UNIVERSIDADE D
COIMBRA

Jorge Manuel Esteves Carvalho Sofia

ESCA COMPLEX IN THE DÃO WINE REGION (PORTUGAL)

Tese no âmbito do Doutoramento em Biociências, ramo de especialização em Biologia Celular e Molecular orientada pela Professora Doutora Maria Teresa Silva Gonçalves e pela Investigadora Engenheira Maria Cecília Nunes Farinha Rego e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Novembro de 2018

Faculdade de Ciências e Tecnologia da Universidade de Coimbra
Departamento de Ciências da Vida

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Novembro de 2018

UNIVERSIDADE D
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To my children

Acknowledgements

A elaboração de uma tese é um projeto solitário e pessoal, como é do conhecimento de todos os investigadores. Este projeto só conseguiu chegar a bom porto com o contributo de muitas pessoas e instituições. Nas linhas seguintes gostaria de expressar os meus agradecimentos a todos os que de forma desinteressada me apoiaram neste projeto pessoal, assim como a todas as pessoas e instituições que demonstraram interesse e empenho, apoiando-me.

Em primeiro lugar, desejo expressar a minha gratidão às minhas duas orientadoras, as Professora Doutora Maria Teresa Silva Gonçalves e Investigadora Engenheira Maria Cecília Nunes Farinha Rego, por toda a confiança depositada em mim, pelo vosso empenho, apoio, ensinamentos e extraordinária paciência ao longo destes anos, na execução de um trabalho que considero uma mais-valia para a região do Dão. Fica uma dívida de gratidão e a minha amizade.

Ao colega Pedro Reis, pelo apoio nos trabalhos laboratoriais, valiosas indicações e inequívoco apoio e amizade.

À Engenheira Teresa Nascimento pelos ensinamentos na área da Biologia Molecular, entre outros.

Ao Doutor António Portugal pelo apoio na concretização do segundo capítulo, valiosas explicações, apoio e amizade que nunca me negou.

Aos colegas João Trovão, Hugo Paiva de Carvalho e Nuno Mesquita pelo apoio nos trabalhos conducentes ao 2º capítulo.

À Doutora Mariana Mota pelo apoio na análise estatística dos resultados do 4º capítulo desta tese.

Ao Doutor Manuel Malfeito Ferreira, pela sua colaboração e ensinamentos.

À Engenheira Vanda Pedroso, do Centro de Estudos Vitivinícolas do Dão da Direção Regional de Agricultura e Pescas do Centro, pelo seu apoio nos trabalhos de campo, disponibilidade permanente e conselhos. Sem o seu apoio incansável e desinteressado este trabalho nunca teria sido levado a cabo.

À Direção Regional de Agricultura e Pescas, na pessoa da Exma. Senhora Diretora Regional, Engenheira Adelina Martins, o meu agradecimento pelas facilidades

concedidas no uso das instalações, equipamentos e meios da DRAPC bem como pelo seu incentivo na prossecução e conclusão deste trabalho.

À la Professeure Florence Fontaine, Université de Reims Champagne Ardenne, chair de l'action COST FA1303 "Sustainable control of GTD", un mot de remerciement pour son soutien et les opportunités offertes dans le cadre de l'action COST.

A todos os colegas das diversas Estações de Avisos do Serviço Nacional de Avisos Agrícolas, que me apoiaram na divulgação deste trabalho.

À minha mulher pela abnegação que demonstrou para levar a bom termo este nosso novo passo.

Aos meus pais, pelo apoio e incentivo que sempre senti, quer agora quer noutras aventuras passadas.

Ao Professor Doutor Vicente Medina Piles da Universitat de Lleida por, em tempos, me ter indicado o caminho e pelo seu apoio e amizade ao longo de todos estes anos de estudo. Moltes Gràcies!

Os agradecimentos não estariam completos sem dedicar uma palavra a todos os agricultores e colegas que de uma forma desinteressada colaboraram nos inquéritos, me facultaram o acesso às suas propriedades, me indicaram locais para recolha de amostras ou me apoiaram na divulgação do meu trabalho.

A todos o meu sentido Bem-Haja.

Resumo

A designação Doenças do Lenho da Videira (DLV) compreende várias patologias, associadas a diferentes fungos fitopatogénicos, que ao afetar a estrutura fixa da planta conduzem ao seu enfraquecimento e morte. As DLV têm em comum uma natureza críptica com manifestação de sintomas característicos ao nível da madeira e da folhagem que podem demorar vários anos até se manifestarem. Estas doenças são comuns a todos os países vitícolas, sendo responsáveis por elevados prejuízos que põem em causa a viabilidade dos vinhedos afetados.

A presente tese procurou contribuir para a melhoria do conhecimento sobre DLV na região vitivinícola do Dão. Numa revisão bibliográfica sobre o estado-da-arte da investigação sobre DLV, identificaram-se a esca, a eutipiose e "dieback" associado a fungos da família Botryosphaeriaceae como as principais DLV referenciadas em videiras adultas enquanto que em plantas jovens a doença de Petri e a doença do pé-negro foram as principais DLV identificadas. O complexo da esca representa uma das DLV mais relevantes, sendo *Phaeomoniella chlamydospora* identificada como a principal espécie envolvida na sua etiologia.

No primeiro capítulo da tese "Contribution for a better understanding of grapevine fungal trunk diseases in the Portuguese Dão wine region" elaborou-se e distribuiu-se, na região do Dão, um folheto acompanhado de um questionário. O folheto com imagens detalhadas dos principais sintomas que caracterizam as diferentes DLV pretendia elucidar os vitivinicultores sobre o tema e permitiu o preenchimento do questionário e a subsequente recolha de dados acerca da presença e relevância dessas doenças nas vinhas dos participantes. Os resultados obtidos representam uma primeira indicação concisa da situação das DLV no Dão. Confirmou-se a presença das DLV, melhorou-se o conhecimento dos viticultores e concluiu-se que a severidade e incidência das DLV nas vinhas do Dão era preocupante. A Esca e a escoriose foram as doenças mais reconhecidas. O complexo da esca foi considerada a DLV mais importante no Dão, embora com baixa severidade na maior parte das vinhas. Os sintomas de "dieback" associado a fungos da família Botryosphaeriaceae e os declínios de jovens videiras, foram considerados problemas menores.

No segundo capítulo da tese, "Molecular and phenotypic characterisation of

Phaeomoniella chlamydospora isolates from the demarcated wine region of Dão (Portugal)", caracterizaram-se 68 isolados de *P. chlamydospora* avaliando a sua diversidade fenotípica e molecular para determinar a sua variabilidade intraespecífica e estrutura populacional. Os isolados formaram dois grupos, tanto nas análises fenotípicas como moleculares, não havendo correspondência entre os grupos formados nas duas abordagens. Este estudo revelou homogeneidade entre todos os isolados, apesar da sua origem geográfica diversa, diferentes anos de isolamento e combinações casta/porta-enxerto, suportando a estratégia de reprodução clonal descrita para esta espécie.

No terceiro capítulo, "Pathogenicity of *Phaeomoniella chlamydospora* isolates", descreve-se um ensaio de patogenicidade em videiras jovens, realizado em condições controladas, usando 22 isolados de *P. chlamydospora*. Todos os isolados demonstraram ser patogénicos ao causar lesões vasculares e ao serem reisolados a partir dessas lesões. As plantas infetadas não manifestaram sintomas foliares típicos de DLV. A maioria dos isolados incluídos por sequenciação de ITS no "Grupo 1" revelou-se mais agressiva, causando lesões mais longas do que os incluídos no "Grupo 2". A frequência de reisolamento de *P. chlamydospora* revelou a mesma tendência, tendo na maioria das amostras infetadas com os isolados do "Grupo 1" sido obtidos valores mais elevados de reisolamento. Estes resultados permitem, em certa medida, algumas inferências relacionando a agressividade dos isolados e os agrupamentos formados.

Por fim, no quarto capítulo intitulado "Grapevine cultivar susceptibility to *Phaeomoniella chlamydospora* infection" foi investigada a suscetibilidade de quatro das castas do encepamento do Dão (Alfrocheiro, Aragonez, Jaen e Touriga Nacional) à infeção por *P. chlamydospora*. Num ensaio, ao longo de três anos, realizado em vinhas estabelecidas, videiras daquelas castas foram infetadas separadamente com três isolados portugueses de *P. chlamydospora*. Os resultados obtidos revelaram a casta Alfrocheiro como a mais suscetível das quatro utilizadas e a casta Jaen a menos suscetível. A variação, ao longo do ensaio dos parâmetros analisados, sugere uma possível relação com os dados meteorológicos. Foram também observadas diferenças na agressividade manifestada pelos diferentes isolados.

Os resultados obtidos nesta tese disponibilizam aos vitivinicultores do Dão informação que constitui uma clara mais-valia para a sua atividade: a) a melhoria do conhecimento das diversas DLV; b) a avaliação concreta da situação dessas doenças,

facilitando a demonstração da sua importância e a necessidade de promover o seu estudo e combate; c) a patogenicidade confirmada dos vários isolados estudados reforça a necessidade de revisão da legislação europeia sobre produção e comercialização de material de propagação vegetativa de videira; d) a confirmação da existência de diferentes suscetibilidades entre algumas das castas locais à infeção por *P. chlamydospora*; providencia uma ferramenta de decisão na constituição de novos encepamentos; e) indicam a necessidade de estudar a diversidade das castas portuguesas quanto ao seu comportamento em termos sanitários; f) sustentam e sugerem ajustes nas estratégias de poda recomendadas para o controle da esca, especificamente deixando talões mais compridos e evitando podas tardias, dificultando a colonização do tronco por *P. chlamydospora*.

Palavras-chave: Alfrocheiro, Aragonez, casta, Dão, Esca, Jaen, “Petri disease”, *Phaeomoniella chlamydospora*, suscetibilidade, Touriga Nacional, *Vitis vinifera*.

Abstract

The designation Grapevine Trunk Diseases (GTDs) comprises several grapevine pathologies, associated with different phytopathogenic fungi, that by affecting the permanent structure of the plant lead to its weakening and death. GTDs share a cryptic nature with characteristic symptoms in wood and on foliage that may take several years to manifest. GTDs are common to all winegrowing countries and are responsible for significant losses by causing premature decline and dieback in vineyards worldwide.

The present thesis seeks to contribute to the improvement of GTDs knowledge in the Dão wine region. In a literature review on the state-of-the-art of GTD research, esca, Eutypa and Botryosphaeria associated diebacks were considered as the main GTDs referenced in adult vines, whereas in young plants Petri disease and black-foot disease were the most common. This review evidenced the esca complex as a major GTD worldwide, with *Phaeomoniella chlamydospora* being recognised as the most important species involved in the aetiology of that complex.

In this thesis first chapter "Contribution for a better understanding of grapevine fungal trunk diseases in the Portuguese Dão wine region", a leaflet and a simple questionnaire were produced and issued to winegrowers. The leaflet, with detailed colour photos of the main symptoms associated with each GTD, aimed to elucidate the winegrowers on the GTDs' theme. While browsing through the information stated on the leaflet, the inquired were invited to fulfil the questionnaire on the presence and relevance of those diseases in their vineyards. The results obtained in the survey represent a first concise indication of the extent of GTDs in the Dão wine region. Besides confirming GTDs' presence, the local winegrower's knowledge on GTDs was improved, concluding that the GTDs situation within that region was a matter of concern. Esca and "Phomopsis cane and leaf spot" were both well known. The esca complex, mainly esca and esca proper, were considered the foremost GTDs within the Dão, although mostly at low frequencies. Botryosphaeria dieback was not as well-known and young grapevine declines were considered lesser problems.

In the second chapter of the thesis, "Molecular and phenotypic characterisation of *Phaeomoniella chlamydospora* isolates from the demarcated wine region of Dão (Portugal)", 68 isolates of *P. chlamydospora* were characterised for phenotypic and

molecular diversity to determine its intraspecific variability and population structure. Morphological and molecular characterisation were performed, and molecular analyses were used to infer phylogenetic relationships. Isolates were in two groups, supported by phenotypic and molecular analyses, but no correspondence was found between the two approaches. Nevertheless, both analyses revealed strong homogeneity among all isolates, despite their diverse geographical origin, year of isolation and scion/rootstock combination, supporting the clonal reproduction strategy described for this species.

In the third chapter, "Pathogenicity of *Phaeomoniella chlamydospora* isolates", a greenhouse pathogenicity assay was performed on two-year potted grapevines, kept under controlled conditions, using 22 of the 68 *P. chlamydospora* isolates. All *P. chlamydospora* isolates proved pathogenic, causing vascular discolouration, and being reisolated from those lesions. However, infected plants did not show GTD related foliar symptoms. Most of the isolates included in ITS sequencing “Group 1” revealed more aggressive, causing longer lesions than the ones included in “Group 2”. The same tendency continued with *P. chlamydospora* reisolation frequencies, where most of the plants infected with “Group 1” isolates had the highest fungal recovery frequencies. These results allow, to a certain extent, some inferences relating the potential aggressiveness of the isolates to each of the formed clusters.

Finally, in the fourth chapter, the response of Dão most common grapevine cultivars (Alfrocheiro, Aragonez, Jaen and Touriga Nacional) to infection by *P. chlamydospora*, was investigated. During a three-year trial, those grapevine cultivars were separately infected with three Portuguese isolates of *P. chlamydospora*. Our results showed cv. Alfrocheiro as the most susceptible, while cv. Jaen was the less susceptible. Variation in parameters like lesion length and fungal recovery from infected spurs within different trial years suggest a relation with weather data. Differences in aggressiveness among isolates were noticed.

The results produced in this thesis are now available to the local winegrowing sector, constituting an added value for their activity: a) improved knowledge of the various GTDs; b) a concrete assessment of the situation of those diseases, facilitating the demonstration of their importance to the public authorities and the need to promote their study and control; c) the confirmed pathogenicity of the various isolates studied reinforces the need to revise european legislation on the production of grapevine vegetative

propagating material to include restrictive measures concerning fungi associated with GTDs; d) confirms the existence of different susceptibilities to *P. chlamydospora* infection among some grapevine cultivars; e) provides a decision tool to the establishment of new vineyards and indicates the need to study the genetic richness of the Portuguese grapevine cultivar pool in relation to its phytosanitary behaviour; f) supports and suggests adjustments to the recommended pruning strategies to control esca, specifically leaving longer spurs and avoiding late winter pruning, thus making difficult the trunk colonization by *P. chlamydospora*.

Keywords: Alfrocheiro, Aragonez, cultivar, Dão, Esca, grapevine, Jaen, Petri disease, *Phaeomoniella chlamydospora*, susceptibility, Touriga Nacional, *Vitis vinifera*.

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List of units and abbreviations

AFLP - Amplified Fragment Length Polymorphism;

ANOVA – Analysis of variance;

a.k.a. – also known as;

BDA – Black Dead Arm;

BLAST - Basic Local Alignment Search Tool;

bp – base pair;

BL – Bottom of the Lesion (lesion end);

°C - degree Celsius;

CE – Common Era;

cm – centimetre;

CEVDão – Centro de Estudos Vitivinícolas do Dão;

DLV- Doenças do Lenho da Videira (Eng. Grapevine Trunk Diseases);

DNA – Deoxyribonucleic Acid;

dNTP – deoxyribonucleotide triphosphate;

DRAPC – Direção Regional de Agricultura e Pescas do Centro (Eng. Centre Portugal Regional Directorate of Agriculture and Fisheries);

EC-European Commission;

EDTA - Ethylenediamine tetra acetic acid;

GLSD - Grapevine Leaf Stripe Disease;

GTD - Grapevine Trunk Diseases;

h – hour;

ICGTD - International Council on Grapevine Trunk Diseases;

ISSR – Inter-Simple Sequence Repeat;

ITS- Internal Transcribed Spacer;

IVV-Instituto do Vinho e da Vinha (Eng. Vine and Wine Institut);

LL - lesion length;

m-meter;

MA - Malt agar;

min - minute;

mm - millimetre;

mM - millimole;

mL - millilitre;

ML - Maximum Likelihood;

ML - middle of the lesion;

NCBI - National Centre of Biotechnology Information;

OIV - International Organisation of Vine and Wine;

PCR – Polymerase Chain Reaction;

PDA – Potato Dextrose Agar;

PDB – Potato Dextrose Broth;

PGI- Portuguese Geographical Indications (Port. Indicação Geográfica);

RAPD - Random Amplified Polymorphic DNA;

rDNA – ribosomal DNA;

ROS - Reactive Oxygen Species;

rpm – Rotations per minute;

s – seconds;

SNP - Simple Nucleotide Polymorphism;

SW - Symptomless Wood;

syn. – synonym;

µg – microgram;

µM – micromole;

µL – microlitre;

U – Unit;

UPGMA – Unweighted Pair-Group Method using Arithmetic Averages;

UP-PCR - Universally Primed - Polymerase Chain Reaction;

U.V. - Ultra-Violet;

TBE – Tris/Borate/EDTA.

Preface

Since the beginning of my career in the early 90s as a plant pathologist, after being hired by DRAPC to collaborate in two projects on grapevine trunk diseases, the subject drew my attention. While working on those projects, local winegrowers started to consult me to solve problems that were slowly decimating their vineyards, mostly within the esca complex spectrum. Without any practical solutions, besides prophylactic measures and the recommendation for spraying with sodium arsenite, not very popular among local winegrowers, I began to study this ailment, its causes and possible solutions. A week stay with Drs. Philipe Larignon, Pascal Lecomte and Bernardette Dubos in 1998 at the INRA facilities in Villenave D'Ornon, Bordeaux allowed me to get a better knowledge of GTDs and lab techniques. Later, in Lisbon, during another training course on GTDs, Cecília Rego presented me to the world of the International Council on Grapevine Trunk Diseases (ICGTD). At the end of the 90s, I started the *periplus* of International Workshops on Grapevine Trunk Diseases (IWGTD), followed by a COST action on GTDs. During this period, meetings in Siena, Lisbon, Cape Town, Florence, Valencia, Reims, Bordeaux, Cognac, Warsaw, Logroño and a "training school" on GTDs in Valencia, led by David Gramaje, Josep Armengol and Artur Alves allowed me to get in touch with the state-of-the-art of knowledge and world top researchers on the theme of GTDs, improving my knowledge and skills.

Meanwhile, I obtained an MSc degree with a thesis on the grapevine esca complex, here at FCTUC, oriented by Maria Teresa Gonçalves (UC) and Helena Oliveira (UL). This work identified the principal agents related to this complex in the Dão wine region. *Phaeomoniella chlamydospora* emerged as the most aggressive of the studied pathogens. Also, from the hundreds of isolations made for local winegrowers, *P. chlamydospora* appeared as one of the most recovered fungi and the principal on young plants presenting symptoms of Petri disease. As field phytosanitary advisor, I also noticed that this disease was progressing in the region, mostly unnoticed, due to the general lack of knowledge on its symptoms and aetiology. The ubiquitous presence of that fungal species, the increasing number of plants affected by esca and the need to help the Dão wine region controling this problem gave me the motivation for the present work.

In the present thesis, based on the previous information, it's intended to give

precise information on the different GTDs to the main grape-growing stakeholders in order to obtain more precise data on their severity and incidence. Considering the esca complex as the main GTD within this region, I intended to highlight the importance of *P. chlamydospora* and the possible different susceptibility of the main Dão red-grape cultivars to the infection by this species, thus providing the grape-growing stakeholders with useful data that can be a tool to avoid those ailments.

Viseu, 27th November 2018

Jorge Manuel Esteves Carvalho Sofia

General introduction

Background

Due to a combination of climatic conditions, soils and culture, Portugal is a country with an inherent ability for wine production. The International Organization of Vine and Wine (OIV) ranks Portugal as the 4th European producer and the 11th highest wine-producing country in the world (OIV, 2017a).

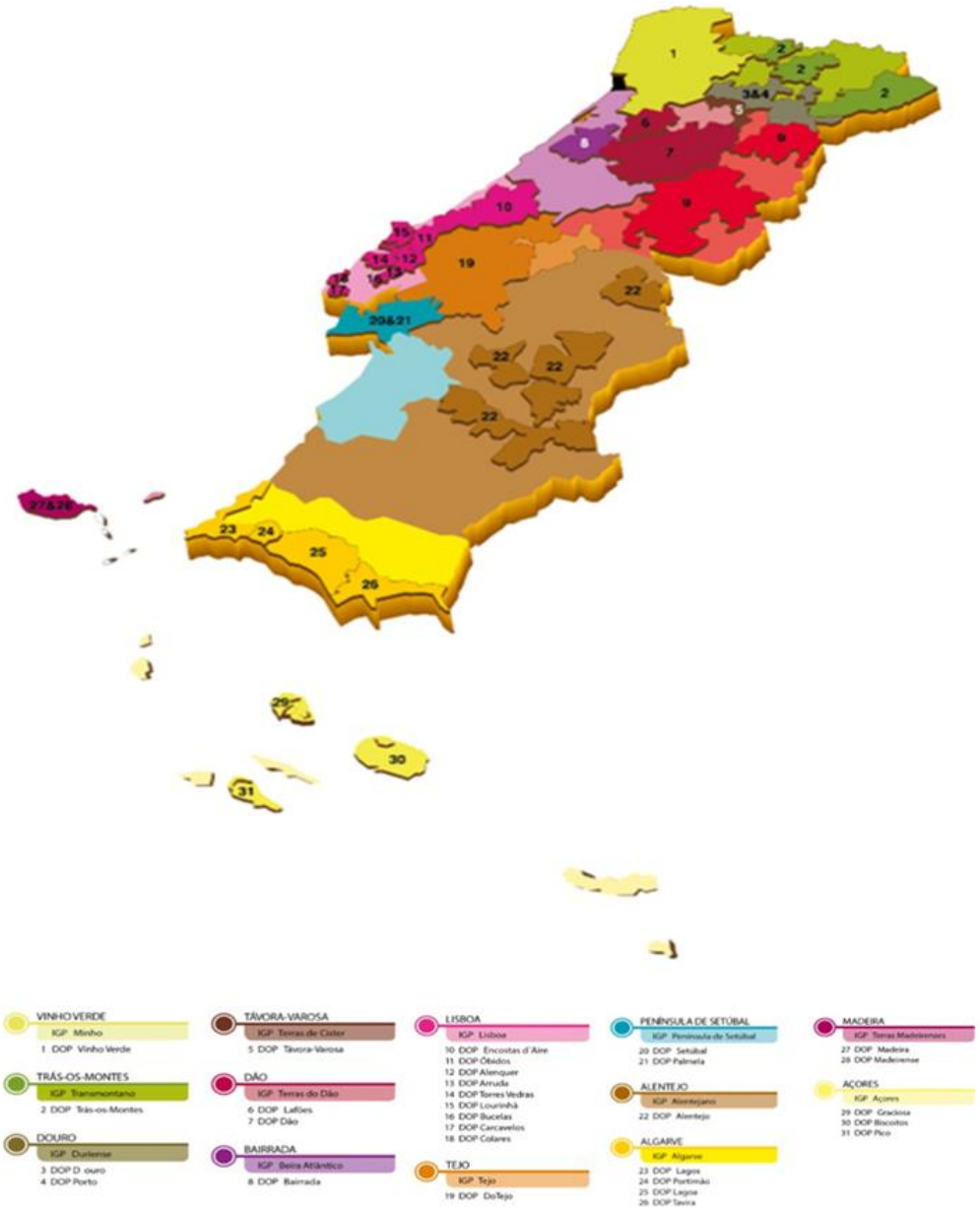


Figure 1. The Portuguese Geographical Indications and Denominations of Origin (adapted from IVV, 2017).

Vineyards are grown throughout 13 broad regions (Portuguese Geographical Indications-PGI) in the mainland and overseas Portugal (Figure 1), representing approximately 190.467 ha of vineyards (OIV, 2017a).

Three hundred and forty three (343) cultivars are approved for wine production under Portuguese law (Decreto-Lei nº 380/2012 of 22 November), from which 240 are considered specific to the country (Cunha *et al.*, 2016). Analysing data for 2016, IVV statistics show that the Portuguese wine sector generated a revenue in exports of *circa* 110 million euros (IVV, 2017), unveiling the noticeable impact of this economic activity for those winegrowing regions and affiliated sectors (e.g. nurseries, glass factories, cork sector, label sector, transport logistics, local employment, grapevine assets industry, and so forth). Within those regions figures the "Dão" Geographical Indication (a.k.a. Dão), with a total area of 376,000 ha, from which 14.467 ha dedicated to grapevine (IVV, 2017).

Historically Dão wine production might go back to Roman times (Loureiro and Cardoso, 1993), though the oldest signs of wine production date from the VI century CE, like winepresses carved on solid granite (Falcão, 2012) found throughout the entire region (Figure 2).

Figure 2. Typical "lagareta" - Winepress carved on a rocky outcrop of granite, located north of Viseu.

Although a basin for some of the most important Portuguese rivers (e.g. Mondego, Vouga, Alva, Dão), the Dão wine region receives its name after the Dão river, that starts, flows and ends within the limits of the region. It is situated on a plateau, protected by a series of mountain ranges from the Atlantic sea and Iberian meseta influences. One of its characteristics is the dense hydrographic network of small rivers and streams, originating on the surrounding mountains that influence its climate, characterised by dry summers with hot days followed by cool nights and mild, rainy winters. Soils are mostly sandy of granitic genesis (Loureiro and Cardoso, 1993). Its viticulture is characterised by a mosaic of small vineyards (Figure 3), unnoticeably installed among pine forests, composed mostly of endemic and locally propagated grapevine cultivars. This physical and historical isolation as well as its unique characteristics, made of this region a grapevine diversity pool and a reservoir for Portuguese grapevine cultivars (Lopes *et al.*, 2006; Almadanim *et al.*, 2007; Cunha *et al.*, 2009; Silva *et al.*, 2018).

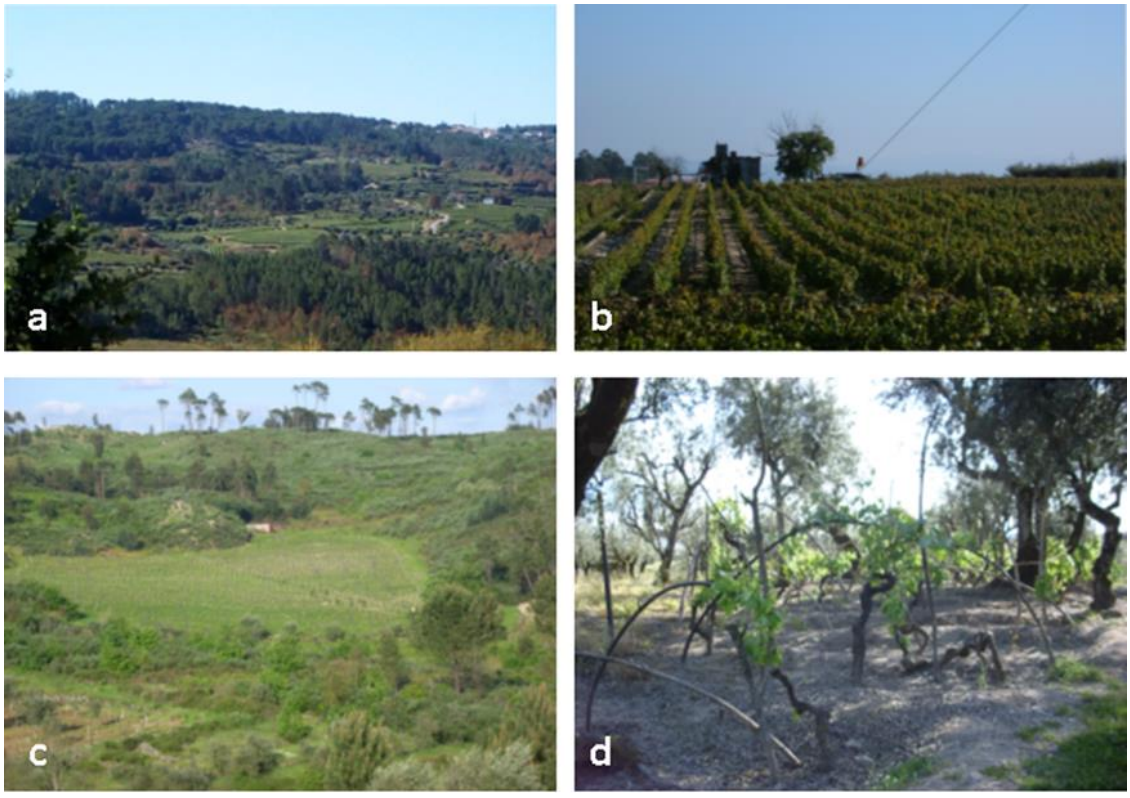


Figure 3. Different aspects of the Dão vineyards landscape: a) Typical landscape of vineyards surrounded by pine trees; b) common disposition of modern Dão vineyards; c) Traditional vineyard on a rocky slope, making complete use of all the available arable land; d) Old and traditional vineyard using pine branches to support the canopy.

Grapevine Trunk Diseases

An overview

Several fungal diseases (e.g. Downy mildew caused by *Plasmopara viticola*; powdery mildew caused by *Uncinula necator*; Botrytis leaf and bunch dry rot caused by *Botrytis* spp.; Black rot caused by *Guignardia bidwellii*) affect grapevines (Figure 4), causing, if not controlled, severe damage and economic losses, but hardly compromising the sustainability of the affected grapevines. All the mentioned fungal diseases have in common a known causal agent, typical symptomatology, known epidemiology and several tools to control their development or consequences, making their impact bearable for the winegrower.

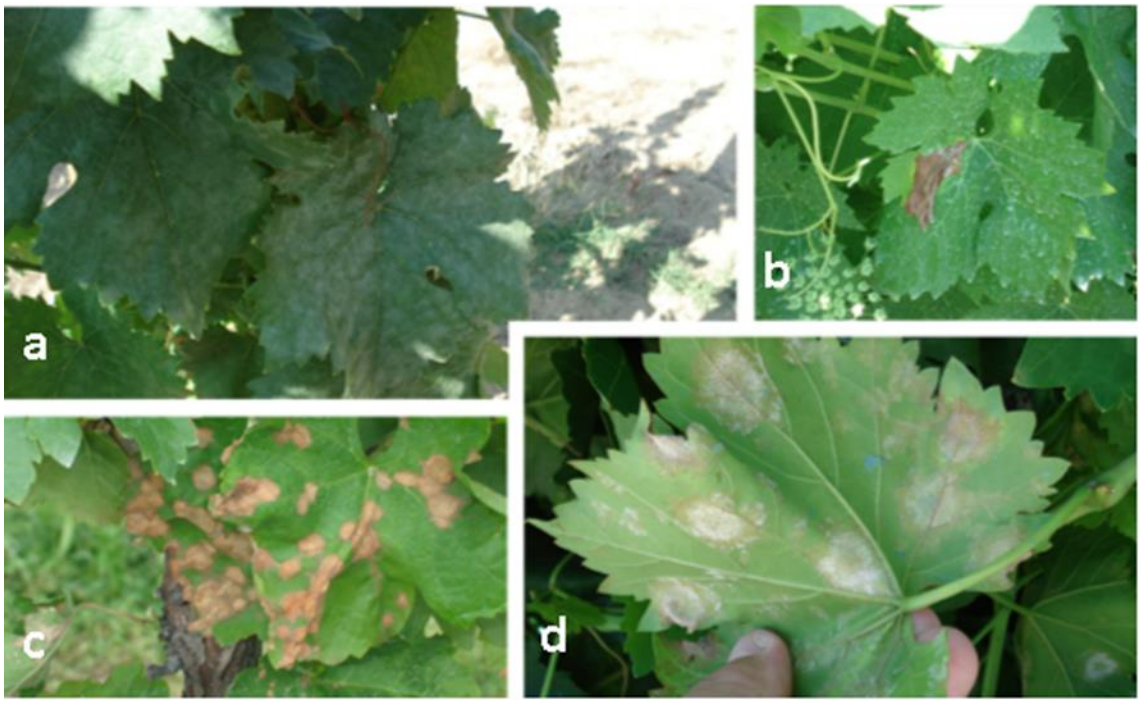


Figure 4. Different symptoms of the main diseases affecting grapevine in the Dão wine region: a) Powdery mildew, caused by *Uncinula necator*; b) Botrytis leaf and bunch dry rot caused by *Botrytis* spp.; c) Black rot caused by *Guignardia bidwellii*; d) Downy mildew caused by *Plasmopara viticola.*

A different group of grapevine diseases, the so-called Grapevine Trunk Diseases (GTDs), however, are causing enormous economic losses in vineyards all over the world, with its incidence reportedly increasing over the last two decades (Bruez *et al.*, 2013; Gramaje *et al.*, 2018) and compromising the sustainability of the wine-growing heritage in short to mid-term. The designation "grapevine trunk diseases", proposed in the late 90s by Luigi Chiarappa and a group of scientists that later formed the International Council

on Grapevine Trunk Diseases (ICGTD) (Mugnai, 2011), englobes a series of symptoms observed in foliage and vascular tissues of grapevine leading to its decline or eventual death (Andolfi *et al.*, 2011; Fontaine *et al.*, 2016; Larignon, 2016). The concern on Grapevine Trunk Diseases is common to all winegrowing countries around the world as it can be seen in Figure 5.

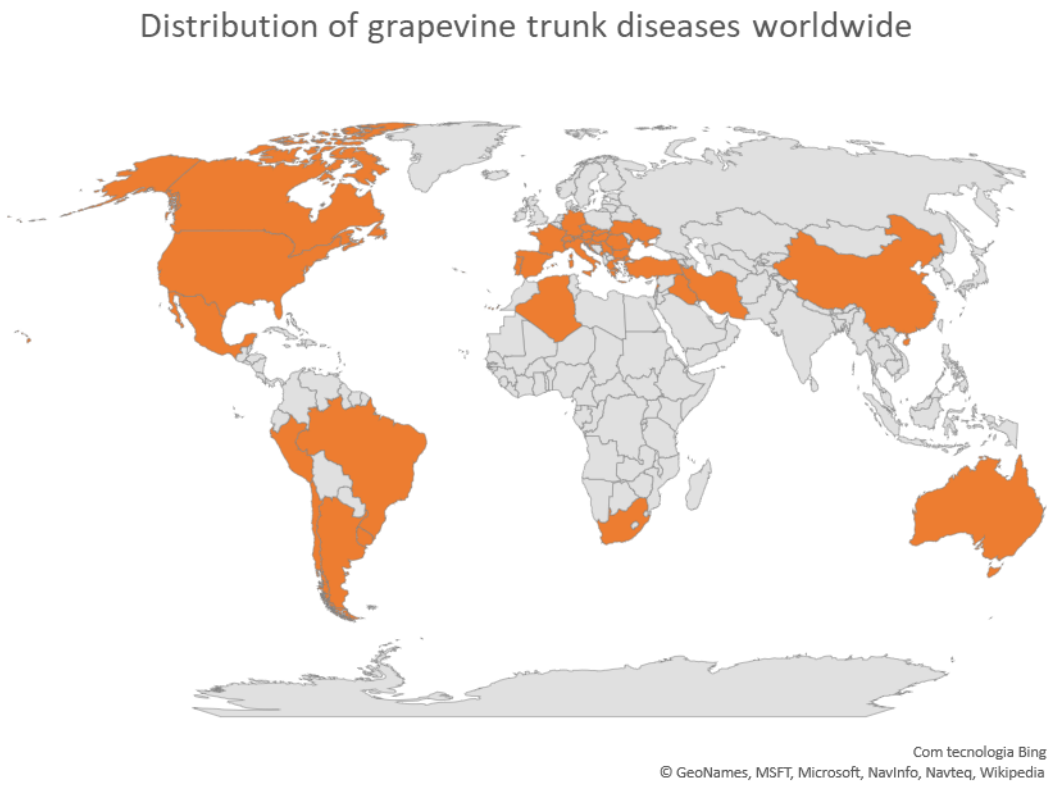


Figure 5. World map of GTDs distribution. In the countries tainted orange, literature has referred to the presence of one or several GTDs in their respective winegrowing regions. Produced by J. Sofia based on data referred by Larignon (2016).

Over the last two decades, the ICGTD has promoted a broad forum gathering research on GTDs leading to a perceptible huge effort to improve knowledge on GTDs, to perceive its situation and possible evolution. Meetings congregating information produced by researchers, technicians and winegrowers of all the continents were held in several locations around the world (i.e., Italy, Portugal, New Zealand, South Africa, USA, Chile, Spain, Australia and France). A COST Action on GTDs (Action FA-1303) took place, gathering leading multidisciplinary academic researchers and institutes within Europe, rethinking the management of GTDs to safeguard European vineyards, promoting the awareness of these ailments by disseminating information to end-users and authorities in the winegrowing sector. The concern due to GTDs, in the last decade, in

France, has originated the "Observatoire National des Maladies du Bois (National Centre for monitoring of Grapevine Trunk Diseases)", in order to monitor and analyse the importance and severity of the trunk diseases (period 2003 to 2008) (Fussler *et al.*, 2008; Bruez *et al.*, 2014). In 2015, an information report was presented by two deputies to the French "Assemblée Nationale" to report the state-of-the-art of GTDs in France and its possible consequences for the wine sector (Quére and Sermier, 2015), revealing the enormous concern around these ailments of grapevine.

The economic impact of GTDs

Investment on a vineyard presumes a huge economic effort to reimburse with a profit within the lifespan of the investment. This effort comprehends, among others, land price, soil preparation and amendments, plants and manpower, pillars, wires, and so forth, as well as all the previous planning and organisation that involves several players (e.g., state financier support, machinery operators, workers, nurserymen), that once engaged have forcibly to be used and paid. These costs in the Dão appellation can easily reach an amount of 15.000€ per hectare. Naturally healthy vineyards will not require much more investment, and the return will start by the third year after plantation. The ones established with poor quality propagation material (i.e., rootstocks, scions or rooted grafts), will have a considerable amount of failing plants resulting on economic losses due to the irregularity of the productions achieved and to the costs of replacing those plants (Waite and Morton, 2007). Concerns with the quality of propagation materials and of vineyards are reflected on the Portuguese and European legislation in a framework of regulations directed to nurserymen, government officials (e.g., Phytosanitary inspectors, economic policies) and commerce (i.e., Decreto-Lei nº 194/2006 of 27 September, transposing the EC directive 2005/43/CE of the 23rd June). The importance given to the phytosanitary quality is clearly stated in the referred directive:

- "*The presence of harmful organisms which reduce the usefulness of the propagation material shall be tolerated only at the lowest possible level*." and that "*Propagation material presenting clear signs or symptoms ascribable to harmful organisms for which there are no efficient treatments shall be eliminated*".

A significant amount of the costs with a vineyard are due to phytosanitary procedures against common pests and diseases. These values, in an average phytosanitary

year, considering that spraying starts in April, englobe usually one treatment for excoriosis, eight for downy mildew, five directed to powdery mildew, two for grey mould and two insecticide sprays for common pests (e.g., *Scaphoideus titanus* Ball. and *Empoasca vitis* Goethe), can easily reach 800 € per hectare. Direct charges for replacing a non-producing grapevine include 1.2 € per grafted plant (2017 values), labour cost per person is about 45€/day (2017 values), not considering other assets like transports, production factors and other services, as well as the time the winegrower will have to wait until that plant starts production.

In Portugal, France, Hungary and Germany, one of the main GTDs, esca, reaches incidences of 20, 15, 11 and 19%, respectively. In Italy, the disease is found in all wine-growing regions with an incidence of up to 50% in old vines (Mugnai *et al.*, 1999).

From our experience, resulting from a 20-year follow-up of the vineyards in the Portuguese wine regions of Dão and Bairrada, it is safe to state that GTDs are present in most of the vineyards of these two regions. It is safe to sustain, that 80% of the vines with more than 15 years within these two regions present more than 5% of plants affected by these diseases. Hofstetter *et al.* (2012a) estimated that the worldwide annual financial cost inherent to the replacement of GTDs' dead plants could easily reach the amount of 1.132 billion euros.

Main GTDs

GTDs affect both young grapevines (less than three years old) as established ones (more than eight years old), and more than 130 fungal species are associated with this group of grapevine diseases (Gramaje, 2017). GTDs commonly affecting young grapevines are described as follow:

- **Black-foot disease**. Characterised by lesions on rootstocks of young grapevines such as black, sunken, necrotic lesions and red-brown discolouration under the bark, leading to the death of affected plants. Aerial organs express either a lack of bud burst or weakened vegetation, which most often dries up during the season (Rego, 2004; Rego *et al.*, 2009; Larignon, 2016). Associated with the genera *Campylocarpon*, "*Cylindrocarpon*", *Cylindrocladiella*, *Ilyonectria, Dactylonectria, Neonectria* and *Telonectria* (Agustí-Brisach and Armengol, 2013; Reis *et al.*, 2013; Carlucci *et al.*, 2017);

- **Petri disease.** In cross-section, the trunks of affected young grapevines present small black dots that in longitudinal cuts correspond to black streaks, caused by the presence of dark phenolic compounds on xylemic vessels (Larignon, 2016; Gramaje *et al.*, 2018). Diseased plants show enfeebled vegetation, and chlorotic leaves with necrotic borders eventually declining and die. Fungi commonly associated are *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams, as well as numerous species of the genus *Phaeoacremonium* (Crous *et al.*, 1996; Mugnai *et al.*, 1999; Mostert *et al.*, 2006a and b; Gramaje *et al.*, 2015; Silva *et al.*, 2017) of which *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous (Teleomorph: *Togninia minima* (Tul. and C. Tul.) Berl. appears to be the most widely distributed and the most common in grapevine (Gramaje *et al.*, 2018). Recently *Pleurostroma richardsiae* (Halleen *et al.*, 2007; Carlucci *et al.*, 2015) and several species of *Cadophora* (Gramaje *et al.*, 2011; Travadon *et al.*, 2015), with *Cadophora luteo-olivacea* the most prevalent (Gramaje *et al.*, 2011), were also associated with this disease.

The most common GTDs affecting established vines are, according to Bertsch *et al.* (2013):

- **Eutypa dieback.** Characterised by stunted shoots with chlorotic leaves, often cupped and with tattered margins. On stunted shoots, bunches are small, ripen unevenly, berries might shrivel and die. Cross sections of affected cordons and trunks show brown sectorial necrosis. External cankers appear as the dieback progresses, characterised by flat areas of the wood with no bark, leading to eventual vine death (Sosnowski *et al.*, 2007; Bertsch *et al.*, 2013; Larignon, 2016). Eutypa dieback is associated with 24 species from the Diatrypaceae (Trouillas *et al.*, 2010; Luque *et al.*, 2012), though only *Eutypa lata* has been found responsible for foliar symptoms (Trouillas and Gubler, 2010);
- **Botryosphaeria dieback (a.k.a. Black Dead Arm).** Its main symptom is the formation of necrotic sectorial cankers on the grapevine framework slowly leading it to death. Yellow to orange (white cultivars) or "bordeaux" (red cultivars) spots appear on leaf margins. As the disease progresses, spots merge forming large brown interveinal necrosis. Removing the bark, it is noticeable, on the underlying wood, the presence of brown streaking associated with a necrotic sector of rotted wood.

Sometimes, inflorescences or fruit clusters shrivel and dry (Bertsch *et al.*, 2013; Larignon, 2016; Reis *et al.*, 2016). Although foliar symptoms tend to make some confusion with those originated by the esca complex, their manifestation is usually from May to June instead of late June to early July (Bertsch *et al.*, 2013). 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; Bertsch *et al.*, 2013; Gramaje *et al.*, 2018);

- **Esca complex.** Characterised by typical foliar symptoms on leaves (i.e. tigre stripe), characteristic internal wood lesions and soft rot and, in some cases, by spots on berries (i.e. black measles). Several fungi are associated with this complex (Bertsch *et al.*, 2013; Gramaje *et al.*, 2018) nevertheless, the most frequent fungal species associated to the Esca complex are *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. (mostly *Phaeoacremonium minimum*) and the lignicolous basidiomycete *Fomitiporia mediterranea* M. Fisch. (Mugnai *et al.*, 1999; Bertsch *et al.*, 2013). Other genera of basidiomycetes as *Inocutis*, *Inonotus*, *Fomitiporella*, *Phellinus*, and *Stereum* have also been reported as associated with soft rot in grapevine (Cloete *et al.*, 2015).

When a grapevine shows symptoms of GTD, the *praxis* recommends its removal and destruction by the fire. If the plant is considered still viable, options are the chirurgical removal of the damaged organs and to retrain new canes to replace the ablated organ (Travadon *et al.*, 2013; Fontaine *et al.*, 2016). Protection against GTDs relies mainly on prophylactic measures and preventive treatments such as pruning wound disinfection, costly, work demanding and not curative.

The Esca complex

Symptomatology

Esca is a long-known GTD, and its symptoms have been described in the classical literature on the theme of agriculture, reporting to Greek civilisation (Mugnai *et al.*, 1999; Larignon, 2016). Two different manifestations of this disease are traditionally described as occurring on standing grapevines: apoplectic/acute form and chronic/mild form (Mugnai *et al.*, 1999; Bertsch *et al.*, 2013; Gramaje *et al.*, 2018). Apoplectic form

or esca proper causes sudden wilt of the entire plant or a branch, leaves scorch, shrivel and eventually drop, clusters dry out. In grapevines presenting the chronic form or "grapevine leaf stripe disease (GLSD)" foliar symptoms are traditionally the "tiger stripe" pattern (Surico, 2009), characterised by red interveinal lesions in red cultivars and yellow to orange interveinal lesions in white cultivars. These lesions are often bordered by small red or yellow blotches. Small spots have been reported on berries, causing the "black measles" symptom (Mugnai *et al.*, 1999). The erratic manifestation of symptoms of the esca complex is well known, with affected plants alternating presence/absence of symptoms in different years, under the influence of environmental, climatic and cultural factors (Sofia *et al.*, 2006; Murolo and Romanazzi, 2014) leading to an underestimation of the real incidence of the disease in the vineyard. Cross-sections of esca and GLSD affected trunks reveal a variety of internal wood symptoms, such as black spots in the xylem sometimes surrounded by pink to brown wood discolouration, brown to black vascular streaking and sometimes the wood may develop a white to soft yellow rot (Mugnai *et al.*, 1999; Surico, 2009; Larignon, 2016).

Aetiology

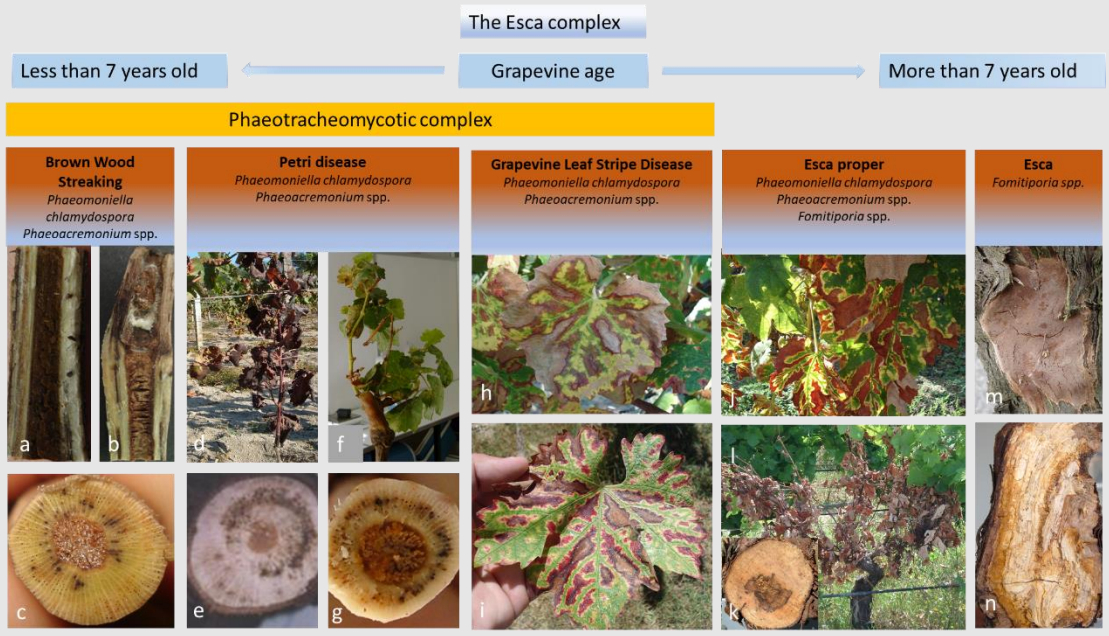
The aetiology of esca disease has been a matter of discussion among scientists since first described at the beginning of the 20th century. Larignon, in 2016, presented a complete review on GTDs theme, where all landmarks leading to the actual knowledge on esca are exhaustively reviewed, as well as symptom description and associated pathogens. The term "esca" initially adapted from the Greek "Iska (tinder)" and later romanized to "esca (i.e., food, bait or tinder ailment)" was used by winegrowers from Smyrna (actual Turkey) and south Italy, respectively, to designate the disease observed on grapevines with internal soft rot that suffered apoplexy. Later, while studying this grapevine problem, the term was adopted by Viala in 1926 (Larignon, 2016). With time, wood rot, apoplexy, chlorosis and interveinal necrosis, become accepted symptoms of esca, although no experimental evidence on the real cause of those symptoms existed (Surico, 2009; Bertsch *et al.*, 2013).

Over the last two decades, systematisation on the knowledge of esca, took place, commencing with the establishment of the International Council on Grapevine Trunk Diseases (ICGTD), back in the late nineties of the 20th century. Since then, a broad range of organisms has been isolated from symptomatic plants, including fungal pathogens and

even endophytic bacteria (Hofstetter *et al.*, 2012b; Bruez *et al.*, 2014) leading to a better understanding of both internal symptoms and aetiology of the disease. The wood rot main causal agent was identified as the lignicolous basidiomycete *Fomitiporia mediterranea* M. Fisch. (Mugnai *et al.*, 1999; Fischer, 2002; Bertsch *et al.*, 2013), while the anamorphic ascomycetes *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. (mostly *P. minimum*) were responsible for vascular symptoms as brown/black wood streaking, observed on esca affected plants (Mugnai *et al.*, 1999; Surico *et al.*, 2006; Surico, 2009; Bertsch *et al.*, 2013). Nevertheless, none of these pathogens has ever been detected in leaves presenting typical esca interveinal necrosis or "tiger striped necrosis", making it impossible to establish a relation pathogen-symptom for this symptomatology of esca. Sparapano *et al.* (2001) reproduced similar symptoms by inoculating the different fungi alone or in combination. Feliciano *et al*. (2004) also obtained them in the vineyard two years after the inoculation of *P. minimum* or *P. chlamydospora* on cv. Thompson Seedless. Similar symptoms were reproduced by Úrbez-Torres *et al.* (2014) on cv. Baco noir on rootstock 3309 C, obtaining an interveinal dryness, giving the leaf an appearance of "tiger stripes" necrosis, by inoculation with *P. chlamydospora, P. canadense, P. iranianum, P. fraxinopennsylvanica* and *P. minimum*. Díaz and Latorre (2014) obtained redness on leaves by inoculating *P. chlamydospora* on cv. Carménère seedlings and cuttings. Pathogenicity tests carried out by artificial inoculation with *P. chlamydospora*, and *P. minimum* on grape berries artificially wounded replicated fruit symptoms (purplish spots on the surface) (Sparapano *et al.*, 2001; Gubler *et al.*, 2004a). It has been hypothesized that the grapevine leaf stripe (GLS) symptom might be due to the action of toxic fungal metabolites produced in colonized wood and transported to the leaves (Andolfi *et al*., 2011; Spagnolo *et al*., 2012) or induced by the disruption of the vessel sap flow (Lecomte *et al*., 2012).

Esca is accepted as a disease complex comprehending five syndromes (**Erro! A origem da referência não foi encontrada.Erro! A origem da referência não foi encontrada.**), esca proper, esca, young esca, Petri disease, and brown wood streaking (Surico, 2009; Bertsch *et al.*, 2013). Petri disease, young esca and black streaking observed on xylem vessels and darkened or brown necrosis circumscribing the pith, are related with *P. chlamydospora* and *Phaeoacremonium* spp., the two most important pathogens in Petri disease (Crous and Gams, 2000; Bruez *et al.*, 2013), while esca, corresponding to the white to soft yellow rot occurring in the trunk of mature grapevines

is associated to *F. mediterranea*. Esca proper, characteristic of mature grapevines, indicates the co-occurrence of Petri disease and esca on the same plant (Bertsch *et al*., 2013).



Box 1. **Esca complex**: The present diagram displays, according to grapevine age, the syndromes grouped under the "umbrella designation" Esca complex. Under each syndrome illustrated, fungi commonly associated are indicated, followed by photos of related symptoms: Brown wood streaking is characterized by the dark wood lesion symptom (Photos a, b and c), proving the presence of the pathogens in young grapevines as the ones coming from the nursery; Petri disease is typical of very young vines, already in new plantations. The typical wood symptoms in both are the vascular discolourations (photos a, b, c, e and g), observed in photos. Petri disease can also cause foliar symptoms (photos d and f) causing the death of the grapevines. The GLSD syndrome is characterised by the typical "tiger-stripe" pattern on leaves (photos h and i) and is often associated with the mild form of esca. It can be found in both young and adult plants, both in grapevines showing only vascular pathogens infections (Photo k) or also presenting wood decay (Photo n), caused by basidiomycetes. Due to the common symptoms observed in these syndromes, in young grapevines, they are commonly grouped under the designation "Phaeotracheomycotic complex". Esca proper is typical of adult grapevines and characterised by the contemporary presence of the main vascular pathogens (Photo k) plus the basidiomycete species (Photo n) and the development of foliar symptoms (Photos j and l). Esca is the term applied to plants presenting exclusively symptoms of white to soft yellow rot (Photo n) due to the action of basidiomycetes (Photo m) of the genera *Fomitiporia* (in Europe, *Fomitiporia mediterranea*). Adapted from Mondello *et al.* (2018).

Recently, a simplification of the above-referred syndromes was proposed: the designation "young esca" was replaced by "grapevine leaf stripe disease (GLSD)", while "esca" was used to designate grapevines showing symptoms in internal woody tissues of white to soft yellow rot, usually due to *F. mediterranea* (Fischer, 2002, 2006; Bertsch *et al.*, 2013) and esca proper [i.e. esca *sensu lato* as described by Viala (1926)]. "Brown wood streaking", "Petri disease" and "grapevine leaf stripe disease" were gathered under the designation "phaeotracheomycotic complex", emphasising the involvement of *P. chlamydospora* sometimes associated to *Phaeoacremonium* spp. in the three syndromes (Bertsch *et al*., 2013). *Phaeoacremonium* spp. are consistently considered to have a minor role in the esca complex than *P. chlamydospora* (Adalat *et al.*, 2000; Halleen *et al.*, 2007; Fischer and Kassemeyer, 2012; Markakis *et al.*, 2017). Moreover, *P. chlamydospora* is endemic to all viticulture areas worldwide and is also the most frequently isolated species, from esca proper and GLSD (Mugnai *et al.*, 1999; Clearwater *et al.*, 2000; Pascoe and Cottral, 2000; Whiteman *et al.*, 2002; Díaz *et al.*, 2013; Díaz and Latorre, 2014), being considered the most important and aggressive fungal organism associated with Petri disease (Wallace *et al.*, 2003; Ridgway *et al.*, 2005; Halleen *et al.*, 2007; Sofia *et al.*, 2007; Zanzotto *et al.*, 2008; Laveau *et al.*, 2009; Pouzoulet *et al.*, 2013). It is consistently recovered from plants within the Dão appellation affected by esca proper and GLSD (Sofia *et al.*, 2006; Sofia, 2007).

The response of cultivars to esca complex

Cultivar susceptibility has been addressed in several studies with diverse grapevine cultivars (Feliciano *et al*., 2004; Travadon *et al*., 2013) and numerous disease susceptibility cultivars rankings have been proposed (Bruez *et al.*, 2013). Evidence of different grapevine cultivar susceptibility to esca has emerged from observations carried out in artificial inoculation trials (Feliciano *et al.*, 2004; Zanzotto *et al.*, 2008; Martin *et al.*, 2009). Feliciano *et al*. (2004), rated cultivar Thompson Seedless more susceptible to esca than cvs. Grenache and Cabernet Sauvignon based on foliar and berry symptoms expression after a three-year artificial inoculation trial. In Italy, Landi *et al*. (2012) artificially infecting cuttings of cultivars Montepulciano, Verdicchio, Sangiovese, Biancame and Cabernet Sauvignon with *P. chlamydospora*, reported the greatest extent of wood colonisation for cvs. Montepulciano and Verdicchio than for C. Sauvignon. Nevertheless, in other works, C. Sauvignon and Verdicchio are considered less

susceptible cultivars (Christen *et al.*, 2007; Borgo *et al.*, 2008; Murolo and Romanazzi, 2014). Zanzotto *et al.* (2008), based on the lower incidence of symptoms on explants of cv. Aglianico considered it less susceptible. In Greece, artificial inoculations with the same species have shown that cultivars Agiorgitiko and Soultanina were susceptible, whereas cvs. Asyrtiko and Xinomavro were tolerant and cv. Roditis displayed an intermediate behaviour (Markakis *et al.*, 2017). Still, data collected under laboratory conditions do not always correspond to field obtained data. Other works ranked cultivars according to their supposed esca susceptibility, based on symptom expression in the field. In France, the French National Grapevine Trunk Diseases Survey (Fussler *et al.*, 2008) reported cultivar Poulsard as presenting the highest levels of esca foliar symptoms, opposing to cv. Merlot with the lowest. In Italy, Borgo *et al*. (2008) surveying symptoms in naturally infected vineyards, yet under different environmental conditions, stated different cultivar susceptibility, in two red cultivars-higher on cv. Cabernet Sauvignon than on cv. Merlot - and in three white ones - higher in Sauvignon blanc reporting to Chardonnay and Pinot blanc. These results were consistent with those reported by Murolo *et al*. (2014) that, also in Italy, presented a wide-ranging assessment of Italian cultivars, concluding that the sensitivity of grapevine genotypes to esca depended on varietal and rootstock factors. In Portugal, cv. Aragonez has been considered more prone to esca than other cultivars (Almeida, 2007). This statement is nevertheless questionable:

On the one hand, this assumption, based on winegrower's day-to-day observations, might be biased by the singular characteristics of the cultivar: erect growth, long internodes, less and bigger leaves than other cultivars, thus highlighting foliar symptoms in the vineyard, particularly on the severe form of esca, when leaves tend to shrivel and dry. On the other hand, cv. Aragonez is the most grown cultivar in Portugal, being transversal to the entire territory, while other cultivars have only a regional expression within the Portuguese vine population (OIV, 2017b), hence the natural occurrence of the disease is probably skewed towards this cultivar. Also in Portugal, and under artificial conditions, Martin *et al*. (2009), compared the variation in individual phenolic compounds produced by cultivars Aragonez, Touriga Nacional and Cabernet Sauvignon, in response to infection by *P. chlamydospora*, reporting a higher accumulation of these compounds on cv. Touriga Nacional. Nevertheless, none of those cultivars did clearly express resistance to infection by *P. chlamydospora*.

Rootstocks are often reported to be more susceptible than *Vitis vinifera* cultivars (Wallace *et al.*, 2003). Retief *et al.* (2006) and Zanzotto *et al.* (2008), isolated *P. chlamydospora* more frequently from rootstock cuttings than from scion cuttings. Nevertheless, Santos *et al.* (2005, 2006b) observed that *in vitro* 3309C rootstocks were less sensitive to inoculation than *V. vinifera* (cvs. Baga and Maria Gomes). Eskalen *et al.* (2001) studying the susceptibility of 20 rootstock's cultivars to *P. chlamydospora* and *Phaeoacremonium* spp. observed that all were susceptible, although in different degrees.

A better knowledge of grapevine cultivars susceptibility to GTDs would allow winegrowers to take into account those cultivars whose characteristics could render more favourable when deciding new vineyards. Until now, no grapevine cultivar is considered resistant to esca. Most of the studies on grapevine cultivars susceptibility are based on symptom expression, which may vary with several influencing factors. Esca complex symptoms manifestation is erratic, and their foliar expression tends to vary with climatic conditions (Surico *et al.*, 2006). Marchi *et al*. (2006) reported that the number of grapevines hiding esca related symptoms during a growing season was inversely related to rainfall in May–June or in summer. Also, chronic esca expression is associated with hot periods in summer following rainfalls whereas, during hot, dry summer periods, apoplexy is more common (Surico *et al.*, 2000). An overview of esca in France (Bruez *et al.*, 2013) reported differences in susceptibility of the same cultivar on different wine regions indicating that besides "varietal susceptibility", other factors, such as climate and soil, might be involved. Studies on the behaviour of a susceptible cultivar (e.g., Cabernet Sauvignon), regarding esca, in different countries around the world, would undoubtedly be useful by characterising factors representative of each area that can influence cultivar's response, thus clarifying the role of cultivar susceptibility. Portugal has 341 regulated grapevine cultivars, from which more than 250 are indigenous (Almadanim *et al.*, 2007; Fraga *et al.*, 2016). Among this genotype wealth, besides its intrinsical characteristics for winemaking, might be some with more interest regarding tolerance to the esca complex.

Phaeomoniella chlamydospora, the main agent of Petri disease and esca complex

Described initially as *Phaeoacremonium chlamydosporum*, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams, is a fungal organism, phylum Ascomycota, classe Eurotiomycetes, order Phaeomoniellales (https://www.mycobank.org; accessed 5th may 2018; https://www.ncbi.nlm.nih.gov,

accessed 5th may 2018), that is morphologically and phylogenetically different from the species included in the genus *Phaeoacremonium* (Crous and Gams, 2000) (Figure 6). Among other characteristics, it is distinguishable by a partly yeast-like growth in culture, prominently green/brown conidiophores, light green to hyaline conidiogenous cells, chlamydospore-like structures and sclerotia produced in culture. *P. chlamydospora* conidia also differ from the ones produced by *Phaeoacremonium* spp. for their straight oblong-ellipsoidal to obovate form and pale brown colouration, in contrast to the dimorphic and hyaline conidia that are typical of *Phaeoacremonium* spp. (Crous and Gams, 2000).

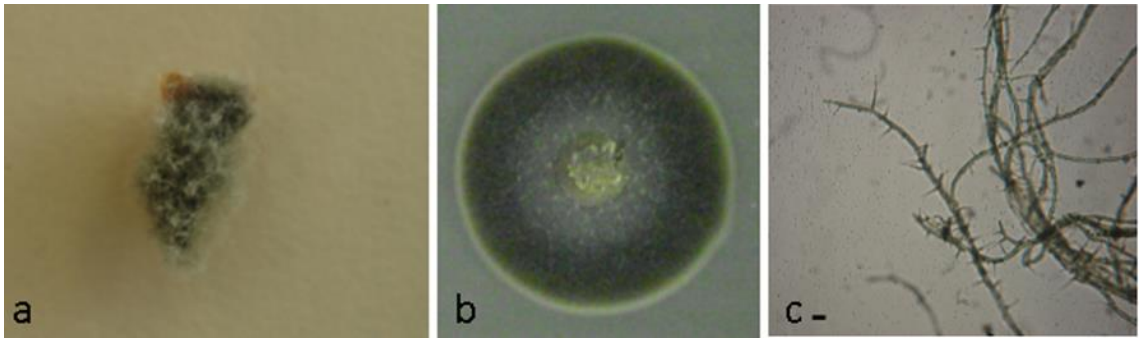


Figure 6. Different aspects of *Phaeomoniella chlamydospora* in culture. a) colony developing on a piece of grapevine tissue in PDA; b) colony obtained after sub-culturing mycelium removed from the previous, also in PDA; c) Conidiophores and conidia of *P. chlamydospora* under optical microscope. Scale bar = 20 µm.

Tello *et al.* (2010) analysing 57 Spanish isolates, revealed that there was consistent phenotypic homogeneity among all *P. chlamydospora* isolates, despite their diverse geographic origins, year of isolation and scion/rootstock combination isolate source. No *P. chlamydospora* teleomorph has been described to date, and it is accepted that the reproduction of this pathogen is strictly clonal. This reproductive strategy is supported by fairly high degrees of genetic homogeneity found among different populations collected in Spain (Cobos and Martin, 2008; Tello *et al.*, 2010), France (Borie *et al.*, 2002; Smetham *et al.*, 2010), Italy (Tegli *et al.*, 2000a, 2000b), South Africa (Mostert *et al.*, 2006a), and New Zealand (Pottinger *et al.*, 2002; Smetham *et al.*, 2010). Tegli *et al.* (2000), using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) analyses, described a high degree of genetic homogeneity among 15 fungal isolates from eight distinct Italian regions. Borie *et al.* (2002) found similar levels of diversity in two French regions, using RAPD analysis to study 72 isolates from one region and 34 isolates from a second; and within a French vineyard, using 47 isolates. Moreover, using RAPD, ISSR, amplified fragment length polymorphism (AFLP) and

universally primed polymerase chain reaction (UP-PCR), Pottinger *et al.* (2002) verified that only slight levels of genetic variability occurred among 39 New Zealand and 6 Italian isolates, and suggested that multiple introductions of foreign fungal populations have occurred in New Zealand vineyards. Mostert *et al.* (2006), using AFLPs, concluded that 63 isolates from South Africa and 25 from Australia, France, Iran, Italy, New Zealand, Slovenia and the USA, presented low genetic diversity, and noted intra-vine, intra- and inter-vineyard variations. Also, genetic variability among different production areas was not significant, concluding that infections occurred via asexual propagation. Cobos and Martin (2008) and Tello *et al.* (2010) observed low genetic diversity between, respectively, 35 and 57 isolates from Spain, using ISSR, RAPD and ITS region, the β-tubulin gene and the elongation factor 1-α gene. More recently, Smetham *et al.* (2010) used 60 isolates from Southern Australia and 67 from Southern France to study 18 microsatellite loci, concluding that limited genetic recombination and essentially clonal structure were present in these populations. However, there is a high level of haplotypic diversity (Comont *et al.*, 2010) suggesting that reproduction in *P. chlamydospora* might not be strictly clonal involving a cryptic sexual phase or a form of parasexuality/somatic recombination.

The life cycle of *Phaeomoniella chlamydospora*

Beside *Vitis* spp., *Phaeomoniella chlamydospora* occurrence has been reported on *Olea europea* (Úrbez-Torres *et al.*, 2013), *Actinidea deliciosa* (Di Marco *et al.*, 2000) and *Convolvulus arvensis* (Agustí-Brisach *et al.*, 2011), suggesting that other hosts may serve as a reservoir for inoculum. Its culture conditions were exhaustively studied and described by Whiting *et al.* (2001) and Valtaud *et al.* (2009).

Without a known sexual stage, its primary sources of inoculum are pycnidiospores produced in pycnidia as well as conidia produced in free conidiophores inside deep cracks and crevices of the wood, that provide a protected humid environment favourable for sporulation (Edwards *et al.*, 2001). Its presence at the surface of different organs (tendrils, spurs, cordons, trunks, berry surfaces), referred by Eskalen and Gubler, (2001) and Rooney *et al.* (2001) suggest its epiphytic character. *P. chlamydospora* produces also a phoma-like synanamorph on canes and litter (Crous *et al.*, 1996; Edwards and Pascoe, 2001; Edwards *et al.*, 2001), with dispersal via rain splash and irrigation, leading to pruning wound infection (Larignon and Dubos, 1997; Pascoe and Cottral,

2000; Eskalen and Gubler, 2001). Spore dispersion is mainly airborne (Larignon *et al.*, 2008) and occurs all year round, mostly during rainfall events. After deposition, spores develop and penetrate the plant through wounds during mild and rainy periods in the winter period (Larignon and Dubos, 2000). Because training the vine causes pruning wounds, they are *P. chlamydospora* most significant entry in the plant. Several studies have shown that the infection of wounds significantly decreases as the length of time between pruning and inoculation increases (Larignon and Dubos, 2000; Eskalen *et al.*, 2007; Serra *et al.*, 2008). There is some controversy with pruning date and wound susceptibility. Larignon and Dubos (2000) and Serra *et al.* (2008) considered that late winter/early spring pruning (February/March in the northern hemisphere) lowered the susceptibility of the pruning wounds to infection by *P. chlamydospora*, and the duration of susceptibility would reduce to 2 weeks. Luque *et al.* (2014) studied the effect of naturally occurring infections of *P. chlamydospora* on standing grapevines and reported that wounds made later in the dormant season were more susceptible to infection than wounds made earlier, what could be due not to the time of the year when pruning was performed, but to the favourable climatic conditions experienced afterwards (I.e. humid and warmer weather), favouring spore release and dispersion, as well as the fungal infection and posterior colonisation of wounds in the pruned grapevine. Later, Elena and Luque (2016), using artificial inoculation, considered wound susceptibility similar in both pruning seasons hypothesising that the seasonal differences reported by Luque *et al.*, (2014) could be related to the availability of inoculum of *P. chlamydospora* in each pruning season. Nevertheless, wounds remain susceptible for up to 4 months after pruning, and dissemination also occurs during the vegetative period (Eskalen *et al.*, 2007; Serra *et al.*, 2008; Luque *et al.*, 2014; Elena and Luque, 2016). The various wounds due to disbudding, leaf stripping, cutting, trimming and thinning may be potential routes for *P. chlamydospora* penetration, as shown in inoculation tests (Larignon *et al.*, 2009). Susceptibility of pruning wounds was also evaluated in Italy (Serra *et al.*, 2008) where it is considered to last for a long time for early pruning. In California, this period is considered until four months after pruning (Eskalen *et al.*, 2007). Dissemination by arthropods, specifically Portuguese millipedes (*Ommatoiulus moreleti* Lucas) and Cocktail ants (*Crematogaster peringueyi* Emery), is also possible (Moyo *et al.*, 2014). Viable propagules of *P. chlamydospora* were obtained from pruning shears and callusing media in nurseries (Aroca *et al.*, 2010). Considered as a potential soil-borne pathogen,

due to its ability to produce chlamydospore-like structures in culture (Crous and Gams, 2000), it has been detected by molecular methods in vineyard soils of New-Zealand (Whiteman *et al.*, 2002) and of South Africa (Damm and Fourie, 2005; Retief *et al.*, 2006). Chlamydospores are thought to form conidia that can penetrate the uninjured roots of vines in nurseries or vineyards (Bertelli *et al.*, 1998; Feliciano and Gubler, 2001). Halleen, *et al.* (2003) sampled for *P. chlamydospora* in nursery vine cuttings, several months after planting finding that although present as an endophyte, in the healthy rootstock material, this species rarely occurred in the roots, which suggested not being a primarily soil-borne pathogen. To date, there is no study on the detection of *P. chlamydospora* on the roots of old vines. In nursery *P. chlamydospora* was isolated from scions, rootstocks, nursery cuttings (surface and wood tissues) (Larignon and Dubos, 2000; Halleen *et al.*, 2003; Rego *et al.*, 2009; Gramaje and Armengol, 2011). Moreover, it has been detected by PCR in the hydration tanks, grafting tools and substrates used for the callusing (Retief *et al.*, 2006; Vigues *et al.*, 2009; Aroca *et al.*, 2010; Gramaje and Armengol, 2011).

Landi *et al.* (2012) and Fischer *et al.* (2016) evaluating grapevine wood colonisation and interaction with several synthetic green fluorescent protein *P. chlamydospora* transformants (Pch-sGFP), found Pch-sGFP mycelium expression localised in the xylem area, especially around the vessels and distant from the inoculation point. Other studies demonstrated that *P. chlamydospora* also invades xylem vessels, and that once established in xylem lumen and fibres it is also able to develop in other tissues, such as the parenchyma or rays, (Valtaud *et al.*, 2009; Pouzoulet *et al.*, 2013), thus suggesting that the main nutrient supply is provided by the xylem sap.

An attempt description of the known life cycle of *P. chlamydospora* is represented on Figure 7.

P. chlamydospora pathogenic ability

The role of *P. chlamydospora* in the esca complex is not yet completely clarified, generating controversy. Authors have questioned whether *P. chlamydospora* is just an endophyte, a latent pathogen, a weak pathogen or a true vascular pathogen. Hofstetter *et al.*, 2012a,b) comparing fungal communities on asymptomatic and symptomatic

Figure 7. *Phaeomoniella chlamydospora* life cycle. Adapted from Gramaje, 2015, COST action FA1303. Available at: managtd.eu/images/uploads/content/171/WG1_Disease%20cycles_DGramaje_ Budapest2015.pdf.

grapevines, stated that *P. chlamydospora* was as frequent and abundant in one group as in the other, concluding that this fungus was not pathogenic but probably specialised in the degradation of already senescent or dead wood. However, Gubler *et al.* (2004b) considered it a revealing pathogenic endophyte when under stress. Regarding Petri disease, of which *P. chlamydospora* is one of the early and main causal agents, the time span between fungi inoculation and foliar symptomatology is of several years, suggesting that there is impairment of the involved pathogens' progress by the host, following defence mechanism activation or specific metabolites production. Since esca pathogens have never been found in the leaves of infected plants, it has been hypothesised that leaf symptoms are actually caused by toxic compounds produced by fungi in the woody tissues and then translocated to the leaves via the transpiration stream (Wagschal *et al.*, 2008).

P. chlamydospora possesses all the traits of a vascular wilt pathogen including systemic host colonisation, spatial restriction to xylem vessels (Fischer *et al.*, 2016) with limited ability to degrade structural plant cell wall polymers, and production of phytotoxins. Pathogenicity tests show that *P. chlamydospora* can reproduce symptoms related to Petri disease, both on the aerial portion of grapevine, like stunted growth, small size leaves, short internodes, and on internal wood as indicated by the darkening of xylem vessels and the production of tyloses resulting in occlusion of the vessels (Larignon and Dubos, 1997; Adalat *et al.*, 2000; Sidoti *et al.*, 2000; Halleen *et al.*, 2007; Zanzotto *et al.*, 2008; Aroca and Raposo, 2009; Valtaud *et al.*, 2009; Díaz *et al.*, 2013; Díaz and Latorre,

2014). Although preliminary works considered it associated with GLSD symptoms (Mugnai *et al.*, 1999; Crous and Gams, 2000), only Sparapano *et al.* (2001) artificially inoculating standing grapevines of cvs. Matilda and Itália and Feliciano *et al.* (2004) inoculating cv. Thompson seedless, with *P. chlamydospora*, reported the production of foliar symptoms of yellowing or reddening of large parts of the lamina and marginal leaf necrosis, two and three years after inoculation. In the vineyard, symptoms reminiscent of those of GLSD were obtained, under controlled conditions, by Úrbez-Torres *et al.* (2014) on cv. Baco noir. Díaz *et al.* (2013) reported reddening of *in vitro* plantlets cv. Carménère 28 days after infection and on two-year canes of the same cultivar, 15 months after infection. Similar foliar symptoms were reported by Sofia *et al.* (2007) on explants of cv. Baga, three months after inoculation. Regarding black measles, Sparapano *et al.* (2001) and (Gubler *et al.*, 2004a) demonstrated the pathogenic effect of *P. chlamydospora* on berries.

Other traits of *P. chlamydospora* pathogenicity

Plants respond to pathogen attack activating some defence effector mechanisms, that include the production of antimicrobial metabolites and proteins and physical reinforcement of cell walls through the production of callose and lignin. Larignon (1991), Larignon *et al.* (2009) and Valtaud *et al.* (2009) considered *P. chlamydospora* unable to degrade cell wall, yet Fischer *et al.* (2016) identified two cellulose degrading glycose hydrolases, an extracellular lipase and a pectinesterase indicating that this species is inducing plant cell wall degradation.

In woody plants, compartmentalisation is instrumental to impair pathogens and ensure the integrity and physiological functions of the xylem, phloem, and cambium (Shigo, 1984). The occlusion of the xylem vessels by tyloses and gums is a well-known mechanism induced in response to aggression (De Micco *et al.*, 2016). Inoculating grapevines with *P. chlamydospora*, Pouzoulet *et al.* (2017), reported that wounding combined with pathogen inoculation, stimulated tyloses formation. Tyloses fill the xylem entering in contact with each other. Narrow spaces remaining become filled with pectic materials. *P. chlamydospora* can metabolise pectin (Marchi *et al.*, 2001; Santos *et al.*, 2006a; Valtaud *et al.*, 2009; Fischer *et al.*, 2016). Histological data obtained by Pouzoulet *et al.* (2017) shown that *P. chlamydospora* was able to actively progress in occluded vessels by colonising pectin pockets and outer tyloses walls. The same authors reported

other aspects of the host-pathogen relationship involving *P. chlamydospora*, as its ability to inhibit vessel occlusion by interfering with the completion of the compartmentalisation process thus impairing tylosis formation. The failed xylem occlusion was followed by local reactions such as cell wall deposition of suberin and non-structural phenolic compounds and depletion of starch on infected tissues, an energy reserve needed by the host defence mechanism.

Sparapano *et al.* (2000) extracted pullulans, an exopolysaccharide (EPS) whose phytotoxicity is well known (Forabosco *et al.*, 2006), from culture filtrates of *P. chlamydospora*. These compounds at very low doses were able to produce foliar symptoms similar to those shown by the esca-affected vines (Bruno *et al.*, 2007). It is thought that these EPSs disturb water movement in the plant tissues by plugging the vessels, thus causing the wilt symptoms in consequence of the size and viscosity of the molecules (Andolfi *et al.*, 2011). Luini *et al.* (2010) also reported on the toxic activity of polypeptides secreted by *P. chlamydospora* in culture media. The structures of these polypeptides have not yet been determined. Even though the electrophoretic patterns of the polypeptides differed from those of the EPSs, their biological activity was very similar. They produced anthocyanins on grapevine leaves and, when applied to grapevine cells in culture, they modified proton fluxes, depolarised the cell membrane, inhibited the transport of sucrose and glutamine and, lastly, caused the death of the cells (Andolfi *et al.*, 2011).

Tabacchi *et al.* (2000) isolated nine metabolites from *P. chlamydospora* culture media, including scytalone and isosclerone. Bruno and Sparapano (2006) and Sparapano *et al.* (2000) also reported that scytalone and isosclerone were also produced in planta by *P. chlamydospora* and Evidente *et al.* (2000) indicated that those substances were able to induce necrosis on leaves, (Santos *et al.*, 2006b) reported a decreased mycelium growth in all strains of this species with resveratrol, being the synthesis of phenolic phytoalexins a recognized defence mechanism in grapevine in response to abiotic or biotic elicitors (i.e., UV irradiation, bacterial and fungal pathogens) (Jeandet *et al.*, 2002; Lima *et al.*, 2012). On the other hand (Gubler *et al.*, 2004b) considered that *P. chlamydospora* could be able to break down toxic phenolic compounds, and Martin *et al.* (2009) considered it the least inhibited by both resveratrol and tannic acid, suggesting the enzymatic conversion of those phenolic compounds into less toxic derivatives.

Rapid production of reactive oxygen species (ROS) following a pathogen attack is a defence mechanism of plants (Glazebrook, 2005). Studying the interaction of *P. chlamydospora* with in vitro grapevine *calli*, Fischer *et al.* (2016) detected the production of oxidoreductases in response to an oxidative burst. The same researchers also reported the induction of several detoxifying enzymes, among which a nitronate monooxygenase that oxidises plant originated nitronates and a dienelactone hydrolase involved in biodegradation of toxic aromatic compounds.

Amalfitano *et al.* (2000) analysing brown-red necrosis, usually associated with *P. chlamydospora* and *Phaeoacremonium* spp., reported the accumulation of phenolic compounds, namely *trans*-resveratrol and ε-viniferin. Both substances were also present in healthy wood but at much lower concentrations than in deteriorated wood. The variation in phenolic compounds resulting from *P. chlamydospora* infection was evaluated in young grapevine plants of cvs. Chardonnay, Touriga Nacional and. Aragonez, by Martin *et al.* (2009), reporting an increase of ε-viniferin and *trans*-resveratrol in the infected tissues. These stilbene polyphenols were also reported by (Amalfitano *et al.*, 2011) on brown-red wood, typically associated with *P. chlamydospora* and *Phaeoacremonium* spp. infection. Qualitative composition of the stilbene polyphenols was identical on brown red wood and asymptomatic wood, whereas quantitative analysis demonstrated higher concentrations of these polyphenols in brown red wood than on asymptomatic wood, evidencing that in the first, *P. chlamydospora* could induce the accumulation of stilbene polyphenols, but was unable to transform/detoxify these compounds, thus tolerating resveratrol and viniferins. These conclusions were supported by the similar qualitative stilbene polyphenol composition of both types of wood and by the fact that the involved species essentially did not induce a boundary/reaction zone of condensed polyphenols in the brown red wood, but merely a general brownish-red discolouration.

Lambert *et al.* (2013) while studying plant defence responses to *P. chlamydospora* on grapevine cuttings of cvs. Merlot, Carignan and Cabernet Sauvignon (the first two less susceptible to esca, while cv. C. Sauvignon is considered susceptible), observed that the first two cultivars responded more strongly and more rapidly by stimulating stilbene accumulation and by overexpressing pathogenesis-related proteins when compared to Cabernet Sauvignon.

Thesis purposes

The broad framework of this thesis is centred on understanding the esca complex and, the role of its main agent *Phaeomoniella chlamydospora* in the Dão wine region taking into account its inherent specificities.

The focus was to determine possible and eventual different susceptibilities of local grapevine cultivars to infection by *Phaeomoniella chlamydospora*, thus contributing to a better knowledge of these cultivars not only benefiting local winegrowers but also promoting their use and dissemination, regarding their other possible advantages.

This thesis addressed the following specific objectives:

I. To evaluate the knowledge of winegrowers on GTDs, and based on their information and indications, evaluate main GTDs frequency within the Dão wine region;

II. To obtain a broad collection of Dão's *Phaeomoniella chlamydospora* isolates to determine its intra-specific variability, regarding morphological, cultural and molecular characteristics;

III. To detect pathogenic variability within the collected isolates of *P. chlamydospora*;

IV. To detect possible different susceptibility responses among Dão's most popular grapevine cultivars to infection by *P. chlamydospora* on productive, standing vineyards subject to natural field conditions, typical of the Dão wine region.

Chapter I

CONTRIBUTION FOR A BETTER UNDERSTANDING OF GRAPEVINE FUNGAL TRUNK DISEASES IN THE PORTUGUESE DÃO WINE REGION

Published as:

Sofia J., T. Nascimento; M.T. Gonçalves and C. Rego, 2013. Contribution for a better understanding of grapevine fungal trunk diseases in the Portuguese Dão wine region. *Phytopathologia Mediterranea* 52, 324–334.

Introduction

The Dão wine region has a total area of 376,000 ha, from which less than 20,000 ha are dedicated to grapevine (Figure I-1). Wine production there might go back to Roman times (Loureiro and Cardoso, 1993). However, the oldest signs of wine production date from the VI century CE, including wine presses carved on solid granite (Falcão, 2012), found throughout the entire region and some are still in use. The Dão region is distinguished by its complex orographic features, soils that are typically low-pH sandy granite with low levels of organic matter, and traditional cultivars, most of them of Portuguese origin.

Figure I-1. Map of Portugal with the Dão wine region in black.

Grown throughout Portugal, cv. Touriga Nacional may originate from this region and due to a clonal selection program (IVV, 2011), is starting to internationalise specifically in Australia (Robinson, 1996; Ambrosi *et al.*, 1997). This unique region remains a reservoir for Portuguese grape cultivar diversity thanks to specific grapevine

preservation projects. In addition, the region hosts an important R&D facility – Centro de Estudos Vitivinícolas do Dão – one of the most important producers of Portuguese grapevine cultivars.

Research to improve knowledge about Grapevine Trunk Diseases (GTD) is taking place around the world. In France, there has been intense work on the evaluation of GTD with the establishment in 2003 of the "Observatoire Nationale des Maladies du Bois de la Vigne", that produces annual reports on GTD surveillance (MAAPAR, 2004). In Portugal, local growers are often incapable of understanding the causes for economic losses due to grapevine decline and mortality in their vineyards, which are often due to GTDs. The observed symptoms are often confused with other diseases (e.g. viruses), occasional plagues (e.g. *Empoasca* leafhoppers), nutritional and water deficits, thereby misleading them on management solutions. The most common GTDs in the Dão, leading to substantial economic losses are esca, *Phomopsis* cane and leaf spot, black dead arm (BDA) and young grapevine declines. Typical foliar symptoms of esca – "tiger striped" leaves (Mugnai *et al.*, 1999) - are obvious, but foliar symptoms of BDA, a disease associated with several species of Botryosphaeriaceae fungi, might resemble those of esca appearing earlier in the season (Larignon *et al.*, 2009). BDA is also associated with wood necrosis, being able to infect both young and mature tissues as well as green shoots causing cankers, vascular discolouration, and/or otherwise dark streaking of the wood (Úrbez-Torres and Gubler, 2009). BDA symptoms in wood may be misattributed to *Eutypa* spp., and on herbaceous organs may be confused with *Phomopsis* cane and leaf spot. This panoply of very similar symptoms, sometimes over the same organs, baffles the winegrower.

In previous related studies done at the Dão wine region, *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams was often isolated both from black and red-brown wood discolouration patches found inside esca affected grapevines and from field spore traps (Sofia *et al.*, 2006). In the present work, the first objective was to increase the knowledge of winegrowers on GTDs, while evaluating its frequency in the Dão wine region, centred on a survey among local winegrowers. The results of the survey will, in a near future, be integrated with field data to provide a more accurate picture of the Dão GTDs situation. To determine the intra-specific variability among *P. chlamydospora* isolates from the Dão wine region,

morphological, cultural and molecular characteristics have been evaluated. Finally, an experiment, using a studied isolate of *P. chlamydospora* was conducted on cv. Touriga Nacional, one of the most important of the Dão wine region, to validate a pathogenicity test procedure for grapevine infection with this fungus, which can become a useful tool to help to detect pathogenic variability within the collected isolates of *P. chlamydospora*, or to help on detection of different cultivars' susceptibility to *P. chlamydospora*.

Materials and methods

Leaflet and survey

A four-page colour leaflet was produced with the key symptoms associated with the main GTDs commonly found in the Dão wine region – esca, *Phomopsis* cane and leaf spot, BDA and young grapevine declines - to promote the grower knowledge on GTDs (Figure I-2). Simultaneously, local growers were invited to fulfil an easy three-step questionnaire (Figure I-3) where the first step was a question acknowledging the existence of any of the four GTD on their vineyards; the second step was also a question meant to evaluate the frequency of the disease(s) based on three numerical boundary categories (level 1: few vines affected; level 2: some vines affected; level 3: many vines affected) and the third step concerned the location of the vineyard within the region.

Fungal isolates

Eighteen of the vineyards identified during the survey were studied, and the frequency of GTDs evaluated, in order to determine the accuracy of the answers given and also to collect some wood samples from esca and Petri disease symptomatic grapevines. From these samples, cross sections were cut from the trunk, and typical dark brown to black discoloured fragments, usually associated with *P. chlamydospora* were extracted. They were surface disinfected by immersion in an 8% solution of NaOCl for 1 min., rinsed with sterile distilled water (SDW), dried with filter paper and placed in Petri dishes containing potato dextrose agar (PDA; Difco, Beckton, Dickinson and Co, Sparks, MD, USA) amended with 250 mg L^{-1} of chloramphenicol (BioChemica, AppliChem, Germany). Inoculated plates were incubated in the dark for eight days, at 25 ± 1ºC. After this period, suspected colonies of *P. chlamydospora* were transferred to PDA to get pure cultures.

Figure I-2. Leaflet produced for divulgation of the main symptoms of grapevine trunk diseases affecting the Dão wine region (English version).

Inquiry on the situation of grapevine trunk diseases on Dão wine region

1. Please, take a look at the leaflet, and if you've found any of the present symptoms on your vine , please check the corresponding box

Disease	Do you find it on your Vineyard?		
	Yes	No	Don't know
Esca			
Phomopsis cane and leaf spot			
Black Dead Arm			
Young Vine Decline			

2. If you have answered positively to question number one, please check, in the table below, the corresponding box, in order to evaluate the frequency of the diseases over your grapevine (1- affects few vines; 2- affects some vines; 3- affects many vines).

Disease	Frequency		
	1	2	3
Esca			
Phomopsis cane and leaf spot			
Black Dead Arm			
Young Vine Decline			

Name of the vineyard
Locality:
Freguesia (Parish):
Concelho (County):

Thank you for your collaboration!

Figure I-3. Questionnaire used to evaluate the situation of grapevine trunk diseases in the Dão wine region (English version).

Phenotypic characterisation

A collection of twenty *P. chlamydospora* isolates obtained from different locations and different scion/rootstock combinations was used: 17 isolates were collected within the Dão wine region plus three studied and classified isolates obtained from Vidigueira, Alentejo (Ph19), from Arruda dos Vinhos, Extremadura (Ph24) and from Dão wine region (Ph30) (Table I-1)

Table I-1. *Phaeomoniella chlamydospora* isolates studied

Isolate	Year of isolation	Geographical origin		Host scion/rootstock
		Location	Wine region	
Ph19[a]	2008	Vidigueira	Alentejo	Petit Verdot/400VO
Ph24[a]	2011	Arruda dos Vinhos	Estremadura	Touriga Nacional/-
Ph26	2011	Lousã	Beiras	Cerceal/-
Ph27	2011	Nelas	Dão	Jaen/-
Ph28	2011	Mangualde	Dão	Jaen/-
Ph29	2012	Mangualde	Dão	Touriga Nacional/-
Ph30	2012	Nelas	Dão	Jaen/S04
Ph31	2012	Nelas	Dão	Tinta Roriz/S04
Ph32	2012	Nelas	Dão	Alfrocheiro/-
Ph33	2012	Seia	Dão	Jaen/-
Ph34	2012	Tondela	Dão	Aragonês/-
Ph35	2012	Mangualde	Dão	Touriga Nacional/-
Ph36	2012	Mangualde	Dão	Encruzado/-
Ph37	2012	Gouveia	Dão	Gouveio/-
Ph38	2012	Nelas	Dão	Touriga Nacional/-
Ph39	2012	Gouveia	Dão	Jaen/-
Ph40	2012	São Martinho da Cortiça	Dão	Baga/-
Ph41	2012	Viseu	Dão	Encruzado/-
Ph42	2012	Mangualde	Dão	Jaen/-
Ph43	2012	Viseu	Dão	Jaen/-

[a] Isolates formerly identified and characterised

All isolates were grown in triplicate on PDA, at 25 ± 1°C, in the darkness for 15 days and phenotypic features (texture, colour, growing margin zonation and hyphal morphology) were described according to Crous and Gams (2000) and González and Tello (2011).

Daily growth and colony mean diameters were obtained after 25 days by

measuring two perpendicular diameters for each colony and calculating mean diameters. For each isolate, six repetitions were taken. The number of conidia produced was evaluated according to the method described by Whiting *et al.* (2001).

Molecular characterisation

For each isolate, DNA was extracted from mycelium grown on potato dextrose broth (PDB; Difco) using the protocol of Cenis (1992) adapted by Nascimento *et al.* (2001). To study the genetic diversity among *P. chlamydospora* isolates the inter-simple sequence repeat (ISSR) analysis was used. The ISSR primers $(AG)_8YT$ (Fang and Rose, 1997), $(CAG)_5$ (Rodriguez and Yoder, 1991), $HVH(TG)_7$ (Gilbert *et al.*, 1999) and MR (5'-GAGGGTGGCGGTTCT-3') (Bridge *et al.*, 1997) were used. Each PCR reaction contained 1× PCR buffer, three mM MgCl2, 200µM of each dNTP, 0.5 µM of each primer, 0.8 U of DreamTaq DNA Polymerase (MBI Fermentas, Vilnius, Lithuania), and 3µL of diluted template DNA in a final volume of 20 µL. Amplifications were performed in a "Biometra T-Gradient", with an initial step of 4 min at 94ºC, followed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 50ºC $(CAG)_5$ and MR or 52ºC $(AG)_8YT$ and $HVH(TG)_7$ for 45 s, and an elongation at 72ºC for 2 min. A final extension was performed at 72ºC for 10 min (Talhinhas *et al.*, 2003). Reactions without DNA were used as negative controls, and each reaction was repeated at least once. Amplification products were separated by electrophoresis in 2% agarose gels in 0.5 × TBE buffer at 40 V for 19 h. A GeneRulerTM 100 bp Plus DNA Ladder (MBI Fermentas) was used as a molecular weight marker. Gels were stained with ethidium bromide and visualised under UV light, followed by digital image capturing using an UVIdoc system (UVItec Limited, Cambridge, UK).

The banding patterns were analysed with GelCompar II Version 5.10 software package (Applied Maths, Saint-Martens-Latem, Belgium). DNA bands detected by the software were verified by visual examination to correct unsatisfactory detection, and the presence (1) or absence (0) of bands was recorded in a binary matrix. Genetic similarities were calculated using the Dice coefficient and dendrograms obtained by clustering according to the unweighted pair-group method using arithmetic averages (UPGMA). The robustness of the branches was assessed by bootstrap analysis with 2,000 replicates.

Pathogenicity experiment

A pathogenicity test was carried out in a vineyard of cv. Touriga Nacional, five years old, trained on bilateral cordon with six spurs. During winter, two spurs in each vine were left with three buds and were inoculated immediately after pruning by depositing a droplet of 40 µL of a 10^5 spores mL^{-1} conidial suspension of strain Ph19. Conidial suspension was obtained by plunging a 3 mm diameter mycelial disk of the isolate in a 250 mL Erlenmeyer flask containing PDB and placed at 20ºC, under darkness, in a reciprocal shaker (90 strokes min^{-1}), for 15 days. The inoculation site was sealed during one week with Parafilm (Parafilm® "M", Pechiney Plastic Packaging, Menasha, USA). Thirty repetitions were made. Control canes were similarly treated, but SDW was used instead of inoculum.

Eight months after the inoculation, brown internal discolourations were visible along a longitudinal cut of last year inoculated spurs. Parameters evaluated were thickness (cm) and length from cut to the base (cm) of inoculated canes and length of necrosis (cm). Four pieces of wood were excised from the border of the necrosis and reisolation procedures were performed as described before. The percentage of reisolation was calculated as the proportion of wood pieces from which *P. chlamydospora* colonies were recovered, versus the total number of pieces of wood plated for each plant. Data obtained were compared using an unpaired T-test, type 2. Calculations were performed with the Statistica 6.1 software package (Statsoft Inc., Tulsa, OK, USA).

Results

Results of the GTD survey

During the 2011/2012 survey, a total of 62 questionnaires were considered completely fulfilled and validated. It was clear from results that esca was the most well-known GTD of the four explained in the leaflet, with positive identification of its presence in more than 88% of the vineyards (Table I-2). Merely 12% of the inquired winegrowers answered that they had never noticed the disease on their vineyards.

Concerning the frequency of esca, level 1 of disease frequency was recorded in 80% of the vineyards, level 2 in 16% and level 3 only in 5% of the vineyards (Table I-3).

Table I-2. Survey on the situation of grapevine trunk diseases in the Dão wine region

Disease	Do you find it in your vineyard? (%)[a]		
	Yes	No	Don't know
Esca	88	12	0
Phomopsis cane and leafspot	82	16	2
Black dead arm	58	34	8
Young vine decline	30	60	10

[a] Results of a total of 62 questionnaires considered completely fulfilled and validated.

Table I-3. Frequency of grapevine trunk diseases in Dão wine region: level 1 - affects few vines; level 2 – affects some vines; level 3 - affects many vines

Disease	Frequency (%)[a]		
	Level 1	Level 2	Level 3
Esca	80	16	5
Black dead arm	72	24	3
Phomopsis cane and leafspot	46	41	12
Young vine decline	87	13	0

[a] For each of the diseases, frequency was determined considering only the number of questionnaires that acknowledged the existence of the disease. Figures rounded to the next integer.

The second most recognisable disease among the inquired was *Phomopsis* cane and leaf spot with 82% of the fulfilled forms confirming its presence. Only 16% of the inquired winegrowers answered that they had never noticed the disease on their vineyards and 2% did not know the disease (Table I-2). Regarding frequency of *Phomopsis* cane and leaf spot, 46% of the inquired winegrowers considered it present although affecting a scarce number of vines (level 1), 41% considered it was affecting an important number of plants (level 2), and 12% considered it a serious problem (level 3) (Table I-3).

The third identifiable disease for the inquired was BDA with 58% of the fulfilled

forms confirming its presence; 34% of the inquired winegrowers answered that they had never noticed the disease on their vineyards and 8% did not know the disease (Table I-2). Concerning the frequency of BDA, 72% of the surveyed winegrowers considered it present on their vineyards but affecting a small number of plants (level 1), for 24% it was affecting some of the plants (level 2) and only 3% considered it a severe problem for their vineyards (level 3) (Table I-3).

Finally, for young vine decline, 30% of the winegrowers recognised its presence on their vineyards, while 60% never acknowledged the disease on their vineyards and 10% were not familiar with the disease (Table I-2). In relation to the frequency of young vine decline, 87% of the inquired considered that it was affecting a negligible number of plants (level 1) and 13% considered it present in some plants (level 2) (Table I-3)

Phenotypic characterisation of the obtained isolates

After 25 days of growth, *P. chlamydospora* isolates produced the characteristic colonies with a felty texture and an absent zonation. However, it was noticeable that the morphology of the colonies was found to be variable among the 20 isolates under study leading to the establishment of four morphological groups (Table I-4). Group I shared colony characters such as an olive-grey colour, an even growing margin and the existence of predominant filamentous somatic hyphae in PDA. Colonies of group II exhibited olive-grey to white colour towards the edge, an even growing margin, producing filamentous, aerial somatic mycelium. Isolates from group III had olive-grey to white colour towards the edge, an uneven growing margin and they produced filamentous, aerial somatic mycelium. Finally, group IV had an olive-grey colour with the pigment concentrically distributed; an even growing margin and it produced filamentous, aerial somatic mycelium. Mycelial growth rates did not differ significantly among *P. chlamydospora* isolates, and not even within the four mentioned groups.

Phaeomoniella chlamydospora isolates produced the characteristic conidia and chlamydospora-like structures of such species. Sporulation rates of the different isolates (Table I-5) showed a broad range of variation (from 2.0 to 14.6 $\times$ 10^6 conidia mL^{-1}). Daily growth rate at 25ºC ranged from 0.70 to 1.40 mm and the growth diameter at 25ºC, after 25 days varied from 17.30 to 34.30 mm.

Table I-4. Distribution of the twenty *Phaeomoniella chlamydospora* isolates among the four morphological groups according to several phenotypic characteristics for each group (after 15 days at 25 ^{0}C in PDA)

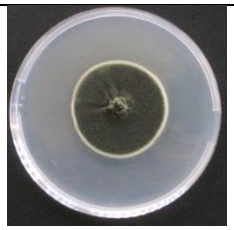
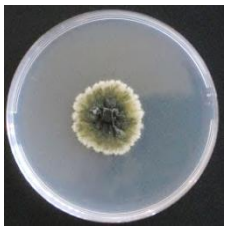
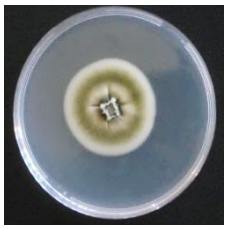
Group	Isolates	Phenotype in PDA culture	Texture	Colour	Growing margin	Zonation	Hyphal morphology
I	Ph19, Ph24, Ph26, Ph28, Ph33, Ph34, Ph37, Ph40, Ph41		Felty	Olive-grey	even	absent	Filamentous somatic hyphae predominant
II	Ph27, Ph32, Ph35, Ph36, Ph39		Felty	Olive-grey to white towards the edge	even	absent	Filamentous somatic hyphae predominant, aerial mycelium scanty
III	Ph38, Ph42, Ph43		Felty	Olive-grey to white towards the edge	uneven	absent	Filamentous somatic hyphae predominant, aerial mycelium scanty
IV	Ph29, Ph30, Ph31		Felty	Olive-grey; pigment distributed concentrically	even	absent	Filamentous somatic hyphae predominant

Table I-5. Mean, maximum and minimum values of the phenotypic variables studied for all the *Phaeomoniella chlamydospora* isolates

Phenotypic variable	Mean [a]	Maximum	Minimum
Sporulation (x 10^{6}conidia mL^{-1})	5.90[a]	14.60	2.00
Daily growth rate (mm) at 25°C	1.20	1.40	0.70
Growth (mm) at 25°C, after 25d	30.00	34.30	17.30

[a] Mean of two independent sets of six replicates for each isolate.

Molecular characterisation

The four ISSR primers tested were able to generate amplification products for all isolates of *P. chlamydospora*. A consensus dendrogram was generated from analysis of all markers (Figure I-4). The isolates studied were clustered with *P. chlamydospora* isolates Ph19 and Ph24 with about 82% similarity. This confirms that all the isolates collected in the Dão wine region belong to the same species. *Phaeomoniella chlamydospora* isolates were clustered into two groups supported by low bootstrap values, 48% and 54% respectively. The similarity level between groups, around 87%, indicates a low intra-specific genetic diversity. No relationship was found between ISSR band patterns and origin or scion/rootstock combination of isolates and the different groups formed.

Pathogenicity experiments

No significant differences were found among thickness and length (from cut to the base) of the control and inoculated spurs (Table I-6). Although *P. chlamydospora* was not recovered from the control spurs, some necroses were noticeable on the tissues of those plants (Table I-6).

Table I-6. Results of pathogenicity experiment carried out in a vineyard of cv. Touriga Nacional

Treatments	Thickness (cm)	Length from cut to the base (cm)	Length of necrosis (cm)	Reisolation (%)
Control	3.06a*	23.6a	2.47a	0.00a
Inoculated	3.10a	23.7a	8.95b	72.41b

*Mean values followed by the same letter are not statistically different at the level 5%.

Significant statistic differences were found in the extension of necrosis among control and inoculated canes. It ranged from 2.47 cm in controls to 8.95 cm in inoculated canes. The reisolation percentage reached 72.4% of the canes inoculated.

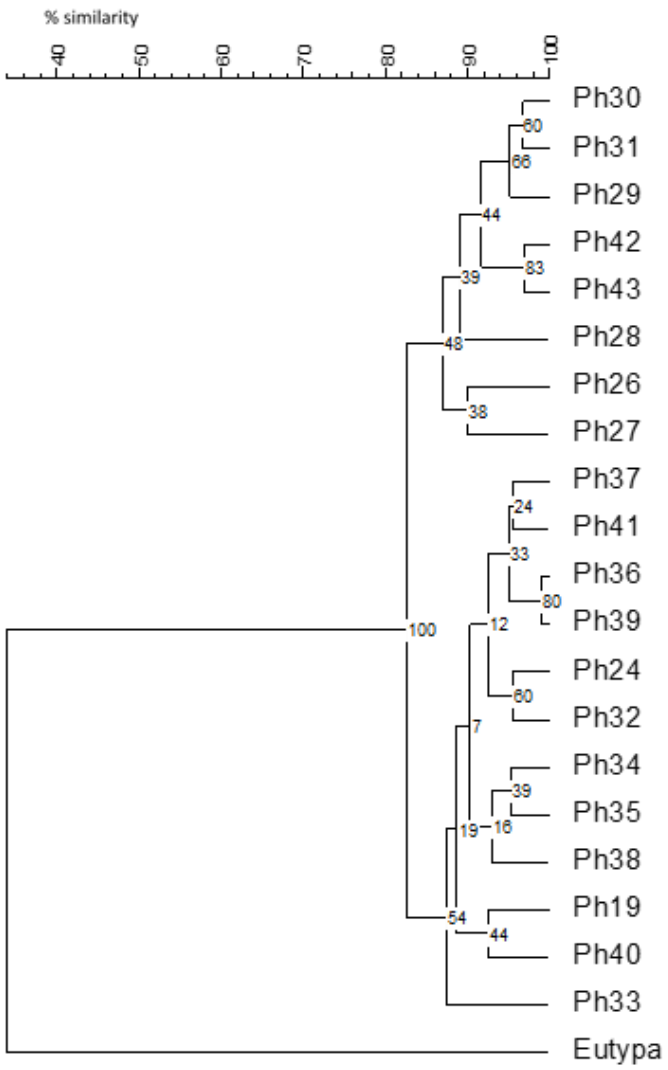


Figure I-4. UPGMA cluster analysis based on Dice coefficient of ISSR fingerprints from a collection of isolates of *Phaeomoniella chlamydospora* with the primers (AG)8YT, (CAG)5, HVH(TG)7 and MR. The numbers at the nodes represent bootstrap support values (2,000 replicates). An *Eutypa lata* isolate was used as outgroup.

Discussion

After one year of public education with the GTDs' leaflet, it was our perception, based on observation of cultural practices like flagging, removing and destruction of symptomatic vines, or in the number of questions on the subject, that winegrowers within the Dão wine region have improved their knowledge of GTD symptoms and general management of the diseases.

Previous works, based on a survey on grapevine trunk diseases (Tomaz *et al.*, 1989), considered esca as the main GTD in Dão having also pointed out for that region the importance of *Phomopsis* cane and leaf spot caused by *Phomopsis viticola* (Sacc.)

Sacc. Also, a newly emerging disease, designated as European excoriose, caused by *Macrophoma flaccida* (Viala & Ravaz) Cavara (*Fusicoccum aesculli* Corda) was also identified in the area (Tomaz and Rego, 1990).

The survey has provided an overview of the phytosanitary status of grapevines within the Dão wine region especially concerning GTDs. The occurrence of these fungal diseases in Dão's vineyards is confirmed, although its frequency and incidence in the vineyards are not high enough to become a matter of urgent concern. Esca and Phomopsis cane and leaf spot are better known due to published research (Tomaz *et al.*, 1989; Tomaz and Rego, 1990). However, BDA caused by Botryosphaeriaceous fungi is not as well known. The abandon of viticulture and the relatively few new plantations registered in this wine region recently (Falcão, 2012; IVV, 2012) has reduced the potential appearance of young plants showing young vine decline symptoms.

Worldwide, *P. chlamydospora* has been regarded as the most important fungus associated with esca and Petri disease (Ridgway *et al.*, 2005; Tello *et al.*, 2010) together with *Phaeoacremonium* spp. In Portugal, several studies have been focused on *P. chlamydospora* isolates (Chicau *et al.*, 2000; Cruz *et al.*, 2005; Rego *et al.*, 2000; Santos *et al.*, 2006b). In the Dão wine region, this fungus is usually isolated from esca symptomatic grapevines (Sofia *et al.*, 2006). Nevertheless, there is a lack of information about the phenotypical and molecular variability of such species.

In our study, analysis of phenotypic characteristics showed that variation among morphological features in culture, such as texture or zonation, concerning *P. chlamydospora* isolates, was low. This pattern was consistent with previous studies (Dupont *et al.*, 1998; Whiting *et al.*, 2005; Tello *et al.*, 2010) in which homogeneity was recorded. However, features like colony colour, growing margin or hyphal morphology were found to be variable, allowing the recognition of four groups of *P. chlamydospora* isolates. Within the four recognised phenotypic groups, the variation of phenotypic characteristics was found to be independent of *P. chlamydospora* isolates geographical origin or scion/rootstock combination. The sporulation and the daily growth rate at 25°C of *P. chlamydospora* isolates were similar to the obtained by Tello *et al.* (2010).

In the ISSR primers analysis, *P. chlamydospora* isolates clustered into two groups although no bootstrap support was found for such grouping. Similar results were previously obtained by Tegli *et al.* (2000b) and Mostert *et al.* (2006a).

The lack of diversity established among the studied isolates might be justified by the short period of time in which the isolates where obtained and by the genetic structure of the population based in asexual reproduction in natural ecosystems (Tegli *et al.*, 2000b; Pottinger *et al.*, 2002; Mostert *et al.*, 2006a). Moreover, ISSR tools did not detect a significant genetic variability which confirms that sexual reproduction does not occur. Further research based on an enlarged collection of isolates and in other molecular tools is needed to confirm the low genetic diversity within *P. chlamydospora* population in Dão region.

Concerning the results obtained in the pathogenic experiment, the use of the necrosis length in inoculated plants, as a measure of disease severity (Adalat *et al.*, 2000; Halleen *et al.*, 2007; Laveau *et al.*, 2009; Gramaje *et al.*, 2010), proved to be an accurate method to evaluate pathogen virulence. The high values obtained on the length of the necrosis formed agrees with the conclusions of Laveau *et al.* (2009) in which *P. chlamydospora* is considered one of the most aggressive pathogens associated with esca. Taking in account that in Dão region, canes are usually pruned to one or two bud spurs on cordons, the extension of the obtained necrosis and the high frequency of reisolation of *P. chlamydospora* strengthens the idea that recently pruned canes may be a potential entrance for *P. chlamydospora* to the main structure of grapevine, in a short period. No esca or Petri disease typical foliar symptoms were observed in inoculated vines. This observation is in accordance with results previously reported by Halleen *et al.* (2007) and Gramaje *et al.* (2010). The inoculation method tested in this pathogenicity test proved to be a successful, simple and practical method to infect plants in field experiments. The known feasibility and simplicity of this method means it will be used in further studies of cultivars' susceptibility to all *P. chlamydospora* isolates obtained, to add other criteria for separation of *P. chlamydospora* strains. Thus, performing a pathogenicity test with the entire collection could lead to a characterisation of hypovirulent or less pathogenic isolates and to a correlation of these data with phenotypic features, geographical origin or ISSR clustering.

Chapter II

MOLECULAR AND PHENOTYPIC CHARACTERISATION OF *PHAEOMONIELLA CHLAMYDOSPORA* ISOLATES FROM THE DEMARCATED WINE REGION OF DÃO (PORTUGAL)

Published as:

Sofia J., J. Trovão, A. Portugal, H. Paiva-de-Carvalho, N. Mesquita, T. Nascimento, C. Rego and M.T. Gonçalves, 2015. Molecular and phenotypic characterisation of *Phaeomoniella chlamydospora* isolates from the demarcated wine region of Dão (Portugal). *Phytopathologia Mediterranea* 54, 403–413

Introduction

The Portuguese Dão wine region or appellation has been afflicted by Grapevine Trunk Diseases (GTD), as have other wine-growing areas around the world. In previous studies (Tomaz *et al.*, 1989; Sofia *et al.*, 2006, 2013) esca was considered the most damaging GTD in this region. Esca is a GTD with complex aetiology (Mugnai *et al.*, 1999; Surico *et al.*, 2006; Bruez *et al.*, 2013). Over the last two decades the "International Council on Grapevine Trunk Diseases (ICGTD)" has promoted a wide forum gathering research on GTDs in general, and esca in particular, from which *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & Mugnai) Crous & W. Gams) and *Phaeoacremonium* spp. [mostly *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous (Teleomorph: *Togninia minima* (Tul. and C. Tul.) Berl], emerged as two of the most important pathogens related to the esca complex and Petri disease, a manifestation of esca in young plants (Crous and Gams, 2000). However, *P. chlamydospora* is the most frequently isolated species (Mugnai, 1998; Clearwater *et al.*, 2000; Pascoe and Cottral, 2000; Whiteman *et al.*, 2002), being considered the most important fungal organism associated with Petri disease (Ridgway *et al.*, 2005; Laveau *et al.*, 2009; Pouzoulet *et al.*, 2013).

Originally described as *Phaeoacremonium chlamydosporum*, *P. chlamydospora* is an anamorphic ascomycete that is morphologically and phylogenetically different from the species included in the genus *Phaeoacremonium* (Crous and Gams, 2000). Among other characteristics, it is distinguished by a partly yeast-like growth in culture, prominently green/brown conidiophores, light green to hyaline conidiogenous cells, chlamydospore-like structures and sclerotia produced in culture. *Phaeoacremonium chlamydospora* conidia also differ from the ones produced by *Phaeoacremonium* spp. for their straight oblong-ellipsoidal to obovate form and pale brown colouration, in contrast to the dimorphic and hyaline conidia that are typical of other *Phaeoacremonium* spp. (Crous and Gams, 2000).

A comprehensive paper on the phenotypical characterisation of 57 Spanish isolates (Tello *et al.*, 2010) revealed that there was consistent phenotypic homogeneity among all *P. chlamydospora* isolates, despite diverse geographic origins, year of isolation and scion/rootstock combination isolate source.

The combination of tradition, geographical isolation and agricultural policies have made the Dão appellation a singularity amid Portuguese wine regions (Falcão, 2012). Dão's winegrowing practice is characterised by the preference for local grapevine cultivars, mainly produced by local nurseries, leading to weak penetration of alien cultivars and foreign propagation material in general. Although previous studies on esca in this region have consistently yielded *P. chlamydospora* from symptomatic plants (Sofia *et al.*, 2006, 2013), the level of diversity of this species within this appellation is still not completely known, considering the highly prevalent use of locally produced plant propagation material.

The Internal Transcribed Spacer (ITS) region of the rDNA is the most extensively sequenced DNA region in fungi (Peay *et al.*, 2008), and has been proposed as the primary fungal barcode marker, due to high accuracy in fungal identification and the strongly defined barcode gap between inter- and intraspecific variation (Xu, 2006; Korabecna, 2007; Bellemain *et al.*, 2010; Schoch *et al.*, 2012). Furthermore, the utility of the ITS region has already been demonstrated for the correct taxonomic classification of *P. chlamydospora* (Crous and Gams, 2000).

No *P. chlamydospora* teleomorph has been described to date, and it is accepted that the reproduction of this pathogen is strictly clonal. The clonal reproduction strategy is supported by fairly high degrees of genetic homogeneity found among different populations, collected in Spain, France (Borie *et al.*, 2002; Smetham *et al.*, 2010), Italy (Tegli *et al.*, 2000a, 2000b), South Africa (Mostert *et al.*, 2006a), and New Zealand (Pottinger *et al.*, 2002; Smetham *et al.*, 2010).

Tegli *et al.* (2000a) using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) analyses, described a high degree of genetic homogeneity among 15 fungal isolates from eight distinct Italian regions. Borie *et al.* (2002) found similar levels of diversity in two French regions, using RAPD analysis to study 72 isolates from one region and 34 isolates from a second; and within a French vineyard, using 47 isolates. Moreover, using RAPD, ISSR, amplified fragment length polymorphism (AFLP) and universally primed polymerase chain reaction (UP-PCR), Pottinger *et al.* (2002) verified that only slight levels of genetic variability occurred among 39 New Zealand and 6 Italian isolates, and suggested that multiple introductions of foreign fungal populations have occurred in New Zealand vineyards. Mostert *et al.*

(2006a), using AFLPs, concluded that 63 isolates from South Africa and 25 from Australia, France, Iran, Italy, New Zealand, Slovenia and the USA, presented low genetic diversity, and noted intra-vine, intra- and inter-vineyard variations. Also, genetic variability among different production areas was not significant, concluding that infections occurred via different inoculum sources. Cobos and Martin (2008) and Tello *et al.* (2010) observed low genetic diversity between, respectively, 35 and 57 isolates from Spain, using ISSR, RAPD and ITS region, the β-tubulin gene and the elongation factor 1-α gene. More recently, Smetham *et al.*, (2010) used 60 isolates from Southern Australia and 67 from Southern France to study 18 microsatellite loci, concluding that limited genetic recombination and essentially clonal structure were present in these populations.

The aim of the present study was to evaluate the intra-specific morphological and molecular variability within a collection of Portuguese isolates. These isolates included 47 from the Dão appellation and 21 from other Portuguese wine-producing regions.

Materials and methods

Isolate collection

The 68 isolates of *P. chlamydospora* were from different Portuguese provenances mostly in the wine-producing region of Dão (Table II-1). For fungal isolation, transverse sections of wood tissues were removed from the trunks of plants that presented symptoms of esca and Petri disease and were screened for the presence of characteristic dark lesions/spots commonly associated with the presence of this pathogen (Larignon and Dubos, 1997). These lesions were carefully separated from surrounding wood tissue using a scalpel. The obtained tissues were surface disinfected for 1 min in 8% NaOCl solution, rinsed with sterile distilled water, dried on sterile filter paper and then placed in Petri dishes containing 2% malt agar (MA, Difco, Beckton, Dickinson and Co.), amended with 250 mg L^{-1} chloramphenicol (BioChemica, AppliChem). Plates were then incubated in the dark at 25°C to allow mycelial growth. After eight days, colonies morphologically identical to those of *P. chlamydospora* were transferred to MA to get pure cultures (Figure II-1).

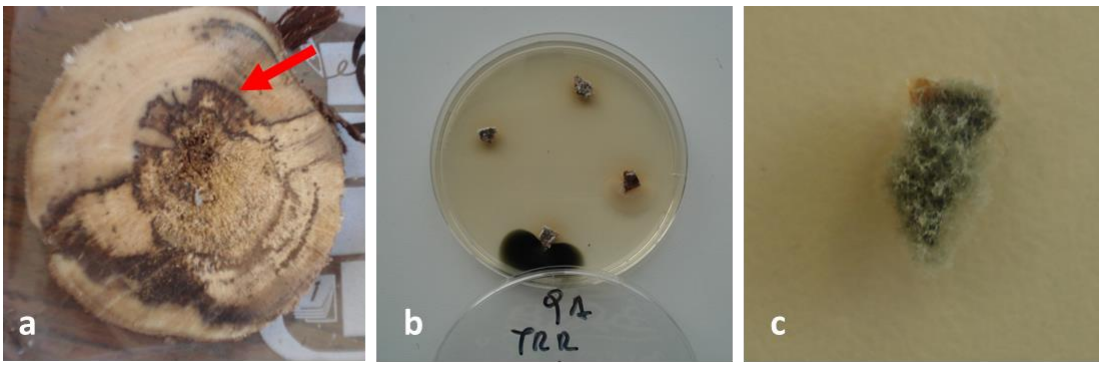


Figure II-1. Details of the isolation procedure: a) cross section of a grapevine trunk showing discolorations associated to esca. The red arrow points the black streaking to be collected; b) Petri dish with four samples evidencing fungal growth; c) detail of a wood sample presenting *P. chlamydospora* mycelial development. (Unpublished).

Morphological characterisation

For phenotypic colony characterisation (texture, colour, growing margin zonation and hyphal morphology), all obtained isolates were grown in triplicate on 2% MA, in the dark, at 25°C. Phenotypic features were described according to Crous and Gams (2000) and González and Tello (2011). To obtain daily growth and mean colony diameters after 30 days, for each isolate, diameters were assessed by measuring two perpendicular diameters per colony and calculating the mean. For the evaluation of the numbers of conidia, a 5 mm mycelial plug from each of the three replicate cultures was extracted from the colony growing margin, placed in a 2 mL vial containing 1 mL of sterile distilled water and vortexed for 5s (Whiting *et al.*, 2001; Tello *et al.*, 2010). Numbers of conidia were counted using a microscope and an improved Neubauer microscope slide cell counting chamber. Values obtained were compared referring to a two-sample *t*-test.

DNA extraction, amplification and sequencing of ITS-rDNA

For DNA extraction, mycelium plugs (each of 5 mm diam.) from each of the isolates were individually plunged into 250 mL flasks containing potato dextrose broth (PDB, Difco), where mycelia were allowed to grow at 22°C. All flasks were placed on reciprocal shakers at 90 rpm in complete darkness. After three weeks, suspensions were filtered using a paper filter disk, and the medium was discarded. For each isolate, 200 mg of the obtained mycelial mass was scraped into a 1.5 mL vial containing 200 µL of NucPrep™ solution (Applied Biosystems). Vials were placed on ice and each

homogenized with a pestle, after which, another 600 µL of NucPrep™ solution were added to each vial and then stored in the freezer at 5°C for 24 h. DNA was obtained using an ABI PrismTM 6100 Nucleic Acid PrepStation (Applied Biosystems), according to the manufacturer's instructions. The obtained genomic DNA was subjected to amplification of the ITS-rDNA region by PCR, using primers ITS1-F and ITS4 (White *et al.*, 1990; Gardes and Bruns, 1993).

PCR reactions consisting of a final amplification volume of 25 µL, with 12.5 µL of JumpStart Taq DNA Polymerase master mix with $MgCl_2$ and dNTP's (Sigma D9307), 0.5 µL of each primer (10 mM), 10.5 µL of ultra-pure water and 1 µL of template DNA, were performed using an ABI GeneAmpTM 9700 PCR System (Applied Biosystems), with the following conditions: initial denaturation at 95ºC for 2 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 53ºC for 1 min, and extension at 72ºC for 1 min, with a final extension at 72ºC for 5 min. Each run included a negative control reaction without template DNA.

Visual confirmation of the overall amplification of the ITS region was performed using agarose gel (1.2%) electrophoresis, stained with Gel Red (Biotium) and photographed under a UV light transilluminator (Bio-Rad Gel Doc XR+). ITS region fragments were purified and sequenced using an ABI 3730 genetic analyser, using the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Molecular identification and phylogenetic analyses

Obtained DNA sequences were deposited in GenBank (with accession numbers: KP886950–KP887017) and compared with the sequences from the National Centre of Biotechnology Information nucleotide databases using NCBI's Basic Local Alignment Search Tool (BLAST), with the option Standard nucleotide BLAST of BLASTN 2,6 (Altschul *et al.*, 1997). Molecular identification was performed following Landeweert *et al.* (2003), with similarity and taxon separations as follows: sequence similarity of 99/100%, identification to the species level; sequence similarity between 95–99%, identification to genus level; and sequence similarity equal or below 95%, identification to family or ordinal level.

Sequence alignment was performed with ClustalW 2.0 (Larkin *et al.*, 2007) and the resulting alignments were checked and manually adjusted using Geneious 7.0.6

software (www.geneious.com). Phylogenetic relationships were inferred with Maximum Likelihood (ML) using Kimura's two-parameter substitution model (Kimura, 1980). The bootstrap analysis involved 1000 replicates (MLB) to verify branches. All ML phylogenetic analyses were conducted using MEGA6 bioinformatics software (Tamura *et al.*, 2013). Phylogenetic trees were viewed and edited using FigTree 1.4.2 and a text editor. *Eutypa lata* (Genbank: KF453558.1) was used as the outgroup for the phylogenetic analyses.

Table II-1. Details of *Phaeomoniella chlamydospora* isolates used in this study

***Phaeomoniella chlamydospora* isolate**	**Genbank ID**	**Reference**	**Year of isolation**	**Geographic origin**		**Cultivar/rootstock**	**Host age (years)**
				Wine appellation	**County**		
1	KP886950	CEVD1	2013	Dão	Mangualde	Tinta Carvalha/-	>50
2	KP886951	CEVD2	2013	Dão	Mangualde	Rufete/-	>50
3	KP886952	CEVD3	2013	Dão	Mangualde	-/-	>25
4	KP886953	CEVD4	2013	Dão	Mangualde	Fernão Pires/-	>50
5	KP886954	CEVD5	2013	Dão	Mangualde	Português Azul/-	>50
6	KP886955	CEVD6	2013	Dão	Viseu	Baga/-	>80
7	KP886956	CEVD7	2013	Dão	Viseu	Dona Branca/-	>80
8	KP886957	CEVD8	2013	Bairrada	Pombal	Fernão Pires/-	>20
9	KP886958	CEVD9	2013	Bairrada	Pombal	Fernão Pires/-	>20
10	KP886959	CEVD10	2013	Dão	Viseu	Aragonês/-	>5
11	KP886960	CEVD11	2013	Dão	Viseu	Jaen/-	>30
12	KP886961	CEVD12	2013	Dão	Viseu	Jaen/-	>30
13	KP886962	CEVD13	2013	Dão	Viseu	Moscatel Hamb./-	10
14	KP886963	CEVD14	2013	Dão	Viseu	Bical/-	25
15	KP886964	CEVD15	2013	Dão	Viseu	Encruzado/-	25
16	KP886965	CEVD16	2013	Dão	Viseu	Arinto Gordo/-	10
17	KP886966	CEVD17	2013	Dão	Viseu	Malvasia Rei/-	25
18	KP886967	CEVD18	2013	Dão	Tondela	Touriga Nacional/-	>50
19	KP886968	CEVD19	2013	Dão	Tondela	Touriga Nacional/-	>50

Table II-1. Details of *Phaeomoniella chlamydospora* isolates used in this study (continued)

***Phaeomoniella chlamydospora* isolate**	**Genbank ID**	**Reference**	**Year of isolation**	**Geographic origin**		**Cultivar/rootstock**	**Host age (years)**
				Wine appellation	**County**		
20	KP886969	CEVD20	2013	Dão	Tondela	Arinto/-	>50
21	KP886970	CEVD21	2013	Dão	Tondela	Aragonês/-	25
23	KP886971	CEVD23	2013	Bairrada	Pombal	Baga/-	20
24	KP886972	CEVD24	2013	Dão	Viseu	Arinto/-	20
25	KP886973	CEVD25	2013	Bairrada	Anadia	Fernão Pires/-	25
26	KP886974	CEVD26	2012	Bairrada	Anadia	Sauvignon Blanc/-	25
27	KP886975	CEVD27	2013	Dão	P. do Castelo	Touriga Nacional/-	1
28	KP886976	CEVD28	2013	Dão	P. do Castelo	Aragonês/-	10
29	KP886977	CEVD29	2013	Açores	Pico	Aragonês/-	10
30	KP886978	CEVD30	2013	Dão	O. Hospital	Aragonês/-	>25
31	KP886979	CEVD31	2013	Dão	Tondela	Jaen/-	>25
32	KP886980	CEVD32	2013	Açores	Pico	Terrantez do Pico/-	20
33	KP886981	CEVD33	2012	Dão	Nelas	Cabernet Sauvignon/-	15
34	KP886982	CEVD34	2013	Dão	Gouveia	Syrah/-	10
35	KP886983	CEVD35	2013	Dão	Tábua	Sauvignon Blanc/-	15
36	KP886984	Ph9	2000	P. Setúbal	Grândola	Periquita/99R	-
37	KP886985	Ph13	2000	Bucelas	Loures	Arinto/-	-
38	KP886986	Ph14	2007	Alentejo	Monforte	Viognier/1103P	-
39	KP886987	Ph15	2007	Alentejo	Monforte	Arinto/1103P	-
40	KP886988	Ph16	2008	Alentejo	Vidigueira	C. Sauvignon/169VO	2
41	KP886989	Ph17	2008	Alentejo	Vidigueira	C. Sauvignon/337MM	2
42	KP886990	Ph18	2008	Alentejo	Vidigueira	Petit Verdot/400MM	2
43	KP886991	Ph19	2008	Alentejo	Vidigueira	Petit Verdot/400VO	2
44	KP886992	Ph20	2008	Alentejo	Vidigueira	Chardonnay/76PB	2
45	KP886993	Ph21	2011	Algarve	Lagoa	Arinto/1103P	-

Table II-1. Details of *Phaeomoniella chlamydospora* isolates used in this study (final)

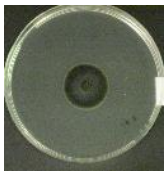
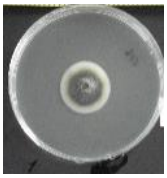
***Phaeomoniella chlamydospora* isolate**	**Genbank ID**	**Reference**	**Year of isolation**	**Geographic origin**		**Cultivar/rootstock**	**Host age (years)**
				Wine appellation	**County**		
46	KP886994	Ph22	2011	Algarve	Lagoa	Arinto/1103P	-
47	KP886995	Ph23	2011	Algarve	Lagoa	A. Bouschet/110R	-
48	KP886996	Ph24	2011	Arruda	A. dos Vinhos	Touriga Nacional/-	-
49	KP886997	Ph26	2012	Dão	Lousã	Cerceal da Bairrada /-	15
50	KP886998	Ph28	2012	Dão	Mangualde	Jaen/-	20
51	KP886999	Ph29	2012	Dão	Mangualde	Touriga Nacional/-	20
52	KP887000	Ph30	2012	Dão	Nelas	Jaen/SO4	15
53	KP887001	Ph31	2012	Dão	Nelas	Aragonês/SO4	15
54	KP887002	Ph32	2012	Dão	Nelas	Alfrocheiro/1103P	20
55	KP887003	Ph33	2012	Dão	Seia	Jaen/-	>50
56	KP887004	Ph34	2012	Dão	Tondela	Aragonês/-	20
57	KP887005	Ph35	2012	Dão	Mangualde	Touriga Nacional/-	25
58	KP887006	Ph36	2012	Dão	Mangualde	Encruzado/-	>50
59	KP887007	Ph37	2012	Dão	Gouveia	Gouveio/-	>50
60	KP887008	Ph38	2012	Dão	Nelas	Touriga Nacional/-	15
61	KP887009	Ph39	2012	Dão	Gouveia	Jaen/-	15
62	KP887010	Ph40	2012	Dão	Arganil	Baga/-	>80
63	KP887011	Ph42	2012	Dão	Mangualde	Jaen/-	25
64	KP887012	CEVD36	2012	Dão	Arganil	Rufete/-	>50
65	KP887013	CEVD37	2012	Dão	Tábua	T. Nacional/1103P	15
66	KP887014	CEVD38	2013	Dão	C. do Sal	T. Nacional/1103P	1
67	KP887015	CEVD39	2013	Dão	C. do Sal	T. Nacional/1103P	1
68	KP887016	CEVD40	2013	Dão	C. do Sal	T. Nacional/1103P	1
69	KP887017	CEVD41	2013	V. Verdes	A. de Valdevez	Loureiro/-	>25

Results

Phenotypic characterisation

All 68 *P. chlamydospora* isolates produced typical colonies, after an incubation period of 30 days. Variability in the morphology of the colonies allowed the establishment of two distinct groups (Table II-2): group I with 45 isolates and group II with 23 isolates. Group I morphology was characterised by olive-grey colour, uniform colony growing margins and filamentous somatic hyphae, while isolates included in group II developed colonies with central olive-grey colour surrounded by white growing margins. In addition, group II isolates also produced filamentous aerial mycelia on 2% MA.

Table II-2. Distribution of the 68 *Phaeomoniella chlamydospora* isolates between the two morphological groups, according to colony phenotypic characteristics (after 30 d at 25°C, in 2% MA)

Group	*Phaeomoniella chlamydospora* isolates	Phenotype in MA culture	Texture	Colour	Growing margin	Zonation	Hyphal morphology
I	1, 2, 3, 4, 5, 9, 13, 14, 15, 16, 17, 18, 21, 23, 24, 26, 27, 28, 30, 33, 35, 39, 40, 42, 43, 44, 45, 47, 48, 49, 50, 51, 52, 53, 55, 56, 58, 59, 60, 61, 62, 64, 65,68, 69		Felty	Olive-grey	even	absent	Filamentous somatic hyphae predominant, aerial mycelium scanty
II	6, 7, 8, 10, 11, 12, 19, 20, 25, 29, 31, 32, 34, 36, 37, 38, 41, 46, 54, 57, 63, 66, 67		Felty	Olive-grey to white towards the edge	even	absent	Filamentous somatic hyphae predominant, aerial mycelium scanty

All *P. chlamydospora* isolates produced the typical conidia and chlamydospore-like structures, displaying a wide range of sporulation rates, from 0.2×10^6 to 10×10^6 conidia mL^{-1}. Daily colony growth rates ranging from 0.48 mm to 0.98 mm were not significantly different, neither among colonies inside each group, nor between the two groups. Mean colony diameters after 30 days of growth ranged from 14.3 to 29.3 mm (Table II-3).

Table II-3. Mean, maximum and minimum values (± standard deviations) of the colony phenotypic characters in *Phaeomoniella chlamydospora* isolates

Phenotypic variable	Group I mean value ± SD[a]	Group II mean value ± SD[a]	Maximum values		Minimum values	
			Group I	Group II	Group I	Group II
Daily growth rate (mm) at 25°C[a]	0.68± 0.13[ns]	0.68± 0.11[ns]	0.98	0.93	0.48	0.50
Growth (mm) at 25°C, after 30 d[a]	20.54± 3.90[ns]	20.52 ±3.50[ns]	29.3	27,87	14.31	14.83

[ns] Non-significant differences according to *t*-test at $\alpha = 0.05$

[a] Colony diameter

Molecular identification

PCR reactions using the universal primers ITS1-F and ITS4 produced a single DNA fragment of ca. 570–600 bp for all *P. chlamydospora* isolates tested. The studied isolates were identified in comparison with reference *P. chlamydospora* ITS sequences deposited in the NCBI GenBank database. The similarity values for all sequences were 99%, with the exception of isolate 61 which presented 100% similarity. These similarity values are adequate for the molecular identification of isolates (Landeweert *et al.*, 2003).

Phylogenetic characterisation

The complete ITS sequences of each isolate were analysed to infer the phylogenetic relationship by using the ML approach (Figure II-2). The results show separation between two distinct groups; one clustering 33 isolates with a bootstrap value of 65% and the second clustering 35 isolates separated from group 1 with a bootstrap value of 80%. The separation of these two groups occurred due to the simple nucleotide polymorphisms (SNPs), in the positions 429 (T/A) and 497 (T/C). In addition, an SNP in position 534 (C/A) separated isolate 37 from the rest of its group (bootstrap value of 100%).

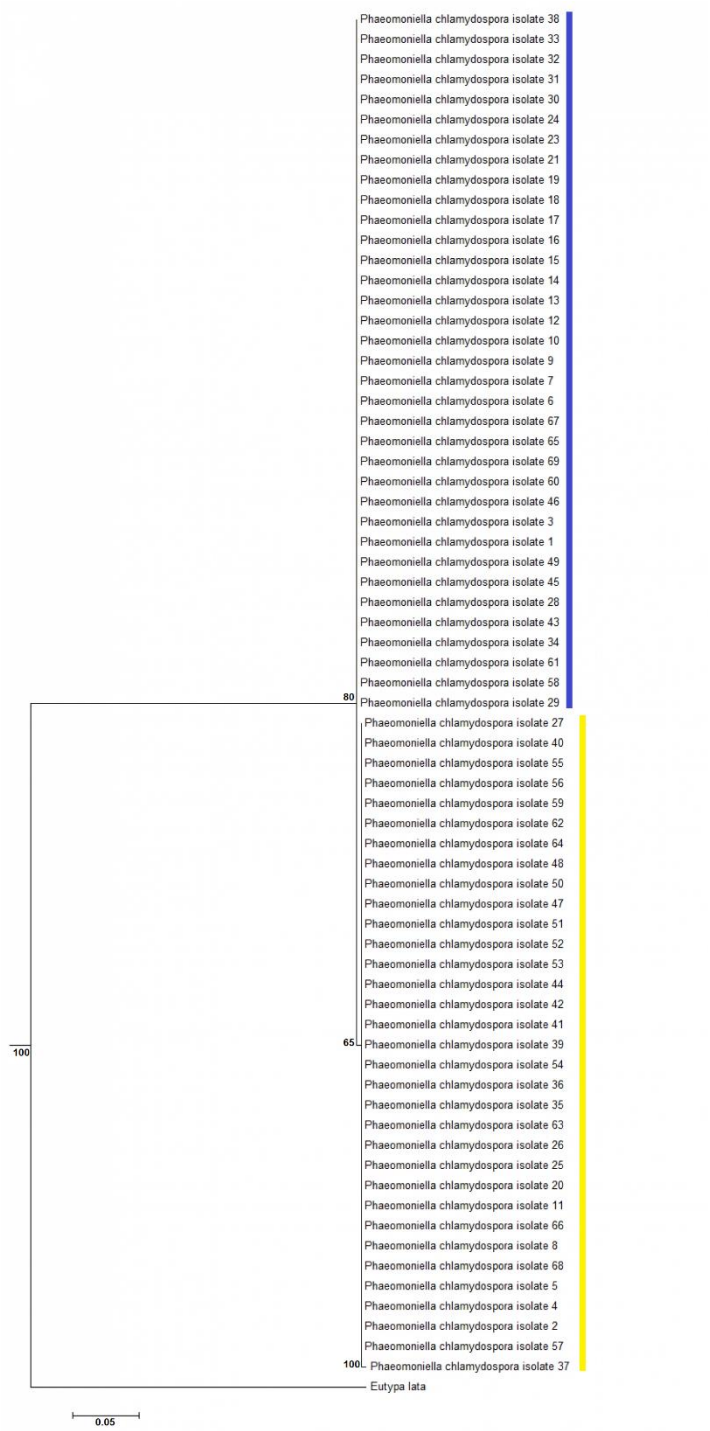


Figure II-2. Maximum Likelihood tree inferred from a character alignment of the 68 rDNA- ITS sequences of *Phaeomoniella chlamydospora* obtained in this study, with *Eutypa lata* (KF453558) as an outgroup. Numbers above branches identify the statistical bootstrap percentages (Maximum likelihood bootstraps from 1000 iterations). Scale represents substitutions per site. Yellow bar-group 1; blue bar-group 2.

Discussion

Several studies have examined *P. chlamydospora* isolates in Portugal (Chicau *et al.*, 2000; Rego *et al.*, 2000; Santos *et al.*, 2006a; Sofia *et al.*, 2013). However, there was little available information about the phenotypical and molecular variability of the species. In this study, a larger collection of *P. chlamydospora* isolates, 47 from Dão appellation and 21 from other Portuguese wine regions was characterized.

Studies concerning morphological features of *P. chlamydospora* isolates, conducted in different countries, have shown a low degree of phenotypic variation (Dupont *et al.*, 1998; Whiting *et al.*, 2001; Santos *et al.*, 2006a; Tello *et al.*, 2010). Moreover, in France, a country with an ancient and ubiquitous viticulture, similar to the Portuguese Dão appellation's viticulture, Comont *et al.* (2010) reported the coexistence of two predominant clonal lineages.

In the present research, phenotypic characteristics displayed little variation among the 68 *P. chlamydospora* isolates from Dão and other Portuguese wine-producing regions. Similar results of low morphological variation were reported in previous studies in which homogeneity was also observed (Dupont *et al.*, 1998; Whiting *et al.*, 2005; Tello *et al.*, 2010). Nevertheless, the morphological features analysed here appeared to divide the 68 isolates into two morphotypes according to the macromorphological appearance of the cultures. Isolates from Dão were separated into the two groups together with isolates from other Portuguese regions. No clear relationships with the source rootstock/scion combination, year of isolation or geographical origin were recorded among the isolates.

Tello *et al.* (2010), for Spanish isolates, and Sofia *et al.* (2013), for Portuguese isolates, registered higher levels of sporulation and daily growth rates than recorded in the present study. These differences are probably mainly due to our usage of malt agar instead of potato dextrose agar, which is a richer growth medium likely to give greater sporulation than malt agar.

The low phenotypic variability observed in the Portuguese populations of *P. chlamydospora*, probably a consequence of predominant clonal reproduction, indicates that different criteria are needed to differentiate the population structure of a large set of isolates.

Multiple alignments of the ITS sequences clustered in two distinct groups, due to changes in the nucleotides from positions 429 (T/A) and 497 (T/C). Similar results have been observed for Spanish populations by Cobos and Martin (2008), with differences occurring in positions 369 (T/A) and 438 (T/C). In addition, isolate 37 was separated from the last group by an SNP in position 534 (C/A).

Since three different clonal lineages of *P. chlamydospora* were detected, results suggest that different sources of inoculum may have been introduced through propagation material, such as mother-plants, rootstock, grafted cuttings and/or scions (Retief *et al.*, 2006; Whiteman *et al.*, 2007).

Our results are in agreement with previous studies conducted in New Zealand, Spain, Australia and France (Tegli *et al.*, 2000a, 2000b; Borie *et al.*, 2002; Pottinger *et al.*, 2002; Mostert *et al.*, 2006a; Cobos and Martin, 2008; Smetham *et al.*, 2010; Tello *et al.*, 2010). There is no clear correlation between the morphological groups and the genetic clusters.

Clonal reproduction has been described for this species (Tegli *et al.*, 2000b; Borie *et al.*, 2002; Pottinger *et al.*, 2002; Mostert *et al.*, 2006a; Cobos and Martin, 2008; Smetham *et al.*, 2010; Tello *et al.*, 2010). Asexual reproduction is predominant. Nonetheless, as suggested by Borie *et al.* (2002), recombination via a parasexual cycle may be involved, explaining the slight genetic variability found.

Chapter III

AGGRESSIVENESS OF *PHAEOMONIELLA CHLAMYDOSPORA* ISOLATES

Introduction

Phaeomoniella chlamydospora is an endophytic fungus well adapted to colonisation and growth in grapevine wood (Eskalen *et al.*, 2001; Halleen *et al.*, 2007). It's able to infect roots, pith, vascular tissue, green cortical tissue, and berries (Gramaje *et al.*, 2010). This pathogen has been thoroughly studied because of its involvement in the esca complex and GLSD in adult grapevines and in the so-called "Grapevine Phaeotracheomycotic complex" in young vines (Mugnai *et al.*, 1999; Surico *et al.*, 2006; Surico, 2009; Bertsch *et al.*, 2013).

External symptoms of Petri disease include late bud break, stunted shoot growth, weak vegetative growth, and occasionally leaf symptoms (interveinal chlorosis, leaf necrosis, and wilting). Internal xylem symptoms include brown to black vascular streaking that in cross sections appear as brown/black spots. Esca internal wood symptoms include areas of soft rot surrounded by wood lesions resembling typical Petri disease wood symptoms (Mugnai *et al.*, 1999). Petri disease wood symptoms associated to *P. chlamydospora* have been successfully replicated in field and greenhouse artificial trials (Mugnai *et al.*, 1999; Halleen *et al.*, 2007; Zanzotto *et al.*, 2008; Aroca and Raposo, 2009; Laveau *et al.*, 2009).

Several inoculation procedures have been successfully used to investigate the role of Esca and Petri disease related pathogens, such as soaking grapevine cuttings or seedlings in spore suspensions (Scheck *et al.*, 1998; Eskalen *et al.*, 2001; Aroca and Raposo, 2009), vacuum-inoculation of conidial suspensions throughout the vascular system of cuttings (Gramaje *et al.*, 2010), inserting mycelial plugs in artificially induced side wounds and deposition of conidial suspensions on top of grapevine freshly pruned spurs (Sofia *et al.*, 2013; Elena *et al.*, 2015; Elena and Luque, 2016). This last procedure is, in our perspective, the one that better emulates infection as it occurs in standing grapevines in the vineyard.

Genetic and morphological differences among *P. chlamydospora* isolates have been described by several authors (Mostert *et al.*, 2006a; Comont *et al.*, 2010; González and Tello, 2011). Also, differences in aggressiveness among *P. chlamydospora* isolates have been reported by Santos *et al.* (2005) and Laveau *et al.* (2009) and González and Tello (2011).

The purpose of the present study was to evaluate and compare the aggressiveness of several isolates of *P. chlamydospora*, randomly chosen from our collection, by inoculating them in young grapevine plants.

Materials and methods

Plant material

Four hundred and sixty, two-years-old potted plants of cv. Touriga Nacional, grafted on rootstock 1103P were used for aggressiveness experiments. Certified plants were acquired to a local nursery, and after delivery, immediately transferred to individual 20-litre plastic woven bags with artificial substrate (Figure III-1). Plants were maintained in a greenhouse at 24ºC ± 5ºC during the day and 18ºC ±2°C by night with 12 h of daylight and watered weekly. Plants were allowed to grow and establish for one year. During this period, plants were assessed for any symptoms resembling those induced by Grapevine Trunk Diseases. The trial took place during their second year of plantation.

Figure III-1. General aspect of trial plants after potting.

Fungal strains

Twenty-two *P. chlamydospora* isolates from our collection (Table II-1), were aleatorily chosen to study their aggressiveness: isolates 15, 26, 27, 28, 29, 30, 31, 36, 42, 43, 48, 51, 52, 54, 56, 57, 58, 59, 60, 61, 62 and 63.

For the preparation of conidial suspensions isolates, kept in PDA (PDA, Difco,

Beckton, Dickinson and Co, Sparks, MD, USA) slants, were transferred to Petri dishes with PDA to promote colony growth. Cultures were incubated at 24°C±1 in complete darkness for 15 days. Conidial suspensions were obtained by removing, with a sterile cork borer, a plug of *circa* 5mm from the border of the culture, which was then plunged in a 250 mL Erlenmeyer flask containing Potato Dextrose Broth (PDB, Difco, Beckton, Dickinson and Co, Sparks, MD, USA) and placed at 20°C, under darkness, in a reciprocal shaker (90 strokes min^{-1}), for 21 days. One day before inoculation, the suspension was homogeneized with a magnetic stirrer and filtered through a sterile gauze cloth. The obtained solution was kept under agitation, in a magnetic stirrer. Conidial suspension was adjusted to a 10^5 conidia mL^{-1} conidial suspension with the help of a hemocytometer, according to Sofia *et al.* (2013). Suspensions were stored overnight at 10°C, until use.

Inoculation procedure

During dormancy, all plants were left with just one cane. Removed canes were sampled for vascular discolouration symptoms, that if found would imply discarding the plant. The remaining canes were then pruned 0.5 cm above the last node, usually the third node so that the resulting spurs would be approximately 10 cm long. Immediately after pruning, a 40 µl droplet of the conidial suspension was carefully deposed on top of the freshly exposed tissue, allowing to soak for about ten to fifteen minutes. The infection point was then covered with a cotton pad soaked in sterile distilled water (SDW) and protected with a stripe of Parafilm (Parafilm® "M", Pechiney Plastic Packaging, Menasha, USA). All protections remained for a week being removed after that period. Twenty repetitions were used per isolate. Control plants were inoculated with a 40 µl droplet of potato dextrose broth (PDB, Difco, Beckton, Dickinson and Co.).

Infection assessment

Nine months after infection, the entire plant above the soil was cut off, packed in freezer bags, and kept in a refrigerator. All samples were processed within two weeks after collection. For analysis, each plant was scrubbed under running tap water to remove all debris and disinfected by immersion in a 1.5 % commercial house bleach solution. Then, plants were open longitudinally, and lesion lengths were measured with an electronic calliper (Figure III-2).

From each lesion, six wood fragments were collected, and surface disinfected,

for 1 minute, by immersion in a 2% sodium hypochlorite solution. Disinfection was followed by elimination of sodium hypochlorite excess by rinsing with SDW; all samples were then allowed to dry for 1 minute on a sterile filter paper pad. After, each fragment was sectioned into four small fragments, and incubated on PDA amended with 250 µg of chloramphenicol (BioChemica, AppliChem, Germany). Two replicates were made per sample. Plates were incubated at 24±1°C under complete darkness and observed every two days until fungal growth permitted the identification of *P. chlamydospora*. Reisolation frequencies and lesion length were recorded.

Figure III-2. Plant of cv. Jaen longitudinally sectioned before analysis.

Statistical analysis

Data of lesion lengths and P. *chlamydospora* reisolation frequency were performed using STATISTICA (StatSoft, Inc. 2007, version 8.0). Homogeneity of variance was tested using Levene's test. One–way analysis of variance (ANOVA) was used to compare differences in mean lesions length among isolates. Means were separated using Tukey's test at the 5% significance level.

Results

Vascular discolorations were visible in both inoculated and control spurs. All *P. chlamydospora* strains were able to infect, colonise and produce vascular discolourations or lesions, and were consistently recovered from the formed lesions.

Table III-1. Average lesion lengths caused by infecting grapevines cv. Touriga Nacional with twenty-two different *P. chlamydospora* isolates. Different letters mean different homogeneity groups (Tukey's HSD)

***P. chlamydospora* Isolate**	**Group***	**Average Lesion Lenght (mm)**	**Homogeneity groups (Tukey's HSD)**					
			1	**2**	**3**	**4**	**5**	**6**
control	–	7.3	a					
29	2	40.6		b				
57	1	41.1		b				
61	2	41.7		b	c			
58	2	43.7		b	c	d		
31	2	44.3		b	c	d		
15	2	44.7		b	c	d		
60	2	45.7		b	c	d		
28	2	47.8		b	c	d		
43	2	51.8		b	c	d	e	
48	1	51.9		b	c	d	e	
30	2	53.6		b	c	d	e	f
56	1	53.7		b	c	d	e	f
63	1	54.3		b	c	d	e	f
51	1	58.1		b	c	d	e	f
52	1	59.0		b	c	d	e	f
62	1	59,0		b	c	d	e	f
27	1	63.0		b	c	d	e	f
42	1	63.9		b	c	d	e	f
36	1	66.5			c	d	e	f
54	1	67.2				d	e	f
26	1	76.7					e	f
59	1	78.5						f

*Group obtained in phylogenetic characterization of *Phaeomoniella chlamydospora* (Sofia *et al*., 2015)

On control plants, although minor lesions were noticeable, no *P. chlamydospora* were ever recovered. Significant statistic differences were found in the extension of necrosis between control and inoculated spurs. Although statistical differences were found among some isolates lesions' length, they didn't differ much among the analysed samples (Table III-1). No foliar symptoms were ever observed on infected plants.

The reisolation percentage ranged from 50 to 78%. No significant statistical differences were found among reisolation percentages between the different isolates. Reisolation from control spurs differed significantly from inoculated spurs (Figure III-3).

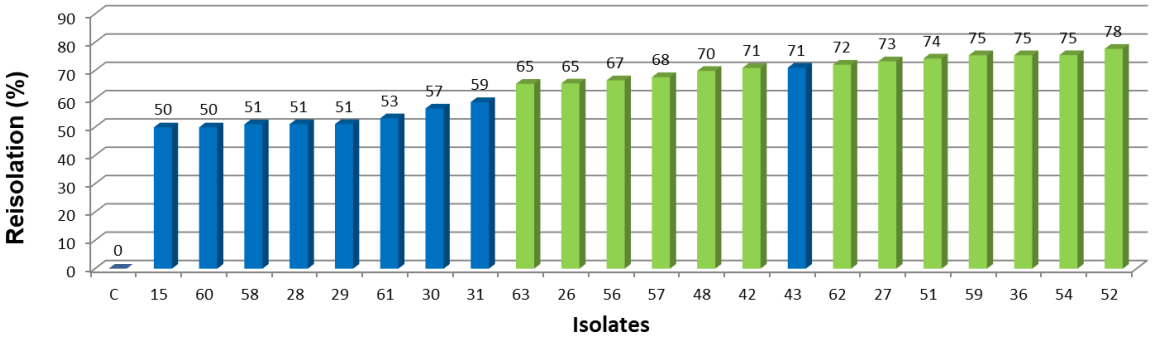


Figure III-3 Isolation frequencies recorded for the twenty-two isolates of *P. chlamydospora*. Columns in light-green corresponded to group 1 isolates and columns in blue to group 2 isolates.

Discussion

The greenhouse experiment on young grapevine plants showed the pathogenic behaviour of the 22 isolates tested.

Symptoms observed in the wood of infected plants were characteristic of vascular diseases. These symptoms have been previously described as some of the main symptoms observed in plants affected by the "phaeotracheomycotic complex", associated with *P. chlamydospora*. Discoloured vascular tissues is a symptom described in plants infected by vascular fungi, which might be associated to the oxidation and translocation of some breakdown products of plant cells attacked by fungal enzymes.

Infected plants showed no foliar symptoms. However, nine months may have been insufficient for symptom expression. Halleen *et al.* (2007) reported similar opinion, considering that symptom expression might depend on the assay time duration. On the other hand, stress conditions might influence the expression of external symptoms. Plants are often affected by GTDs without showing foliar or fruit symptoms (Fischer and

Kassemeyer, 2012). External symptoms expression is not strictly correlated with inner symptoms, and their occurrence may be highly related with environmental stress like extreme temperatures or water shortage (Fischer and Kassemeyer, 2012; Lecomte *et al.*, 2012), that did not occur in this experiment hence plants were maintained in a controlled artificial environment, thus avoiding stress conditions.

Our results show that not all isolates had the same behaviour regarding severity, evaluated by the length of the internal lesions. Some differences were noticed, on their ability to progress inside the plant. Previous results on molecular intra-specific variance of several isolates of *P. chlamydospora* (Sofia *et al.*, 2015) using multiple alignments of the ITS sequences clustered those isolates in two distinct groups: "Group 1", clustering 33 isolates with a bootstrap value of 65% and a "Group 2", clustering 35 isolates with a bootstrap value of 80%. The 22 isolates used in the present study, although chosen randomly amid the referred collection, were distributed by those two groups: isolates 26, 27, 36, 42, 48, 51, 52, 54, 56, 57, 59, 62 and 63 belonged to "Group 1", while isolates 15, 28, 29, 30, 31, 43, 58, 60 and 61 belonged to "Group 2". All isolates included in "Group 1", with the sole exception of isolate 57 (included in "Group 2"), caused longer lesions than the ones included in "Group 2". The same tendency continued with *P. chlamydospora* reisolation frequencies, with plants infected with "Group 1" isolates exhibiting the higher recovery frequencies, except isolate 43, included in "Group 2". These results can, to a certain point, allow some inferences relating the aggressiveness of the isolates with the formed cluster's groups.

Finally, this model, which is a simpler and faster method of evaluation than testing on standing grapevines could be used to study the aggressiveness of *P. chlamydospora* isolates.

Chapter IV

RESPONSE OF FOUR PORTUGUESE GRAPEVINE CULTIVARS TO INFECTION BY *PHAEOMONIELLA CHLAMYDOSPORA*

Sofia J., M. Mota, M.T. Gonçalves and C. Rego, 2018. Response of four Portuguese grapevine cultivars to infection by *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea. Accepted for publication.*

Introduction

Dão Protected Geographical Indication (PGI) is a very traditional and distinct Portuguese wine region, where, unlike other Portuguese wine appellations, local cultivars are predominant. Due to their oenological and consequent economic interest, four cultivars dominate – Alfrocheiro, Aragonez (Tempranillo), Jaen (Mencia), and Touriga Nacional - therefore, there is a huge interest in understanding both its agronomical behaviour and its susceptibility to certain diseases. Previous works (Tomaz *et al.*, 1989; Sofia *et al.*, 2006, 2013) have shown esca as one of the most important grapevine trunk diseases (GTDs) in that region.

Esca, a GTD leading to the decline and eventual death of affected plants, has been considered a complex of several syndromes - esca proper, esca, young esca, Petri disease, and brown wood streaking (Surico, 2009; Bertsch *et al.*, 2013). This complex is commonly associated with several fungal species such as the anamorphic ascomycetes *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams and *Phaeoacremonium* spp. and the basidiomycete *Fomitiporia mediterranea* M. Fisch. (Mugnai *et al.*, 1999; Bertsch *et al.*, 2013). Esca proper, characteristic of mature grapevines (i.e. 7 years and older), indicates the co-occurrence of Petri disease (young esca, a manifestation of esca in plants less than 6 years old) and esca on the same plant (Surico *et al.*, 2006; Surico, 2009; Bertsch *et al.*, 2013). *P. chlamydospora* and *Phaeoacremonium* spp. are the two most important pathogens related with brown wood streaking of xylem vessels, Petri disease and young esca (Crous and Gams, 2000; Bruez *et al.*, 2013), whereas esca is associated with several basidiomycetes, mostly *F. mediterranea*. Recently, a simplification of the above-referred syndromes has been proposed: The designation "young esca" was replaced by the designation "grapevine leaf stripe disease" (GLSD), while "esca" was used to designate grapevines showing symptoms in internal woody tissues of soft white rot, usually due to *F. mediterranea* (Fischer 2002, 2006; Bertsch *et al*. 2013), and esca proper, brown wood streaking, Petri disease and grapevine leaf stripe disease where grouped under the designation "phaeotracheomycotic complex", emphasizing the involvement of *Phaeomoniella. chlamydospora* and/or *Phaeoacremonium minimum* in the three syndromes (Bertsch *et al.*, 2013). *Phaeoacremonium* spp. are consistently considered to have a less important role in the esca complex than *P. chlamydospora* (Adalat *et al.*, 2000; Halleen *et al.*, 2007;

Fischer and Kassemeyer, 2012; Markakis *et al.*, 2017). Also, *P. chlamydospora* is the most frequently isolated species (Mugnai *et al.*, 1999; Clearwater *et al.*, 2000; Pascoe and Cottral, 2000; Whiteman *et al.*, 2002), and considered the most important and aggressive fungal organism associated with Petri disease (Halleen *et al.*, 2007; Laveau *et al.*, 2009; Pouzoulet *et al.*, 2013; Ridgway *et al.*, 2005; Sofia *et al.*, 2007; Wallace *et al.*, 2003; Zanzotto *et al.*, 2008). It is consistently recovered from plants in the Dão appellation affected by the esca syndromes (Sofia *et al.*, 2006; Sofia, 2007). Considered a solution to control esca symptoms (Mugnai *et al.*, 1999), sodium arsenite was banned from all European winegrowing countries at the beginning of the 21st century due to its toxic effects. Several active ingredients were reported as effective in control of *in vitro* growth of *P. chlamydospora* and *Phaeoacremonium* spp., nevertheless no chemical treatment has been considered effective against any of the esca syndromes (Gramaje *et al.*, 2009; Martín and Martín, 2013; Travadon *et al.*, 2013). When a grapevine shows symptoms of GTDs, *praxis* recommends its removal and destruction (burned or composted) followed by replacement with a new plant. If the plant is considered still viable, options are the chirurgical removal of the damaged organs and the retraining of new canes to replace the ablated organ (Travadon *et al.*, 2013; Fontaine *et al.*, 2016). Protection against GTDs relies mainly on prophylactic measures (e.g., pruning wound disinfection) or remedial surgery, both costly and work demanding. Viticulture is under pressure for change. Environmental costs due to phytochemical protection, public health concerns, new stringent legislation restraining the use of pesticides (e.g., Directive 2009/128/EC of the 21 October), all point the direction to new environmental-friendly practices. The use of resistant varieties is the least expensive, easiest, safest and one of the most effective means of controlling plant diseases in crops. Artificial inoculation of standing vines is a potential tool to improve knowledge on the susceptibility to esca of different cultivars (Feliciano *et al.*, 2004). Regarding control of *P. chlamydospora*, employing less susceptible genotypes has, until now, received little attention. Some works, based on the external manifestation of symptomatology hierarchized the susceptibility of some cultivars to esca-related fungi (Larignon *et al.*, 2009; Bertsch *et al.*, 2013). Also, evidence of cultivar susceptibility to esca has emerged from observations carried out under controlled conditions (Feliciano *et al.*, 2004; Martin *et al.*, 2009), but there are few reports based on field infection of standing grapevines with *P. chlamydospora*, and none on Portuguese cultivars. The purpose of the present study was to evaluate the susceptibility

responses of Dão most popular cultivars to infection by *P. chlamydospora*, using an infection method emulating natural contamination by that species. To our knowledge, the present work constitutes the first attempt to evaluate such responses, on typical Dão productive vineyards under field conditions.

Material and methods

Plant material

Trials took place in the experimental facilities of CEVDão in Nelas [UTM coordinates (Datum WGS 84): 29 T 596837, 4486566] Portugal, on 15 years old standing grapevines of cultivars Aragonez, Alfrocheiro, Jaen and Touriga Nacional, grafted on rootstock 110 Richter, trained in bilateral cordons, spur-pruned. In the first two trials (2012 and 2013) 18 plants of each cultivar were used per treatment while in the last trial (2015) 26 plants were used per treatment. Before each trial, during the summer season, all plants were assessed for esca symptom expression. Plants showing foliar or wood symptoms that could be related to GTDs were discarded, whereas those asymptomatic were included in the trial. Each trial year, grapevine growth stages were recorded according to Baggiolini's scale (Baggiolini, 1952).

Fungal isolates

Three different isolates of *P. chlamydospora* were compared for infection promotion over the different grapevine cultivars on trial: Isolate 43 (GenBank KP886991), obtained of a 2-year-old "Petit Verdot" grapevine from Vidigueira, Alentejo, Southern Portugal; isolate 48 (GenBank KP886996) obtained from a Touriga Nacional grapevine from Arruda-dos-Vinhos, Centre Portugal and isolate 52 (GenBank KP887000) obtained from a 15-year-old Jaen grapevine from Nelas (Sofia *et al.*, 2015). Isolates were maintained in PDA (PDA, Difco, Beckton, Dickinson and Co, Sparks, MD, USA) slants and transferred to Petri dishes with PDA to promote colony growth. Cultures were incubated at 24°C±1 in complete darkness for 15 days.

Conidial suspensions were obtained by removing, with a sterile cork borer, a plug of *circa* 5mm from the border of the culture, which was then plunged in a 250 mL Erlenmeyer flask containing Potato Dextrose Broth (PDB, Difco, Beckton, Dickinson and Co, Sparks, MD, USA) and placed at 20°C, under darkness, in a reciprocal shaker (90

strokes min^{-1}), for 21 days. One day before inoculation, formed lumps were smashed with a sterile glass rod, and the solution was filtered through a sterile gauze pad. The obtained solution was kept under agitation, with a magnetic stirrer. Conidial suspension was adjusted to a 10^5 conidia mL^{-1} conidial suspension with the help of a hemocytometer, according to Sofia *et al.* (2013) and chapters II and III of this thesis. Suspensions were stored overnight at 10°C, until use.

Inoculation methodology

Infection was performed during the late pruning seasons of the trial years on freshly pruned canes of standing vines, respectively on the 25th March 2012, 5th April 2013 and 20th March 2015. Pruning for immediate infection was only performed after the beginning of the bleeding in all cultivars. For each cultivar, infection was performed on distinct groups of plants with each one of the three different fungal isolates and with a control group of plants inoculated with sterile PDB. Under this training system, canes are typically pruned to the two first buds forming a spur with about five to seven cm high; spurs predestined for infection were left with about 15 cm high but with just two buds- the bottommost and uppermost ones- to allow development of potential necrosis and to avoid complete destruction of the fruiting unity, when of the recovery of the infected wood, once the trial was on a productive vineyard. All intermediate buds were cut off with a disinfected pruning scissors. Spurs were infected immediately after pruning, in the afternoon, with fair weather, no wind and temperature above 10°C. A 40 µl droplet of the conidial suspension, containing approximately 4×10^3 conidia, was carefully deposed on top of the pruning wound, allowing to soak for about ten to fifteen minutes. The infection point was then covered with a cotton pad wet with sterile distilled water and protected with a stripe of Parafilm (Parafilm® "M", Pechiney Plastic Packaging, Menasha, USA) to avoid natural infection and other contaminations. All protections were due to stay for a week and removed after that period, allowing the spur to stay in normal vineyard conditions (Figure IV-1).

Lesion lenght and recovery rates

To assess infection, approximately nine months after infection, infected spurs were cut off one cm above the bottommost bud, packed into freezer bags, and refrigerated at 5ºC, until further use. All samples were processed within a threshold of 72 hours after

collection. To avoid external contamination, for each sample, the bark was peeled off

Figure IV-1. Illustration of field methodology: a) Erlenmeyer flask containing the conidial suspension; b) freshly pruned cane ready for infection; c) deposition of the conidial suspension; d) droplet on top of pruning wound; e) Protection in place; f) physical protection of the freshly infected spurs against possible dry out or eventual drift from spray of nearby vineyards; g) bud bursting on infected spur; h) collecting the sample nine months after infection.

with a sterile knife, and wood bellow disinfected by spraying with a 70% ethanol solution. With pruning scissors, thin slices of the sample were taken upwards, beginning from the lowest end of the spur until black dot lesions were visible inside the wood. All slices were kept in the sequential order they were taken off, for further reisolations. With an electronic calliper, distance from the infection point to the previously detected end of the lesion was measured and recorded. Obtained values were rounded to the unit. All samples were then longitudinally sectioned to verify the presence and continuity of the putative lesions.

From each sample, three subsamples were taken: in the length direction, one from the middle of the lesion (ML), one from the lesion end (BL) and one from symptomless wood collected approximately 0.5 cm below the lesion end (SW) (Figure IV-2). From the end of each subsample, two transversal cuts, approximately 1 mm thick, were cut off with a disinfected pruning scissors, and surface disinfected for one minute by immersion on a 2% sodium hypochlorite solution and thereupon rinsed with sterile distilled water and allowed to air dry for one minute on a sterile filter paper pad. Each piece was then sectioned into four fragments and plated in PDA amended with 250 µg chloramphenicol (BioChemica, AppliChem, Germany), four fragments per PDA plate, sealed and placed to incubate at 24°±1°C, under complete darkness. Plates were observed every two days until fungal growth permitted the identification of *P. chlamydospora*. Recovery percentages were calculated as the percentage of fragments from which the

pathogen was recovered out of the total number of samples.

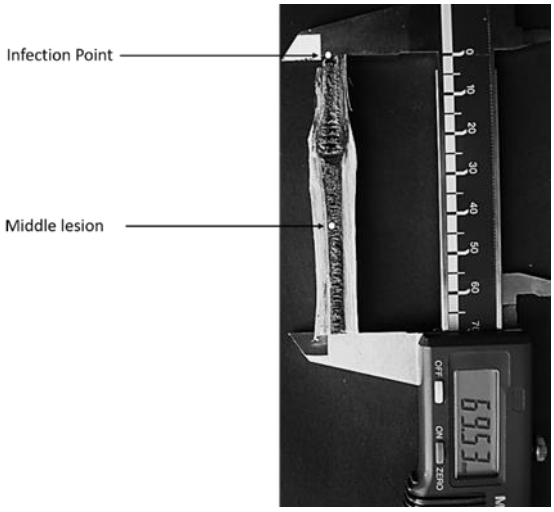


Figure IV-2. Infection assessment: white dots indicate infection point and middle lesion (ML), symptomless wood (SW) was removed 0.5 cm below the lesion end. Calliper reading shows lesion length (LL) in mm.

Weather data

Daily values of temperature and rainfall were collected with a "Campbell CR 510" automatic weather station, located on the trial site and property of Centre Portugal Regional Directorate of Agriculture and Fisheries (DRAPC).

Statistical analysis

Assumptions for variance analyses were assessed with software R (www.r-project.org). When the assumptions for variance analysis were not at all accomplished, the influence of the distinct levels of one factor was assessed using the non-parametric test of Kruskal-Wallis. In this case, when significant differences were found ($P<0.05$), the comparison between the distinct levels was made using the ranks.

Results

All *P. chlamydospora* isolates produced lesions, characterised by black/brown streaking on longitudinal cuts of the infected spurs and were recovered from those lesions. Control plants evidenced minor internal discoloration (ranging from 9.5 to 12.3 mm), usually associated to desiccation of the internode, rarely showing black streaking on longitudinal cuts, and *P. chlamydospora* was never recovered from those lesions. Therefore, control values were not included in subsequent analysis.

The results of artificial inoculations with *P. chlamydospora* carried out in four cultivars in a three-year trial show that the percentage of recovery of *P. chlamydospora* was significantly higher in cv. Alfrocheiro (47.0%, 35.1%, 17.4%) than in the other three cultivars. Cultivars Touriga Nacional and Aragonez performed similar whereas cv. Jaen showed the lowest recovery percentage (26.3%, 16.1%, 6.9%). Considering the length of the lesions, the longest lesions were observed in Touriga Nacional (54.9 mm) but not significantly longer than those observed in Alfrocheiro (50.1 mm). Aragonez showed significantly smaller lesions (48.9 mm) than cv. Touriga Nacional, and Jaen repeated this pattern (41.7 mm) towards Aragonez. Cultivars Alfrocheiro and Touriga Nacional did not differ significantly (Table IV-1).

Table IV-1. Percentage of *Phaeomoniella chlamydospora* colonies recovered from the middle of the lesion, bottom of the lesion and from symptomless wood and lesion length, recorded for the four cultivars (three fungal isolates and three years trials considered)

Cultivars	***Phaeomoniella chlamydospora* recovered colonies (%)**			**Average lesion length (LL) (mm)**
	Middle of the lesion (ML)	**Bottom of the lesion (BL)**	**Symptomless wood (SW)**	
Alfrocheiro	47.0 a*	35.1 a	17.4 a	50.1 ab
Touriga Nacional	30.8 bc	21.2 b	8.0 b	54.9 a
Aragonez	33.8 b	18.4 bc	7.8 b	48.9 b
Jaen	26.3 c	16.1 c	6.9 b	41.7 c

* - Different letters in column correspond to significant differences (α=0.05) based on ranks assessed by Kruskal-Wallis analysis.

The distribution of recovery rates (Figure IV-3) shows undoubtedly that the percentage of *P. chlamydospora* colonies recovered from ML ranged in all cultivars from 0 to 100%, assuming tendentially lower values in BL and even lower values in SW. Median values, indicating the recovery percentage that includes half of the sorted samples analysed, show the same tendency. In the sections corresponding to ML, median values of about 50% for cv. Alfrocheiro indicate that 50% of the samples had up to 50% of recovery percentage, whereas in cv. Aragonez and cv. Touriga Nacional up to 50% of the samples had less than 20% of recovery percentage. In cv. Jaen 50% of the samples analysed did not allow the recovery of *P. chlamydospora*. Considering BL, the pattern is

tendentially similar, with Alfrocheiro recovery percentage reaching much higher values than the other cultivars. In this case, no *P. chlamydospora* colonies were recovered from 50% of the samples from cultivars Aragonez, Jaen and Touriga Nacional, opposite from what it could be observed in cv. Alfrocheiro. In symptomless wood, the tendency was reinforced; for all cultivars, 50% of the samples did not allow recovery of *P. chlamydospora*, but still Alfrocheiro samples range more consistently through higher values than other cultivars.

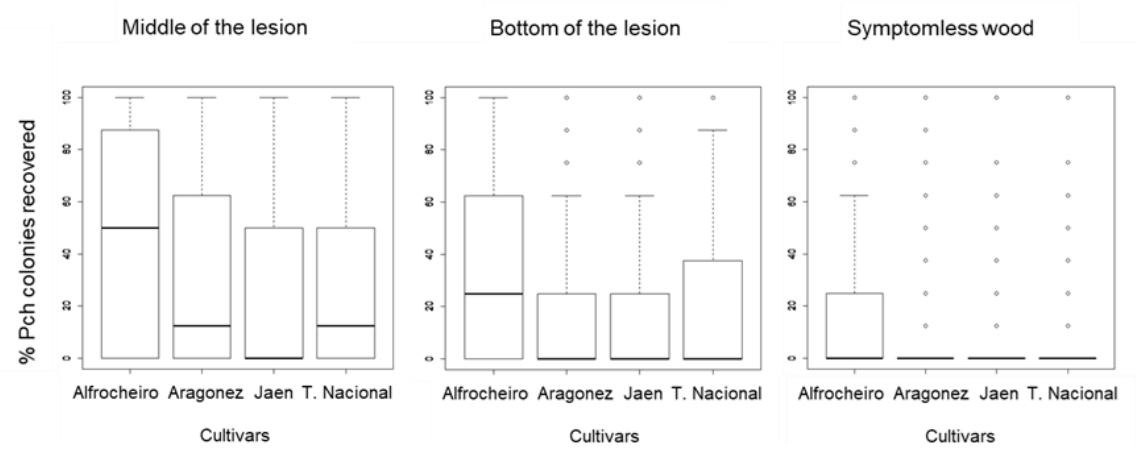


Figure IV-3. Boxplots of the percentage of *Phaeomoniella chlamydospora* colonies recovered from the middle of lesion, the bottom of the lesion and from symptomless wood, for the four cultivars.

A similar non-parametric analysis considering the length of the lesion (Figure IV-4) corroborates the results presented in Table IV-1, with cvs. Alfrocheiro and Touriga Nacional showing similar and tendentially higher values than those of cvs. Aragonez or Jaen. The distribution of values for cv. Jaen suggests a tendency again to smaller lesions, with 50% of the samples harbouring lesions smaller than 40 mm and 75% of the samples lesions smaller than 60 mm.

The present study employed three *P. chlamydospora* isolates, from our collection: isolate 43, isolate 48 and isolate 52. The percentage of *P. chlamydospora* colonies recovered from ML showed significant differences between all isolates, while for BL, only isolate 48 was significantly different from the other two, which performed similarly. In both cases, isolate 48 recovery values were higher and significantly different from the obtained with the other two isolates (Table IV-2).

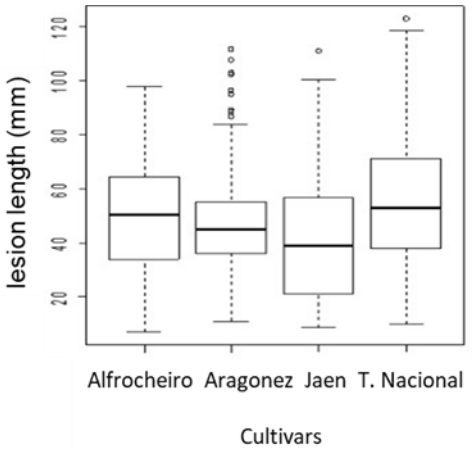


Figure IV-4. Boxplots of the average lesion length recorded for the four cultivars. The median is represented by the solid line.

The percentage of *P. chlamydospora* colonies recovered from SW was significantly higher for isolate 48 than for isolate 43, isolate 52 had an intermediate behaviour. Lesion length values were significantly higher for isolate 48 than for isolates 52 and 43, which didn't differ from each other (Table IV-2).

Table IV-2. Percentage of *Phaeomoniella chlamydospora* isolates (48, 52 and 43) recovered from the middle of lesion, bottom of the lesion and from asymptomatic wood and lesion length, recorded for the three isolates (four cultivars and three trial years considered altogether)

Isolates	***Phaeomoniella chlamydospora* recovered colonies (%)**			**Average lesion length (LL)(mm)**
	Middle of the lesion (ML)	**Bottom of the lesion (BL)**	**Symptomless wood (SW)**	
43	27.2 c*	18.6 b	8.5 b	47.6 b
48	42.2 a	29.1 a	12.6 a	52.8 a
52	34.9 b	21.3 b	9.5 ab	46.4 b

* Different letters in column correspond to significant differences (α=0.05) based on ranks assessed by Kruskal-Wallis analysis.

The analysis of range and values distribution points out again a decreasing tendency of the recovery percentage from the middle lesion samples to the bottom of the lesion sections and further to the symptomless wood sections (Figure IV-5). Regarding the ML sections, it becomes clear that isolate 43 was less aggressive than the two others, with 50% of the samples not enabling any recovery of colonies. Isolates 48 and 52 behaved similarly. Concerning BL sections, it is already possible to distinguish isolate 48

from isolate 52; in these sections, isolate 52 behaved less aggressive than isolate 48 and closer to isolate 43. In symptomless wood, almost no colonies were recovered from isolate 43; again, isolates 48 and 52 behaved quite similarly and more aggressive than isolate 43.

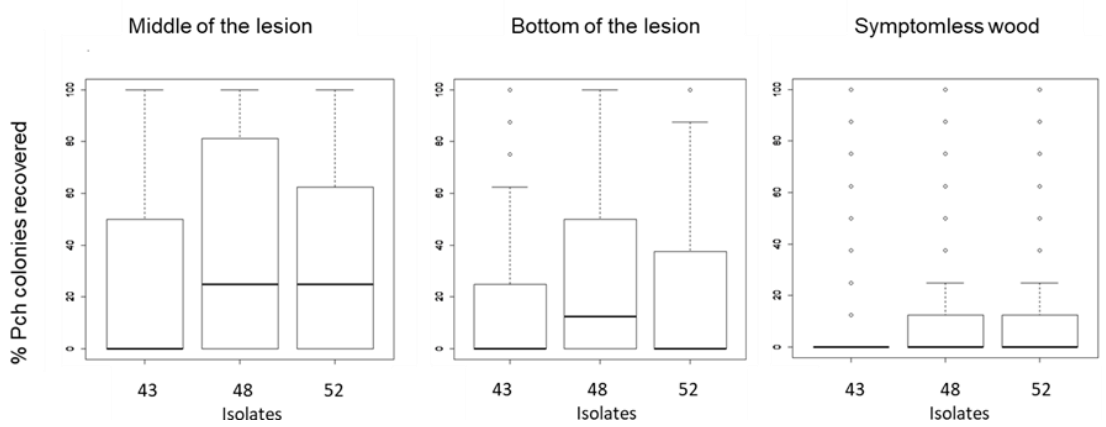


Figure IV-5. Boxplots of the percentage of *Phaeomoniella chlamydospora* colonies recovered from the middle of lesion, the bottom of the lesion and from symptomless wood, for the three isolates.

Regarding lesion length (Figure IV-6), median value was slightly higher for isolate 48. Considering the distribution of the values of the lesion lengths, there is a slight difference between isolate 48 to the other two isolates that behaved similarly.

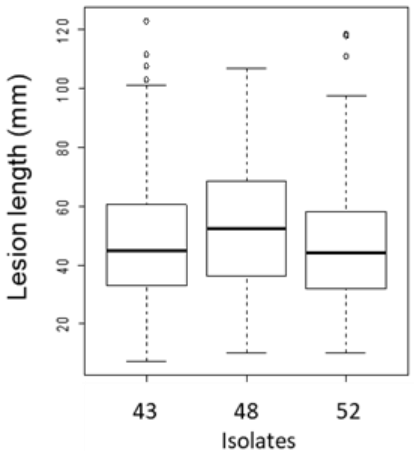


Figure IV-6. Boxplots of the average lesion length registered for the three isolates.

A joint analysis combining isolates and cultivars evidences a global tendency to isolate 48 to cause generally larger lesions on all cultivars. However, this tendency was not observed on Touriga Nacional, where the largest lesions were obtained with isolate 43. Cultivars Alfrocheiro and Jaen showed similar patterns, and in both cases, isolate 48 is considerably more aggressive to the host than the two other isolates, that behave similarly regarding the size of the lesions that they cause. In respect to cv. Aragonez, all

three isolates seemed to be equally aggressive, concerning the lesions they caused (Figure IV-7).

Regarding the effect of the different trial years, results suggest that infections were more effective in 2012 as the recovery percentages were significantly higher on this year in the sections corresponding to ML and BL. For SW, the tendency was followed, but this difference was not so perceptible, as the recovery percentage of 2013 was not significantly different neither from the one of 2012 nor from the one of 2015 (Table IV-3).

Considering the average values, 2012 behaved as the year with the highest efficient inoculations, 2015 as the year with lowest efficient ones and 2013 showed an intermediate behaviour.

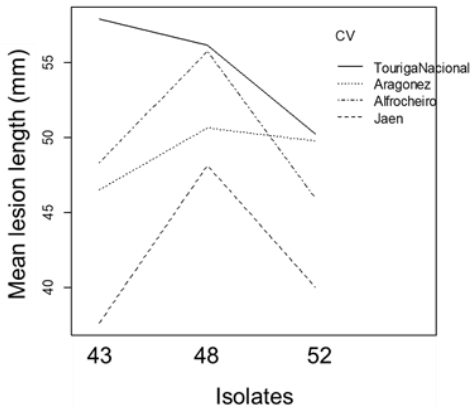


Figure IV-7. Lesion length (mm): tendency between isolates (43, 48 and 52) and cultivars Alfrocheiro, Aragonez, Jaen and Touriga Nacional.

Considering the lesion length, the observed results further support this pattern as infections conducted in years 2012 and 2013 were larger than those obtained from the inoculations of 2015.

Table IV-3. Percentage of *Phaeomoniella chlamydospora* colonies recovered from the middle of lesion, bottom of the lesion, and from symptomless wood and lesion length, for three years of trials 2012, 2013 and 2015 (all isolates and cultivars considered)

Year	***Phaeomoniella chlamydospora* recovered colonies (%)**			**Average lesion length (LL)(mm)**
	Middle of the lesion (ML)	**Bottom of the lesion (BL)**	**Symptomless wood (SW)**	
2012	47.6 a*	34.2 a	16.7 a	50.3 ab
2013	35.6 b	23.6 b	10.9 ab	52.5 a
2015	26.1 c	15.7 b	5.7 b	45.9 b

* - Different letters in column correspond to significant differences (α=0.05) based on ranks assessed by Kruskal-Wallis analysis.

Median value was highest in 2013, but lesion lengths achieved higher values in 2012. A closer analysis to the range and values distribution of the percentage of *P. chlamydospora* colonies recovered from ML, BL, and SW (Figure IV-8) corroborates the above-presented results. There is again a decreasing tendency of the recovery percentage values from the ML samples to BL sections and further to SW sections. For the symptomatic tissues, the results of years 2013 and 2015 distribute and range quite similarly, indicating slightly less efficient inoculations than 2012 (for ML sections, in years 2013 and 2015, 50% of the samples delivered less than about 10% *P. chlamydospora* colonies; in the BL sections, 50% of the samples collected in these 2 years did not allow recovery of *P. chlamydospora*). The lower efficiency of the inoculations carried out in 2015 becomes more visible in the analysis of the symptomless tissues, that shows that the standard observation was 0% of *P. chlamydospora* recovery percentage, indicating less effectiveness of the inoculation (Figure IV-8).

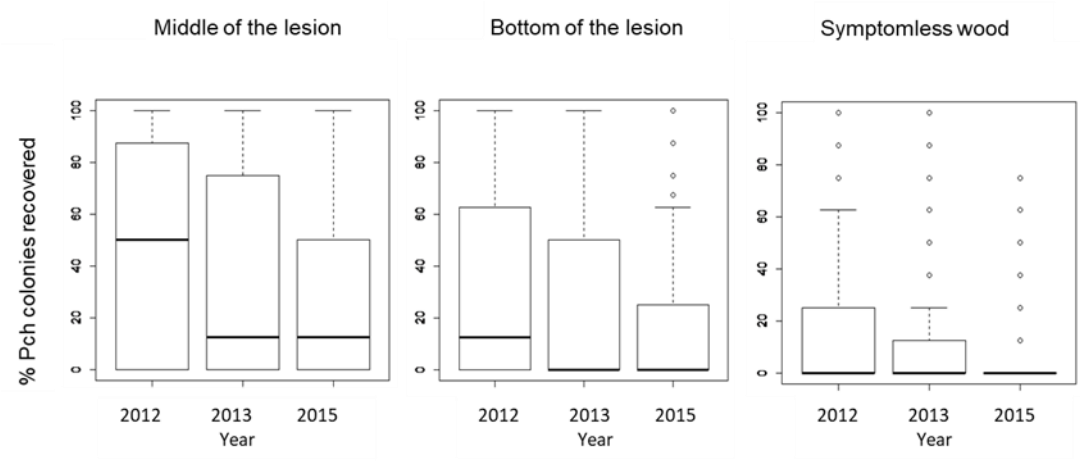


Figure IV-8. Boxplots of percentage of *Phaeomoniella chlamydospora* colonies recovered from the middle of lesion, the bottom of the lesion and from symptomless wood, for the three trial years.

Regarding the distribution of the lesion length values (Figure IV-9), the behaviour is quite homogeneous in the three years, with a slight tendency for lower values for the infections performed in the year 2015.

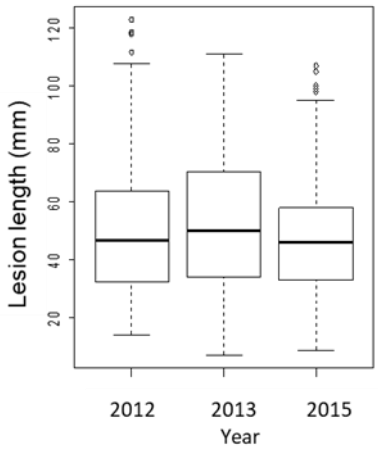


Figure IV-9. Boxplots of the average lesion length for the three trial years.

A joint analysis combining inoculation year and isolates revealed that isolates 43 and 52 behaved similarly, with smaller lesions in 2012 and 2015 and larger lesions in 2013, whereas isolate 48 behaved opposite (Figure IV-10).

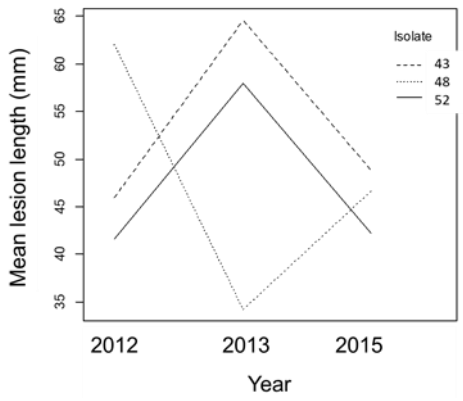


Figure IV-10. Lesion length (mm): tendency of relations between years (2012, 2013 and 2015) and isolates (43, 48 and 52).

Analysis combining trial years and cultivars, showed a discrepant response from cultivars in the different years, being noteworthy the smaller dimension of the lesions in cv. Touriga Nacional in 2013 when compared with the two other trial years (Figure IV-11). The distinct pattern showed by each cultivar suggests that meteorological conditions during inoculation and colonization process could cause a different reaction in host-pathogen relation. In fact, rainfall amount and distribution (Figure IV-12) were similar in 2012 and 2015 (481 and 459 Lm^{-2}), while in 2013, accumulated rainfall almost doubled the other years's amounts (894 Lm^{-2}), happening mostly in the three first months of 2013.

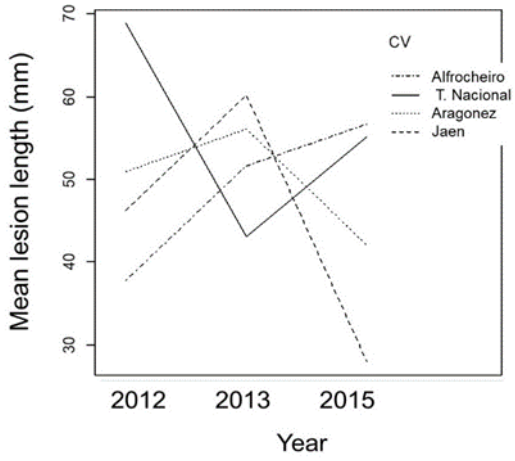


Figure IV-11. Lesion length (mm): tendency of relations between years (2012, 2013 and 2015) and cultivars (Alfrocheiro, Aragonez, Jaen and Touriga Nacional).

In 2012, an exceptional period of mild temperatures occurred during March until two weeks after infection. Maximum temperatures recorded reached more than 25°C during a week after inoculation, with the minimum temperature never dropping below 10°C for the same period. In the two weeks after infection, average temperature reached from 19°C

to 12 °C never going below 10°C. In 2013, due to heavy rain, inoculation was delayed and took place at the beginning of April. Maximum temperatures after inoculation stayed below 16°C during the following week, while minimum temperature for the same period reached from 0°C to 8°C and average temperature for the two weeks period after infection reached from 7°C to 14°C attaining this value only by the end of that period. In 2015, temperatures in the first week period after infection suffered a clear drop with maximum temperature going from 20°C to 10°C and minimum temperature lowering from 10°C to 2°C for the same period. Recorded values for the average temperature during the two weeks after infection never rose above 11°C, reaching most of the time values below 8°C.

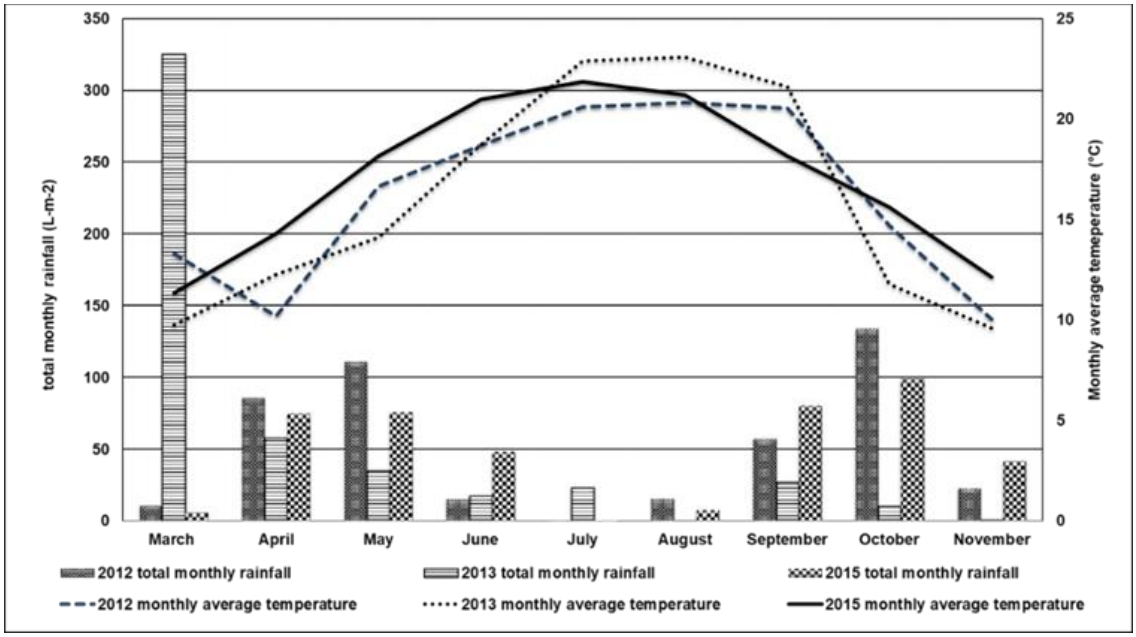


Figure IV-12. Monthly rainfall and average temperature for the three trial years.

Discussion

The present research is the first comprehensive study to assess, *in situ*, the susceptibility of the four most common Dão appellation grapevine cultivars to *P. chlamydospora*. Assessment of the cultivars conducted along three years, revealed different levels of cultivar susceptibility to colonization by *P. chlamydospora*. Cultivar Alfrocheiro had the highest recovery of *P. chlamydospora*, also exhibiting the longest lesions, while the lowest rate of *P. chlamydospora* recovery was achieved with cv. Jaen, which also showed the smallest lesions. Concerning cultivars Touriga Nacional and Aragonez/Tempranillo, our results demonstrated an intermediate behaviour, with slightly longer lesions and recovery percentages in Touriga Nacional. These results point out

clearly that Jaen behaved as the less susceptible cultivar and Alfrocheiro as the most susceptible one, while the other two cultivars showed intermediate susceptibility. Martin *et al*. (2009) considered cv. Aragonez clearly more susceptible and more prone to infection by *P. chlamydospora* than cultivars Touriga Nacional and Cabernet Sauvignon, which is not in accordance with our results, but great differences among the experimental conditions of these two assays, namely, the age of the plants, trial condition, duration of the trial and aggressiveness of isolates, could explain those differences. Our results also contradict the long-established empirical idea, among Portuguese winegrowers, that cv. Aragonez is more prone to esca than other cultivars, a biased opinion possibly since cv. Aragonez is ubiquitous in Portugal, while cvs. Touriga Nacional, Alfrocheiro and Jaen have less expression within the Portuguese vine population (OIV, 2017b). Resistance of grapevine cultivars to infection by *P. chlamydospora* is not yet completely understood. One reason for this different tolerance to *P.chlamydospora* among cultivars could lay on the hypothesized by Pouzoulet *et al.* (2017), that considered that the outcome of grapevine cultivar resistance regarding *P. chlamydospora* infection could be related to xylemic vessels diameter. These authors proposed that a larger amount of small diameter vessels could impair *P. chlamydospora* progression as well as toxins spread, by efficiently isolating infected sections of the xylemic vessels through deposition of gels and tyloses formation. Regarding Esca, cv Merlot, anatomically characterized by narrow diameter xylemic vessels is usually considered less susceptible (Christen *et al.*, 2007) while Thompson seedless, which harbours wider xylemic vessels, is regarded as very susceptible (Murolo and Romanazzi, 2014). Portugal has 341 regulated grapevine cultivars from which, more than 250 are indigenous (Fraga *et al.*, 2016). Anatomical and morphological studies on these Portuguese cultivars, deciphering their vessel configuration and other features, could lead to a better comprehension of their susceptibility to esca, allowing winegrowers to take into account those characteristics when deciding new vineyards.

In our experiments the lesion length values, in average, ranged from 41.7 mm to 54.9 mm, depending on the cultivar, yet *P. chlamydospora* was also recovered from symptomless wood, although in relatively low percentage, an observation also reported by Landi *et al.* (2012). These values, attained in just 9 months after infection, demonstrate the standing risk of *P. chlamydospora* reaching the permanent structure of grapevine (arms and trunk), stressing the proposed by Elena and Luque (2016), that a pruning

strategy of increasing the length of the pruned spurs, might difficult arm and trunk invasion by *P. chlamydospora*.

Phaeomoniella chlamydospora recovery values evidenced a decrease from the middle portion of the lesions downwards, on all samples obtained from all cultivars. This pattern was expected as the fungus develops in xylem from the inoculation point downwards (Pascoe and Cottral, 2000; Feliciano and Gubler, 2001; Serra *et al.*, 2008) evidencing the advance of the infection and becoming quite visible when recovery percentage results are analysed regarding range and distribution in each of the wood sections assessed.

All three *P. chlamydospora* isolates were able to infect, colonise and produce vascular discolourations or lesions, demonstrating their aggressiveness and were reisolated from the formed lesions and subjacent tissues. Our results show that not all isolates had the same behaviour regarding infection: isolate 43 revealed lower aggressiveness than isolates 48 and 52. Differences in aggressiveness among fungal strains are well known (Sneh, 1998; Boland, 2004) and have been reported among *P. chlamydospora* isolates by Santos *et al.* (2005) and Laveau *et al.* (2009). Although the reasons for this behaviour among *P. chlamydospora* isolates were not the subject of the present work, it is interesting to notice that previous studies (Sofia *et al*., 2015), focusing on the ITS sequences of *P. chlamydospora* isolates from distinct Portuguese regions, that included the present ones, revealed two distinct phylogenetic groups: group 1 and group 2. Isolate 43 was included in group 2 while isolates 48 and 52 belonged to group 1. In a two-year trial, pathogenicity tests were carried out on potted plants of cv. Touriga Nacional grafted in 1103 Paulsen (Chapter 3, data not published) with 22 *P. chlamydospora* isolates belonging to both groups. It was evident that isolates included in group 1 produced longer lesions and had higher recovery frequencies regarding those included in group 2, evidencing a tendency to be more aggressive, that the ones included in group 2. Another hypothesis for explaining the difference on aggressiveness verified among isolates could be the age of the isolate and consequent high number of consecutive sub-culturing that it forcibly undergoes. Indeed, isolates 52 and 48 were four years younger than isolate 43. Reduction of aggressiveness on fungal strains under continuous sub-culturing has been reported for *Eutypa lata*, the causal agent of Eutypa dieback (Laveau *et al.*, 2009) and entomopathogenic fungi (Kary and Alizadeh, 2017). Further

studies will be needed to fully explain the aggressiveness differences found among the isolates under study. Nevertheless, isolate 48, the most aggressive, was not obtained in the Dão wine region, showing no relationship between terroir and strains aggressiveness which follows the stated by Comont *et al.* (2010) and by Sofia *et al*. (2015) about the absence of significant geographic structuring of the *P. chlamydospora* populations thus reinforcing the risks inherent to the transit of potentially infected material between regions.

Mild temperatures verified in the period after infection might justify the high rate of *P. chlamydospora* recovery obtained in 2012, and the low recovery percentages observed in 2015, as optimal temperature for *P. chlamydospora* growth is 25°C (Whiting *et al.*, 2001; Valtaud *et al.*, 2009). Optimal growth conditions were observed in the post infection period of 2012, while in 2015, the low temperatures recorded after infection did not favour the obtention of high infection rates. Luque *et al.* (2014) expressed an identical opinion on the advantages of forestalling pruning, hypothesising that colder and drier conditions occurring after early pruning could hamper fungal infection and development. In the same work it was also proposed that seasonal variance in pruning wound susceptibility was not due to the time of the year pruning was performed, but to the favourable meteorological conditions experienced afterwards (I.e. rain and warmer temperatures), that would favour spore release and dispersion, as well as fungal infection and colonisation of pruning wounds. Serra *et al.* (2008) also observed that regularly distributed rainfall promoted both grapevine growth and the pathogen infection process. Both premises were observed during 2012 trial.

In the Dão wine region, good management standard practices advice late pruning as the best option to avoid esca related fungi from infecting pruning wounds. Temperature in this time of the year (February/March) tend to rise accompanied by higher relative humidity. Results obtained in 2012, together with the recorded temperature and rainfall, evidence the need of further research to support the benefits or misfits of the standard pruning practice. Pruning recommendations should be more flexible and adapted to regional conditions, following the suggested by Elena and Luque (2016), observing the discrepancy in pruning wound susceptibility among geographic regions and the influence of local abiotic and biotic factors, that pruning wound susceptibility should be studied on a local or regional basis to better understand host–pathogen interaction within the

infection process, in order to define the better rules for that practise.

Meteorological features of the three years naturally influenced grapevine development. While grapevine growth stages developed identically in 2012 and 2015, in 2013, due to heavy rain and low temperatures bud burst suffered a delay of two weeks. These conditions caused an extent of the bleeding. In 2013, bleeding started late and lasted for a longer period than in the other two trial years. This occurrence is expected to hinder fungal infection (Larignon and Dubos, 2000; Serra *et al.*, 2008) although it is difficult to relate this data type with infection. Extended bleeding could be an explanation for the smaller lesions obtained in Touriga Nacional in 2013 as this cultivar is characterised by late burst and delayed phenological development in the Dão wine region which could indicate that infection in 2013 could have been hampered by sap flow.

Conclusions

Dissemination of knowledge

In the Dão wine region the Esca complex and associated syndromes have always been known, but as a problem affecting mainly old vines. In the last decades, these problems met a huge escalade becoming a major concern for the local winegrowing industry.

At the beginning of this thesis, we have published a colourful leaflet characterising the main Grapevine Trunk Diseases most common symptoms. This publication, with a broad distribution, helped to instruct winegrowers on the typical characteristics of those diseases. In addition, a query was elaborated to characterise the presence and importance of the different GTDs within the Dão region. This work also helped us locate places for GTDs prospection, facilitating the prosecution of the present thesis. It provided the first overview of GTDs within the Dão wine region with esca and *Phomopsis* cane and leaf spot (excoriosis) being the most well-known, affecting more than 80% of the inquired. Also, we realized that Botryosphaeria dieback and young grapevine declines were not as well-known. Another achievement was the success met by that leaflet, distributed not only within the Dão wine region but also in some other Portuguese wine regions, referenced by some of the Portuguese Phytosanitary Alert System stations in their bulletins. The divulgation of the leaflet and survey publicized GTDs and our work, resulting in several lectures for technicians and winegrowers, in the Dão wine region and in other Portuguese (Alentejo, Algarve, Lisboa, Vinhos Verdes, Douro, Beira Interior and Bairrada) and in a Spanish wine region (Ribera del Duero), naturally leading to a better knowledge of those diseases by the winegrowers. Indeed, notes on the esca complex, Botryosphaeria dieback and young vine declines, on phytosanitary alert bulletins become common after this effort which reached even the smallest winegrower.

Pathogen characterization

As stated by our studies, the esca complex is the most important GTD within the Dão wine region. The frequent isolation of *Phaeomoniella chlamydospora* from grapevines affected by esca or Petri disease in the Dão wine region has been a focus of our attention during the past twenty years. Although several fungal species are associated with this complex, *P. chlamydospora* is the main fungal species associated with esca and

GLSD. Limited information was available on the phenotypical and molecular variability of *P. chlamydospora* isolates obtained from grapevines within the Dão wine region. To this aim, a collection of 68 *P. chlamydospora* isolates was gathered, by rejoining 47 *P. chlamydospora* isolates collected in the Dão wine region, five from contiguous regions, two from the isolated Azores islands and 14 of other continental Portuguese origins. These isolates were studied using phenotypical characters, phylogenetical analyses and pathogenicity tests.

As reported by other works, in different countries, little morphological variation was found among the isolates of *P. chlamydospora*. Even so, two groups were formed based mainly on colony characters and pigment production. However, these morphological groups did not have correspondence with genetic clusters. Moreover, no relationship was found with the rootstock/scion combination, year of isolation or geographical origin among the isolates. Multiple alignments of the ITS sequences of the 68 *P. chlamydospora* isolates revealed two distinct groups, detecting three different clonal lineages of *P. chlamydospora*. The presence of only three clonal lineages suggests that asexual reproduction is predominant in this species, and the introduction of the different sources of inoculum through infected propagation materials, emphasises the risks associated with the commerce and transport of propagation material among different regions.

Pathogenicity tests were performed with some of the isolates selected from our collection, confirming their ability to colonise grapevine wood and cause typical vascular discoloration usually found in GLSD and Petri disease. Most of the isolates included in the phylogenetical "Group 1" (Chapter 2) caused longer lesions and had higher recovery frequencies, seeming tendentially more aggressive than the ones included in "Group 2", thus establishing a possible link between the genotypes and aggressiveness.

Susceptibility of the cultivars

Field inoculations carried out with three isolates selected from our collection (43, 48 and 52) revealed different levels of grapevine cultivar's susceptibility to infection by *P. chlamydospora*. Cultivar Alfrocheiro revealed to be the most susceptible, Jaen the least, while cultivars Touriga Nacional and Aragonez revealed an intermediate behaviour, with slightly longer lesions and higher recovery percentages in Touriga Nacional than in

Aragonez.

Our results also confirm, based on the length of the black streaking formed, that the adoption of a pruning strategy of increasing the length of the pruned spurs (cultural practice), and consequently double pruning, might difficult arm and trunk invasion by *P. chlamydospora*.

Although all isolates were aggressive, their performance showed different degrees of severity, with isolate 43 being the less aggressive while isolate 48 was the most aggressive one. The lack of relationship between terroir and strains aggressiveness reinforces the idea that the transit of propagation material between regions is a risk.

Meteorological conditions influenced *P. chlamydospora* infection and the colonisation of the plants.

The optimal infection results obtained in 2012, resulting from an exceptional period of mild temperatures occurred during March until two weeks after infection, confirm the need of further research to evaluate the benefits or misfits of the early pruning of grapevines in the winter to reduce infection by esca complex associated fungi, contradicting the actual practice.

All the knowledge generated by this thesis will be available in Portuguese language to the Dão winegrowers and local nurseries. The results provide tools to minimize the impact of *P. chlamydospora* and associated GTDs on the long-term sustainability of this particular region vineyards by: a) improving knowledge of the various GTDs; b) assessing the real situation of these diseases, facilitating the demonstration of their importance to the public authorities and the need to promote their study and control; c) confirming the small diversity and the aggressiveness of the studied isolates which reinforces the need to revise european legislation on the production of grapevine vegetative propagation material; d) demonstrating the different susceptibilities among the main grapevine cultivars to *P. chlamydospora* infection; e) providing a decision tool to the establishment of new vineyards and demonstrating the need to study the genetic richness of the Portuguese grapevine cultivar pool in relation to its phytosanitary behaviour; f) suggesting adjustments to the recommended pruning strategies to control the esca complex, specifically leaving longer spurs and avoiding late

winter pruning, thus making difficult the trunk and spur colonization by *P. chlamydospora*.

Finally, the results of this thesis may contribute to reduce economical losses for the winegrowers and to diminish social and environmental impacts for the Dão wine region, with an economy centred on wine production, and in the short-term will help the Dão viticulture sector to remain productive by contributing to a sustainable viticulture.

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