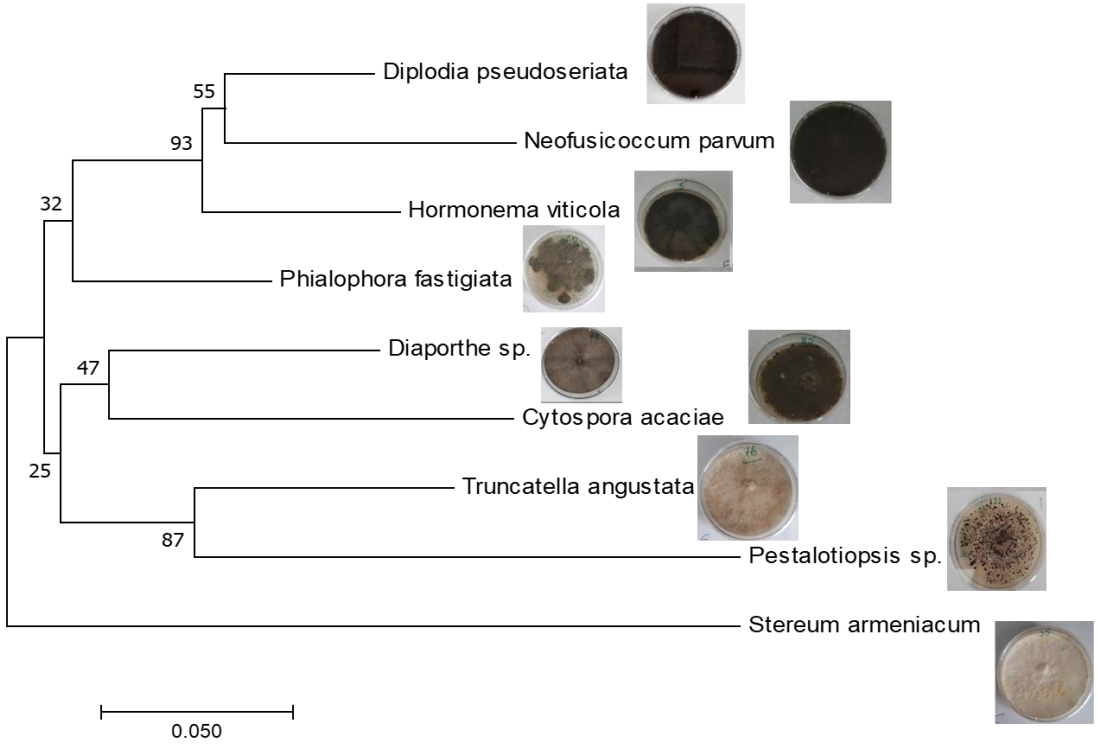

Figure 11. Phylogenetic tree generated from the alignment of ITS sequences from the identified grapevine trunk disease pathogenic fungi, using the Neighbour-joining method. Numbers at each node indicate the bootstrap confidence value.

Phylogenetically*, Stereum armeniacum* is situated in a different cluster, isolated from the others GTDs fungi, explained due to be the only fungi belonging to *Division Basidiomycota* (Figure 11 and Table 7). *Diplodia Pseudoseriata* and *Neofusicoccum parvum* are in the same cluster, once they belong to the same Family (Figure 11 and Table 7). *Truncatella angustata* and *Pestalotiopsis sp.* are both from *Amphisphaeriaceae* Family. *Diaporthe sp.* and *Citospora acaciae* are from *Diaportales* Order (Table 7).

Table 7. GTD pathogen identified by specie, genera, family, order, class and division.

Fungus	Genera	Family	Order	Class	Division
Hormonema viticola	*Hormonema*	Dothioraceae	Diaporthales	Sordariomycetes	Ascomycota
Truncatella angustata	*Truncatella*	Amphisphaeriaceae	Xylariales	Sordariomycetes	Ascomycota
Stereum armeniacum	*Stereum*	Stereaceae	Russulales	Agaricomycetes	Basidiomycota
Phialophora fastigiata	*Phialophora*	Herpotrichiellaceae	Chaetothyriomycetidae	Eurotiomycetes	Ascomycota
Cytospora acaciae	*Cytospora*	Valsaceae	Diaporthales	Sordariomycetes	Ascomycota
Diplodia pseudoseriata	*Diplodia*	Botryosphaeriaceae	Botryosphaeriales	Dothideormycetes	Ascomycota
Diaporthe sp.	*Diaporthe*	Diaporthaceae	Diaporthales	Sordariomycetes	Ascomycota
Pestalotiopsis sp.	*Pestalotiopsis*	Amphisphaeriaceae	Xylariales	Sordariomycetes	Ascomycota
Neofusiccoccum sp.	*Neofusiccoccum*	Botryosphaeriaceae	Botryosphaeriales	Dothideormycetes	Ascomycota

Overall, almost all GTDs fungi identified belong to *Division Ascomycota* (8 fungi), represented by three classes, with the class *Sordariomycetes* the most representative (5 fungi), followed by *Dothideomycetes* (2 fungi), *Eurotiomycetes* (1 fungus). Only one fungus was identified belonging to *Phylum Basidiomycota*, which is represented by *Class Agaricomycetes* (Table 7).

4.2 GTDs fungi identification in the vegetal material

The incidence of each GTDs fungus identified, by number of isolates, in all the vineyards studied can be seen in Figure 12. Relating the number of isolates with each fungus obtained from asymptomatic plants, there were 53 isolates of *Hormonema viticola*, 13 isolates of *Diaporthe* sp. and 11 of *Neofusicoccum parvum*. The remaining fungi presented less them 10 isolates. In the symptomatic plants analysed it was verified 97 isolates of *Diaporthe* sp., 73 isolates of *Hormonema viticola,* 62 of *Neofusicoccum parvum,* 15 of *Phialophora* sp.*,* 11 of *Cytospora aceciae and* 11 of *Truncatella angustata.* Other identified fungi are represented by less than 10 isolates (Figure 12). In general, more GTDs fungi isolates were obtained from symptomatic than from asymptomatic plants.

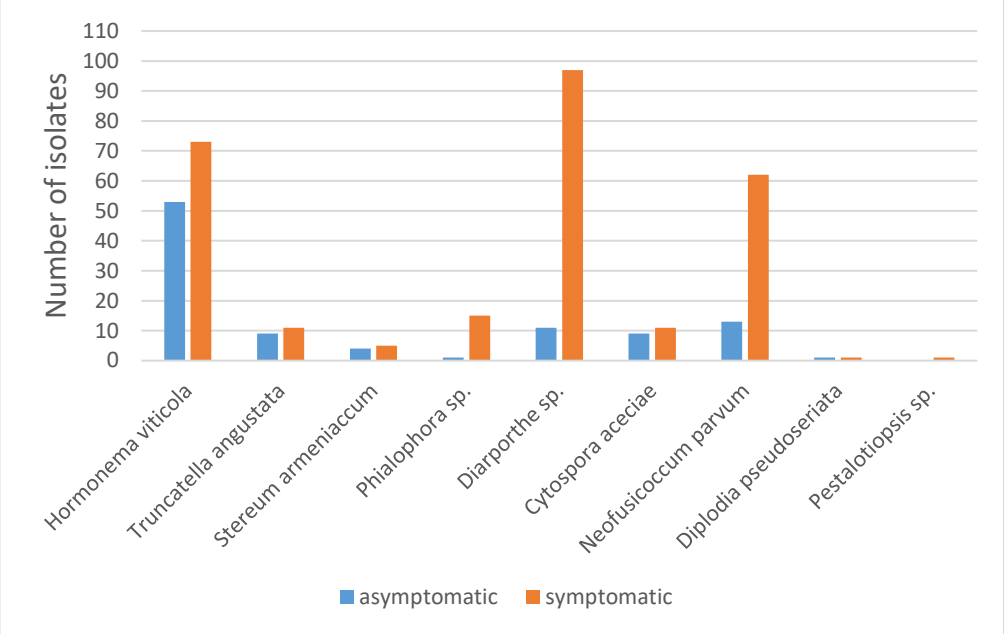

Figure 12. Number of GTDs fungi isolates identified in asymptomatic and symptomatic plants from the three vineyards studied.

Asymptomatic plants of VRH vineyards engendered 41 GTDs fungi isolates: 22 of *Hormonema viticola,* 8 of *Neofosicoccum parvum*, 7 of *Truncatella angustata,* 2 of *Diaporthe sp*. and 2 of *Cytospora acacia.* From symptomatic plants of the same vineyards, it was collected in total 64 isolates: 28 of *Hormonema viticola,* 21 of *Diaporthe sp.,* 9 of *Neofosicoccum parvum,* 1 of *Diplodia pseudoseriata and* 1 of *Pestalotiopsis sp.* (Figure 13A).

From FEA, 49 GTDs fungi isolates were obtained in asymptomatic plants: 24 of *Hormonema viticola*, *7* of *Cytospora acacia*, 6 of *Diaporthe sp*., 5 of *Neofosicoccum parvum* and 4 of *Sterium hirsutum.* From symptomatic plants, of the same vineyards, a total of 104 isolates were collected: 41 of *Diaporthe sp.*, 23 of *Hormonema viticola*, 19 of *Neofosicoccum parvum*, 15 of *Phialophora sp.*, 4 of *Sterium hirsutum* and 2 of *Cytospora acacia* (Figure 13B).

Analysing VCM GTDs fungi isolates from asymptomatic plants, it was verified 11 isolates in total: 7 of *Hormonema viticola*, 3 of *Diaporthe sp*. and 1 of *Phialophora sp.* The number GTDs fungi isolates from symptomatic plants in the same vineyard was 102: 35 of *Diaporthe sp*., 34 of *Neofosicoccum parvum*, 22 of *Hormonema viticola,* 6 of *Truncatella angustata,* 5 of *Cytospora acacia and* 1 of *Sterium hirsutum* (Figure 13C).

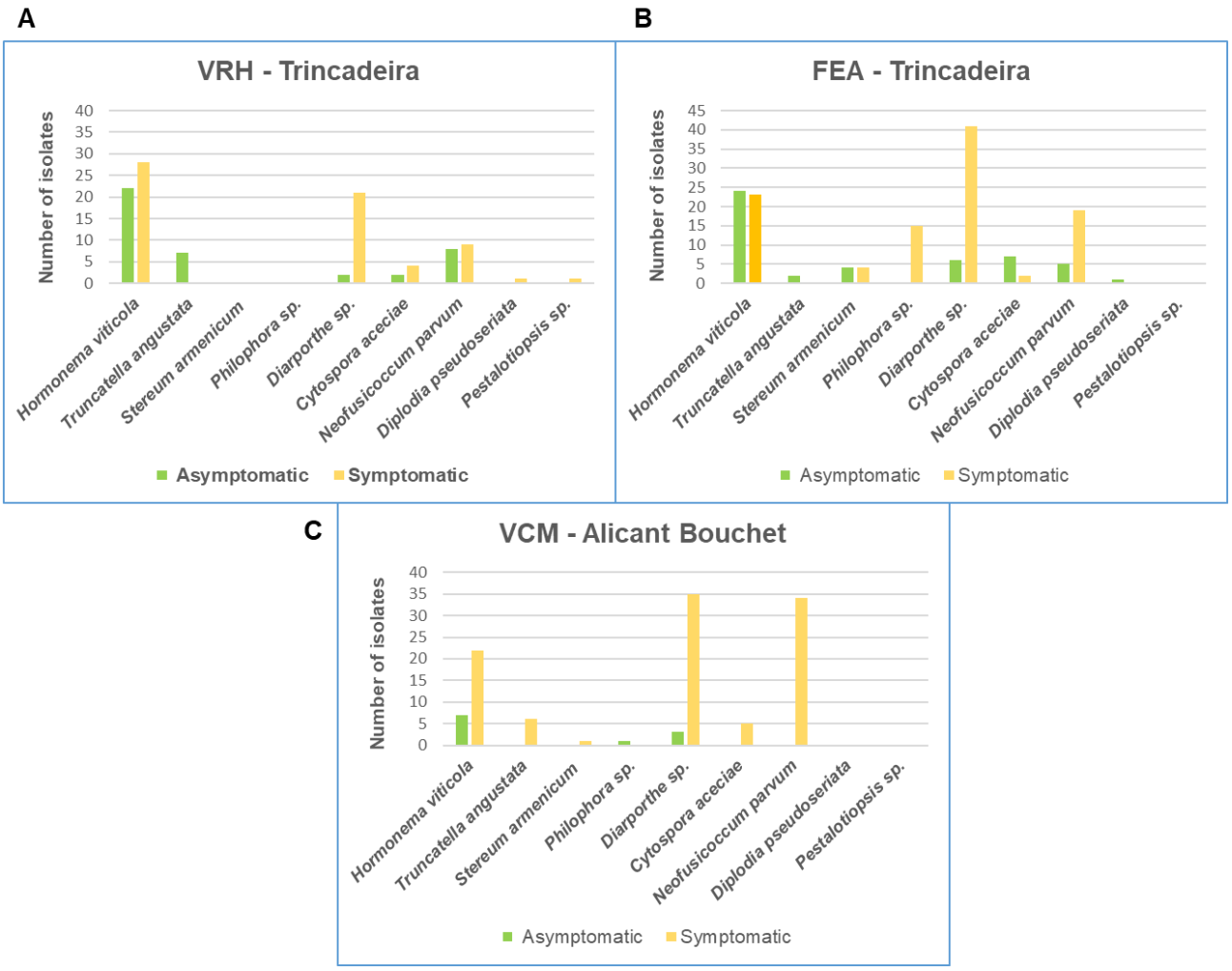

Figure 13. Number of GTDs fungi isolates collected in asymptomatic and symptomatic plants of each vineyards studied. A) Fundação Engénio de Almeida – cultivar Trincadeira, B. Vale do Rico Homem – cultivar Trincadeira, C. Vinhas de Campo Maior - cultivar Alicant Bouchet.

Truncatella angustata and *Stereum armenicum* were identified only in roots in both, symptomatic and asymptomatic plants. The presence of *Hormonema viticola* was verified in all plant organs of asymptomatic and symptomatic plants, however its incidence was predominantly observed in the petioles with more than 25 isolates, whereas in symptomatic plants the predominance was in the roots. The incidence of *Diaporthe* sp. was observed in asymptomatic and symptomatic plants, mainly in the offshoots. *Neofusicccum parvum* was also verified in all plant organs, in asymptomatic and symptomatic plants, however its incidence was mainly observed in offshoots in symptomatic plants (Figure 14A and Figure 14B).

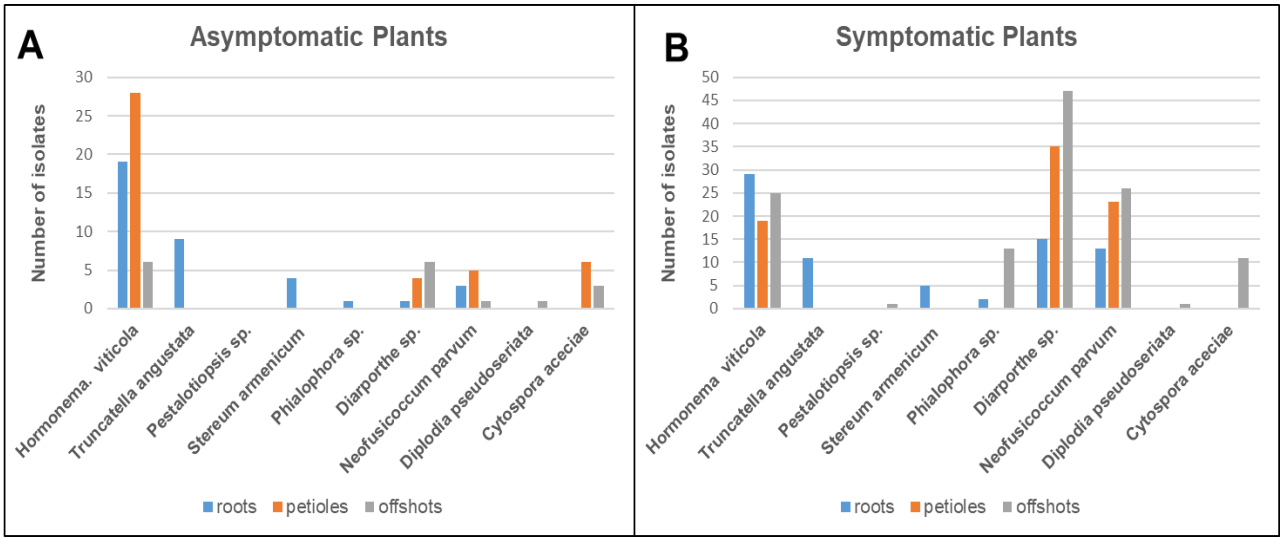

Figure 14. Number of GTDs fungi isolates collected in different organs of the plants (roots, petioles and offshoots) in asymptomatic (A) and symptomatic (B) plants studied.

4.3 SYBR@ Green assay specificity *for Diaporthe* sp.

Due to the lack of information in the bibliography regarding the use of qPCR to identify *Diaporthe sp.*, one of the fungi responsible for GTD identified in the present work, this fungus was chosen for our qPCR assays. A gene-specific primer set was designed to target β-actin gene region for *Diaporthe* sp. β-actin, based on the alignment of its β-actin gene sequence together with other fungi β-actin collected from NCBI GenBank database.

For validation of the method, the specificity of the *Diaporthe sp.* forward (5'-TTTTCGTAAGTCACCCCCGC-3') and reverse (5'-TAGCCTTCATGGTCGTTGCA-3') primers were firstly verified *in silico* against NCBI databases. Secondly, the specificity of the assay was evaluated experimentally, against the DNA of the other 40 DNAs identified fungi (Table 6), trough qPCR (chapter 3.5.1). However, the specificity could not be demonstrated, with several other fungi being target by the *Diaporth*e sp. SYBR@ Green assay, as can be observed in Figure 15.

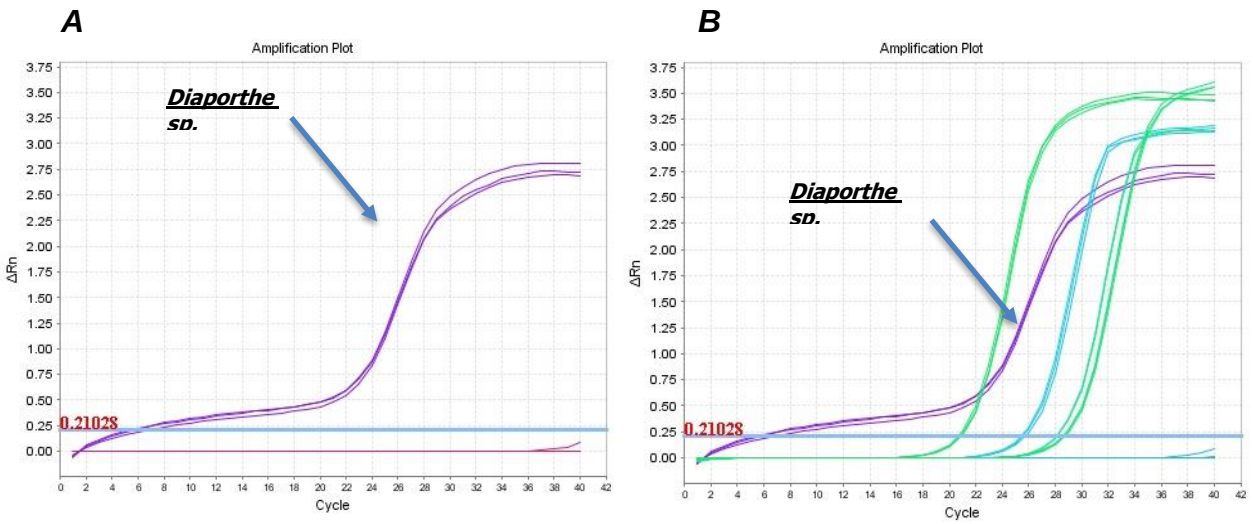

Figure 15.. Amplification plots to assess the specificity of the D*iaporthe* sp. SYBR@ Green assay. **A)** Amplification of *Diaporthe* sp. positive control. **B)** Cross-reactivity with amplification of several other identified fungi.

4.4 TaqMan assay specificity for *Diaporthe sp.*

The non-specificity of the *Diaporthe* sp. SYBR@ Green assay from the experiment previously described, led to another attempt, with the design of a TaqMan assay, with specific primers and probe to anneal the ITS gene region of *Diaporthe* sp. A TaqMan primer set and probe were designed based on *Diaporthe* sp. ITS sequences previously isolated by ITS1 and ITS4 primers (Table 4). A BLAST query at NCBI was performed to ascertain TaqMan primer set and probe usefulness which includes all the fungi ITS sequences obtained in this study.

To validate the specificity of the primer set and the probe a TaqMan qPCR assays were performed according to 3.5.2 Chapter.

Even though TaqMan primers and probe designed were able to amplify *Diaporthe* sp. in qPCR conditions, they also amplified the DNA of the nontarget species (Figure 16), and so the specificity of the assay could not be demonstrated.

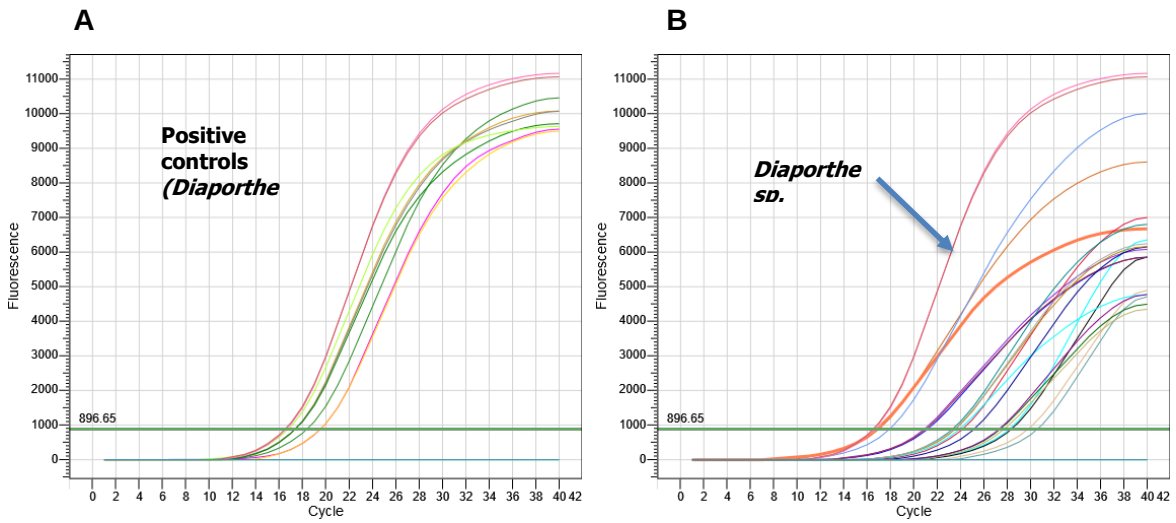

Figure 16. Amplification plots to assess the specificity of the *Diaporthe* sp. TaqMan assay. **A)** Amplification of *Diaporthe* sp. positive controls. **B)** Cross-reactivity with amplification of several other identified fungi.

4.5 Direct inhibition antagonism tests

Previously in this study, 40 different endophytic fungi were successfully identified based on their ITS sequences and 9 of them were considered GTDs pathogen (4.1 Chapter). Direct inhibition antagonism tests were carried out using three GTDs pathogens, chosen randomly, and endophytic fungi which were already reported to have antagonist activity against pathogens.

The antagonism was tested by direct inhibition between three different GTDs pathogen (*Diaporthe* sp., *Phialophora* sp. and *Diplodia pseudoseriata*) and six endophytic fungi (*Fusarium oxysporum*, *Aspergilus niger*, *Penicillium* sp., *Trichoderma sp*., *Clonostachys rosea* and *Epicocum nigrum*) for 9 days.

The fungal growth was observed and measured from the 1st to the 9th day of *Diaporthe* sp. (Figure 17), *Diplodia pseudoseriata* (Figure 20), and *Phialophora* sp. (Figure 23). ANOVA statistical analyses were performed comparing the mycelial growth of *Diaporthe* sp. (Figure 18), *Diplodia pseudoseriata* (Figure 21), and *Phialophora* sp. (Figure 24) in the 3th, 6th and 9th day of the tests. The growth of the negative control fungi was also observed and measured (Table 8).

Analysing the average growth of negative control fungi over the days, it was verified that: the growth of *Diaporthe* sp. started on the 3th day (0.67 cm) and stopped on the 6th (3.80 centimetres). *Phialophora* sp. started its growth on the 1th day (0.70 cm) and stopped it on the 5th day (4.10 cm), when the margins of the fungus reached the edge of the Petri dish.

Diplodia pseudoseriata started its growth on the 1th day (0,70 cm) and continued growing until the 4th day (4.00 cm). *Fusarium oxysporum* started its growth on the 1th day (0.23 cm) and on the 9th day was still growing (2.80 cm). The growth of *Aspergilus niger* started on the 1th day and in the 9th reached 1.13 cm. *Penicillium* sp. had not started growing until the 3th day (0.27 cm) and on the 9th reached 1.17 cm. *Trichoderma sp*. had a growth from 1th (0.10 cm) to 9th day (1.78 cm). *Clonostachys rosea* started growing on the 2th day (0.30 cm) and on the 9th day was still growing (2.30 cm). *Epicocum nigrum* grown 0.43 cm on the first day and reached 4.03 cm on the 9th (Table 8).

Table 8. The average growth of the negative control fungi over the days, in cm.

Fungus	Day								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Diaporthe sp.	0,00	0,00	0,67	1,50	2,83	3,80	3,80	3,80	3,80
Diplodia pseudoseriata	0,70	1,53	2,83	4,00	4,00	4,00	4,00	4,00	4,00
Phialophora sp.	0,70	1,33	2,77	3,90	4,10	4,10	4,10	4,10	4,10
Fusarium oxysporum	0,23	0,50	0,90	1,17	1,53	1,77	2,13	2,37	2,80
Aspergilus niger	0,03	0,20	0,37	0,57	0,73	0,87	0,97	0,97	1,13
Penicillium sp.	0,00	0,00	0,27	0,53	0,90	1,00	1,07	1,10	1,17
Trichoderma sp.	0,10	0,30	0,60	0,87	1,07	1,20	1,40	1,68	1,78
Clonostachys rosea	0,00	0,30	0,80	1,07	1,57	1,67	1,97	2,05	2,30
Epicocum nigrum	0,43	0,70	1,23	1,70	2,20	2,73	3,17	3,70	4,03

4.5.1 Antagonism action against *Diaporthe* sp.

Direct inhibition antagonism tests were carried out to verify the antagonist action of six endophytic fungi against the mycelial growth of *Diaporthe* sp. whith wasmeasured for 9 days (Figure 17). Growth values were also statistically analysed, by ANOVA, on the 3th, 6th and 9th of the assuming significant differences for a value of $p < 0.01$.

The ANOVA analysis revealed that the growth of *Diaporthe* sp. in the negative control was always significant higher ($p < 0.01$) compared to the *Diaporthe* sp. growth in the antagonism assay, showing that the mycelial growth of the pathogen did not reach the maximum when in the presence of others endophytic fungi (Figure 17 and Figure 18). The average growth ± SE of *Diaporthe* sp. in the negative control dish ranged from 0,63 cm ± 0.09 on the 3th day to 3.80 ± 0.15 on the 9th day (Figure 18).

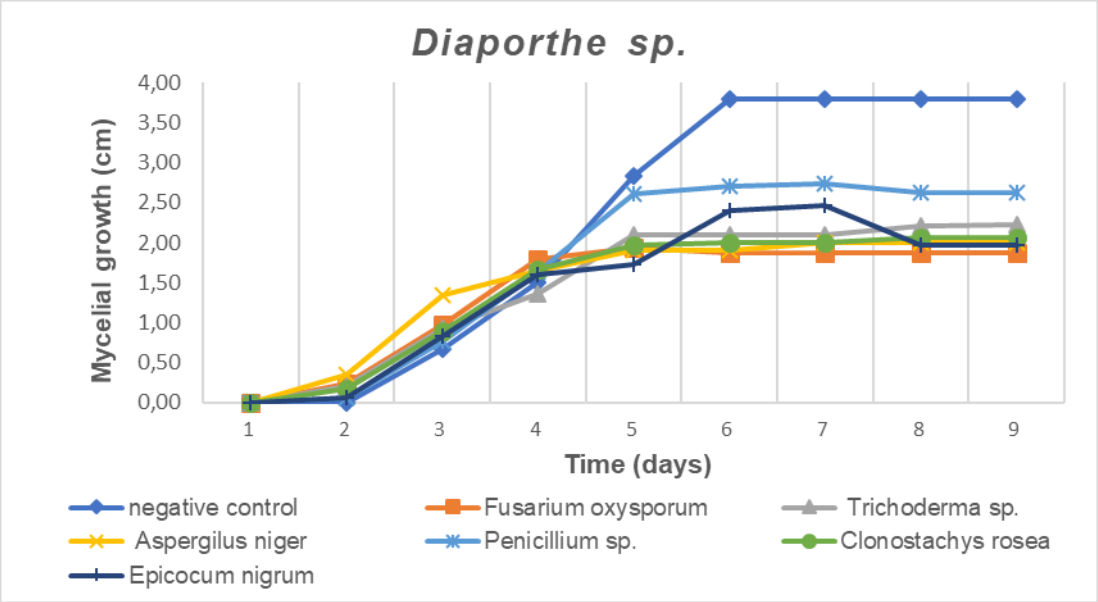

Figure 17. *Diaporthe* sp. radial mycelial growth (cm) over time (days), registered in direct inhibition tests with some endophytic fungi.

In addition, the ANOVA analysis revealed that the *Penicillium* sp. showed significant lower antagonistic effect compared to *Fusarium oxysporum* (p< 0,0052), *Trichoderma* sp. (p< 0,0162), *Aspergilus niger* (p< 0,0172) and *Clonostachys rosea* (p< 0,0177) The average growth ± SE of *Diaporthe* sp. versus *Fusarium oxysporum* ranged from 0.97 cm ± 0.07 on the 3th day to 1.87 ± 0.07 on the 9th day. The average growth ± SE of *Diaporthe* sp. versus *Trichoderma* sp. ranged from 0.93 cm ± 0.07 on the 3th day to 2.23 ± 0.03 on the 9th day. The average growth ± SE of *Diaporthe* sp. versus *Aspergilus niger* in the antagonism assay ranged from 0.90 cm ± 0.10 on the 3th day to 2.00 ± 0.08 on the 9th day. The average growth ± SE of *Diaporthe* sp. versus *Penicillium* sp. in the antagonism assay ranged from 0.77 cm ± 0.09 on the 3th day to 2.63 ± 0.09 in the 9th day. The average growth ± SE of *Diaporthe* sp. versus *Clonostachys rosea* ranged from 0.90 cm ± 0.10 on the 3th day to 2.07 ± 0.12 on the 9th day. The average growth ± SE of *Diaporthe* sp. versus *Epicocum nigrum* ranged from 0.83 cm ± 0.09 on the 3th day to 1.97 ± 0.15 on the 9th day (Figure 18).

The ANOVA analysis also revealed no significant differences between the remaining fungi (p> 0.05): *Fusarium oxysporum vs Aspergilus niger, Fusarium oxysporum vs Clonostachys rosea, Fusarium oxysporum vs Epicocum nigrum, Trichoderma* sp. *vs Aspergilus niger, Trichoderma* sp. *vs Clonostachys rosea, Trichoderma* sp. *vs Epicocum nigrum, Aspergilus niger vs Clonostachys rosea, Aspergilus niger vs Epicocum nigrum, Penicillium* sp. *vs Epicocum nigrum and Clonostachys rosea vs Epicocum nigrum.*

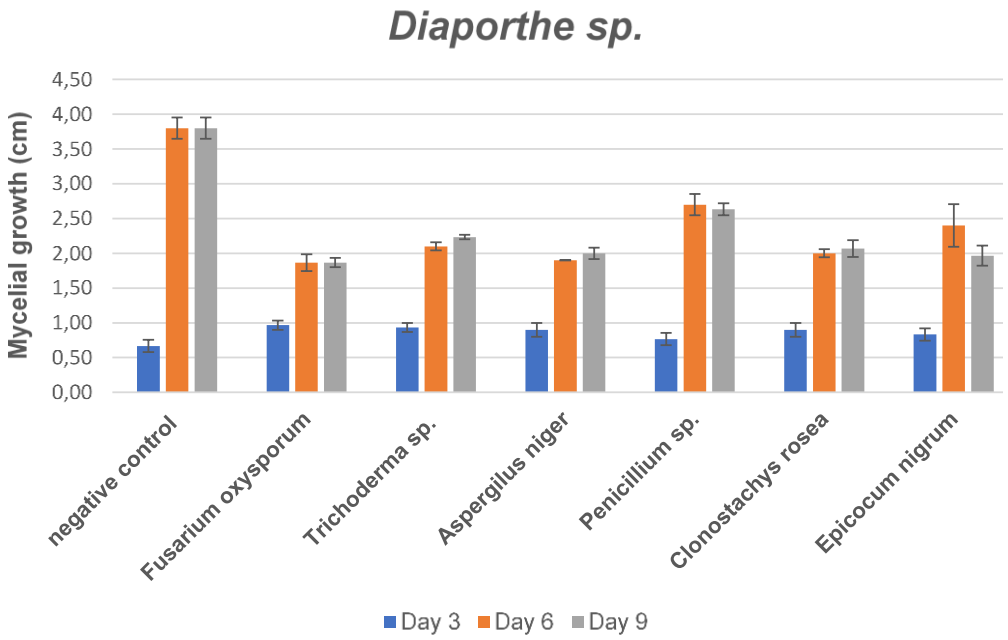

Figure 18. Radial growth values of *Diaporthe sp.* (cm) (r *Diaporthe sp.* ± SE) in the presence of different endophytic fungi on the 3th, 6th and 9th day of direct antagonism test.

During interspecific mycelial interactions all the endophytic fungi were able to stop the growth of the *Diaporthe* sp., once they were fighting for space. Some of them, like *Trichoderma* sp*. and Aspergilus niger* did not even touched the pathogen to stop its growth. *Fusarium oxysporum, Penicillium* sp*., Clonostachys rosea and Epicocum nigrum* started touching margins of the pathogen on the 5th day. Changes on *Diaporthe* sp. mycelium pigmentation was also observed during interspecific mycelial interactions. Margins of *Diaporthe* sp. colonies became lighter brown pigmented in the contact zone with *Fusarium oxysporum* and dark green pigmented in the contact zone with *Clonostachys rosea and Epicocum nigrum* which was best observed on the reverse side of the colony (Figure 19).

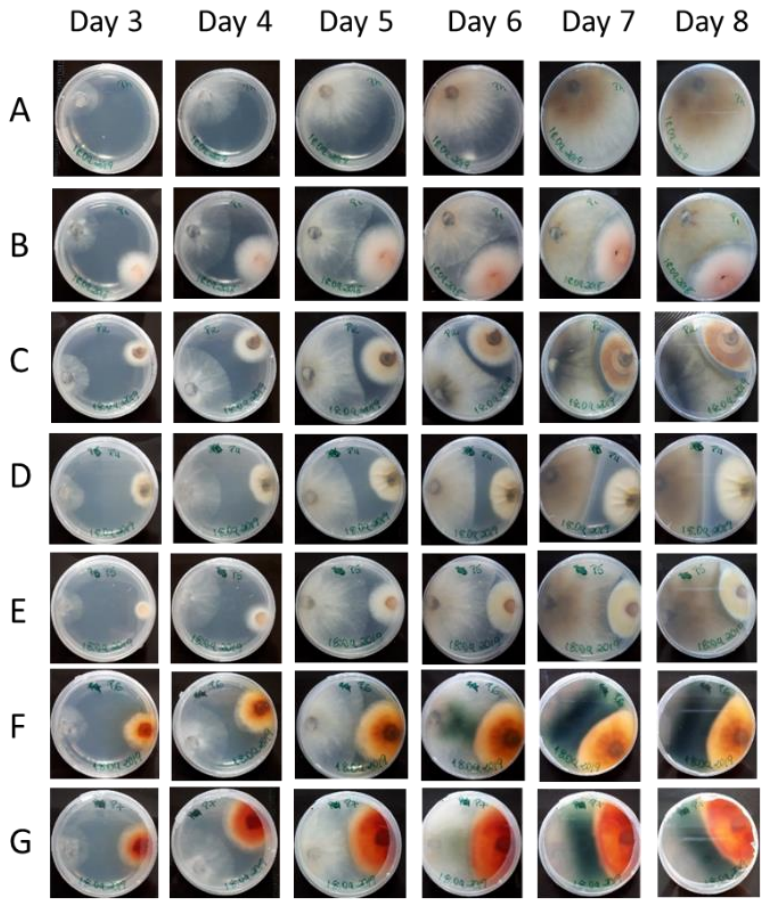

Figure 19. Interactions of *Diaporthe* sp*.,* over the days, with grapevine endophytic fungi. *Diaporthe sp* on the left side and the endophytic on the right side of the Petri dish. (A) *Diaporthe* sp*.* (negative control)*,* (B) *Diaporthe sp.* X *Fusarium oxysporum, (C) Diaporthe* sp*. X Trichoderma* sp*., (D) Diaporthe* sp. *X Aspergilus niger, (E) Diaporthe* sp. *X Penicillium* sp*., (F) Diaporthe sp. X Clonostachys rosea, (G) Diaporthe* sp. *X Epicocum nigrum.* Pictures from the reverse side of the colonies.

4.5.2 Antagonism action against *Diplodia pseudoseriata*

Analysing the mycelial growth of *Diplodia pseudoseriata* over the days, it was verified that: from the 3th day there was a slowdown in the pathogen growth caused by the presence of the endophytic fungi comparing to the negative control. After the 4th day, all the growth radius values of *Diplodia pseudoseriata* were lower than the radius values of the negative control (Figure 20), what was confirmed by statistical analyses (Figure 21).

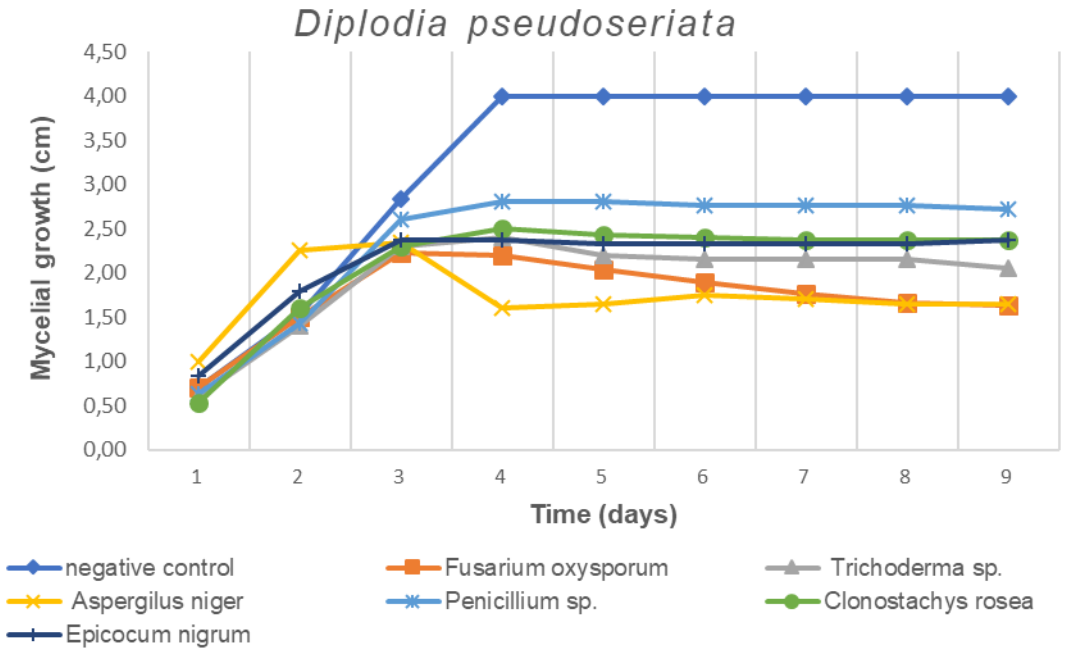

Figure 20. *Diplodia pseudoseriata* radial mycelial growth (cm) over time (days), registered in direct inhibition tests with some endophytic fungi.

The statistical analyses of the results presented on the Figure 21 allowed us to ascertain the differences between *Diplodia pseudoseriata* radial growth on the control dish and on PDA dishes with the presence of endophytes fungi. The ANOVA analysis revealed that the growth *D. pseudoseriata* in the negative control was always significant higher ($p < 0.01$) compared to the *D. pseudoseriata* used in the antagonism assay.

The average growth ± SE of *Diplodia pseudoseriata* in the negative control dish ranged from 2.83 cm ± 0.03 on the 3th day to 4.00 ± 0.00 on the 9th day. The average growth ± SE of *Diplodia pseudoseriata* versus *Fusarium oxysporum* ranged from 2.23 cm ± 0.03 on the 3th day to 1.63 ± 0.03 on the 9th day. The average growth ± SE of *Diplodia pseudoseriata* versus *Trichoderma* sp. ranged from 2.30 cm ± 0.08 on the 3th day to 2.05 ± 0.04 on the 9th day. The average growth ± SE of *Diplodia pseudoseriata* versus *Aspergilus niger* on the antagonism assay ranged from 1.57 cm ± 0.09 on the 3th day to 1.65 ± 0.04 on the 9th day. The average growth ± SE of *Diplodia pseudoseriata* versus *Penicillium* sp. in the antagonism assay ranged from 2.60 cm ± 0.06 on the 3th day to 2.72 ± 0.04 on the 9th day. The average growth ± SE of *Diplodia pseudoseriata* versus *Clonostachys rosea* ranged from 2.30 cm ± 0.15 on the 3th day to 2.37 ± 0.32 on the 9th day. The average growth ± SE of *Diplodia pseudoseriata* versus *Epicocum nigrum* ranged from 2.37 cm ± 0.09 on the 3th day to 2.37 ± 0.17 on the 9th day (Figure 21).

The ANOVA analysis revealed that the *Penicillium* sp. showed significant higher antagonistic effect compared to *Fusarium oxysporum* ($p< 0.0006$), *Trichoderma* sp. ($p< 0.0093$) and to *Aspergilus niger* ($p< 0.0011$). *Epicocum nigrum* showed significant higher antagonistic effect compared to *Aspergilus niger* ($p< 0.01$). *Trichoderma* sp. showed significant higher antagonistic effect compared to *Fusarium oxysporum* ($p< 0.0353$) and *Aspergilus niger* ($p< 0.0137$). *Fusarium oxysporum* showed significant higher antagonistic effect compared to *Aspergilus niger* ($p< 0.017$) and lower antagonistic effect compared to *Epicocum nigrum* ($p< 0.0123$). The ANOVA analysis also revealed no significant differences between the remaining fungi ($p> 0.05$): *Fusarium oxysporum vs Clonostachys rosea*, *Trichoderma* sp. *vs Clonostachys rosea*, *Trichoderma* sp. *vs Epicocum nigrum*, *Aspergilus niger vs Clonostachys rosea*, *Penicillium* sp. *vs Clonostachys rosea*, *Penicillium* sp. *vs Epicocum nigrum* and *Clonostachys rosea vs Epicocum nigrum* (Figure 21).

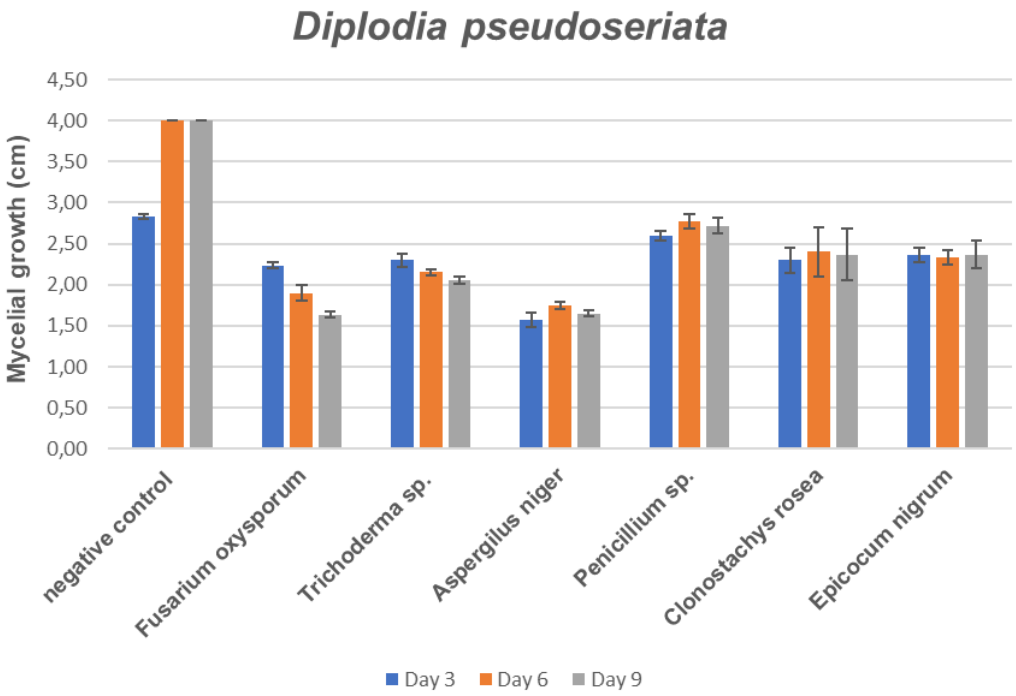

Figure 21. Radial growth values of *Diplodia pseudoseriata* (cm) (r *D. pseudoseriata* ± SE) in the presence of different endophytic fungi on the 3th, 6th and 9th day of direct antagonism test.

Mycelial growth of *Diplodia pseudoseriata* was stopped by all the endophytic fungi studied in the antagonism tests. However, the endophytic fungus *Aspergilus niger* did not even touched the pathogen to stop its growth. *Epicocum nigrum* started touching the margins of the pathogen on the 3th day; *Fusarium oxysporum* and *Penicillium* sp. on the 4th day; *Clonostachys rosea* on the 5th day and *Trichoderma* sp. on the 6th day (Figure 22).

Changes on *Diplodia pseudoseriata* mycelium pigmentation was also observed during interspecific mycelial interactions. *Diplodia pseudoseriata* colony did not change colour when interacting with *Penicillium* sp*.* In all the other interactions, the colonies of *Diplodia psudoseriata* became dark brown (Figure 22).

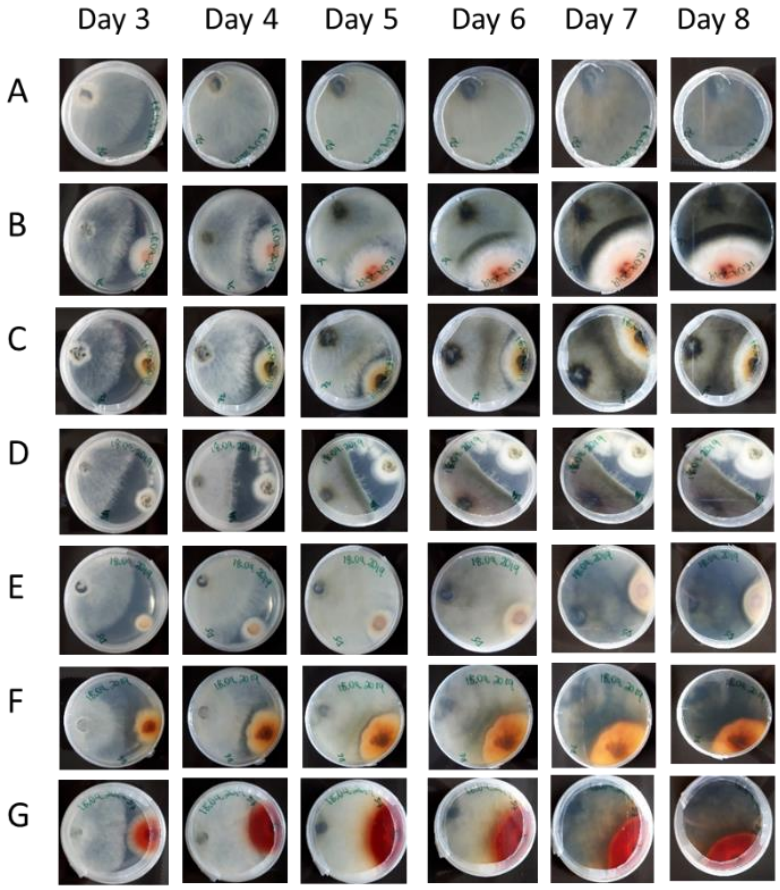

Figure 22. Interactions of *Diplodia pseudoseriata,* over the days, with grapevine endophytic fungi. *Diplodia pseudoseriata* on the left side and the endophytic on the right side of the Petri dish. (A) *Diplodia pseudoseriata* (negative control), (B) *Diplodia pseudoseriata* X *Fusarium oxysporum, (C) Diplodia pseudoseriata X Trichoderma* sp., *(D) Diplodia pseudoseriata X Aspergilus niger, (E) Diplodia pseudoseriata X Penicillium* sp., *(F) Diplodia pseudoseriata X Clonostachys rosea, (G) Diplodia pseudoseriata X Epicocum nigrum.* Pictures from the reverse side of the colonies.

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4.5.3 Antagonism action against *Phialophora* sp.

Analysing the mycelial growth of *Phialophora* sp**.** over the days was verified a slowdown in the pathogen growth from the 3th day comparing to the negative control, caused by the presence of the endophytic fungi. After the 4th day, all the growth radius values of *Phialophora* sp**.** were lower than the radius values of the negative control (Figure 23), the same was observed for *Diplodia pseudoseriata* growth (Figure 20). The ANOVA analysis

revealed that the growth *Phialophora* sp. in the negative control was always significant higher ($p < 0.01$) compared to the *Phialophora* sp. used in the antagonism assay (Figure 24).

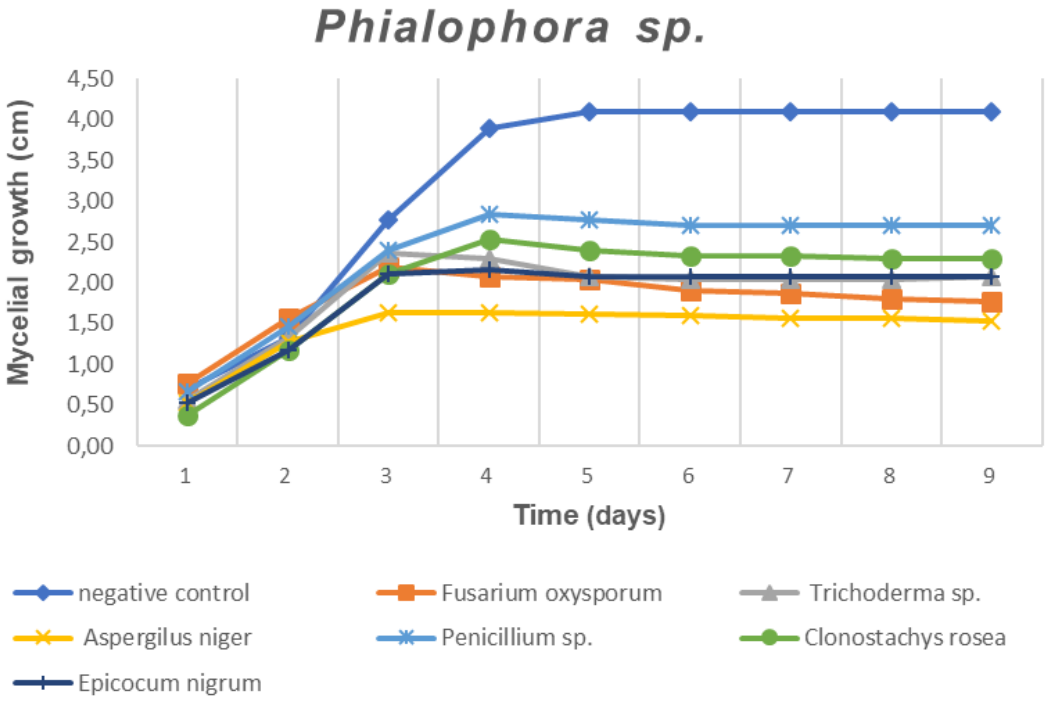

Figure 23. *Phialophora* sp. radial mycelial growth (cm) over time (days), registered in direct inhibition tests with some endophytic fungi.

The average growth ± SE of *Phialophora* sp. in the negative control dish ranged from 2.77 cm ± 0.09 on the 3th day to 4.10 ± 0.10 on the 9th day. The average growth ± SE of *Phialophora sp.* versus *Fusarium oxysporum* ranged from 2.20 cm ± 0.00 on the 3th day to 1.77 ± 0.13 on the 9th day. The average growth ± SE of *Phialophora sp.* versus *Trichoderma sp.* ranged from 2.37 cm ± 0.15 on the 3th day to 2.07 ± 0.22 on the 9th day. The average growth ± SE of *Phialophora sp.* versus *Aspergilus niger* on the antagonism assay ranged from 1.63 cm ± 0.13 on the 3th day to 1.53 ± 0.12 on the 9th day. The average growth ± SE of *Phialophora sp.* versus *Penicillium sp.* in the antagonism assay ranged from 2.40 cm ± 0.15 on the 3th day to 2.70 ± 0.25 on the 9th day. The average growth ± SE of *Phialophora sp.* versus *Clonostachys rosea* ranged from 2.10 cm ± 0.06 on the 3th day to 2.30 ± 0.06 on the 9th day. The average growth ± SE of *Phialophora* sp. versus *Epicocum nigrum* ranged from 2.10 cm ± 0.06 on the 3th day to 2.07 ± 0.15 on the 9th day (Figure 24).

In addition, the ANOVA analysis revealed that the *Penicillium* sp. showed significant higher antagonistic effect compared to *Fusarium oxysporum* ($p< 0.0392$) and to *Aspergilus niger* ($p< 0.0087$). *Aspergilus niger* showed significant lower antagonistic effect compared to

Clonostachys rosea (p< 0.0059), *Epicocum nigrum* (p< 0.0277) and *Trichoderma* sp. (p< 0.0381). *Fusarium oxysporum* showed significant lower antagonistic effect compared to *Clonostachys rosea* (p< 0.0293). The ANOVA analysis also revealed no significant differences between the remaining fungi (p> 0.05): *Fusarium oxysporum vs Trichoderma* sp., *Fusarium oxysporum vs Aspergilus niger, Fusarium oxysporum vs Penicillium* sp., *Fusarium oxysporum vs Clonostachys rosea*, *Fusarium oxysporum vs Epicocum nigrum*, *Trichoderma* sp. *vs Aspergilus niger*, *Trichoderma* sp. *vs Penicillium* sp., *Trichoderma* sp. *vs Clonostachys rosea*, *Trichoderma* sp. *vs Epicocum nigrum, Aspergilus niger vs Penicillium* sp., *Aspergilus niger vs Clonostachys rosea*, *Aspergilus niger vs Epicocum nigrum*, *Penicillium sp. vs Clonostachys rosea*, *Penicillium* sp. *vs Epicocum nigrum* and *Clonostachys rosea vs Epicocum nigrum* (Figure 24).

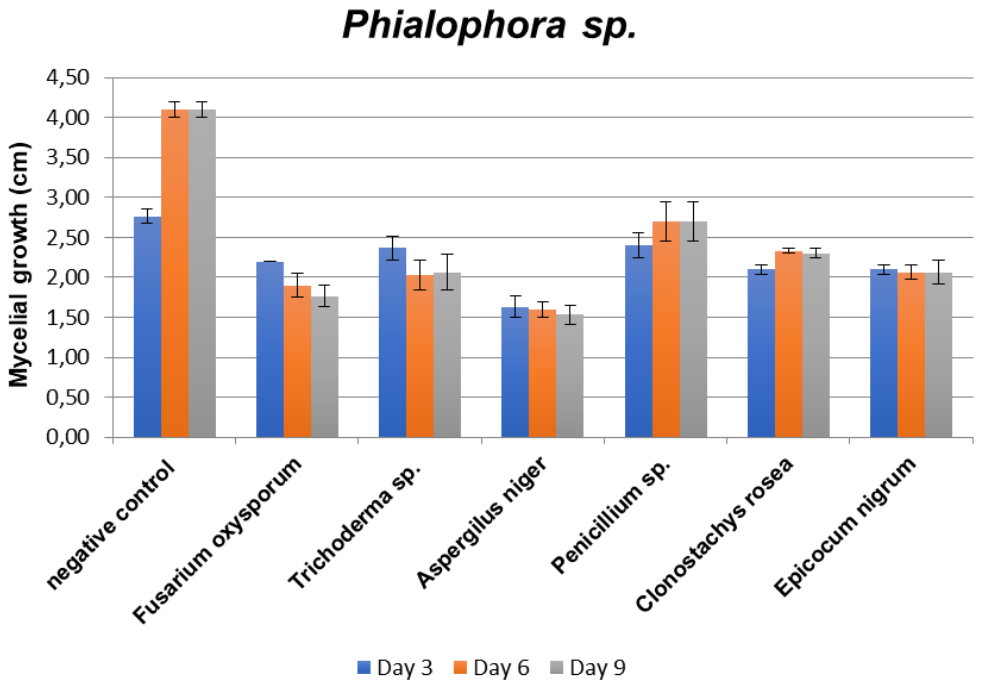

Figure 24. Radial growth values of *Phialophora* sp. (cm) (r *Phialophora* sp. ± SE) in the presence of different endophytic fungi on the 3th, 6th and 9th day of direct antagonism test.

Mycelial growth of *Phialophora* sp. was stopped by all the endophytic fungi studied in the antagonism tests, once they were fighting for space. In the all interactions, the pathogen touched the margins of the endophytic fungi. *Fusarium oxysporum* started touching the margins of the pathogen on the 3th day; *Trichoderma sp.* and *Epicocum nigrum* on the 4th day; *Penicillium* sp. and *Clonostachys rosea* on the 5th day and *Trichoderma* sp. on the 8th day (Figure 25).

Changes on *Phialophora* sp. mycelium pigmentation was also observed during interspecific mycelial interactions. The pathogen did not change colour only in the interaction with *Trichoderma sp.* In all the other interactions, the colonies of *Phialophora sp.* became darker brown (Figure 25).

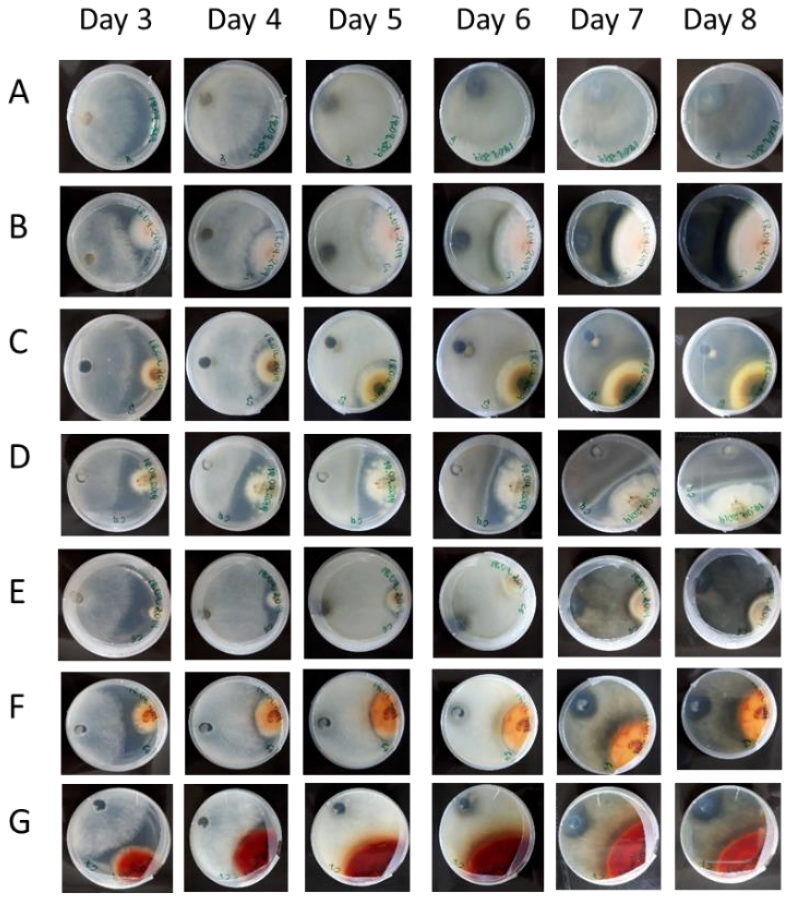

Figure 25. Interactions of *Phialophora sp.,* over the days, with grapevine endophytic fungi. *Phialophora sp.* on the left side and the endophytic on the right side of the Petri dish. (A) *Phialophora sp.* (negative control), (B) *Phialophora sp.* X *Fusarium oxysporum, (C) Phialophora sp. X Trichoderma sp., (D)* Phialophora sp. *X Aspergilus niger, (E) Phialophora sp. X Penicillium sp., (F) Phialophora sp. X Clonostachys rosea, (G) Phialophora sp. X Epicocum nigrum.* Pictures from the reverse side of the colonies.

4.5.4 The growth inhibition of GTDs pathogens.

The growth inhibition percentage of *Diaporthe sp., Diplodia pseudoseriata* and *Phialophora* sp. was calculated on the 9th day of the direct inhibition test, using the formula described on the 3.6.1. Chapter.

The inhibition percentages calculated for fungal isolates ranged from 30.70% to 62.60% (Table 9), showing that all endophytic fungi had some inhibitory action against the growth of the GTDs pathogens used for the test.

On the 9th day of the interspecific mycelial interactions the inhibition percentages values over the growth of *Diaporthe* sp. was: 50.88%; 41.23%; 47.37%; 30.70%; 45.61% and 48.25%; respectively in the presence of *Fusarium oxysporum*, *Trichoderma sp., Aspergilus niger, Penicillium sp., Clonostachys rosea, Epicocum nigrum.* The inhibition percentages over the growth of *Diplodia pseudoseriata* were 59.17%; 48.75%; 58.75%; 32.08% in the presence of *Fusarium oxysporum*, *Trichoderma sp., Aspergilus niger, Penicillium sp.,* respectively*;* and 40.83% in the presence of *Clonostachys rosea* and *Epicocum nigrum.* The inhibition percentages over the growth of the growth of *Phialophora sp.* were 56.91%; 49.59%; 62.60%; 34.15%; 49.59%; 43.90% in the presence of *Fusarium oxysporum*, *Trichoderma sp., Aspergilus niger, Penicillium sp., Clonostachys rosea, Epicocum nigrum.*; respectively (Table 9).

Table 9. Inhibition percentage values observed 9 days after inoculation of both fungi in PDA plates during direct inhibition test.

	Inhibition percentage		
	Diaporthe sp.	*Diplodia pseudoseriata*	*Phialophora sp.*
Fusarium oxysporum	50,88%	59,17%	56,91%
Trichoderma sp.	41,23%	48,75%	49,59%
Aspergilus niger	47,37%	58,75%	62,60%
Penicillium sp.	30,70%	32,08%	34,15%
Clonostachys rosea	45,61%	40,83%	43,90%
Epicocum nigrum	48,25%	40,83%	49,59%

5. Discussion

5.1. Identification of fungi responsible for GTDs

In the summer of 2017, visual analysis of some vineyards in the Alentejo region detected vines showing evident symptoms of water stress, sparse and chlorotic foliage, delayed budburst, dead buds, dieback and apoplexy, symptoms usually associated to GTDs creating an opportunity to investigate the main phytopathogenic fungi associated with those diseases in Alentejo region. Since some GTDs fungi can be, during a time in their lives, endophytes that do not cause symptoms in the vine, asymptomatic plants of the same cultivars located in the same plots were also analysed.

GTDs are an important diseases complex which affect all the vineyard areas and cause economic loss around the globe. Many researches have been carried out to understand these diseases complex in order to find better solutions to stop their spread and to control their incidence. The management of GTDs is very difficult due to the wide amount

of phytopathogenic fungi involved, variety of symptomatology that can be often confused with other type of biotic or abiotic disease, the low efficiency of pesticides, the lack of care with the equipment, especially with the pruning tools. The control of this complex is based on prevention and mitigation; thus, several researchers believe that an effective management is done using a group of strategies such as physical, chemical and biological control.

From different parts of sampled plants (roots, petioles and outshoots), 3054 organisms were isolated with fungal-like morphological characteristics. Fungi were grouped based on morphological analyse characteristics and 101 morphologically different fungi were submitted to an extraction of genetic DNA which allowed an amplification of the ITS region. After sequencing of the ITS region, 40 different fungi were successfully identified and 67.5% of the identification were at species level, higher than the 40% of the isolates identified in a study involving fungal endophytic communities associated to grapevine cultivars (Varanda *et al*., 2016). Despite being suitable and the primary genetic marker for molecular identification at species-level (Nilsson *et al*., 2014), ITS regions present low taxonomic resolution for some species delimitations (Porras-Alfaro *et al*., 2014) and cannot sometimes work well, mainly in some highly speciose genera; such as *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichoderma*; due to narrow barcode gaps or lack of them (Raja *et al*., 2017).

In the study here presents, among the 40 fungi identified by molecular techniques, 9 were associated to GTDs, being 6 identified at species level (*Hormonema viticola, Truncatella angustata, Stereum hirsutum, Phialophora fastigiata, Cytospora acaciae, Diplodia pseudoseriata*) and 3 to genera level (*Diaporthe* sp*., Pestalotiopsis* sp*., Neofusiccoccum* sp.).

GTDs are primarily caused by ascomyceteous fungi. However, some basideomiceteous taxa are also thought to play an important role in this disease complex (Fischer, 2002; Gamaje et al., 2018), such as *Stereum armenicum* from *Stereaceae* family, the only fungus belonging to the *Basidiomycota* Division identified in this study. Among the ascomyceteous identified in this work two belong to *Botryosphaeriaceae* family, two to *Dothioraceae*, two to *Amphisphaeriaceae*, one to *Herpotrichiellaceae*, one to *Valsaceae*.

Fungi from *Botryosphaeriaceae* family can cause cankers and consequent grapevine dieback in the most important grape growing areas in the world; and are associated to the disease "Botryosphaeria dieback" of grapevines (Úrbez-Torres *et al*., 2009). *Diplodia seriata* and *Neofusicoccum parvum*, fungi identified in this work, are two of the most frequently isolated *Botryosphaeriaceae* fungi in grapevine areas worldwide (Úrbez-Torres, 2011).

Hormonema viticola and *Diaporthe* sp. belong to *Dothioraceae* family. *Hormonema viticola* is a new fungus associated to GTDs and it was first identified in Canary Islands from

grapes in *Vitis vinifera* cv. Malvasia (Crous *et al*., 2015). According to Guarnaccia *et al*. (2018), the generic names *Diaporthe* and *Phomopsis* are no longer used to distinguish different morphs of this genus and *Diaporthe* is the denomination used nowadays (Rossman *et al*., 2015). Pathogenicity of several *Phomopsis spp./Diaporthe spp*., including *Phomopsis viticola* (Ascomycota, *Diaporthales*; syn. = *Diaporthe ampelina*), on grapevines was identified in green shoots of new vegetative growth (van Niekerk *et al*., 2004). However it is clear that *Phomopsis Viticola,* the principal pathogen associated to Phomopsis dieback disease, can be also associated with cankers (Úrbez-Torres *et al*., 2009; Barba *et al*., 2018).

Truncatella angustata and *Pestalotiopsis* sp., commonly known as pestalotioid, belonging to *Amphisphaeriaceae* family, have been reported from grapevines with decline symptoms (Arzanloua et al., 2013). *Pestalotiopsis* sp. and *Truncatella* sp. were associated with grapevine cankers in Texas. Pathogenicity of a *Truncatella* sp. showned low virulence and low percentage recovery from necrotic tissue indicate that this specie may act as a weak and/or opportunistic pathogen on grapevine (Úrbez-Torres et al., 2009). *Phialophora fastigiata*, originally described as *Cadophora fastigiata* (Cole & Kendrick, 1979), belongs to Herpotrichiellaceae family. The role of *Cadophora/Phialophora* in the decline of grapevine (*Vitis vinifera L.*) had been reported from many grapevines growing countries causing wood lesions and black streaking in longitudinal stem sections, the typical internal symptoms of esca and Petri disease (Travadon *et al.*, 2014). *Cytospora acaciae* belongs to the genus *Cytospora*, family Valsacea. According to Lawrence *et al.* (2016), *Cytospora sp*. canker shows some of the same general dieback-type symptoms as botryosphaeria, eutypa and phomopsis diebacks, placing these fungi in the grapevine trunk-disease complex.

In the present study, it was possible to identified GTDs pathogens in symptomatic and asymptomatic plants. Environmental conditions can alterate the plant-fungus balance leading to the activation of the virulence factors of fungi (Kusari et al., 2012), resulting in disease in the host. Plant diseases may result from continuous irritation generated by a pathogen what causes malfunction of host cells and their tissues (Agrios, 2005), and it leads to the development of symptoms. However, if the environmental conditions are not favourable and the pathogenicity of the fungus is not activated, the fungus can enter in a latent state and remain inside the host without causing any symptoms (Aly et al., 2011). The presence of antagonistic microorganisms can also difficult the development of the disease, stopping the colonization of the pathogen by the competition for nutrition and space or by the production of secondary metabolites that inhibit fungal growth (Gonzalez and Tello, 2011; Landum et al., 2016).

In this study, the most commonly identified fungus associated with GTDs disease in asymptomatic plants was *Hormonema viticola*, presented in all plant tissues studied, mainly in plants belonging to Trincadeira cultivar. However, the most incidence of this fungus in asymptomatic plants was identified in the petioles of the plants. *Diaporthe* was the second most identified fungi in symptomatic plants. Fungi belonging to this genera were already reported to be pathogen also saprobic and endophyte (Udayanga *et al*., 2014); therefore, fungi from this genus are frequently present in asymptomatic plant tissues as endophyte fungus (Sessa *et al*., 2018). It was possible to identify incidence of fungi belonging to the *Diaporthe* genera in samples from asymptomatic plants in the three areas, the two cultivars and in all plant organs studied. Despite the fact that, there is a lack of information about *Hormonema viticola*, especially linked to grapevine plants, this fungus belongs to the same family as *Diaporthe*, which can be an interesting beginning for future studies.

Neofusicoccum species, Truncatella angustata, Stereum hirsutum, Cytospora acaciae were verified in asymptomatic plants belonging mainly to Trincadeira cultivar. Once inside the host, endophytes enter a latent state (Aly *et al*., 2011), which can be the mainly reason for the late onset of symptoms caused by the infection of GTDs pathogens. Plant-endophyte balance can be altered by environmental conditions what can activate virulence factors leading to pathogenicity of the fungus (Kusari *et al*., 2012), which explains the presence of phytopathogenic fungi identified in this study inside also of asymptomatic plants.

In symptomatic plants, the most predominated GTDs fungi in the three areas studied were: *Diaporthe sp., Hormonema viticola* and the fungi associated to *Neofusiccoccum genera. Phomopsis viticola* was also one of the most dominant species identified in a field study by several vineyards in Portugal *(*Phillips*,* 1998*).* The fungus *Phialophora fastigiata* was identified mainly in symptomatic plant of Trincadeira cultivar in FEA site. *Truncatella angustata, Stereum armeniaccum, Cytospora acaciae, Diplodia pseudoseriata* and the fungi from *Pestalotiopsis genera* were identified on only a few occasions.

GTDs pathogens were verified in all plant organs, however their incidence in the organs showed differences, once vascular fungi like GTDs pathogens do not colonize systemically (Pouzoulet *et al.,* 2014). In other words, once inside the plant, vascular fungi responsible for cankers colonize the organ which was infected, and their spores do not spread throughout the plant. *Diaporthe* sp. was the only fungus whichtheincidence was observed similarly in asymptomatic and symptomatic plants, in which offshoot was the most infected organ, follow by petiole and root, respectively, showing that the infection of the pathogen in the plant could possibly happened mainly by pruning wounds (Gramaje, 2018). Even though,

GTDs fungi infection happen primarily by pruning wound, the infection by those pathogens can also happen through any type of open wound such as those caused by retraining, trimming, and de-suckering (Makatini et al., 2014), what can possible explain the identification of GTDs fungi in different organs of the plants. According to Gramaje *et al.,* 2018, some GTDs pathogen, like fungi responsible for black foot, are soilborne and are commonly found in nursery fields and soils. Therefore, inoculum may already exist in soils before planting and infection can happen by some wounds caused by culture management what explain the incidence of some phytopathogens in roots, like what happened to *Truncatella angustata* and *Stereum armeniaccum that* were verified only in the roots of both, asymptomatic and symptomatic plants.

5.2 Endophyte antagonism activity

To provide an efficient control against GTDs, researches and specialists have been testing control techniques set, which can involve also biological alternatives. Biological control against fungi have been studied in the last decades and some researchers believe that it can be a good alternative to maintain GTDs fungi under control. Several studies have already shown that some fungal endophytes have beneficial effects on their hosts, such as in grapevine, showing antagonistic effects against some important pathogens (Varanda *et al*., 2016). Antagonist microorganisms can be used as biological control agents, contributing to achieve productive and sustainable agriculture. Thus, many researches have still been conducting about the diversity, distribution and influence of endophytic fungi on the development and/or prevention of certain fungal diseases (Núñez-Trujillo et al., 2012).

Since the present work had the aim to identified and study the GTDs fungi with the intention of contributing on alternatives for reducing the incidence of the diseases, it was pertinent in the frame of this research a better understanding about the role of endophytes in this GTDs complex. The variety of endophyte fungi identified in this research led to a study of the interaction, *in vitro*, between possible antagonistic endophyte and GTDs phytopathogenic fungi as a start for a development of possible biological control. Therefore, direct inhibition antagonism was tested *in vitro*, using fungi identified in this research: three GTDs phytopathogenic randomly chosen (*Diaporthe sp*., *Phialophora sp*. and *Diplodia pseudoseriata)* and 6 possible antagonistic endophytes (*Fusarium oxysporum*, *Aspergillus niger*, *Penicillium sp*., *Trichoderma sp*., *Clonostachys rosea* and *Epicoccum nigrum*).

All the endophyte used for the direct inhibition antagonism tests were able to negatively affect the growth of the GTDs fungi, showing that non-pathogenic microorganisms

can possibly protect hosts through their competition with phytopathogen for space and nutritional resources. However, it is not possible to confirm that the competition for space and nutritional resources is the only mechanism responsible for the pathogen growth inhibition, once the antagonistic properties of biocontrol agents are based on the activation of multiple mechanisms, not only competition for nutrients and space but also mycoparasitism, antibiosis, metabolite production or volatile compounds production (Heydari & Pessarakli, 2010; Nunez-Trujillo *et al*., 2012; Landum *et al*., 2016).

Aspergilus niger was the only fungus that did not even touch the mycelia of the pathogen *Diaporthe* sp. and *Diplodia pseudoseriata*, however it still stopped the mycelia growth of those pathogen. The growth inhibitory effects before physical contact between fungi may suggest the antagonistic action can also occur due to the production of certain metabolites, rather than just competition or parasitism (Sezões**,** 2016).

In the 9th day of the test, inhibition percentage were calculated based on negative control of each phytopathogen. *Fusarium oxysporum* was the most effective antagonist fungi for growth inhibition of *Diaporthe* sp. and *Diplodia seriata*, and it presented inhibition rate above 50% of *Phialophora* sp. mycelial growth. *Fusarium oxysporum* is known to be a soil pathogen and responsible for grapevine decline and death (Highet and Nair, 2008; Vilvert *et al.*, 2017). *Fusarium* species were reported also to have antagonistic activity against *Colletotrichum acutatum* in olives trees (Landum *et al*., 2016).

Many antagonistic microorganisms have been proved to be active *in vitro* or *in vivo.* Among this list, the most known are the fungi from genus *Trichoderma*, genus *Aspergillus* and *Penicillium* species (Boughalleb-M'Hamdi *et al*., 2018). In this study, *Aspergilus niger* was the most potent inhibitor for mycelia growth of *Phialophora sp.*. Landum *et al*. (2016) also confirmed the success of the antagonistic activity of *Aspergilus niger* in a study with *Colletotrichum acutatum* in *Olea europaea* L. trees and the antagonistic efficiency was assigned to the rapid growth and competition for space and nutritional resourches. *Penicillium sp*. was the least efficient antagonism fungi against the growth inhibition of the three GTDs pathogen, arranging inhibition rate between 30% and 35%, however fungi belonging to this species are considered having interesting antagonistic activity against diverse pathogenic fungi due to the production of secondary metabolites with antibiotic activity (Nunez-Trujillo *et al*., 2012).

In this study, *Trichoderma* sp. was effective in pathogen grown inhibition, however it showed mycelial inhibition rate under 50% in all tests. *Trichoderma* spp. was considerate to be the most widely studied biological control agents for root and shoot pathogens for

Hajieghrari *et al*. in 2008, and in the last decades, have been the most common fungi applied as biological control agents (BCA) to combat a wide range of plant diseases. Trichoderma-based biocontrol mechanisms is mainly relying on mycoparasitism, production of antibiotic and/or hydrolytic enzymes, competition for nutrients, as well as induced plant resistance; numerous secondary metabolites which can act directly or indirectly against the targeted plant pathogen (Nusaibah & Musa, 2019).

Clonostachys rosea and *Epicocum nigrum* presented inhibition percentage around 40% and 50% in mycelia growth of all pathogen studied. *Epicoccum* species have also showed antagonism activity against some grapevine phytopathogen like *Plasmopara viticola* and *Botrytis cinerea* (Varanda *et al*., 2016). *Epicoccum nigrum* was considerate a biocontrol agents and biological control using it was developed commercially due to its capability to produce secondary metabolites with antibiotic activity (Martini *et al*., 2009). *Clonostachys rosea* has already been used as biological control in some crops. This biocontrol agent acts by two forms of antagonism: parasitism of hypha and competition for space and nutrition. During parasitism, the antagonistic fungi can remove pathogenic hyphae from the substrate and the previously colonized tissues (Haleem *et al*., 2016).

Changes pathogen mycelium pigmentation were observed during almost all interspecific mycelial interactions. *Diaporthe sp*. changed mycelia pigmentation from light brown to almost black when in contact with *Trichoderma* sp.. *Diaporthe* sp. mycelia also changed pigmentation, light brown to dark-green, when in contact with C*lonostachys rosea* and *Epicoccum nigrum*. *Diplodia pseudoseriata* mycelia pigmentation did not change only when interacting with *Penicillium* sp.. In all the interactions test with *Diplodia pseudoseriata*, the colonies of the pathogen changed color from light to dark brown. In the interaction between *Phialophora sp.* and the endophytes, only *Trichoderma* sp. did not affect the pathogen mycelia pigmentation. *Phialophora* sp. colonies cchanged color to dark brown in the interaction with the other endophytes. Preto *et al*. (2017) also observed, during interspecific mycelial interactions, changes on mycelium pigmentation of *Colletotrichum acutatum* in the contact zone with *Epicoccum nigrum, Asergillus brasiliensis and Aspergillus sp.* colonies*;* and alleged that the formation of pigments in the fungi mycelium can be a mechanism of the pathogen to protect hyphae from the antagonistic fungi by preventing access by cell wall degrading enzymes.

5.3 Molecular methods for GTDs diagnosis

The wide diversity of symptomatology and fungi involved in GTDs complex makes diseases diagnosis more difficult (Martín *et al*., 2012). Morphological similarity of some fungi and time-consuming of classical methods (Guo *et al*., 2000; Hartman et al., 2017) leaded the development of faster, easier and more precisive methods for fungi detection and identification based on molecular DNA techniques (Ma and Michailides, 2012). In the last decades, molecular methods for GTDs fungi identification have shown to be efficient tools for early diagnosis, helping in the GTDs management control, and avoiding the establishment of the diseases in the vineyard (Shirahatti *et al*., 2015).

Molecular tools, such as PCR techniques, have been used to identify endophytic and phytopathogenic fungi in grapevine plants (Overton *et al.,* 2004; Romanazzi *et al*., 2009; Pouzoulet *et al*., 2013; Varanda *et al*., 2016). Fungi identification using specific primers preceded by DNA extraction from plant tissues, allow researchers to skip intermediate steps (culturing and isolation) which is timing and money consuming (Ridgway *et al*., 2002; Martín *et al.*, 2012). Morphological methods for identification of fungi, especially Phomopsis/Diaporthe, are impressive due to the amount of cultural variation within the species in terms of colony colour, growth and sporulation rate based on different geographical areas, substrate and the external conditions (Shirahatti *et al*., 2015). The qPCR approach, through specific designed primers has shown to be a useful technology to evaluate grapevine susceptibility to *Eutypa lata* invasion and it can be adapted to be used for other pathogens associated with GTDs (Pouzoulet *et al*., 2013). An early detection of the infection before the pathogen can establish itself in the host, through specie-specific primers, is an efficient tool to establish management control against fungi (Shirahatti *et al*., 2015).

Due to the high incidence of *Diaporthe* species in this study, the absence of *Diaporthe* sp. molecular studies in grapevine (Gomes *et al.,* 2013), and lack of primers published for these fungi; specific primers and probe were designed, in this research, for fungi from *Diaporthe* genera. A first attempt was made using β-actin gene to design the specific primers. SYBR®Green qPCR assays were performed using *the* specific primers, which were designed after alignment of full-genome β-actin sequence from *Diaporthe* together with other β-actin sequences collected from NCBI database. The primers designed were successfully able to amplify the target. However, they didn't show specificity for *Diaporthe* sp. once they amplified all the other fungi tested in the assays performed in this study. Real-time SYBR®Green primers were already designed for some GTDs pathogen, such as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp*.* based on ITS region (Overton *et al.*, 2004).

A second approach was performed by the design of Taqman primers and probe for detection of *Diaporthe* sp. based on the ITS sequences previously isolated by ITS1 and ITS4 primers. The ITS region has an importance for fungal diagnostics, due to its areas of high conservation and areas of high variability, what make this region an ideal starter for the development of specific PCR primers for identification of fungal species (Atkins & Clark, 2004). qPCR assays were performed to test TaqMan primers and probe, which was positive for the amplification of the DNA of the target. However, TaqMan primers and probe designed were not also specific and could amplify all the fungi tested by qPCR assay as well. According to Porras-Alfaro *et al.* (2014), ITS region has high variability what makes difficult the alignment and the design of fungus-specific PCR in this region. During a study with *Diaporthe* in *Phaseolus vulgaris* L.*,* Santos *et al*. (2016) identified high similarities in the nucleotide sequences of the ITS rDNA region and notice that multi-locus phylogenetic analysis resulted in a more robust identification, at both the species and genus levels comparing with the use of the rDNA ITS region alone.

6. Conclusion and Future Perspectives

Through the results obtained in this present work, it was possible to conclude that: the studied vines in the Alentejo region presented diversity of endophyte and fungi responsible for GTDs. Among the fungi identified, nine were associated to GTDs phytopathogen, causers of diseases like esca, Petri disease, Phomopsis dieback and Botryosphaeria dieback. The incidence of these fungi in the two cultivars and plant organs were different, whereas *Diaporthe* sp., *Neofusicoccum* sp. and *Hormonema viticola* were the most prevalent fungi in symptomatic plants and their incidence were verified in the two cultivars and in all plant organs studied. *Hormonema viticola* was the most prevalent fungus in asymptomatic plants of Trincadeira cultivar, however its role in the complex GTDs has not been much explored yet. *Diaporthe* sp. and other GTDs phytopathogen were also verified in asymptomatic plants showing that some pathogen can also survive, part of their lives, as endophytes without causing any symptoms in the host, what can also explain the long latency time of the diseases.

The presence of GTDs pathogen in roots, petioles and offshoots lead the conclusion that those pathogens are easily spread in the vineyard and in the plant. GTDs pathogen were also presented inside the two cultivars, Trincadeira and Alicant Bouchet, showing that both are susceptive to GTDs pathogen.

In addition to the verification of GTDs phytopathogenic fungi identified, some fungal genus/species with characteristics of biological antagonists were detected and their antagonist activity were verified through direct inhibition. All endophytic fungi tested in the interaction endophyte/pathogen presented of pathogens mycelia grown inhibition, verified by competition for nutrients and space. Thus, plant hosts can be considerate the best places to obtain good antagonistic fungi against GTDs pathogens, which showed to be potential bio-control agents.

This work showed a high incidence of *Diaporthe* sp. in the vineyards studied, showing the increase importance of those fungi in Alentejo region and the necessity of further studies. The lack of publication of primers and probe designed for *Diaporthe* sp. can be possibly due to the difficulty of finding specific sequences for these fungi, as happened in this work, which the region and gene chosen to design specific primers were not efficient. Therefore, there is a need for isolation of other genes more specific for the design of new primers and probes for *Diaporthe* sp. detection.

This study has increased the knowledge of grapevine GTDs fungal communities in the Alentejo region, which is one of the most important vine productor region of Portugal. It also showed the importance of the study of the antagonism activity of some endophytic fungi in the GTDs complex. However, it certainly opens the possibility for new lines of studies based on:

- *in vivo* assays with disease-free grapevine to perform Koch Postulates and confirm the relation between GTDs phytopathogenic fungi isolated in this study and the symptoms observed in the plants.
- further exploration of grapevine endophytic diversity and their possible antagonistic activity against the GTDs pathogen in the vines for understanding their role and influence in this complex and future development of bio-control against GTDs.
- the design of specific primers and probe, based in one or more genes of GTDs fungi, to increase the specificity and allow the development of molecular diagnosis assays that can later contribute for more effective strategies against GTDs.
- the use of the New Generation Sequencing (NGS) as molecular based technique to identify also GTD fungi that are still unknown and are not cultivable through microbiological approaches.

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