

**New insights on Botryosphaeria dieback of grapevine:
a focus on cultivars response and sustainable control of
Lasiodiplodia spp.**

Pedro Miguel Calhás Jorge Reis

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TESE ELABORADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM
ENGENHARIA AGRONÓMICA

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Abstract

Botryosphaeria dieback caused by several species of the family *Botryosphaeriaceae* is one of the most impactful grapevine trunk diseases (GTDs) affecting vineyards worldwide. Wedge-shaped perennial cankers, intervein leaf chlorosis, mummified berries and death of the plant have been associated with its infection, being these symptoms usually observed in late spring/early summer. Occasionally, during the early stages of grapevine growth, symptoms such as necrosis on green shoots, dried inflorescences and flowers, were observed. Upon isolation from samples taken from these contaminated organs, *Neofusicoccum parvum*, *Diplodia seriata* and *Diplodia mutila* were recovered and identified. The inoculation of these species on greenhouse and field assays reproduced the symptoms observed on the field and confirmed its pathogenicity. Optimised methods and protocols used in GTDs researching were compiled which might be useful for other researchers for their approaches on this subject. *Lasiodiplodia theobromae* have been reported as one of the most aggressive *Botryosphaeriaceae* species in several grape growing regions and have been previously reported from Portuguese vineyards. Greenhouse and field assays revealed that Touriga Nacional appeared to be the most susceptible cultivar, while Aragonez showed the lowest response to inoculation with *Lasiodiplodia* spp. The control of Botryosphaeria dieback pathogens is extremely difficult, especially considering the increasing restrictions on effective products. The combination of a biological pruning wound protectant, *Trichoderma atroviride* strain I-1237 (Esquive®), with a new copper-based product, namely LC2017 (CuSPHy+HA), did not revealed a great increase on controlling *Lasiodiplodia* spp. Nevertheless, further research on the impact of this product on plant defense, confirmed an interesting elicitation effect by inducing several genes linked to grapevine defense mechanisms. The new insights on Botryosphaeria dieback symptoms, and more specifically for *Lasiodiplodia* spp., in relation to cultivar response and susceptibility, may not only lead to new research programs and opportunities, but may also help growers improving and optimizing vineyard installation and management.

Keywords: Grapevine trunk diseases, *Botryosphaeriaceae*, early symptoms, cultivar response, gene expression.

Resumo

A botriosferiose, causada por várias espécies da família *Botryosphaeriaceae*, tem sido referida a nível mundial como uma das mais importantes doenças do lenho da videira (DLV). Com o objetivo de uniformizar e agilizar a abordagem às DLV compilaram-se os métodos e protocolos utilizados no diagnóstico e proteção destas doenças.

Os sintomas habitualmente associados à botriosferiose são cancrios no tronco e ramos, cloroses entre nervuras nas folhas, cachos mumificados e morte de plantas e, surgem habitualmente no fim da Primavera e início do Verão. Recentemente, observaram-se no início do ciclo vegetativo necrose nos sarmentos, inflorescências e flores secas. Os isolamentos realizados a partir dos órgãos infetados conduziram à identificação de *Neofusicoccum parvum*, *Diplodia seriata* e *Diplodia mutila* cuja patogenicidade foi comprovada em estufa e no campo.

Espécies de *Lasiodiplodia* têm sido descritas como sendo bastante agressivas em várias regiões vitivinícolas mundiais. Ensaio de suscetibilidade, realizados em estufa e no campo, revelaram que a cultivar Touriga Nacional era a mais suscetível enquanto Aragonez era a menos suscetível a infeções por *Lasiodiplodia* spp.

A proteção das videiras contra as DLV e, em particular à botriosferiose, é extremamente difícil tendo em conta as crescentes restrições à utilização de produtos fitofarmacêuticos da UE. A combinação de um produto novo à base de cobre, LC2017, com um biofungicida protetor de feridas de poda, *Trichoderma atroviride* estirpe I-1237 (Esquive®), não revelou um aumento de eficácia no controlo de *Lasiodiplodia* spp. Contudo, o produto LC2017 (CuSPHy+HA) revelou um efeito eliciador induzindo a expressão de vários genes ligados aos mecanismos de defesa da videira.

Os resultados obtidos contribuem para o diagnóstico precoce da botriosferiose, para esclarecer a interação *Lasiodiplodia* -videira e também representam um forte contributo e avanço para o desenvolvimento de novas estratégias de planeamento na instalação e gestão da vinha.

Palavras-chave: Doenças do lenho da videira, *Botryosphaeriaceae*, sintomas precoces, resposta de cultivares, expressão de genes.

Resumo alargado

A cultura da videira é uma atividade de importância mundial, com grande relevância económica em Portugal. Todos os anos, as videiras são infetadas por diversos agentes patogénicos cujos estragos e prejuízos dependem essencialmente da suscetibilidade das cultivares e das condições edafoclimáticas. Algumas doenças são causadas por um único patogéneo, como por exemplo, o oídio causado por *Erysiphe necator* enquanto outras resultam da ação de um complexo de patógenos, como é o caso das doenças do lenho da videira (DLV). Estas doenças estão atualmente entre as mais devastadoras a nível mundial representando uma ameaça à sustentabilidade da vinha a longo prazo. A complexidade das DLV, para além da diversidade de fungos que lhe estão associados, resulta da possível ocorrência de um período de latência indeterminado dos agentes patogénicos (videiras assintomáticas), da expressão errática anual dos sintomas foliares e, principalmente, de más práticas agrícolas adotadas e da ausência de proteção fitossanitária adequada.

Os ensaios biológicos *in vitro* (suspensões de células, *calli*, discos foliares, folhas adultas ou plântulas) e os ensaios biológicos *in vivo* (enxertos prontos na estufa, videiras artificialmente infetadas na vinha) são os métodos mais utilizados na investigação relacionada com o diagnóstico e a proteção fitossanitária de DLV. Apresenta-se uma compilação dos diversos métodos disponíveis, a descrição exaustiva dos procedimentos experimentais, os principais resultados obtidos, as vantagens e desvantagens de cada método. Esta síntese permite uniformizar e agilizar a investigação em DLV e inclui alguns dos métodos utilizados nesta tese.

A botriosferiose, causada por várias espécies da família *Botryosphaeriaceae*, é uma das mais importantes doenças do complexo das DLV. Os sintomas externos vulgarmente associados à doença surgem no fim da Primavera / início do Verão e são cancrios nos ramos e varas, cloroses entre as nervuras das folhas, cachos mumificados e, eventualmente, morte de plantas. Em 2018, no início do ciclo vegetativo, foram observadas necroses nos sarmentos, nas inflorescências e nas flores nalgumas plantas de videiras da região centro de Portugal. Estes sintomas poderiam ser facilmente confundidos com os atribuídos a outras doenças como por exemplo a podridão cinzenta. Realizaram-se isolamentos microbiológicos a partir de órgãos tendo-se obtido um

conjunto de 23 isolados de *Botryosphaeriaceae*. As características morfológicas e culturais, a análise do ITS e da sequência parcial do EF-1 α permitiu a identificação de *Diplodia seriata*, *Diplodia mutila* e *Neofusicoccum parvum*. Testes de patogenicidade realizados em estufa e no campo reproduziram os sintomas observados nas vinhas e confirmaram a identificação dos agentes patogénicos envolvidos.

Na família *Botryosphaeriaceae*, as espécies do género *Lasiodiplodia* são muito agressivas e predominam em climas quentes e húmidos. Em Portugal, à data deste trabalho têm uma distribuição reduzida e localizada, contudo, no panorama das alterações climáticas, podem vir a representar uma ameaça para os vinhedos nacionais. Neste contexto, pretendeu-se avaliar a suscetibilidade de algumas das cultivares mais cultivadas em Portugal (Touriga Nacional, Touriga Franca, Alvarinho, Aragonez e Cabernet Sauvignon) a estas espécies. Isolados de *Lasiodiplodia* spp. (*L. theobromae* e/ou *L. mediterranea*) obtidos em Portugal e no Perú foram inoculados em sarmentos de videira com dois anos de idade em estufa e com sete anos em vinha. Cinco meses após a inoculação avaliou-se o comprimento das necroses produzidas nas varas. Os resultados obtidos revelaram que todos os isolados produziram necroses no lenho das videiras. Procedeu-se reisolamento e confirmação identidade dos isolados. Detetaram-se diferenças significativas em todas as combinações de isolado/cultivar. Em ambos os ensaios (estufa e campo), as maiores lesões surgiram na cultivar Touriga Nacional enquanto as menores na cultivar Aragonez. Verificou-se ainda que os isolados nacionais foram mais agressivos do que os isolados peruanos. O conhecimento antecipado da suscetibilidade de cultivares nacionais importantes a espécies bastantes agressivas, como é o caso de *Lasiodiplodia* spp., permitirá não só uma melhor gestão das práticas culturais na vinha como também apoiar a tomada de decisão relativamente ao planeamento e instalação de novas vinhas.

A proteção das feridas de poda da videira é, sem dúvida, uma das práticas mais eficazes no combate à botriosferiose. Avaliou-se a eficácia de *Trichoderma atroviride* estirpe I-1237 (Esquive®), agente de biocontrolo protetor de feridas, aplicado exteme ou em combinação com um novo produto à base de nanopartículas de cobre (CuSPHy+HA), LC2017, e, boscalide+piraclostrobina (Tessor®), produto químico de síntese que foi utilizado como produto padrão. O ensaio foi instalado em videiras das cultivares Touriga

Nacional e Cabernet Sauvignon, foram inoculados três isolados de *Lasiodiplodia* spp. e este decorreu ao longo de dois anos. Os produtos, Esquive® e Tessior®, foram aplicados diretamente nas feridas de poda enquanto o produto LC2017 foi aplicado ao longo do ciclo vegetativo de acordo com as instruções do fabricante. Tanto o Esquive® como o Tessior® revelaram eficácia na proteção das feridas de poda com variação entre os anos e as cultivares. A aplicação, em combinação com LC2017 não demonstrou melhorar a eficácia na proteção das feridas de poda relativamente à aplicação extreme de Esquive®. Porém, ensaios realizados *in vitro* com LC2017 revelaram um efeito fungistático relativamente a *Lasiodiplodia* spp., característica que representa uma aposta promissora na mitigação dos estragos produzidos por botriosferiose.

Paralelamente, realizou-se em estufa um ensaio com as cultivares Cabernet Sauvignon e Touriga Nacional para (i) avaliar a eficácia relativamente a *L. theobromae* da combinação de Esquive® e LC2017 através do comprimento das lesões internas produzidas e, (ii) investigar o efeito da aplicação destes produtos na expressão de um conjunto de genes associados aos mecanismos de defesa da videira. Os resultados confirmaram a maior dimensão das lesões em Touriga Nacional e, que, o comprimento das lesões não foi reduzido quando o produto LC2017 foi aplicado extreme ou em combinação. A análise da expressão genética confirmou o efeito eliciador de LC2017, e a inexistência de diferenças significativas entre as plantas tratadas com LC2017 extreme ou em combinação. Foram ainda detetadas diferenças significativas entre as cultivares, na expressão do conjunto de genes estudados, especialmente na resposta inicial de ativação de genes de defesa causada pela aplicação do produto. É fundamental prosseguir a investigação para aprofundar o conhecimento sobre a interação entre o produto LC2017, a cultivar (Touriga Nacional, Cabernet Sauvignon) e o patógeno *L. theobromae*.

Em suma, os resultados obtidos nesta tese colocam à disposição da comunidade informação que constitui uma clara mais-valia para: a) melhorar o conhecimento sobre os métodos disponíveis para a diagnose e proteção das DLV; b) complementar o conhecimento sobre os sintomas associados à botriosferiose facilitando o diagnóstico precoce no início do ciclo vegetativo da videira; c) comprovar as diferenças de suscetibilidades entre as principais cultivares nacionais de videira à infeção por

Lasiodiplodia spp.; d) testar soluções de proteção mais sustentáveis; e) avaliar a expressão dos genes associados aos mecanismos de defesa da videira na interação produto (biofungicida; elicitador), a cultivar (Touriga Nacional e Cabernet Sauvignon) e o fungo patogénico *L. theobromae*.

Table of contents

Acknowledgements.....	iii
Abstract.....	vi
Resumo	vii
Resumo alargado	viii
Abbreviations List	xxv
CHAPTER 1.	27
INTRODUCTION	27
1. Introduction	28
1.1 Grapevine and viticulture.....	28
1.2 Grapevine trunk diseases	29
1.2.1 Current situation	29
1.2.2 Symptoms and causal agents of grapevine trunk diseases.....	31
1.2.2.1 Black-foot disease.....	32
1.2.2.2 Petri disease	33
1.2.2.3 Esca disease.....	34
1.2.2.4 Phomopsis dieback.....	36
1.2.2.5 Eutypa dieback.....	37
1.3 Botryosphaeria dieback.....	38
1.3.1 Symptoms	38
1.3.2 Aetiology.....	41
1.3.3 Epidemiology	42
1.3.4 Cultivar response to <i>Botryosphaeriaceae</i> infection	45
1.3.5 Control	47
1.3.5.1 Cultural practices.....	47
1.3.5.2 Chemical control.....	49

1.3.5.3 Biological control	51
1.4 Objectives and thesis outline	52
1.5 References.....	55
CHAPTER 2.	77
RESEARCH METHODS IN GRAPEVINE TRUNK DISEASES.....	77
2. <i>Vitis</i> Methods to Understand and Develop Strategies for Diagnosis and Sustainable Control of Grapevine Trunk Diseases	79
2.1 Introduction.....	80
2.2 Cell Suspension and callus cultures to detect molecules associated with phytotoxicity or aggressiveness of GTD pathogens and to describe early host signaling events.....	82
2.3 Plantlets and foliar explants to decipher the molecular and physiological effects of molecules associated with aggressiveness produced by GTD pathogens at different plant integrative levels.....	86
2.4 <i>In vivo</i> artificially inoculated plant assays, to reproduce wood and foliar symptoms and to validate pathogen contribution to natural GTD symptomatology	92
2.5 Field validation by artificial inoculation of pruning wounds and stems, the last experimental step	100
2.6 Discussion of the strengths, weaknesses, and complementarities of the methods	104
2.6.1 The use of <i>in vivo</i> nonsterile plants.....	107
2.7 Author contributions.....	112
2.8 Funding.....	112
2.9 Acknowledgements.....	112
2.10 References.....	112
CHAPTER 3	123
NEW INSIGHTS ON <i>BOTRYOSPHAERIACEAE</i> SYMPTOMS IN GRAPEVINE.....	123

3. Early Season Symptoms on Stem, Inflorescences and Flowers of Grapevine Associated with <i>Botryosphaeriaceae</i> Species.....	125
3.1 Introduction.....	126
3.2 Material and methods.....	127
3.2.1 Sampling and Fungal Isolation.....	127
3.2.2 Morphological Characterization and DNA Analysis for Fungal Identification	128
3.2.2.1 Morphological Characterization	128
3.2.2.2 DNA Analysis	128
3.2.3. Pathogenicity Tests.....	129
3.2.3.1. Pathogenicity tests on Tendrils and Leaves	130
3.2.3.2. Pathogenicity Tests on Green Stems	130
3.2.3.3. Field Pathogenicity Tests on Clusters	131
3.3 Results	131
3.3.1 Sampling and Fungal Isolation.....	131
3.3.2 Morphological Characterization and DNA Analysis for Fungal Identification	133
3.3.3 Pathogenicity Tests.....	134
3.3.3.1 Pathogenicity Tests on Tendrils and Leaves	134
3.3.3.2 Pathogenicity Tests on Green Stems	134
3.3.3.3 Field Pathogenicity Tests on Clusters	136
3.4 Discussion.....	138
3.5 Author Contributions	141
3.6 Funding.....	141
3.7 References.....	141
CHAPTER 4	149

GRAPEVINE-PATHOGEN INTERACTION:	149
RESPONSE TO <i>LASIODIPLODIA</i> SPP. INFECTION	149
4. Response of different grapevine cultivars to infection by <i>Lasiodiplodia theobromae</i> and <i>Lasiodiplodia mediterranea</i>	151
4.1 Introduction.....	152
4.2 Material and methods.....	154
4.2.1 Greenhouse experiment:.....	154
4.2.2 Field experiment:.....	156
4.2.3 Statistical analysis.....	157
4.3 Results	158
4.3.1 Greenhouse experiments	160
4.3.2 Field experiment.....	165
4.4 Discussion.....	165
4.5 Author contributions.....	170
4.5 Funding.....	171
4.6 References.....	171
CHAPTER 5	179
DISEASE MANAGEMENT:.....	179
SCREENING NEW CONTROL STRATEGIES FOR <i>LASIODIPLODIA</i> SPP. INFECTION IN GRAPEVINE	179
5.1 Combining an HA + Cu (II) Site-Targeted Copper-Based Product with a Pruning Wound Protection Program to Prevent Infection with <i>Lasiodiplodia</i> spp. in Grapevine	181
5.1.1 Introduction.....	182
5.1.2 Materials and Methods.....	185
5.1.2.1 In vitro Assays.....	185
5.1.2.1.1 Mycelial Growth Inhibition Assay	185

5.1.2.1.2 Dual Culture Antagonism Assay	186
5.1.2.2 Field Assays.....	186
5.1.2.2.1 Experimental Field.....	186
5.1.2.2.2. Fungal Isolates Used and Inoculum Preparation	187
5.1.2.2.3. Experimental Design and Treatment Plan	187
5.1.2.2.4 Product Application and Pathogen Inoculation	188
5.1.2.2.5 Pathogen Recovery and Identification	189
5.1.2.2.6 Meteorological Data	190
5.1.2.2.7 Statistical Analysis	191
5.1.3 Results.....	191
5.1.3.1 In vitro Assays	191
5.1.3.1.1 Mycelial Growth Inhibition Assays.....	191
5.1.3.1.2 Dual culture antagonism assays	193
5.1.3.2 Field assays	194
5.1.4 Discussion	199
5.1.5 Author contributions	207
5.1.6 Funding.....	207
5.1.7 Acknowledgments	207
5.1.8 References.....	208
5.2 Effect of the Combined Treatments with LC2017 and <i>Trichoderma atroviride</i> Strain I-1237 on Disease Development and Defense Responses in Vines Infected by <i>Lasiodiplodia theobromae</i>	219
5.2.1 Introduction	220
5.2.2 Material and methods	222
5.2.2.1 Fungal isolates and inoculum preparation.....	222
5.2.2.2 Plant material, experimental design and treatments.....	223

5.2.2.2.1 Plant material	223
5.2.2.2.2 Tested products	223
5.2.2.3 Product application and pathogen inoculation	224
5.2.2.4 Gene expression analysis	225
5.2.2.5 Determination of internal lesions and pathogen recovery.....	226
5.2.2.6 Statistical analysis	226
5.2.3 Results.....	226
5.2.3.1 Relationship between the lesion length induced by <i>L. theobromae</i> and treatments.....	226
5.2.3.2 Pathogen recovery	228
5.2.3.3 Plant responses at gene expression levels according to the treatments .	230
5.2.3.3.1 Effect of LC2017 treatment alone	230
5.2.3.3.2 Effect of the double treatment Esquivel and LC2017	231
5.2.3.3.3 Influence of cultivar on defense responses	231
5.2.4 Discussion	233
5.2.5 Author Contributions	236
5.2.6 Funding	237
5.2.7 Acknowledgments	237
5.2.8 References	237
CHAPTER 6	243
FINAL CONSIDERATIONS.....	243
6. Final considerations	244
SUPPLEMENTARY MATERIAL	249
Supplementary material Sub-Chapter 5.2.....	250

List of Figures

Figure 1. 1 - Black-foot and Petri disease foliar and vascular symptoms: A – Poor vigour and declining grapevines (young vine decline); B- Necrosis on the basal end of a rootstock; C – “J-root” symptom on a grapevine collected in a vineyard showing symptoms of young vine decline; D and E – Cross section of a grafted grapevine showing dark xylem vessels infected by black-foot pathogens (D) and Petri disease pathogens I disease; F – Cross section of a 4 year-old rootstock showing black goo caused by Petri disease.	32
Figure 1. 2 – Esca symptoms. A – Grapevine “tiger-stripe” disease (GLSD) symptoms on a red cultivar; B - Internal symptoms of black vascular streaking and black spot in the xylem; C – Black spots on the xylem vessels; D – Acute or apoplectic form characterized by sudden wilting of plant; E – White rot develop on the centre of mature grapevines; F – Basidiomycete’s colonization of esca infected grapevine woody tissues.	35
Figure 1. 3 – Phomopsis cane and leaf spot and Phomopsis dieback symptoms. A, B – Lesions of Phomopsis cane and leaf spot on green shoots; C – Wedge shaped canker of Phomopsis dieback similar to those found associated with Botryosphaeria and Eutypa diebacks.	37
Figure 1. 4 – Eutypa dieback symptoms. A, B - Stunted shoots with chlorotic and often cupped leaves; C – Wedge shapes internal necrosis on an infected cordon.	38
Figure 1. 5 – Botryosphaeria dieback symptoms. A – Perennial canker on infected green shoot; B – Canker on the base of green shoot; C – Grapevine showing reduce growth and dieback and foliar symptoms; D – Dried fruit rot on green shoot showing a canker; E – Non-uniform to circular central staining of wood; F – Orange/brown stripes located in the outer xylem, appearing longitudinally when the bark is removed.	40
Figure 1. 6 - Disease cycle of Botryosphaeria dieback on grapevine.	44
Figure 2. 1 - Callus assay for <i>Vitis vinifera</i> to test pathogen aggressiveness. A , Callus medium in an Erlenmeyer flask and supernatant of pathogens in a tube to test toxicity of the extracellular compounds and proteins. B , Three-week-old calli. C , Coculture of callus with extracellular compounds or proteins incorporated in the culture medium. D , Calli subcultured for 6 days in Petri dishes with malt medium proteins (D1) native protein fractions produced by <i>Neofusicoccum parvum</i> isolate Bourgogne S-116 (D2) and <i>Diplodia seriata</i> isolate 98.1 (D3) after 6 days of contact.	83

Figure 2. 2 - Leaf-drop and foliar disc methods for <i>Vitis vinifera</i> . A , On a Petri dish, a drop with fungal toxins is deposited on the adaxial side of leaf to test their toxicity. B , With a cork borer, leaf discs are excised and C , transferred to a plate containing liquid medium with fungal compounds to test their toxicity.	88
Figure 2. 3 - Plantlet model for <i>Vitis vinifera</i> treatment with the toxins associated with grapevine trunk disease fungi. Left side shows the plantlet model (photo and diagram) as described by our team. Right side shows the plantlet transferred to a new tube containing fresh liquid incubating medium supplemented with toxin.	90
Figure 2. 4 – Process developed to reproduce <i>Eutypa lata</i> symptoms with unrooted or rooted cuttings of <i>Vitis vinifera</i> , as described by Péros and Berger (1994) and Chapuis (1995). A , Inoculation preparation. A pellet of <i>E. lata</i> sampled on a 1-week-old mycelium culture in petri dish. B , Perforation of the woody stem. On the 1-year-old lignified stem, a hole (4 to 5 mm in diameter) under the upper bud produced with a drill. C and D , Artificial inoculation of rooted cutting. The pellet, spores or a plug of <i>E. lata</i> is put in the stem hole. E and F , Foliar symptoms of <i>E. lata</i> on 1-year-old rooted cuttings.	93
Figure 2. 5 - Cutting inoculation procedure for <i>Vitis vinifera</i> wood with esca-associated fungi. The left side describes the internodal model and the right side the nodal model inoculation methods. I , Plants carrying one or two branches are maintained in a growth chamber for 1 week. The internodal infection consists of wounding damage at the middle of an internode. I bis , For the nodal model, the intermediate branch is cut with a blade. II , Wounding damage is done using a drill bit. III , Esca associated fungi are deposited in the wound.	96
Figure 2. 6 – Inoculation of grapevine plants under controlled conditions with <i>Neofusicoccum parvum</i> and <i>Diplodia seriata</i> , as described by Reis <i>et al.</i> (2016). A , Perforation of the green stem. A wound is created by removing 3 mm of epidermis with a corkborer at the third internode of the green shoot. B , Artificial inoculation. A 3-mm mycelial plug is cut from the actively growing margin of <i>N. parvum</i> or <i>D. seriata</i> colonies and placed on the wound. C , The plug is covered with moist cotton wool and sealed with Parafilm for 2 weeks. D , External lesions observed in the green shoots of inoculated plants. E , Foliar symptoms expressed on inoculated plants.	99
Figure 2.7 – Pruning wound methods in the vineyard, as described by Lecomte <i>et al.</i> (2003). A , In the vineyard, inoculation with 50 to 250 spores of <i>Eutypa lata</i> at the pruning	

of the canes composed of 2 to 3 internodes. B , In the laboratory, 5 pieces of wood from the pruning wound inoculation site were deposited onto culture medium and grown for 4 to 7 days.....	101
Figure 3.1 - Symptoms observed on tendril and green shoots: (A) necrotic tendrils obtained after inoculation with <i>D. mutila</i> (Bt218); (B) necrotic apex of the shoot after inoculation with <i>D. seriata</i> (Bt204); (C) necrotic leaf after inoculation with <i>N. parvum</i> (Bt216); (D) lesion obtained after inoculation with <i>D. seriata</i> (Bt204); (E) lesion obtained after inoculation with <i>N. parvum</i> (Bt216); (F) control inoculation.	135
Figure 3.2 - Percentage of plants showing symptoms on tendrils and leaves, after inoculation with the <i>Botryosphaeriaceae</i> isolates under study. Ten plants were used per isolate while control plants were sprayed with sterile distilled water.	135
Figure 3.3 - Mean canker areas (cm ²) in green stem caused by artificial inoculation with <i>Botryosphaeriaceae</i> isolates under study. Different letters in column correspond to significant differences ($P \leq 0.05$) based on ranks assessed by Kruskal-Wallis analysis. Ten plants were used per isolate while control plants were sprayed with sterile distilled water.....	136
Figure 3.4 - (A) best cluster from each inoculation/isolate; (B) worst cluster from each inoculation/isolate.....	137
Figure 3.5 - Boxplot of the percentage of infected area on clusters artificially inoculated with <i>Botryosphaeriaceae</i> isolates under study. Scale of evaluation of area infected—1 = no symptoms; 2 = 1–5%; 3 = 5–25%; 4 = 25–50%; 5 = >50%. The median is represented by the solid line. Top and bottom lines of the box correspond to the 25th and 75th percentiles of the data, respectively. Circles represent outliers.	137
Figure 4.1 – Necrosis on shoots inoculated for the greenhouse (A, B, C) and field (D, E, F) experiments: A – Isolate Bt103 inoculated on Touriga Nacional (2017); B – Isolate CBS124060 inoculated on Aragonez (2019); C- Mock inoculation (Control) on Alvarinho (2019); D – Isolate Bt105 inoculated on Touriga Nacional (2018); E – Isolate LA-SOL3 inoculated on Touriga Franca (2019); F – Mock inoculation (Control) on Aragonez (2018).	159
Figure 4.2 – Principal component analysis (PCA) of the response of five different grapevine varieties inoculated with eight <i>Lasiodiplodia</i> sp. isolates, during the two years	

of greenhouse experiment (A – 2017; B - 2019). Percentage value within parentheses represent the variance explained by each principal component.....	163
Figure 5.1.1 - Diagram showing the several steps of the field assay. Information on the products used can be found in Table 5.1.2, and a description of the different treatments can be found in Table 5.1.3. A: Marked sample collected from the field prior to analysis; B: Sample with the bark removed showing sign of necrosis; C: Sample collected about 1 cm below the pruning wound, to be divided into four pieces and plated onto PDA; D: Isolate Bt105 (<i>Lasiodiplodia theobromae</i>) recovered from infected pruning wounds; E: Isolate LA-SOL3 (<i>Lasiodiplodia theobromae</i>) recovered from infected pruning wounds; F: Isolate CBS124060 (<i>Lasiodiplodia mediterranea</i>) recovered from infected pruning wounds; G: Petri dish containing wood obtained from non-inoculated control plants showing no signs of pathogen growth.	190
Figure 5.1.2 - Mean inhibition percentage of growth radius for <i>Trichoderma atroviride</i> strain I-1237 against <i>Lasiodiplodia theobromae</i> isolates Bt105 and LA-SOL3 and <i>Lasiodiplodia mediterranea</i> isolate CBS124060. Dots and triangles represent data from both experiments, black dots represent the mean percentage of growth inhibition for the total replicates of both experiments and black bars represent the standard error of means. Columns with the same letter (a, b) are not significantly different according to Tukey's test ($P \leq 0.05$).	193
Figure 5.1.3 - Dual culture antagonism assay of <i>T. atroviride</i> strain I-1237 and the three <i>Lasiodiplodia</i> spp. isolates under study. A: isolate Bt105; B: isolate LA-SOL3; C: isolate CBS124060.....	194
Figure 5.1.4 - Monthly rainfall and average temperature for the two years of the trial.	196
Figure 5.1.5 - Principal component analysis (PCA) for the interactions of all treatments, 15 variables including all the product/isolate combinations (T represents the treatment numbers which are stated in Table 5.1.5), as well as inoculated controls on both variables and the meteorological variables (Figure 5.1.4). Red marks represent the observations for Cabernet Sauvignon (CS) and green marks represent the Touriga Nacional (TN) observations. A-PCA analysis for 2019; B-PCA analysis for 2020.	197

Figure 5.2.1 - Diagram showing the several steps of treatment and inoculation of greenhouse kept grapevine of cultivars Cabernet Sauvignon and Touriga Nacional. Information on the treatments and products used can be found on table 5.2.1..... 224

Figure 5.2.2 - Boxplot representing the average lesion lengths recorded for the inoculation of cuttings from two different grapevine cultivars Cabernet Sauvignon and Touriga Nacional with an isolate of *L. theobromae*, under greenhouse conditions. The median is represented by the solid line. Mean lesion lengths of isolates with different letter are significantly different at $P < 0.05$, Tukey's test. Lower-case letters for Cabernet Sauvignon and upper-case letters for Touriga Nacional. Dots represent outliers..... 227

Figure 5.2.3 - Boxplot representing the re-isolation percentage of *L. theobromae* recorded from the cuttings of two cultivars, Cabernet Sauvignon and Touriga Nacional. The median is represented by the solid line. Mean lesion lengths of isolates with different letter are significantly different at $P < 0.05$, Tukey's test. Lower-case letters for Cabernet Sauvignon and upper-case letters for Touriga Nacional. Dots represent outliers..... 229

Figure 5.2.4 - Expression levels of selected genes (determined with quantitative reverse-transcription polymerase chain reactions) and relative standard deviation values (sd) recorded on leaves cv Cabernet Sauvignon at T0 + 2d and T0 + 5d. The values (each the mean of three technical replicates) represent expression levels ($\Delta\Delta Ct$) of treatments relative to the control (condition Esquive® T0 + 2d). The expression of each gene was considered up-regulated when the value was >2-fold compared to the controls or down-regulated when the value was <0.5-fold compared to the controls..... 230

Figure 5.2.5 - Expression levels of selected genes (determined with quantitative reverse-transcription polymerase chain reactions) and relative standard deviation values (sd) recorded on leaves cv Touriga Nacional at T0 + 2d and T0 + 5d. The values (each the mean of three technical replicates) represent expression levels ($\Delta\Delta Ct$) of treatments relative to the control (condition Esquive® T0 + 2d). The expression of each gene was considered up-regulated when the value was >2-fold compared to the controls or down-regulated when the value was <0.5-fold compared to the controls..... 230

Figure 5.2.6 - Overexpressed genes (values > 2) at T₀ + 2d in Cabernet Sauvignon cuttings with single (LC2017) or double (LC2017 + Esquive®) treatment, not inoculated or inoculated with *L. theobromae* strain Bt105. Statistical analysis with the Mann-Whitney

U test showed no significant differences between medians of LC2017 and LC2017 + Esquive® and between LC2017 + Bt105 and LC2017 + Esquive® + Bt105. Only a significant effect of pathogen presence was observed in some genes in LC2017-treated vines. The asterisk indicates a statistical significance (Mann-Whitney U test) between the medians of the two considered conditions. * = $p < 0.05$; ** = $p < 0.01$ 232

Figure 5.2.7 - Overexpressed genes (values > 2) at $T_0 + 2d$ in Touriga Nacional cuttings with single (LC2017) or double (LC2017 + Esquive®) treatment, not inoculated or inoculated with *L. theobromae* strain Bt105. Statistical analysis with the Mann-Whitney U test showed no significant differences between the medians of LC2017 and LC2017 + Esquive® and between LC2017 + Bt105 and LC2017 + Esquive® + Bt105. Only a significant effect of pathogen presence was observed in some genes in LC2017-treated vines and in double-treated vines. The asterisk indicates a statistical significance (Mann-Whitney U test) between the medians of the two considered conditions. * = $p < 0.05$; ** = $p < 0.01$ 233

Figure S5.2.1 - Comparison of the 9 targeted genes with induction values >2 recorded at $T_0 + 5d$ on the different condition of treatments (LC2017 alone, LC2017 and Esquive) between Cabernet Sauvignon (CAB) and Touriga Nacional (TN) not infected or inoculated with *L. theobromae* (Bt105). The asterisk indicates a statistical significance (Mann-Whitney U test) between the medians of the two considered condition. * = $p < 0.05$; ** = $p < 0.01$ 252

List of Tables

Table 2.1 – Set of methods to be reviewed and their use	82
Table 2. 2 - Synthesis of the <i>Vitis</i> methods available to understand grapevine trunk diseases.....	83
Table 3.1 - <i>Botryosphaeriaceae</i> isolates obtained during the study, respective GenBank accession numbers and percent identity when compared to reference sequences...	132
Table 3. 2 - Conidial dimension of the <i>Botryosphaeriaceae</i> species under study.	133
Table 4. 1 - <i>Lasiodiplodia</i> spp. isolates used for inoculation of the grapevine cultivars	155
Table 4.2 – Mean internal lesion length (cm) obtained five months after inoculations of five different cultivars with six isolates of <i>L. theobromae</i> and one isolate of <i>L. mediterranea</i> for the greenhouse and two <i>L. theobromae</i> isolates for the field experiments.....	161
Table 4.3 – Mean re-isolation percentage obtained five months after inoculations of five different cultivars with six isolates of <i>L. theobromae</i> and one isolate of <i>L. mediterranea</i> for the greenhouse and two <i>L. theobromae</i> isolates for the field experiments.....	164
Table 5.1.1 - <i>Lasiodiplodia</i> spp. isolates used for pruning wound inoculation.	186
Table 5.1.2 - Treatments tested for control of <i>Lasiodiplodia theobromae</i> and <i>Lasiodiplodia mediterranea</i> under field conditions.	188
Table 5.1.3 - Treatment plan designed to assess the efficacy of three products against pruning wound infection by <i>Lasiodiplodia theobromae</i> and <i>Lasiodiplodia mediterranea</i> . Combinations of products used and inoculations spore solution volume.....	188
Table 5.1.4 - Mycelial growth inhibition rate (%) of product LC2017 on all the <i>Botryosphaeriaceae</i> isolates under study (Bt105, LA-SOL3, CBS124060) and the <i>Trichoderma atroviride</i> strain I-1237 (Esquive).....	192
Table 5.1.5 - Efficacy of treatments used solely as pruning wound protectants and in a combination with the copper-based product, LC2017, to reduce <i>Lasiodiplodia</i> sp. as part of an integrated disease management.....	195
Table 5.2.1 – Treatments tested on cultivars Cabernet Sauvignon and Touriga Nacional.	223
Table S5.2.1. Primers of genes analyzed by quantitative real-time reverse-transcription polymerase chain reaction.	251

Abbreviations List

BCA – Biocontrol Agent

BDA – Black Dead Arm

CHIT4C - Chitinase class V

CuSPHy + HA - Hydroxyapatite loaded with Copper (II) Sulphate Pentahydrate

cv – cultivar

DLV – Doenças do Lenho da Videira

DNA – Deoxyribonucleic Acid

dNTP - Deoxyribose Nucleotide Triphosphate

EPPO - European and Mediterranean Plant Protection Organization

FAO - Food and Agriculture Organization of the United Nations

gfp- Green fluorescent protein

GLSD – Grapevine Leaf Stripe Disease

GLUC - β -1,3 glucanase

GTDs – Grapevine Trunk Diseases

GTS1 - Glutathione s-transferase 1

HA – Hydroxyapatite

HPLC – High-Performance Liquid Chromatography

HYD2 - ABA 8' hydroxylase 2

IPM – Integrated Pest Management

ITS – Internal Transcribe Spacer

IVV- Instituto da Vinha e do Vinho

MDPC – Mean Percentage of Disease Control

MPR – Mean Percentage Recovery

MS medium - Murashige and Skoog medium

MSR - Peptide methionine sulfoxide reductase

PAL - Phenylalanine ammonia lyase

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

PDA – Potato Dextrose Agar

PME25 - pectin esterase inhibitor

PPP – Plant Protection Products

PR -protein - pathogenesis-related protein

RT-PCR - Reverse Transcription Polymerase Chain Reaction

SDW – Sterile Distilled Water

STS - Stilbene synthase

tef1-α - Translation Elongation Factor 1α

WRKY - transcription factors

CHAPTER 1.
INTRODUCTION

1. Introduction

1.1 Grapevine and viticulture

Grapevine, *Vitis vinifera* L., has been cultivated through all recorded history, being the birth of viticulture dated as far back as 6000 B.C. Although the most ancient winery discovered is in Armenia (4100 B.C.), the Egyptians were one of the first major civilizations of Bronze Age (3500 – 1200 B.C) to use viticulture practices (Hidalgo and Hidalgo, 2019). Viticulture tradition was spread through Europe by the Romans that had inherited the knowledge on cultivating grapevines from the Greeks and wine and table grape production has, since then, expanded to almost all continents. It is currently considered one of the most important crops worldwide, having a high commercial value for fresh table grape, dried fruit and wine production (Gramaje *et al.*, 2018). Grape growing countries can greatly benefit economically from the grape and wine industries, with revenues from the larger industries going as high as USD\$161 billion in 2005, in the USA and AUD\$40.2 billion in 2015 in Australia (Gramaje *et al.*, 2018). In Portugal, although the grapevine cultivated area has slightly decreased in the last 20 years, a huge increase in production has been verified during the last few years mainly due to a large re-qualification of the planted area, and the adoption of new and more state-of-the-art cultural practices. The planted area in Portugal is now close to 190.000 ha, with a revenue of around EUR€ 1.7 billion in 2020, from which about 50% of this value represents exports revenues (IVV, 2022a, 2022b).

Grapevine is also host to the broadest range of pathogens of all woody crops with agronomic interest (Martelli *et al.*, 1997). According to Wilcox *et al.* (2015), grapevine is susceptible to 29 fungal diseases. Viticulture, and especially European viticulture, has gone through several epidemics due to some of the referred fungal diseases. *Plasmopora viticola* the agent responsible for downy mildew and *Erysiphe necator*, the causal agent of powdery mildew, have caused huge constraints on viticulture throughout history, although both are now controlled with the use of both chemical fungicides (Gessler *et al.*, 2011), and biocontrol agents (Compant and Mathieu, 2017). More importantly the arrival in Europe of the phylloxera infestation (*Daktulosphaira vitifoliae*) in the 19th century, from North America, left European viticulture almost devastated. There were huge crop losses, the near destruction of

viticulture in some European regions, including French producers which were left close to bankruptcy (Garnett *et al.*, 2001; Álvarez-Ramos and Villarías-Moradillo, 2012). Fortunately, research has led to the discovery that vine stock from the American *Vitis riparia*, *Vitis berlandieri*, *Vitis rupestris*, *Vitis cinerea*, *Vitis rotundifolia* and the Asian *Vitis amurensis*, were phylloxera resistant due to the largest lignification of their roots and more dense tissues. Therefore, the practice of grafting was able to control this pest, and it is still in practice today. Currently, grapevine trunk disease (GTDs), although known since the 19th century, are considered as the major threat to the future economic sustainability of modern viticulture, causing severe losses, due to yield reduction, increased production costs and shortened life span of the grapevines (Bertsch *et al.*, 2013; Kaplan *et al.*, 2016; Gramaje *et al.*, 2018).

1.2 Grapevine trunk diseases

1.2.1 Current situation

The first reliable report of GTDs can be found in French literature from the late 19th century where foliar symptoms were described and named as “folletage” and “apoplexy” (Ravaz, 1898, 1909; Surico 2009). Nevertheless, it was only in the beginning of the 1990’s that Professor Luigi Chiarappa in collaboration with other scientists, created the term grapevine trunk disease to describe a series of symptoms observed both in the foliage and vascular system of grapevines, supposedly caused by a group of fungi that infected mainly through pruning wounds (Mugnai *et al.*, 1999).

GTDs are currently considered to be the most important challenge for viticulture (Bertsch *et al.*, 2013; De la Fuente *et al.*, 2016). These destructive diseases cause severe damage every year because their causal agents attack the perennial organs, ultimately killing the vines in either the short or long term. This causes severe impairments to the sustainability of winemaking heritage and are of rapidly growing concern in all wine-producing countries of the world. The increased importance that GTDs had during the last three decades, being probably the most relevant threat for the vitivinicultural sector nowadays, can be traced to several reasons:

- I) The 1990s saw a huge increase on planted area, increasing the movement of possibly infected propagation material and allowing the grapevines to reach an age where symptoms are expressed (Gramaje and Armengol, 2011).
- II) The increased number of infections brought to vineyards by previously infected planting material (Rubio and Garzón, 2011; De la Fuente *et al.*, 2016)
- III) Changes in cultural practices, especially in the adoption of training systems that increased planting density and consequently the number of pruning wounds (Gramaje *et al.*, 2018). Moreover, the mechanization of vineyard practices and poor pruning wound protection strategies (Rubio and Garzón, 2011; De la Fuente *et al.*, 2016) are also considered important factors on the higher expression of GTD infection.
- IV) The ban of effective fungicides due to health and environmental concerns, such as sodium arsenite, the only effective fungicide against Esca, as well as benzimidazoles and methyl bromide (Decoin, 2001; Larignon *et al.*, 2008, Mondello *et al.*, 2018).

These diseases pose a significant threat to sustainable viticulture worldwide since the cost associated with GTD losses has been increasing during recent years. Most grape production worldwide is targeted for the wine industry, so any factor leading to yield decrease and /or quality loss, associated with increased production costs can have a huge impact on profits. For example, in Spain, there was an increase of 1.8% of incidence in vineyards in 2003 up to 10.5% in 2007 (Rubio and Garzón, 2011). In fact, just for La Rioja region, in Spain, the estimated cost of replacing 1% of dead “Tempranillo” plants is of €7.16 million (Martínez-Diz *et al.*, 2019). In France, it is estimated that the equivalent of 1 billion euros is lost every year due to these diseases (De la Fuente *et al.*, 2016), while in Australia, the economic impact can reach up to 8.3 billion AUD (Ridgway *et al.*, 2014). In California, the difference of profit between healthy and diseased vineyards is estimated in approximately US\$33,000/ha over a 25-year life span. In Portugal, although there is no official estimation on the monetary value of economic impact of GTDs, it is known that they also cause severe damage and economic impact (De la Fuente *et al.*, 2016). GTDs have been reported throughout all the wine appellations of the country and are known to sometimes occur as frequently as more

than 60% of the established vineyards (Guérin-Dubrana *et al.*, 2019). Nevertheless, the real extension of vineyards affected by GTDs is still somewhat uncertain, since infections develop in the wood, and external symptoms may not appear for several years (Bertsch *et al.*, 2013; De la Fuente *et al.*, 2016). Also, until this day, no curative measures are available to mitigate the impact of GTDs once grapevines are already infected, making it extremely difficult and complex to manage on the field.

To the present date 136 species from 35 genera have been associated with a total of six different GTDs (Gramaje *et al.*, 2018; Lawrence *et al.*, 2019; Aigoun-Mouhos *et al.*, 2019; Berlanas *et al.*, 2020) affecting both young and mature grapevines: Black-foot, Petri disease, Esca, Botryosphaeria, Eutypa and Phomopsis diebacks. The number of different species continues to increase almost daily due to the continuous improvement of molecular tools for their identification. This number of different pathogens responsible for GTDs, the fact that it is common for one single grapevine can be infected with several GTDs at the same time (Gramaje *et al.*, 2018) and the banning of several effective chemical active ingredients makes it hard for growers to control the impact and spread of GTDs. Thus, the main focus of researchers during the last years has been to develop new active ingredients (biological and chemical) and new innovative cultural practices that can help control this problem (Úrbez-Torres, 2011).

1.2.2 Symptoms and causal agents of grapevine trunk diseases

Grapevine trunk diseases are caused by several ascomycetous and basidiomycetous fungi and their pathosystem is extremely complex due, not only to the high number of species of pathogens involved, but also that to the fact that both young and mature grapevines are affected by common pathogens. This leads to young GTD infected vines to only show external symptoms when they mature, after a latency period. These diseases are cryptic, and the fact that internal and external symptoms of these diseases sometime overlap, when several infections occur on the same plant and can resemble abiotic disorders, can make it extremely difficult to accurately identify a specific pathogen in the field (Gramaje *et al.*, 2018; Mondello *et al.*, 2018). A brief description of each known GTD is provided below, except for Botryosphaeria dieback, for which a more detailed picture is provided, since it constitutes the focus of this work.

1.2.2.1 Black-foot disease

Black-foot disease commonly affects grapevine planting material and young vineyards (< 5-year-old), usually infecting roots and the rootstock (Gramaje and Armengol, 2011). Characteristic external symptoms of this disease are well established and include reduced vigour with shortened internodes, uneven wood maturity, sparse foliage and small leaves with interveinal chlorosis and necrosis (Figure 1.1 A) (Agustí-Brisach and Armengol, 2013). Internal symptoms are usually characterized by black, sunken, necrotic lesions on roots, causing a reduction of root biomass (Rego *et al.*, 2000; Halleen *et al.*, 2006). Sometimes, on plants affected by this disease, a second crown of root develops close to the soil surface to try to compensate the loss of the functional roots (Agustí-Brisach and Armengol, 2013). Also, the affected wood tissue develops a dark brown to black coloration, which gives the plant a necrotic appearance immediately above the soil (Halleen *et al.*, 2006; Gramaje *et al.*, 2018).

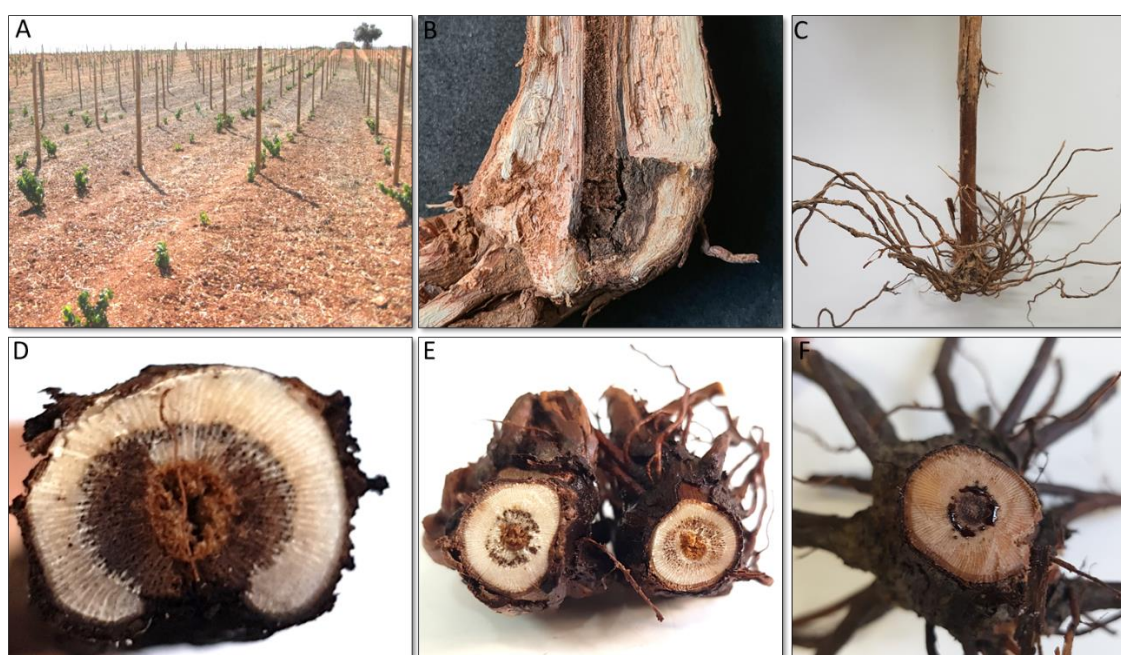


Figure 1. 1 - Black-foot and Petri disease foliar and vascular symptoms: A – Poor vigour and declining grapevines (young vine decline); B- Necrosis on the basal end of a rootstock; C – “J-root” symptom on a grapevine collected in a vineyard showing symptoms of young vine decline; D) and E – Cross section of a grafted grapevine showing dark xylem vessels infected by black-foot pathogens (D) and Petri disease pathogens (E); F – Cross section of a 4 year-old rootstock showing black goo caused by Petri disease.

Photos by P. Reis and C. Rego

The longitudinal and cross cutting of affected plants usually reveals black streaks and necrosis of the basal wood vascular tissues (Fig. 1.1 B and 1.1 D). This disease can usually

be found in association with the so called “J-symptom” (Fig 1.1 C) which results from poor planting practices (Gubler and Petit, 2013).

Black-foot disease has been attributed to several causal agents belonging to up to 32 species of *Campylocarpon* Halleen, Schroers & Crous, *Cylindrocladiella* Boesew, *Dactylonectria* L. Lombard & Crous, *Ilyonectria* P. Chaverri & C. Salgado, *Neonectria* Wollenw., *Pleiocarpon* L. Lombard & D. Aiello and *Thelonectria* P. Chaverri & C. Salgado (Carlucci *et al.*, 2017; Gramaje *et al.*, 2018; Lawrence *et al.*, 2019; Aigoun-Mouhous *et al.*, 2019; Berlanas *et al.*, 2019, 2020). Currently, *Dactylonectria torresensis* is considered to be the most common and prevalent species infecting European grapevines (Reis *et al.*, 2013; Carlucci *et al.*, 2017; Berlanas *et al.*, 2017)

1.2.2.2 Petri disease

Petri disease also contributes to the young vine decline, and can affect propagating material in the nurseries, as well as young grapevines (<7-8 years old) (Serra *et al.*, 2000; Halleen *et al.*, 2003; Gramaje *et al.*, 2018). Foliar symptoms of this disease include general stunting, reduced foliage and wilting of leaves or entire shoots, retarded and absent sprouting, shortened internodes and dieback (Gramaje and Armengol, 2011; Gramaje *et al.*, 2018). These symptoms can resemble those of abiotic stresses, such as spring frost, winter damage and water and/or nutrient deficits, which makes a visual diagnostic extremely fallible. Internal symptoms associated with Petri disease are more characteristic allowing for a simpler disease recognition. Usually, black discoloration of the xylem vessels occurs (Fig 1.1 E), with dark-coloured phenolic compounds being produced inside the referred vessels, in response to the fungus growth. These compounds can exude upon cutting of the plant in cross sections (commonly known as black goo) (Fig. 1.1 E) (Rooney-Latham *et al.*, 2005; Gramaje and Armengol, 2011).

The most common species associated with Petri disease is *Phaeomoniella chlamydospora* (W. Gams, Crous, Wingf. & Mugnai) Crous & W. Gams (Mostert *et al.*, 2006) but several other species have been isolated from symptomatic grapevine tissue. These include 29 species of *Phaeoacremonium*, as well as *Pleurostoma richardsiae* (Nannfeldt) Réblová & Jaklitsch and six species of the genus *Cadophora* (Gramaje *et al.*, 2018). Currently, *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert &

Crous and *Cadophora luteo-olivacea* (J.F.H. Beyma) Harr. & McNew are considered the most common species worldwide (Mostert *et al.*, 2006; Gramaje *et al.*, 2011). Infection by these pathogens usually occurs through root wounds, the base of the rootstock and grafting zone (Gramaje and Armengol, 2011; Gramaje *et al.*, 2018).

1.2.2.3 Esca disease

Esca can be considered as one of the oldest diseases affecting cultivated grapevines, with references dating back to the ancient Greeks (Mugnai *et al.*, 1999). However, as referred previously, the first reliable report of Esca was made on the late 1800s/early 1900s, in France and Italy and was first named as “apoplexy” or “folletage” (Ravaz, 1898, 1909; Mugnai *et al.*, 1999). Esca disease affects mainly mature grapevines with more than 8 years-old, and has traditionally, been described as having two distinct forms, chronic/mild and acute/apoplectic (Lecomte *et al.*, 2012; Gubler *et al.*, 2015; Gramaje *et al.*, 2018). The most iconic, recognizable and characteristic of esca’s mild form is the foliar symptom known as grapevine leaf stripe disease (GLSD) (Fig. 1.2 A) (Gubler *et al.*, 2015). This symptom is described by leaves showing a “tiger-stripe” pattern, i.e., multiple banding discolouration surrounding dry, light, or red-brown necrotic tissue on the leaf blade, often bordered by narrow red or yellow blotches, whereas on white cultivars the red colour is usually absent (Gramaje *et al.*, 2018). The reasons behind GLSD symptoms are not yet clear, but it has been hypothesized that are a host defence response due to the translocation via sap flow of fungal produced metabolites and/or products from wood degradation (Evidente *et al.*, 2000; Tabacchi *et al.*, 2000; Abou-Mansour *et al.*, 2004; Andolfi *et al.*, 2011). There is also description on the literature of other leaf symptoms of esca chronic form such as drying, dropping, reddening and yellowing (Lecomte *et al.*, 2012). There is also the possibility of symptoms on the berries, called “black-measles”, where small reddish spots appear on the surface of the epidermis (Surico, 2009). The apoplectic form of esca can be characterized by a sudden wilting of the entire plant or one or several arms (Fig. 1.2 D), usually leading to the removal of the grapevine as sanitary measure (Mondello *et al.*, 2018). During this process, leaf symptoms include scorching, dropping and shrivelling, and grape clusters can also dry and stay attached to the vine (Mugnai *et al.*, 1999). For both forms of esca, foliar symptoms appear late in the spring or early summer, but the expression of these

symptoms can be erratic and be expressed on one year, but during the next year, no symptoms are shown by the infected grapevine (Gramaje *et al.*, 2018). Cross-sections of esca affected grapevines can reveal several internal symptoms such as, black spots in the xylem, sometimes surrounded by pink to brown wood discoloration (Figure 1.2 B, C), longitudinal black vascular streaking, silvery or dry wood. In older, infected grapevines, wood may develop a soft yellow to white rot (Fig. 1.2 E, F) (Fischer, 2002; Gramaje *et al.*, 2018).



Figure 1. 2 – Esca symptoms. A – Grapevine “tiger-stripe” disease (GLSD) symptoms on a red cultivar; B - Internal symptoms of black vascular streaking and black spot in the xylem; C – Black spots on the xylem vessels; D – Acute or apoplectic form characterized by sudden wilting of plant; E – White rot develop on the centre of mature grapevines; F – Basidiomycete’s colonization of esca infected grapevine woody tissues.

Photos by P. Reis and C. Rego

Esca aetiology has been a matter of debate amongst scientists during the last two decades, but the current hypothesis is that young grapevines infected with *Phaeomoniella chlamydospora* and/or species of *Phaeoacremonium* can later develop symptoms of esca after colonization by several basidiomycetes species from the genera *Inocutis* Fiasson & Niemelä, *Inonotus* P. Karst, *Fomitiporella* Murrill, *Fomitiporia* Murrill, *Phellinus* Quél, and *Stereum* Hill ex Pers. (Cloete, 2015; Guerin-Dubrana *et al.*, 2019). Nevertheless, Koch’s postulates are yet to be completed to fully support the role of

basidiomycetes on the symptomatology of esca disease (Gramaje *et al.*, 2018). Recently, studies by Brown *et al.* (2020) suggested that basidiomycetes do not require previous infection by *P. chlamydospora* to colonize grapevine wood, but further and more in-depth research is still required. Also, several species of *Cadophora* have been recently associated to esca infected grapevines (Travadon *et al.*, 2015).

1.2.2.4 Phomopsis dieback

Phomopsis dieback has been associated with several species from the genus *Diaporthe* Nitschke (Baumgartner *et al.*, 2013; Úrbez-Torres *et al.*, 2013; Dissanayake *et al.*, 2015; Guarnaccia *et al.*, 2018), being *Diaporthe ampelina* (Berkeley & M.A. Curtis) R.R. Gomes, C. Glienke & Crous (formerly known as *Phomopsis viticola*) considered as the most virulent and the primary cause for the disease. This pathogen has historically been identified as the causal agent of the disease known as Phomopsis cane and leaf spot in the USA, or excoriosis in Europe (Bugaret, 1984). Several symptoms have been associated with this disease on all herbaceous part of the plant (shoots, basal wood, leaves, stems or fruits). On green shoots, small black spots that later develop in well-individualized blackish-brown crusts or brown lesions with strips of corky appearance are common on infected grapevines (Fig. 1.3 A, B). In branches, it can appear as a strangulation at their base, which can lead to breakage under wind or crop weight stresses. During the dormant season, canes show a white appearance with black points at internode zones. Fruits turn brown and wither, with mummies or shrivelled berries close to harvest (Larignon, 2012; Úrbez Torres *et al.*, 2013). Nonetheless, this pathogen has also been associated with perennial cankers, on surveys conducted on the USA (Úrbez-Torres *et al.*, 2013; Baumgartner *et al.*, 2013). The wood symptoms caused by *D. ampelina* are characterized by sectorial necrosis (Fig.1.3 C) and some punctuations of brown colour, which closely resemble the wood symptoms of *Botryosphaeria* and *Eutypa* dieback. This led to a re-evaluation of this fungus as a canker-causing agent, by Úrbez-Torres *et al.* (2013), naming the disease Phomopsis dieback.

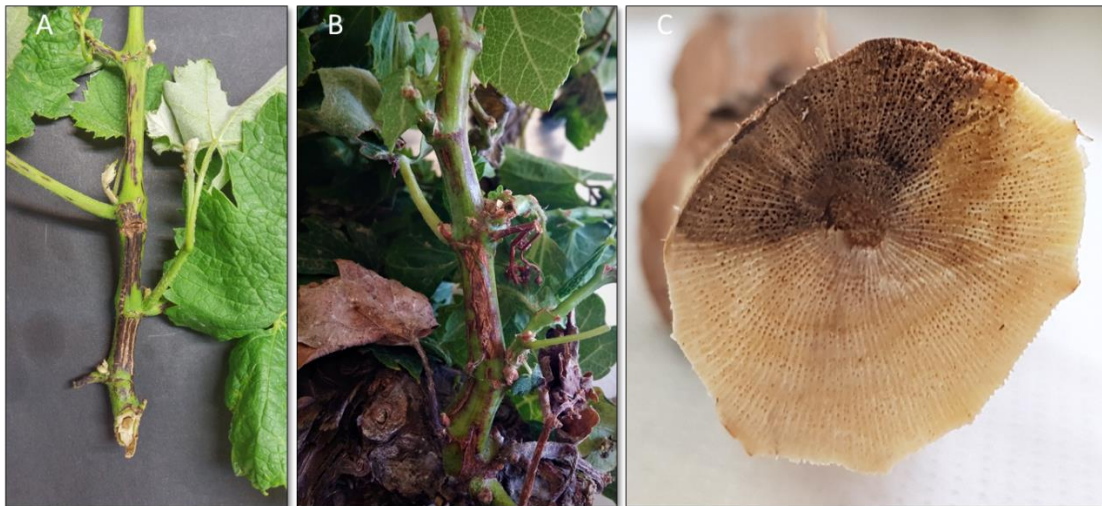


Figure 1. 3 – Phomopsis cane and leaf spot and Phomopsis dieback symptoms. A, B – Lesions of Phomopsis cane and leaf spot on green shoots; C – Wedge shaped canker of Phomopsis dieback similar to those found associated with Botryosphaeria and Eutypa diebacks.

Photos by P. Reis and C. Rego

1.2.2.5 Eutypa dieback

Eutypa dieback affects mainly mature vineyard (>8 years-old) (Gubler *et al.*, 2013), and its symptoms are characterized by stunted shoots with short internodes and usually with chlorotic leaves, often cupped and with tattered margins (Fig 1.4 A). On stunted shoots, bunches are small, ripen unevenly, berries might shrivel and die (Sosnowski *et al.*, 2007; Bertsch *et al.*, 2013, Gramaje *et al.*, 2018). Eutypa dieback has been previously associated with 24 *Diatrypaceae* species (Trouillas *et al.*, 2010; Luque *et al.*, 2012; Rolshausen *et al.*, 2013a; Pitt *et al.*, 2013a), being *Eutypa lata* (Pers.) Tul. & C. Tul. considered as being the most virulent and common species causing symptoms (Trouillas and Gubler, 2010). In fact, only this species has been found as responsible for the described foliar symptoms, which are caused by toxic metabolites produced by the fungi (Mahoney *et al.*, 2005; Trouillas and Gubler, 2010). Like in esca disease, these symptoms are also known to vary from year to year (Sosnowski *et al.*, 2007, 2016).

Internal symptoms on wood include brown sectorial necrosis on cross sections of infected cordons (Fig. 1.4 C), and cordon dieback with loss of spurs. As the disease advances, external cankers appear, characterised by flat areas of the wood with no bark, leading to eventual vine death (Bertsch *et al.*, 2013; Gramaje *et al.*, 2018).



Figure 1. 4 – *Eutypa* dieback symptoms. A, B - Stunted shoots with chlorotic and often cupped leaves; C – Wedge shapes internal necrosis on an infected cordon.

Photos by P. Reis and C. Rego

1.3 Botryosphaeria dieback

Botryosphaeria dieback is currently one of the most important GTDs worldwide (Bertsch *et al.*, 2013), caused by fungi in the family *Botryosphaeriaceae*. Fungi belonging to this family have been found worldwide as endophytes, saprophytes or pathogenic in many perennial and ornamental plants (Smith *et al.*, 1996a, 1996b; van Niekerk *et al.*, 2006; Slippers and Wingfield, 2007; Bellé *et al.*, 2017). Several species of this family are significant plant pathogens causing leaf spots, fruit rots, dieback, perennial cankers, and eventual death in economically important woody perennial crops and ornamental plants as well as both in native and introduced forest trees (Far and Rossman, 2011; Úrbez-Torres *et al.*, 2011). Among all the agricultural species affected by *Botryosphaeriaceae*, grapevines are currently of highly importance and relevance (Úrbez-Torres *et al.*, 2011). Nowadays, *Botryosphaeria* dieback is considered one of the most important GTDs worldwide, and it is reported in vineyards throughout the globe. In Portugal, it is present in all the wine appellations of the country, being reported as frequently occurring in 60% of the established vineyards (Guérin-Dubrana *et al.*, 2019). *Botryosphaeria* dieback alongside with Esca are considered the major GTDs affecting Portuguese vineyards causing considerable damages and economic losses (De la Fuente *et al.*, 2016).

1.3.1 Symptoms

Botryosphaeria dieback, formerly known as black dead arm (BDA) was firstly described in Hungary, in the Tokaj grape growing region, in 1974, at the time associated with *Diplodia mutila* (Lehoczky, 1974; Úrbez-Torres, 2011). At the time, Lehoczky (1974) observed that buds from affected grapevine did not burst and eventually died. In

addition, the bark tissue of the disease cordons and/or trunks became discoloured to dark brown, a characteristic symptom that gave BDA (Black Dead Arm) its name. In 1986, a different disease caused by a botryosphaeriaceous fungus, was reported as cankers caused by *Botryosphaeria dothidea* in Chile in 1986 (Larorre *et al.*, 1986), and was later named as Botryosphaeria canker in 1990 (Leavitt, 1990) to describe symptoms caused by *Lasiodiplodia theobromae* in Southern California. However, since several *Botryosphaeriaceae* species are currently known to cause several dieback symptoms worldwide, and most of the times, the symptoms described for both diseases overlapped, it was concluded that both designations were probably referring to the same symptoms and hence disease (Úrbez-Torres, 2011). Thus, it was suggested by Úrbez-Torres (2011) that symptoms associated with *Botryosphaeriaceae* infection in grapevines should be designated as simply Botryosphaeria dieback, a name which is nowadays used and completely accepted both by growers and researchers.

Although the symptomatology of Botryosphaeria dieback is consequently somewhat complex, the most common symptoms include no or limited burst, trunk dieback, lack of spring growth, bud and xylem necrosis, perennial cankers on green shoots (Fig. 1.5 A, B) and plant death (Larignon and Dubos, 2001; Phillips, 2002; van Niekerk *et al.*, 2004; Úrbez-Torres *et al.*, 2006; Úrbez-Torres, 2011; Linaldeddu *et al.*, 2015, Gramaje *et al.*, 2018). Infection of grapevines by *Botryosphaeriaceae* can also lead to leaf symptoms where yellowish-orange or wine-red spots on leaf margins and blades can appear depending on the cultivar (white or red) (Fig 1.5 C) and inflorescences wilt, fruit rot (Fig. 1.5 D) and cane bleaching. (Phillips, 2000; Larignon *et al.*, 2001, 2009; Úrbez-Torres, 2011; Bertsch *et al.*, 2013; Spagnolo *et al.*, 2014; Reis *et al.*, 2016). Symptom expression of *Botryosphaeriaceae* usually appears 1 to 2 years after infection by the pathogen (Leavitt, 1990; Úrbez-Torres, 2006) and in different countries, species occurring on grapevines have been shown to differ in pathogenicity, in their epidemiology, and in the symptoms produced (van Niekerk *et al.*, 2004; Larignon *et al.*, 2009). Internal symptoms on wood infected by Botryosphaeria dieback are characterized by wedge-shaped perennial cankers which are indistinguishable from those of Eutypa dieback (Fig. 1.5 E), and non-uniform to circular central staining of wood, observable in cross section of

affected cordons and vascular tissue, usually beginning in pruning wounds (Úrbez-Torres *et al.*, 2006, 2008; Úrbez-Torres and Gubler, 2011; Gramaje *et al.*, 2018).



Figure 1. 5 – *Botryosphaeria* dieback symptoms. A – Perennial canker on infected green shoot; B – Canker on the base of green shoot; C – Grapevine showing reduce growth and dieback and foliar symptoms; D – Dried fruit rot on green shoot showing a canker; E – Non-uniform to circular central staining of wood; F – Orange/brown stripes located in the outer xylem, appearing longitudinally when the bark is removed.

Photos by P. Reis and C. Rego

Infection by *Botryosphaeria* dieback pathogens has also been commonly associated with orange/brown stripes located in the outer xylem, appearing longitudinally when the bark is removed (Fig. 1.5 F) These stripes can extend from the base of the infected shoots to the graft unions, or even to the rootstock (Larignon *et al.*, 2001; Abou-Mansour *et al.*, 2015).

Although *Botryosphaeria* dieback is mainly observed in vineyards above 8 years-old, it has also been associated with planting material in grapevine nurseries (Gramaje and

Armengol., 2011) and in younger vineyards (3-5 years-old) (Úrbez-Torres *et al.*, 2008), and can lead to graft failure and central, bud, pith and root necrosis (Úrbez-Torres, 2011).

1.3.2 Aetiology

Several *Botryosphaeriaceae* species are associated worldwide with Botryosphaeria dieback (Moller and Kasimatis 1978; Larignon and Dubos 1997; Graniti *et al.*, 2000; Fischer 2006; Larignon *et al.*, 2009; Úrbez-Torres, 2011; Spagnolo *et al.*, 2014; Larignon *et al.*, 2015). Currently up to twenty-six species in different *Botryosphaeriaceae* genera have been associated with this disease (Úrbez-Torres, 2011; Pitt *et al.*, 2013b, 2013c; Rolshausen *et al.*, 2013b; Yang *et al.*, 2017; Gramaje *et al.*, 2018), namely from the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria* and *Spencermartinsia*.

Nevertheless, the most common species isolated from grapevines are *Diplodia seriata* (Cristinzo, 1978; Castillo-Pando *et al.*, 2001; Larignon *et al.*, 2001; Phillips *et al.*, 2007; Rovesti and Montermini, 1987; Savocchia *et al.*, 2007; Úrbez-Torres *et al.*, 2008, Úrbez-Torres and Gubler 2009a), *Diplodia mutila* (Lehoczky, 1974; Taylor *et al.*, 2005), *Neofusicoccum parvum* (Crous *et al.*, 2006), and *Lasiodiplodia theobromae* (Úrbez-Torres *et al.*, 2008; Luque *et al.*, 2009; Rodríguez-Gálvez *et al.*, 2015).

The development and improvement of DNA sequencing methods, during the last three decades, has allowed the determination of more accurate phylogenetic relationships at various taxonomic levels from species to order (Phillips *et al.*, 2013). Consequently, the taxonomy of *Botryosphaeriaceae* has seen deep changes, which occurred until recently (Phillips *et al.*, 2019) and the observation of only morphological characteristics is no longer sufficient to identify different species of *Botryosphaeriaceae*. Like so, DNA sequencing is today mandatory, to identify the specific *Botryosphaeriaceae* species infecting grapevine. Although the internal transcribed spacer (ITS) region alone is usually enough to separate species within each genus of *Botryosphaeriaceae*, the inclusion of the translation elongation factor 1-alpha (*tef1*- α) is highly recommended to obtain a more robust separation of species, especially in some genera such as *Diplodia*, *Dothiorella*, *Lasiodiplodia* and *Neofusicoccum* (Phillips *et al.*, 2013)

To this day, a great amount of research has been made worldwide to detect and identify *Botryosphaeriaceae* from diseased grapevines. Currently, there are species of fungi responsible for Botryosphaeria dieback, affecting grapevines in all the grape producing regions of the world. Fungi from the referred genera has been isolated and identified from Algeria (Linaldeddu *et al.*, 2015), Australia (Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005; Wood and Wood, 2005; Savocchia *et al.*, 2007; Pitt *et al.*, 2013b, 2013c), Bolivia (Kaiser *et al.*, 2009), Brazil (Correia *et al.*, 2013), Chile (Auger *et al.*, 2004), China (Yan *et al.*, 2013), France (Larignon *et al.*, 2001, 2015; Larignon and Dubos, 2001; Kuntzmann *et al.*, 2009), Hungary (Lehoczy, 1974), Italy (Cristinzo, 1987; Rovesti, 1987; Burruano *et al.*, 2008; Mondello *et al.*, 2013; Linaldeddu *et al.*, 2015, Carlucci *et al.*, 2015), Mexico (Úrbez-Torres *et al.*, 2008), New Zealand (Ridgway *et al.*, 2014), Peru (Rodríguez-Galvez *et al.*, 2015), Portugal (Phillips, 2002; Rego *et al.*, 2010), Spain (Aroca *et al.*, 2008; Luque *et al.*, 2009), South Africa (van Niekerk *et al.*, 2004, 2010), Tunisia (Linaldeddu *et al.*, 2015; Rezgui *et al.*, 2018), and USA (Úrbez-Torres *et al.*, 2006, 2008, 2012; Úrbez-Torres and Gubler, 2009a; Rolshausen *et al.*, 2013b).

From all the pathogenicity and aggressiveness studies performed on *Botryosphaeriaceae* species isolated from infected grapevine, Úrbez-Torres (2011) suggested that species infecting grapevines could be divided into three different virulence rankings including, highly virulent species (*Lasiodiplodia* spp. and *Neofusicoccum* spp.), moderately virulent (*B. dothidea* and *Diplodia* spp.) and slightly virulent (*Dothiorella* spp. and *S. viticola*).

1.3.3 Epidemiology

Grapevine pathogens responsible for Botryosphaeria dieback are primarily spread through the dispersion of airborne spores. Pycnidia of *Botryosphaeriaceae* species are found embedded in disease woody parts of vines, as well as in wood debris left in the vineyard after pruning (Fig. 1.6) (Lehoczy, 1974; Leavitt, 1990; van Niekerk *et al.*, 2004; Úrbez-Torres *et al.*, 2006; Úrbez-Torres, 2011; Elena and Luque, 2016). Under humid conditions, pycnidia of *Botryosphaeriaceae* spp. produce pycnidiospores that are exuded in a gelatinous matrix or forming cirrhi, a dry ribbonlike mass of spores (Ahimera *et al.*, 2004; Michailides *et al.*, 1993; Phillips, 2002; Úrbez-Torres *et al.*, 2006, 2010). Consequently, once the impact of water droplets over pycnidia cease following the

termination of rain or irrigation, *Botryosphaeriaceae* pycnidiospores may not be dispersed. Therefore, the dissemination of spores happens primarily through water splash and occurs over relatively short distances (Fig. 1.6) (Ahimera *et al.*, 2004; Baskarathevan *et al.*, 2010; Úrbez-Torres *et al.*, 2010; Úrbez-Torres, 2011). Although *Botryosphaeriaceae* fungi are found as endophytes or parasites and saprophytes on a vast number of both annual and perennial plants, it is currently well accepted that most of the inoculum leading to new infections is produced within the vineyards (Úrbez-Torres, 2011). Seasonal patterns of conidial release have been shown to vary from one grapevine growing region to another; however, conidial release has always been shown to be correlated with rain events (Kuntzmann *et al.*, 2009; Serra *et al.*, 2007; Úrbez-Torres *et al.*, 2010).

To infect and colonise grapevine tissues, it is well assumed that botryosphaeriaceous fungi require fresh wounds as entry points. Each successful infection initially occurs with the simultaneous occurrence of inoculum and a wound, although it is still unclear whether some fungi can enter through uninjured tissues (Feliciano and Gubler, 2001), or if infections could be initiated from endophytic or latent propagules after being triggered by some environmental changes as occurs in forest trees (Baum *et al.*, 2003; Claverie *et al.*, 2020). While other types of wounds (caused by biotic or abiotic stresses) can also play roles, winter pruning wounds are the major point of entry for *Botryosphaeria* dieback fungi (Figure 1.6). Nonetheless, Shafi *et al.* (2019) recently showed by fluorescence microscopy that these pathogens can remain latent on the grapevine bark, and even without any type of wounding, germinating conidia and mycelium could be observed near lenticels, as well as mycelia in the underlying wood, demonstrating that the pathogens had infected through the lenticels.

Úrbez-Torres and Gubler (2011) presented evidence that pruning wound infections by species of *Botryosphaeriaceae* occur mainly during the dormant season in California, especially when vines were pruned from November to January. In a similar study conducted in Italy, conidia of *Diplodia seriata* were shown to infect grapevine pruning wounds up to late spring/early summer (Serra *et al.*, 2007). Consequently, it is likely that *Botryosphaeriaceae* spores germinate and infect grapevines under a wide range of different environmental conditions including temperature. In fact, Úrbez-Torres *et al.*,

(2010) concluded that *Botryosphaeriaceae* species that have the highest optimal temperatures for conidial germination, such as is the case of *L. theobromae*, where usually the dominant species in vineyards established in warmer grape-growing regions and were only rarely isolated from diseased vines in cold regions. *Diplodia seriata* showed the greatest germination percentage and optimum mycelial growth under the widest range of temperatures (10 - 40°C), which may explain why this species is probably the most cosmopolitan botryosphaeriaceous fungus infecting grapevines (Úrbez-Torres, 2011). This relation between spore release and geographical location and weather conditions, and therefore high-risk infection periods, have been reported to vary throughout the growing season and from year to year, but mainly overlaps with dormant seasons both in both hemispheres (Larignon and Dubos, 2000; Amposah *et al.*, 2009; Kuntzmann *et al.*, 2009; Úrbez-Torres *et al.*, 2010; van Niekerk *et al.*, 2010; Valencia *et al.*, 2015).

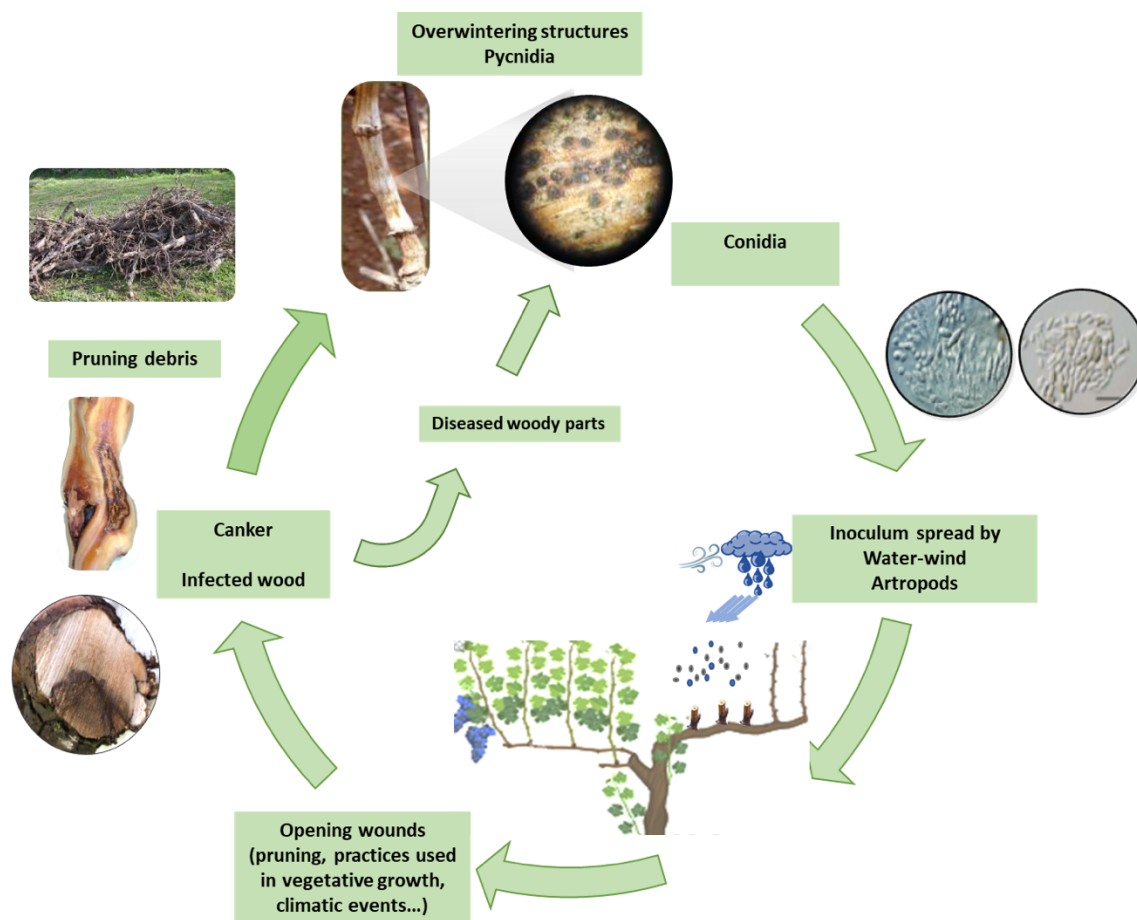


Figure 1. 6 - Disease cycle of *Botryosphaeria* dieback on grapevine.

Nevertheless, these fungi can also infect grapevines during the grafting and production processes in nurseries. Wounds created during the production of ready-to-sell plants have shown to be a port of entry for botryosphaeriaceous fungi, and the analysis of plants obtained from nurseries have previously allowed for the detection of fungi responsible for Botryosphaeria dieback (Vigues *et al.*, 2007; Larignon *et al.*, 2009; Spagnolo *et al.*, 2011). This suggests that some steps in the nursery processes (rehydrating, grafting, callusing and in nursery field rooting) may be important for infection with *Botryosphaeriaceae* (Claverie *et al.*, 2020).

1.3.4 Cultivar response to *Botryosphaeriaceae* infection

Studies on cultivar response to *Botryosphaeriaceae* infection, based on lesion length of several cultivars of *V. vinifera* and other *Vitis* spp., have been performed revealing variation in susceptibility (Úrbez-Torres *et al.*, 2008; Úrbez-Torres and Gubler, 2009a; Savocchia *et al.*, 2007; Pitt *et al.*, 2013a; Travadon *et al.*, 2013; Billones-Baaijens *et al.*, 2014; Carlucci *et al.*, 2015; Guan *et al.*, 2016; Ramírez *et al.*, 2018). Sosnowski *et al.* (2016) also reported a vast variation in GTD symptoms on mature grapevines that were part of germplasm repository, where they found significant differences in the rate of pathogen colonization of grapevine canes among cultivars and rootstocks, to try to find less susceptible or even tolerant cultivars. Travadon *et al.* (2013) showed on their work that Thompson seedless was the most susceptible to infection by *N. parvum*, while the least susceptible cultivars tested were Merlot and Concorde. In this study, there was a trend of greater susceptibility of Thompson seedless compared to the wine cultivar Chardonnay to *L. theobromae* and *N. parvum*. Nevertheless, these results were in contrast with past studies in which Thompson seedless that was found to be more resistant than Chardonnay to *L. theobromae* (Úrbez-Torres *et al.*, 2008). Amponsah *et al.* (2011) showed that the detached green shoots of Sauvignon Blanc, Riesling, Chardonnay, Pinot noir and Cabernet Sauvignon were all equally susceptible to four *Botryosphaeriaceae* species. In fact, the high number of species of *Botryosphaeriaceae* affecting grapevine, and the variability of aggressiveness between them makes assessing cultivar responses rather complex. This high variability in aggressiveness among isolates of *Botryosphaeriaceae*, such as *N. parvum*, *N. luteum* and *N. australe*, for example has

been consistently reported in several studies (van Niekerk *et al.*, 2004; Úrbez-Torres and Gubler, 2009a; Billones-Baaijens *et al.*, 2013). Therefore, the use of one isolate for a susceptibility test may not reflect the general level response of the teste cultivar.

Several hypotheses could arise to explain these discrepancies. First and foremost, since there are numerous cultivars of grapevines, differences on susceptibility may come from intrinsic properties of each cultivar such as vessel dimension. Morphological analysis by Pouzolet *et al.* (2014) showed that vessel dimension varied among cultivars, which may influence and/or restrict the pathogen progression in the grapevine. Also, grapevine cultivars may also possess several different clones and thus, variation among different clones of the same cultivar, may also contribute to different cultivar response (Travadon *et al.*, 2013). Recently, Berlanas *et al.* (2017) showed that there was clear variation of response of several clones of “Tempranillo” to GTDs, which creates further evidence of the influence of this factor on grapevine response to *Botryosphaeriaceae* infection. Other factor also to be considered is plant age. Plant age is considered a key factor in biotic and abiotic stress responses, as the effect of the combination of stress on plants is largely dependent on their age (Pandey *et al.*, 2015), existing a positive correlation between grapevine age and GTD incidence (Brown *et al.*, 2016; Fischer and Peighami-Ashnaei, 2019). Also, climatic and edaphic factor have been confirmed as influencing the expression of several GTDs (Graniti *et al.*, 2000; Sosnowski *et al.*, 2011; van Niekerk *et al.*, 2011; Calzarano *et al.*, 2018). This leads to the hypothesis that solely the pathogen is not responsible for the expression of disease symptoms (Fischer and Peghami-Ashnaei 2019). Such abiotic factors of stress can have an impact on plant defence mechanisms, which strongly impact resistance and/or susceptibility (Amtmann *et al.*, 2008; Kulday and Yates, 2000; Mittler and Blumwald, 2010). In fact, the impact of abiotic stresses, such as drought and/or thermal stress, have been previously reported to influence grapevine response to *Botryosphaeria dieback* (Songy *et al.*, 2019). Hence, the many factors underlying the response of cultivar s, not only to *Botryosphaeria dieback* but to GTDs in general, are extremely complex, not only due to biotic and abiotic stresses, but also due to the large number of cultivars, planted worldwide, that may present only minor differences from each other.

1.3.5 Control

Although great advances have been made in developing and implementing control techniques against *Botryosphaeria* dieback, the wide range of different species of pathogens still constitutes a challenge for the management of this GTD (Úrbez-Torres, 2011). Recent research has generated knowledge on identifying high-risk infection and susceptibility periods throughout the year, which can assist on developing and maintaining effective management strategies against *Botryosphaeria* dieback (Gramaje *et al.*, 2018). Nevertheless, although these breakthroughs on susceptibility periods, and several other promising solutions currently exists, it still seems impossible to control this disease using just one approach. Presently, an integrated pest management (IPM) strategy is recommended from the nursery to the vineyard, integrating cultural practices, biological control agents (BCAs) and responsible and sustainable use of fungicides (Sosnowski and McCarthy, 2017; Mondello *et al.*, 2018; Gramaje *et al.*, 2018).

1.3.5.1 Cultural practices

As stated previously, *Botryosphaeria* dieback causing fungi can infect grapevine during the nursery processes, namely rehydrating, grafting, callusing and in nursery fields (Claverie *et al.*, 2020). Several cultivation practices in mother plants can have a direct effect on this disease incidence, and therefore on the quality of grafting material (Gramaje *et al.*, 2018). Although, on nursery mother blocks, overhead sprinkling has been considered a good method of irrigation, it has been considered that this method can trigger the release of *Botryosphaeriaceae* conidia in some vineyard site in California (Gubler *et al.*, 2013; Úrbez-Torres *et al.*, 2010, Gramaje *et al.*, 2018) and should therefore be avoided. Also, burial and/or removal of dead wood and pruning debris in source blocks is currently highly recommended since they can become huge sources of inoculum for new infections (Elena and Luque, 2016). The hydration process where plant material undergoes long soaking periods can be a threat to the phytosanitary condition of the cuttings, since this process can promote infection by pathogens (Pollastro *et al.*, 2009; Aroca *et al.*, 2010; Gramaje *et al.*, 2011; Agustí-Briasch *et al.*, 2013). The following process of callusing, where cuttings are subjected to high temperatures, can further increase the susceptibility to *Botryosphaeria* dieback infection (Waite *et al.*, 2005; Gramaje *et al.*, 2018).

On established vineyards, the practice of removing pruning debris and all dead or infected tissues is also highly recommended (Elena and Luque, 2016; Gramaje *et al.*, 2018) as a sanitation practice. The pycnidia of botryosphaeriaceous fungi are primarily developed on the referred dead and infected wood, and therefore pruning debris left on the vineyard are a reservoir of inoculum (Úrbez-Torres *et al.*, 2010; Elena and Luque, 2016). This wood can be destroyed by burning, burying, mulching and incorporation into the soil, or composting. Since burning has obvious environmental implications, presently mulching or composting are recommended (Gramaje *et al.*, 2018).

The management of *Botryosphaeria* dieback has also been traditionally achieved by remedial surgery. This method includes pruning out all the infected wood and cordons and/or trunk, at least 10 cm below the vascular symptoms, and vines should be re-trained from healthy shoots (Úrbez-Torres, 2011). This method has proven to control *Botryosphaeria* dieback in USA (Leavitt, 1990) and more recently studies on the use of remedial surgery in Australian vineyards, also pointed out the extreme importance of low trunk cuts to ensure that all the infected wood is removed (Savocchia *et al.*, 2014). Although remedial surgery is considered an expensive operation (Epstein *et al.*, 2008), it still remains cheaper and more cost effective than uprooting and re-planting an entire vineyard (Sosnowski and McCarthy, 2017). However, the success of remedial surgery can be limited if the contamination has reached the ground or graft union. Moreover, the possibility of fungi being present in asymptomatic tissue (Gonzalez and Tello, 2011; Bruez *et al.*, 2014) constitutes another limitation to the success of this procedure.

One of the most important cultural practices on grapevine is pruning. It is considered the main point of entry for *Botryosphaeria* dieback pathogens, and therefore a good pruning management is of paramount importance for vineyard sustainability. Spore release from *Botryosphaeriaceae* fruiting bodies is shown to be linked to rainfall events. Therefore, epidemiological studies around the world have allowed for the establishment of what are considered the most high-risk periods for inoculum pressure (Gramaje *et al.*, 2018), and consequently pruning in wet conditions should be avoided. Double pruning has shown to be effective in reducing *Botryosphaeria* dieback pathogen infections in Californian vineyards (Úrbez-Torres and Gubler, 2009b, 2011; Úrbez-Torres *et al.*, 2010). The concept behind this method is that the final wound is to be made when the

pathogens are less likely to successfully colonize exposed wounds, either because their presence in the environment is low, or due to low pruning wound susceptibility (Úrbez-Torres, 2011). In California, the period of less inoculum levels is during late winter, which will reduce the risk of infection of fresh pruning grapevines (Gramaje *et al.*, 2018). However, recent epidemiological studies in Spain showed that in this case the highest levels of infection by *Botryosphaeria* dieback pathogens were in late winter, in opposition with late fall, which contradicts the pruning recommendations from the Californian studies. In this case, early pruning was recommended to mitigate pathogen infection (Luque *et al.*, 2014; Elena and Luque, 2016). These variation in inoculum availability among grape growing regions throughout the world leads to the conclusion that epidemiological studies should be conducted on a regional basis, to optimize pruning strategies (Gramaje *et al.*, 2018). Also, to complement a good management strategy (training system and pruning methods), a minimal pruning system, where the number of wounds per vine is minimized is also recommended.

1.3.5.2 Chemical control

Since, as referred previously, *Botryosphaeriaceae* infect mainly through wounds, the majority of strategies developed to control these pathogens have been based on protection of pruning wounds (Úrbez-Torres, 2011). Pruning wound protection is considered the most valuable strategy to control *Botryosphaeria* dieback, especially if adopted since the vineyard implementation (Kaplan *et al.*, 2016; Sosnowski and McCarthy, 2017; Gramaje *et al.*, 2018). Extensive research on the chemical control of *Botryosphaeria* dieback, has been performed over the last three decades. Leavitt (1990) was the first to have some success in reducing incidence of *Botryosphaeria* dieback pathogens, by applying benomyl, captan, iprodione and penconazole paints as pruning wound protectants. Since then, the field efficacy of chemical wound protectants has been demonstrated in several grape-growing regions worldwide. The now banned benzimidazoles, such as benomyl, carbendazim and thiophanate-methyl have shown a high efficiency in controlling *Botryosphaeria* dieback pathogens, both *in vitro* and on the field, despite the geographical location, and were, and are still used as positive controls in several research studies (Mondello *et al.*, 2018). These active substances proved to be effective in vineyards in Australia (Pitt *et al.*, 2012), Chile (Díaz and Latorre, 2013),

New Zealand (Amponsah *et al.*, 2012; Sosnowski and Mundi, 2019), Portugal (Rego *et al.*, 2014), South Africa (Bester *et al.*, 2007; Mutawila *et al.*, 2015), Spain (Martínez-Diz *et al.*, 2021) and USA (Rolshausen *et al.*, 2010). These active ingredients were considered to be optimally used as preventive pruning wound treatments, and only to a lesser extent, as curatives (Mondello *et al.*, 2018). Fungicides like fluazinam have also shown good efficiency in protecting pruning wounds against *Botryosphaeriaceae* in Australia (Pitt *et al.*, 2012) and New Zealand (Amponsah *et al.*, 2012; Sosnowski and Mundi, 2019). It is thought that this efficiency is due to this contact fungicide being broad-spectrum with high blocking activity of conidial germination (Mondello *et al.*, 2018). Also, pyraclostrobin has shown some effective reduction of *Botryosphaeriaceae* in California (Rolshausen *et al.*, 2010), and other active substances such as the organic salt fosetyl-Al has shown some promising results in reducing pathogen infection on protected pruning wounds (Di Marco *et al.*, 2011; Díaz and Latorre, 2013; Mondello *et al.*, 2018). The use of organic disinfectants has also been researched, especially for use in nurseries, where didecyldimethylammonium chloride has shown positive effects against *Botryosphaeria* dieback (Fourie and Halleen, 2006; Gramaje *et al.*, 2009; Gramaje and Armengol, 2011).

Another well researched and proven method to be highly effective in controlling *Botryosphaeriaceous* fungi is the application of pastes and paints amended or not with fungicides. These products can provide a physical barrier that can provide the germination of spores on the pruning wound, and in case this barrier fails due to any external factor, the presence of a fungicide, will also act on the pathogen (Gramaje *et al.*, 2018). Pitt *et al.* (2012) have shown that protecting pruning wounds with a paste formulated with cyproconazole and iodocarb was effective against *Botryosphaeria* dieback pathogens, and acrylic paints amended with thiophanate methyl also provide good control of these pathogens (Díaz and Latorre). More recently a formulation of pyraclostrobin + boscalid with a sealing polymer was tested in Germany (Kuhn *et al.*, 2017; Lengyel *et al.*, 2019), Greece (Kühn *et al.*, 2017; Samaras *et al.*, 2019) and Spain (Kühn *et al.*, 2017; Martínez-Diz *et al.*, 2019) where it showed to be highly effective in controlling *Botryosphaeria* dieback pathogens. The only downside to the application of this type of products is that they must be applied by hand, on every pruning wound, which can be very expensive and time consuming (Sosnowski and McCarthy, 2017).

The application of site targeted fungicides has also been recently investigated (Fischer *et al.*, 2019; Battiston *et al.*, 2018, 2021; Wu *et al.*, 2020; Mondello *et al.*, 2021). The most promising results against *Botryosphaeria* dieback pathogens have been reported by Mondello *et al.* (2021) where they were able to reduce lesion length on grapevines inoculated under controlled conditions, with the application of a copper sulfate pentahydrate salt (Cu^{2+} 35 g/L) distributed throughout the plant with carrier molecules of hydroxyapatite. The treatment with this new formulation also showed an ability to stimulate plant growth and photosynthesis, as well as an elicitation effect on several genes linked to plant defense (Mondello *et al.*, 2021). This complex category of biostimulants and elicitors is currently under revision at EU regulation and, although elicitation alone cannot be considered a miracle solution to control *Botryosphaeria* dieback and other plant diseases, it appears to be a good solution to use in combination with other management strategies (Delaunoy *et al.*, 2014; Mondello *et al.*, 2021).

1.3.5.3 Biological control

The increase consumer demands for products free of chemical use have created a peak on the interest of natural products such as biocontrol agents (BCAs). Several microorganisms have been evaluated both *in vitro* and *in planta*, such as *Pseudomonas*, *Bacillus* and *Enterobacter* species as well as fungi such as *Fusarium* and *Trichoderma* (Hunt *et al.*, 2001; Di Marco *et al.*, 2004; John *et al.*, 2004; Compant *et al.*, 2013; Compant and Mathieu, 2017; Mondello *et al.*, 2018; Trotel-Aziz *et al.*, 2019; Leal *et al.*, 2021; Martínez-Diz *et al.*, 2021). *Trichoderma* species are the most studied as bio-pesticides (Úrbez-Torres *et al.*, 2020), and several products based on these fungi are currently used as pruning wound protectants against *Botryosphaeria* dieback (Gramaje *et al.*, 2018; Mondello *et al.*, 2018). *Trichoderma* species are known to directly antagonize pathogens by competition for nutrients and space, mycoparasitism and antibiosis (Harman, 2006; Vinale *et al.*, 2008; Waghunde *et al.*, 2016). They have great potential as pruning wound protectants since they can maintain activity against *Botryosphaeria* dieback pathogens for extended periods of time, which is the main disadvantage of chemical wound protectants that do not confer protection throughout the whole contamination period, that can last from 2 to 4 months (Kotze *et al.*, 2011; Mondello *et al.*, 2018). Several studies have been made using different *Trichoderma* species and strains against

Botryosphaeriaceae (Mondello *et al.*, 2018) in South Africa (Mutawila *et al.*, 2011, 2015, 2016), Spain (Bergal *et al.*, 2020; Martínez-Diz *et al.*, 2021; Leal *et al.*, 2021), France (Mounier *et al.*, 201, 2016; Leal *et al.*, 2021) and Portugal (Reis *et al.*, 2017). However, results obtained with these *Trichoderma*-based products have been inconsistent not only for *Botryosphaeria dieback*, but also to the generality of GTDs and have been reported by several authors (John *et al.*, 2005; Pitt *et al.*, 2012; Mutawila *et al.*, 2011; Halleen *et al.*, 2016; Martínez-Diz *et al.*, 2021). For example, the exact same strain of *T. atroviride* (strain I-1237) has shown good efficacy in reducing *Botryosphaeria dieback* pathogens in both Portuguese (Reis *et al.*, 2017) and French (Mounier *et al.*, 2014), but recently, Martínez-Diz *et al.* (2021) reported that practically no control was obtained with the same product in Spanish vineyards. The efficacy of *Trichoderma*-based products is linked with the ability of the fungi to establish themselves within the wound. Therefore, one of the reasons for this discrepancy in efficacy can be related with the myriad of grapevine cultivars planted worldwide as was pointed by Mutawila *et al.* (2015). It has been hypothesized that these differences can be due to the plant defense response triggered by the plant to the presence of *Trichoderma*, that can differ among cultivars which has been recently corroborated by Leal *et al.* (2021) that verified different priming stimulus towards different cultivars.

1.4 Objectives and thesis outline

As stated in the general introduction, grapevine trunk diseases (GTDs) are considered the most important challenge to modern viticulture. It can cause large yield reductions and increase production costs adding to the already existing costs of annual viticultural operations.

These diseases are caused by several ascomycetous and basidiomycetous fungi, that can cause a slow or rapid decline because of vascular colonization and/or toxin production. In Portugal, *Botryosphaeria dieback* and Esca are considered the major disease affecting grapevines, causing considerable economic losses, but *Botryosphaeria dieback* is also, currently considered one of the most important GTDs worldwide, but the symptomatology of *Botryosphaeria dieback* is somewhat complex. Moreover, there are several different species of *Botryosphaeriaceae* currently associated with *Botryosphaeria dieback*. The genus *Lasiodiplodia* includes pathogenic species that can

cause damages in a wide range of hosts worldwide, but with higher incidence in the tropical and sub-tropical regions. Species of this genus, especially *Lasiodiplodia theobromae*, have recently been more commonly associated to Botryosphaeria dieback symptoms in grapevines, not only on warmer regions, but also on regions with more temperate climates. The reason for this phenomenon is still not clear, but it has been hypothesized that it may be due to the introduction of viticulture in new regions, trade globalization and consequent spread of pathogens to new areas, or even as consequence of climate change. Despite the species involved, the management of Botryosphaeria dieback is currently a challenge, especially with the increasing restrictions on pesticide usage. It is well known that there is an increasingly higher demand for more sustainable practices, based on integrated pest management (IPM) incorporating several, such as biocontrol agents and tolerant cultivars is required to be further researched and posteriorly implemented to try to minimize the impact of Botryosphaeria dieback in vineyards.

Thus, the relevance of Botryosphaeria dieback is already established, but the impact that more uncommon species such as *Lasiodiplodia* spp. may pose to Portuguese and worldwide viticulture is still fairly unknown. Although these species have only been reported as pathogens with lower incidence than species from the *Diplodia* and *Neofusicoccum* genera, they still constitute an important research topic due to, firstly, been considered as some of the most aggressive species associated with Botryosphaeria dieback, and secondly the more common appearance in wine regions with temperate climates. Therefore, this doctoral thesis set out to address questions as:

Are there symptoms that may have been overlooked or associated with other pathogens, that may be in fact related to Botryosphaeria dieback pathogens?

How does grapevines respond to *Lasiodiplodia* spp. infections, specifically in economically important cultivars?

Is it possible to mitigate the impact of *Lasiodiplodia* spp. through the combination of new, improved and more sustainable control strategies?

To achieve these goals, and to try to answer these questions this PhD thesis is divided into five chapters. On each chapter we tried to address each one of the questions above.

Each chapter corresponds to one or more published or submitted paper to peer-review journals. Below, a brief summary describing each chapter is provided.

Chapter 2. In this chapter we did a review of several methods that have been developed for laboratory and field work used to answer the numerous questions on the complexity of GTD infection and symptom development. These methods have been used to test various hypotheses, and the main objectives of this chapter were to illustrate the methods currently used and their respective purpose. The protocol used is described, its values and limitations, and its vantages and disadvantages are discussed. This set out the basis for all the methods used throughout this work to try to reach and unlock our objectives.

Chapter 3. In this chapter we characterized symptoms, that grapevine growers and field technicians in the central region of Portugal started to observe during the spring of 2018. These symptoms appeared even after the planned fungicide applications against other grapevine diseases, which was puzzling for the growers. The objectives of this chapter were to identify the pathogens responsible for these symptoms by means of morphological features and DNA analysis. We also fulfilled Koch's postulates therefore ensuring the pathogenicity of the pathogens found.

Chapter 4. *Lasiodiplodia* species have been increasingly isolated from grapevines grown not only in tropical and subtropical regions, but also in regions with more temperate climates, including Portugal. The status of this genus is still unclear, so we set out to evaluate the pathogenicity and virulence of *Lasiodiplodia* isolates obtained in Portugal or collected in several different grapevine growing worldwide regions. We inoculated several of the most planted cultivars in Portugal, both on plants kept in a greenhouse and field grown, in order to test their response to infection with these pathogens and, therefore, have an idea of a scale of cultivars susceptibility. This will provide knowledge on the possible impact that the further progression of this genus in Portugal may represent.

Chapter 5. Disease management: screening new control strategies for *Lasiodiplodia* spp. infection on grapevine. We tested new solutions for a more sustainable mitigation of infection by *Lasiodiplodia* spp. The work presented on this chapter covers the evaluation

of a biocontrol agent and a new site-targeted copper formulation, and the combination between both, in providing an elicitor effect to plant defenses, and also as a strategy for pruning wound protection. Therefore, this chapter is divided into two sub chapters: Sub-Chapter 5.1. a field test, over two growing seasons, to evaluate a BCA formulation applied alone or in combination with the new site-targeted copper formulation. We compared the efficacy of this strategy with a more conventional strategy of applying a commercially available chemical pruning wound protectant alone and again in combination with the copper formulation; Sub-Chapter 5.2. A greenhouse assay was set up in order to evaluate the potential of both products in controlling *Lasiodiplodia theobromae*. Also, the induction of defense response of grapevines was assessed by looking into the expression of targeted genes, to look for a possible elicitor effect.

Chapter 6. This chapter contains a general summarizing of the aims achieved on this thesis as well as concluding remarks, on how the output of this work have led to suggestions for future research projects and opportunities and what practical applications they may have.

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CHAPTER 2.
RESEARCH METHODS IN GRAPEVINE TRUNK DISEASES

***Vitis* Methods to Understand and Develop Strategies for Diagnosis and Sustainable Control of Grapevine Trunk Diseases**

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2. *Vitis* Methods to Understand and Develop Strategies for Diagnosis and Sustainable Control of Grapevine Trunk Diseases

Abstract

Vitis vinifera is affected by many diseases every year, depending on causal agents, susceptibility of cultivars, and climate region. Some are caused by a single agent, such as gray mold caused by *Botrytis cinerea*, or powdery mildew caused by *Erysiphe necator*. Others result from the actions of a complex of pathogens such as grapevine trunk diseases (GTDs). GTDs are presently among the most devastating diseases in viticulture worldwide because both the economic losses and the long-term sustainability of vineyards are strongly affected. The complexity of GTDs results from the diversity of associated fungi, the undetermined period of latency within the vine (asymptomatic status), the erratic foliar symptom expression from one year to the next, and, probably correlated with all of these points, the lack of efficient strategies to control them. Distinct methods can be beneficial to improve our knowledge of GTDs. *In vitro* bioassays with cell suspensions, calli, foliar discs, full leaves, or plantlets, and *in vivo* natural bioassays with cuttings, grafted plants in the greenhouse, or artificially infected ones in the vineyard, can be applied by using progressive integrative levels of *in vitro* and *in vivo*, depending on the information searched. In this review, the methods available to understand GTDs are described in terms of experimental procedures, main obtained results, and deliverable prospects. The advantages and disadvantages of each model are also discussed.

Keywords: Botryosphaeria dieback, callus, cell suspension, cutting, *Diplodia seriata*, esca disease, Eutypa dieback, *Eutypa lata*, grafted plant, *Neofusicoccum parvum*, *Phaeoacremonium minimum*, *Phaeomoniella chlamydospora*, plantlet, pruning wound, vineyard.

2.1 Introduction

Vitis vinifera L. is one of the oldest cultivated crops, with important human nutritional and health benefits and of great economic impact worldwide. However, it is susceptible to attack by a diverse range of phytopathogens that compromise the productivity and longevity of the vines, affect rooting capacity, plant architecture and vitality and ultimately, resulting in mortality (Ferreira *et al.*, 2004). Among these diseases, grapevine trunk diseases (GTDs) are currently considered to be the most important challenge for viticulture (Bertsch *et al.*, 2013; De la Fuente *et al.*, 2016). These destructive diseases cause severe damage every year and they are of rapidly growing concern in all wine-producing countries of the world. GTDs harm the sustainability of winemaking heritage because the causal agents attack the perennial organs, ultimately killing the vines in either the short or long term. Esca disease, which is defined as a disease complex associated with vascular and wood decay pathogens, and Eutypa and Botryosphaeria diebacks are the main decay diseases, while Petri disease, black foot disease, and Phomopsis dieback are seen mostly in young vineyards.

Most GTD pathogens colonize the perennial woody tissues of vines, infecting via pruning wounds (Rolshausen *et al.*, 2010). Wood symptoms consist of brown wood streaking, internal necrotic lesions, brown stripes in the outer xylem, or perennial cankers, with symptoms differing according to the disease (Mugnai *et al.*, 1999; Mondello *et al.*, 2018). Associated with symptoms in the wood, foliar symptoms may appear, although their expression is not systematic and can be discontinuous from one year to the next due to environmental, climatic, and cultural changes (van Niekerk *et al.*, 2011; Murolo and Romanazzi, 2014). For Eutypa dieback, foliar symptoms are stunted shoots and small, chlorotic leaves with cup-shaped deformation appearing early in the growing season (Mondello *et al.*, 2018). Symptoms of the esca disease complex are visible in summer, when leaf stripe symptoms can be seen on the entire plant as well as a sudden wilting of part or all of the plant (i.e., apoplexy) (Mugnai *et al.*, 1999; Lecomte *et al.*, 2012; Gramaje *et al.*, 2018; Mondello *et al.*, 2018). Minute black spots called “black measles” are frequently seen on the berries. The first symptoms of Botryosphaeria dieback are visible from budburst in spring and consist of dead spurs or buds associated with stunted or delayed growth, as is sometimes observed for Eutypa dieback. According to some

authors, a severe form similar to esca apoplexy can also appear (Larignon *et al.*, 2001). Foliar symptoms, when present, include chlorosis and discoloration similar to esca. Eutypa dieback is now known to be associated with 24 species in the family *Diatrypaceae*, of which the most virulent and common is *Eutypa lata* (Carter, 1991; Gramaje *et al.*, 2018; Mondello *et al.*, 2018). Typical foliar symptoms of Eutypa dieback have been reproduced following artificial infection with *E. lata* (Petzoldt *et al.*, 1981; Péros and Berger, 1994; Sosnowski *et al.*, 2007; Camps *et al.*, 2010). Pioneer fungi of esca disease are *Phaeomoniella chlamydospora* and species of the genus *Phaeoacremonium*, with *Phaeoacremonium minimum* being the most prevalent and virulent (Gramaje *et al.*, 2018). To a lesser extent, other fungi belonging to the genera *Fomitiporia* and *Stereum* are also associated with esca disease (Cloete *et al.*, 2015). At least 26 botryosphaeriaceous taxa are associated with GTDs, especially Botryosphaeria dieback (Úrbez-Torres, 2011; Gramaje *et al.*, 2018), but the most frequently isolated and virulent are from the genera *Diplodia*, *Lasiodiplodia*, *Neofusicoccum*, and *Neoscytalidium*. All of these GTD pathogens can produce phytotoxins (Andolfi *et al.*, 2011; Abou-Mansour *et al.*, 2015; Cimmino *et al.*, 2017), which are translocated to the leaves via the xylem and may be responsible for the emergence of foliar symptoms (Mugnai *et al.*, 1999). Several hypotheses have been proposed relating to the role of the various pathogens in symptom expression, their lifestyle, the flexibility of their aggressiveness according to their associated metabolites, the molecular dialog between the grapevine and the microbiome, and their role in the development of occlusions or other hydraulic consequences. The complexity of GTDs could be explained by the diversity of fungi and the metabolites they produce (toxins and exopolysaccharides), especially their contribution to the expression of several symptoms associated with GTDs. These factors, together with the incomplete knowledge about the influence of environmental factors on the development of GTD symptoms, have resulted in a lack of information on the connection between the actors conditioning GTDs expression. In some cases, Koch's postulates are needed to validate the role of GTD pathogens. Over the years, several methods have been developed and transferred from the laboratory to the field to answer questions about GTDs and test various hypotheses. In this review, the methods currently used, and their respective purpose are described (Table 2.1).

An overview of each method will be provided by describing the protocol used, and their values, limitations, advantages, and disadvantages will be described and discussed. The main information that each method can provide is also discussed.

Table 2.1 – Set of methods to be reviewed and their use

Cell suspension and callus cultures	Plantlet and foliar explants	Entire plant assay with artificial inoculation	Field assays for validation through artificial inoculation
Necrosis development	Toxin effects	Foliar symptom expression	Fungal colonization and disease expression
Growth inhibition of callus	Necrosis development	Necrosis development	Necrosis development
Molecular effects	Physiological effect	Microscopy analysis	Molecular effect
	Molecular effect	Molecular effect	Protein expression

2.2 Cell Suspension and callus cultures to detect molecules associated with phytotoxicity or aggressiveness of GTD pathogens and to describe early host signaling events

The causal agents of GTDs can produce several metabolites, some of which might be considered to be toxins (Evidente *et al.*, 2011; Abou-Mansour *et al.*, 2015). They are translocated to the leaves via the xylem and are suspected of being involved in the expression of foliar symptoms (Mugnai *et al.*, 1999). A great diversity of pathogen-derived biochemicals is currently under study to decipher their role in GTD foliar symptom expression, such as secreted proteins (Bénard-Gellon *et al.*, 2015), exopolysaccharides (Bruno and Sparapano, 2006), and secondary metabolites (Abou-Mansour *et al.*, 2004). Cell suspensions and calli, composed of undifferentiated cells, can be useful to easily and quickly test the toxicity of these compounds and relate them to their roles in pathogen virulence (Fig. 2.1; Table 2.2).

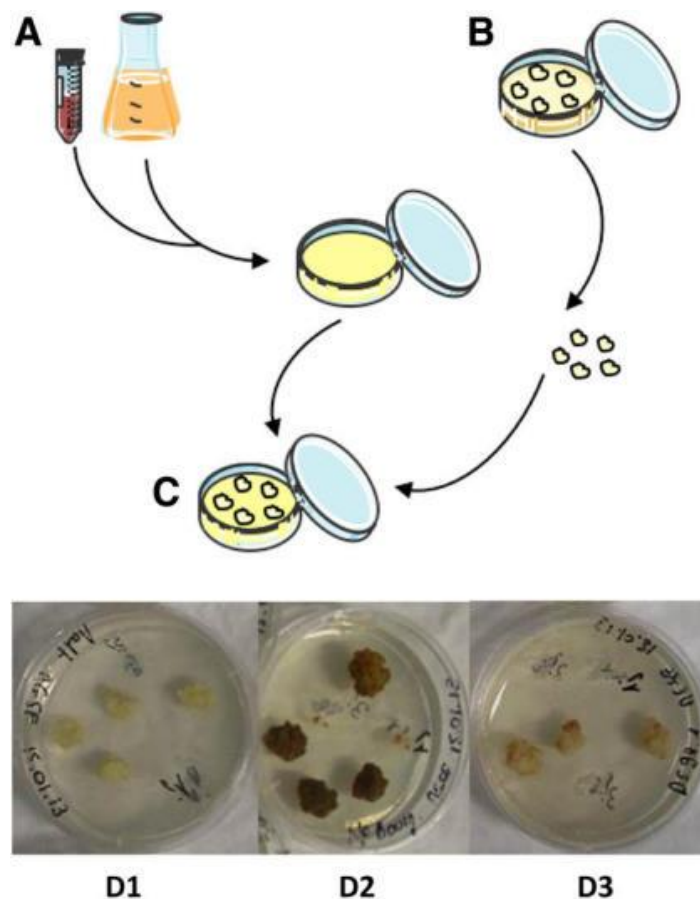


Figure 2. 1 - Callus assay for *Vitis vinifera* to test pathogen aggressiveness. **A**, Callus medium in an Erlenmeyer flask and supernatant of pathogens in a tube to test toxicity of the extracellular compounds and proteins. **B**, Three-week-old calli. **C**, Coculture of callus with extracellular compounds or proteins incorporated in the culture medium. **D**, Calli subcultured for 6 days in Petri dishes with malt medium proteins (**D1**) native protein fractions produced by *Neofusicoccum parvum* isolate Bourgogne S-116 (**D2**) and *Diplodia seriata* isolate 98.1 (**D3**) after 6 days of contact.

Table 2. 2 - Synthesis of the *Vitis* methods available to understand grapevine trunk diseases

Methods, diseases, pathogens	Treatments	Parameter studied	References
Cell suspension			
<i>Neofusicoccum parvum</i> , <i>Diplodia seriata</i>	Co-culture of the secreted secondary metabolites and the cells	Phytoalexins and phytohormones production, defense genes expression, programmed cell death	Stempien <i>et al.</i> , 2017
Callus			
<i>N. parvum</i> , <i>D. seriata</i>	Co-culture of the secreted secondary metabolites and the calli	Visual observation of necrosis, defense gene expression	Abou-Mansour <i>et al.</i> , 2015; Ramírez-Suero <i>et al.</i> , 2014
<i>Phaemoniella chlamydospora</i> , <i>Phaeoacremonium minimum</i> , <i>Fomitiporia mediterranea</i>	Inoculation of the calli	Evaluation of the host/pathogen interactions	Bénard-Gellon <i>et al.</i> , 2015

Table 2. 2 - Synthesis of the Vitis methods available to understand grapevine trunk diseases (cont.)

Methods, diseases, pathogens	Treatments	Parameter studied	References
Callus			
<i>P. angustiu</i> , <i>P. chlamydospora</i>	Inoculation of the calli	Fungal Growth rates, malondialdehyde production, chlorophyll content and fluorescence	Sparapano <i>et al.</i> , 2001; Santos <i>et al.</i> , 2005
<i>Togninia minima</i> , <i>Fomitiporia mediterranea</i> , <i>P. chlamydospora</i>	Co-culture of the secreted secondary metabolites and the calli	Fungal Growth rate	Bruno and Sparapano, 2006
Leaf methods			
<i>P. chlamydospora</i> , <i>P. minimum</i> , <i>N. parvum</i> , <i>D. seriata</i> , <i>Lasiodiplodia sp.</i> , <i>N. australe</i>	Detached leaves of grapevine cv. Italia, leaf disc assay	Necrosis development	Abou-Mansour <i>et al.</i> , 2015; Andolfi <i>et al.</i> , 2011, 2016
Plantlet (Chardonnay cultivar)			
Botryosphaeria virulence factors, toxins (mellein, terremutin)	Incubation at root level	Toxin removal (medium) & plant uptake, physiological parameters & plant gene expression	Trotel-Aziz <i>et al.</i> , 2019
Cutting			
<i>Eutypa dieback</i> , <i>Eutypa lata</i> ; the model can be adapted to all other ascomycete wood pathogenic fungi	Insertion of a mycelial disc or injection of a spore suspension into a hole (3-5 mm)	Development of necrosis	Péros and Berger, 1994; Chapuis, 1995
<i>P. minimum</i> and <i>P. chlamydospora</i>		Gene expression study	Pierron <i>et al.</i> , 2016; Trotel-Aziz <i>et al.</i> , 2019
Botryosphaeria dieback		Molecular diagnostic	Pouzolet <i>et al.</i> , 2013
<i>N. parvum</i>		Microscopic analysis of woody tissues	Pouzolet <i>et al.</i> , 2013, Pierron <i>et al.</i> , 2015a
		Development of control tools	Pierron <i>et al.</i> , 2015b, 2016
<i>Botryosphaeria dieback</i> , <i>N. parum</i> , <i>D. seriata</i>	Inoculation on the green stem	Foliar symptoms expression, size of necrosis, gene expression	Reis <i>et al.</i> , 2016
Pruning wound in field			
<i>Eutypa dieback</i> , <i>Eutypa lata</i> and other <i>Diatrypaceae</i> ; the model can be adapted to other ascomycete wood pathogenic fungi	Artificial contamination of the pruning wound surface	Development of infection in the underlying wood tissues	Carter, 1991; EPPO, 2017; Lecomte and Bailey, 2011; Lecomte <i>et al.</i> , 2003; Sosnowski <i>et al.</i> , 2008

Table 2. 2 - Synthesis of the Vitis methods available to understand grapevine trunk diseases (cont.)

Methods, diseases, pathogens	Treatments	Parameter studied	References
Stem in field			
Botryosphaeria dieback , <i>N. parum</i> , <i>D. seriata</i>	Inoculation in the green stem (third internode)	Size of necrosis, gene expression, proteins	Spagnolo <i>et al.</i> , 2017

The procedures with cell suspensions and calli to screen toxic compounds are as follows and described according to Ramírez- Suero *et al.* (2014) for calli and Stempien *et al.* (2017) for cell suspensions. Calli of cultivar Chardonnay clone 96 were obtained from anthers, as described by Perrin *et al.* (2004). All calli were subcultured every 3 weeks on MPM1 medium and maintained in the dark at $25 \pm 0.5^\circ\text{C}$ with $70 \pm 10\%$ relative humidity. For each test, 30 yellowish white calli, with a diameter of 10 ± 2 mm, were used. The toxicity test can consist of a global approach by using the liquid culture medium filtrate of fungi, or a targeted approach in which the purified molecules were used. When using the culture medium filtrate of fungi, the supernatant is recovered and filtered through membranes with a $0.20 \mu\text{m}$ pore size to eliminate spores and to sterilize the solution. After optimization of the calli culture conditions, the culture medium filtrate from each fungus was incorporated into the MPM1 calli medium and corresponded to a 40% (vol/vol) final concentration, which was established as the sublethal concentration (Ramírez-Suero *et al.*, 2014). For the purified molecules, the appropriate concentration of the tested molecule was incorporated into the MPM1 calli medium. For example, to evaluate the effects of protein samples on calli, a final concentration of proteins at $300 \mu\text{g ml}^{-1}$ was added. This protein concentration was estimated on the basis of the protein concentration present in the previous tests performed with culture medium filtrate from the fungus at 40% (vol/vol). To evaluate the toxic effects of mellein, a purified toxin isolated from the liquid culture media of *Diplodia seriata* was added at different concentrations (25, 200, and $500 \mu\text{g ml}^{-1}$) to the MPM1 calli medium. Mellein added to the MPM1 media at 25 and $200 \mu\text{g ml}^{-1}$ did not induce any necrosis at 1, 3, and 6 days. However, at $500 \mu\text{g ml}^{-1}$, slight and partial necrosis was observed on calli after 6 days of contact. Three-week old *V. vinifera* 'Chardonnay' calli were then subcultured onto the amended MPM1 media and incubated at 25°C in the dark. Toxicity of the secreted extracellular compounds or the purified molecules was assessed visually by the

appearance of necrosis on the calli after 0, 1, 3, and 6 days and by analysis of defense gene expression (Ramírez-Suero *et al.*, 2014; Bénard-Gellon *et al.*, 2015). Five calli (from five different Petri dishes) were collected for each condition and at each time point (0, 1, 2, and 6 days). To investigate early signaling events such as ion fluxes and reactive oxygen species production, cell suspensions were used. Cell cultures were obtained from non-embryogenic callus. Suspension cell cultures of cultivar Gewurztraminer were maintained in adapted Murashige and Skoog (MS) liquid medium in the dark and at $25 \pm 0.5^{\circ}\text{C}$ on an orbital shaker at 120 rpm. Cells were subcultured every week by transferring 20 ml of the cell culture into 80 ml of fresh medium. Six days after subculture, cells were treated with fungal culture filtrates or purified molecules. This method can be used to study defense reactions such as production of phytoalexins and phytohormones, expression of defense genes, and programmed cell death (Stempien *et al.*, 2017). In vitro callus bioassays were used to understand the inhibitory effect of toxins from *Phaeoacremonium angustius* or *Phaeomoniella chlamydospora* in callus growth (Abou-Mansour *et al.*, 2004; Santos *et al.*, 2005). This model was also used to study the virulence of *P. minimum* and *P. chlamydospora* strains (Santos *et al.*, 2005) evaluated by their effect on the growth of calli and the severity of induced necrosis. For example, Bruno and Sparapano (2006) observed various degrees of necrosis in *V. vinifera* 'Italia' and 'Mathilde' calli grown with extracellular compounds from three different esca-associated fungi (*P. chlamydospora*, *P. minimum*, and *Fomitiporia mediterranea*), suggesting a different level of susceptibility for these two cultivars. Callus methods have also been used to quickly assess the toxicity of purified (R)-mellein (details on chemical structures are provided by Andolfi *et al.* (2011) and Abou-Mansour *et al.* (2015), a characteristic toxin present in the extracellular compounds of taxa in *Botryosphaeriaceae* (Bénard-Gellon *et al.*, 2015; Ramírez-Suero *et al.*, 2014). Callus methods are also often used to study expression of defense genes in different diseases or to study the effects of elicitors (Wielgoss and Kortekamp, 2006).

2.3 Plantlets and foliar explants to decipher the molecular and physiological effects of molecules associated with aggressiveness produced by GTD pathogens at different plant integrative levels

Cell suspensions and calli are useful tools to prescreen the toxicity of some GTD fungal metabolites by observing their ability to cause necrosis or modulate plant defense

responses, especially during the early stages of the diseases. Nevertheless, these preliminary toxicity bioassays are far from the whole-plant organizational level needed to ascertain any final conclusions. Following this first screening, the use of in vitro plantlets (i.e., plants with root system, herbaceous stems, and leaves without reproductive organs and growing in a sterile tube) or foliar explants (i.e., leaf-disc or full leaf) can be used to decipher the molecular and physiological effects of the molecules associated with pathogen aggressiveness on plant responses at different plant organizational levels (Table 2.2). Among plant responses, research focused on the balance between the primary metabolism and the defense responses, especially the detoxification processes, including the metabolization of phytotoxins, their cell sequestration, and their transport within the whole plant.

Leaf methods to evaluate the toxic nature of secreted compounds: From leaf discs to full leaves. Early-stage investigations of phytotoxic substances produced in cultures of microorganisms invariably demand the use of complementary bioassays to monitor purification procedures. We also assume that the best fungal culture media to use are those that favor toxin production. Grapevine leaf assays can supply, at a microscale, information on the chemical profiles of extracts and fractions. They are useful in the early stages of purification and, therefore, significantly reduce the time for phytotoxin identification. These procedures are described according to Abou-Mansour *et al.* (2015) and E. Abou-Mansour (personal communication). Leaves of plantlets are more sensitive than older ones taken from plants in the vineyard. Therefore, for the assay, intact, fully expanded young leaves taken from the vineyard or greenhouse should be used. Immediately after they are cut from the vines, they should be immersed in distilled water. When screening culture filtrates, care should be taken to adjust the culture medium to neutral pH before proceeding with the leaf assay. Extracts, fractions, or pure compounds may be diluted in 2 to 4% ethanolic or 2 to 4% aqueous dimethyl sulfoxide. It is necessary to include negative and positive controls in the assays in order to properly estimate the response regardless of the quantity of compounds analyzed. Concentration load by unit of leaf surface should be in a linear range of increased toxicity and should correspond to natural concentrations detected in the field. Three kinds of leaf assays can be considered and are discussed below: full-leaf, leaf-disc, or a leaf-drop method.

Leaf-disc method. Leaf discs were cut out in deionized water with a cork borer, avoiding major veins (Fig. 2.2). Water (2 ml) containing a serial dilution of culture filtrate or known concentration of dissolved phytotoxins (100 to 500 $\mu\text{g ml}^{-1}$), as described before, was placed into each well of a 12-well plate. Three to four discs (5 mm) or one disc (9 mm) were deposited in each well. The extent of necrosis was assessed after 24 and 48 h of incubation at room temperature. Eutypine at 200 $\mu\text{g ml}^{-1}$ was used as a positive control.

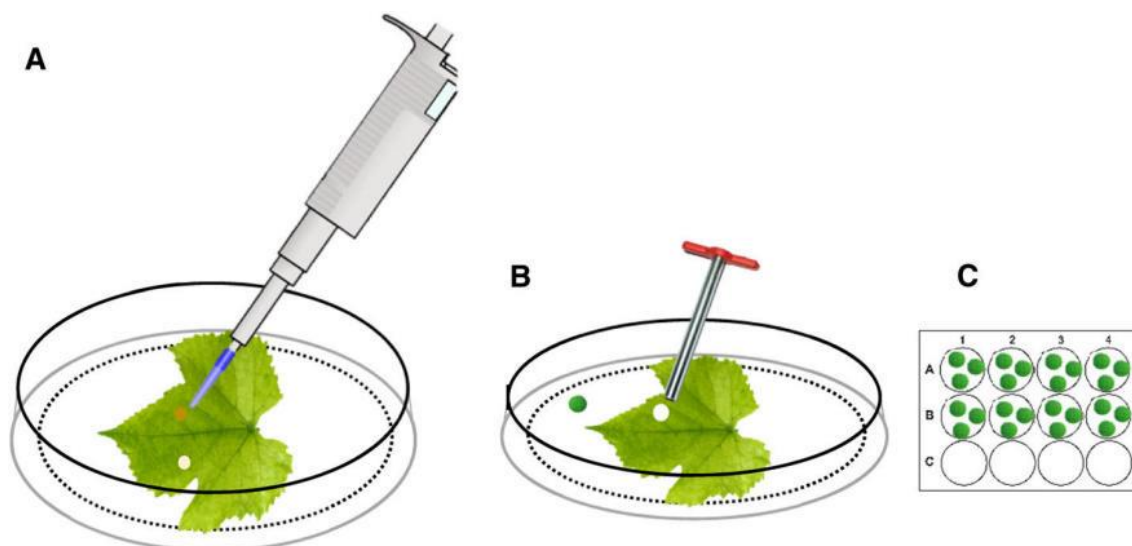


Figure 2. 2 - Leaf-drop and foliar disc methods for *Vitis vinifera*. **A**, On a Petri dish, a drop with fungal toxins is deposited on the adaxial side of leaf to test their toxicity. **B**, With a cork borer, leaf discs are excised and **C**, transferred to a plate containing liquid medium with fungal compounds to test their toxicity.

Full-leaf method. In this assay, phytotoxicity screening was conducted on leaves by absorption of the toxins through the petioles. Leaves were cut from the vine and the petiole was immediately immersed in distilled water for 12 h. Leaves were transferred to the phytotoxin solution for a defined time, then transferred to distilled water until necrosis appeared. Kinetics of the manifestation of the foliar symptoms, and of the volume of solution uptake, can be monitored. Symptoms such as browning, or reddening followed by necrosis were observed with the toxins. Wagschal *et al.* (2007) reported that, with the higher concentrations of toxins identified from *E. lata*, there was a significantly greater uptake of solution by the detached leaf, suggesting increased transpiration.

Leaf-drop method. This method can be applied on a full leaf or a leaf disc with or without injury. Light injury was performed by gently pressing the tip of a pipette onto the leaf surface while applying the drop. In the case of full leaves, the petiole was cut close to

the leaf base in a way that would allow maximum vascular contact with the water. The leaf, adaxial side up, was placed in sterile plates containing filter paper soaked in distilled H₂O (2 ml), ensuring contact of the cut petiole with water (Fig. 2.2). The Petri dishes were kept in a laminar flow hood until the upper surface of the leaf had dried. The methods consist of depositing drops of 10 to 50 µl of culture filtrate, culture extract, or phytotoxin solution on one side of the leaf surface. Drops of a negative control were placed to the other side. The dishes were sealed with parafilm and stored under bright fluorescent light with 16 h of light per day at 20°C until necrosis appeared. An additional volume of H₂O (1.5 ml) was added to the Petri dish after 24 h. A similar protocol can be applied to leaf discs where drops of phytotoxic solutions are deposited at the center of the leaf disc. Recording the intensity of lesions induced by different concentrations per droplet per leaf disc can be used to determine the minimal concentration that induces necrosis. For all three leaf methods, the qualitative assessment of toxicity is based on visual observations of the extent of the necrotic lesions by a grading key from 0 to 4, where 0 indicates no observable necrosis and 4 indicates complete necrosis. An alternative method is the measurement of chlorophyll levels, which provides a relative measure of tissue damage in the leaf disc (Smith *et al.*, 2003). Today, open-source image processing programs designed for scientific multidimensional images such as Image J developed at the National Institutes of Health or APS Assess 2.0 software for plant disease quantification are available. These can be used for simple and rapid measurements of leaf area, lesion count, or necrosis percentage (Abou-Mansour *et al.*, 2015; Andolfi *et al.*, 2016).

To investigate whether the phytotoxic activity of an isolated extract or compound is specific to vines, additional test plants such as tomato cuttings or *Arabidopsis* leaves should be used. If lesion development is observed on all three plants, it indicates that metabolites are nonhost-specific toxins. Several phytotoxins have been reported from GTD pathogens, apparently with different modes of action. Methods based on cell cultures are very rapid methods for screening large numbers of samples under highly controlled conditions and can also be very useful to help understand the specific interactions between GTD pathogens and grapevines on a molecular level. However, the way they interact with cellular methods based on cell cultures, alter membrane

integrity, modify gene expression, or interact with plant enzymes to counteract metabolite biosynthesis are all aspects that remain to be elucidated.

In vitro whole-plantlet method to investigate detoxification and host defenses. *In vitro* whole-plantlet methods provide accurate data to fine tune our understanding of the toxin or the physiological and molecular effects of fungal toxins on grapevines. Indeed, the whole plant is considered in its simplest vegetative organization, making it easier to target and study the direct effects of toxins on the plant in *in vitro* entirely controlled conditions (Fig. 2.3). This method is often used to study the changes in grapevine basal or induced immunity in relationship with different diseases or elicitors (Gruau *et al.*, 2015).

The procedures are as follows, according to Trotel-Aziz *et al.* (2019). Plantlets of Chardonnay clone 7535 were produced from nodal explants and transferred to 15 ml of modified MS medium in 25-mm-diameter test tubes. Plantlets were grown at 25°C day and night, with a photoperiod of 16 and 8 h, respectively, under white fluorescent lamps ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$) and, after 8 weeks, they were ready to be used for the experiments (Fig. 2.3).

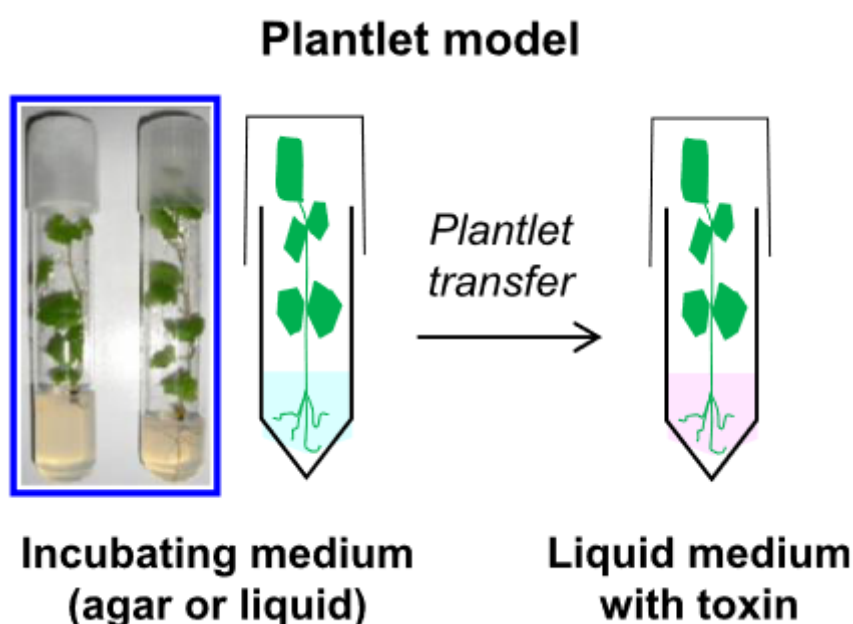


Figure 2. 3 - Plantlet model for *Vitis vinifera* treatment with the toxins associated with grapevine trunk disease fungi. Left side shows the plantlet model (photo and diagram) as described by our team. Right side shows the plantlet transferred to a new tube containing fresh liquid incubating medium supplemented with toxin.

This method is useful for toxicity assessment, which is measured in terms of maximum quantum yield of photosynthesis (F_v/F_m), which can be a sensitive indicator of the physiological condition of the plant, growth rate, and pigment concentrations after 72 h of exposure to GTD toxins such as (R)-mellein or (-)-terremutin added to liquid MS medium (Fig. 2.3) in a culture chamber at 20°C with a photoperiod of 16 h of light and 8 h of darkness. All experiments should be repeated four times, with each sample at least in triplicate. None of these markers changed below 1,500 $\mu\text{g liter}^{-1}$, enabling investigation of the detoxification process within a nontoxic range of (0 to 1,500 $\mu\text{g liter}^{-1}$). For detoxification studies, 8-week-old plantlets were transferred to test tubes containing liquid MS medium supplemented or not with GTD toxins and incubated for 3 days under identical conditions in a growth chamber. Because no significant toxicity was noticed below 1,500 $\mu\text{g liter}^{-1}$ with (R)-mellein or (-)-terremutin, all experiments using these phytotoxins were performed with (R)-mellein at 350 $\mu\text{g liter}^{-1}$ or with (-)-terremutin at 750 $\mu\text{g liter}^{-1}$ (Trotel-Aziz *et al.*, 2019). To perform detoxification studies, the applied phytotoxin concentration should be as weak as possible but above the high-performance liquid chromatography (HPLC) detection threshold if the aim is to quantify it over time. To investigate grapevine responses or defenses, plantlets for RNA extraction and reverse transcription polymerase chain reaction (PCR) analysis were collected 3 days post inoculation and stored at -80°C until extraction, as described by Gruau *et al.* (2015). To investigate grapevine detoxification capacity, phytotoxins were extracted and quantified as described by Trotel-Aziz *et al.* (2019). Two different controls were required: the living plants in a toxin-free medium and the medium containing only toxin without living plants. Toxin removal from the plant-incubating medium was also investigated by directly injecting an aliquot of the (-)-terremutin-containing medium into the HPLC system, while (R)-mellein was extracted with hexane (10:2 [vol/vol]) before analysis. Thus, this last in vitro plantlet model can refine the toxicity tests (micrograms per liter compared with micrograms per milliliter applied to calli) but it is most interesting for detoxification studies, investigating both the host physiological and molecular changes as well as the transport, bioaccumulation, and metabolization of molecules delivered by a GTD pathogen. This in vitro method also offers the potential to first screen the efficient plant response associated or not with beneficial microorganisms (Trotel-Aziz *et al.*, 2019). Nevertheless, all of these interesting and

valuable informative in vitro models do not allow direct experimentation with the GTD pathogens; only the in vivo models allow such an approach.

2.4 *In vivo* artificially inoculated plant assays, to reproduce wood and foliar symptoms and to validate pathogen contribution to natural GTD symptomatology

This laboratory method was first developed to directly investigate pathogen contribution to GTD symptomatology and ways of possible control. Such bioassays are also suitable to investigate trunk microbial ecology, useful for nursery diagnosis and in field conditions. In most cases, 1-year-old cuttings were used to focus on early events in grapevine–GTD pathogen interactions. This model aims to (i) to study multidirectional interactions at tissue, cellular, and molecular levels between pathogens themselves, pathogens and vine, and pathogens and vine-microbiome and (ii) provide an easy to-handle biological tool to test any control compound or agent to limit the progression of GTD-associated fungi. The model is adapted depending on the GTD pathogen; therefore, they are described individually (Table 2.2).

A method for *Eutypa dieback*, the first one available. At least two simple approaches are currently used to reproduce *E. lata* infections and typical foliar symptoms in cuttings. The earliest methods for this pathogen were probably those described by Mur (1979) in Europe with two methods: (i) using rooted plants of Sauvignon Blanc grafted onto *Rupestris* du Lot rootstocks and inoculated by placing mycelium of a strain isolated from grapevine into the graft union, and (ii) using unrooted cane segments inoculated with *Eutypa* mycelium close to the upper bud after taking out the pith. Petzoldt *et al.* (1981) inoculated pruning wounds of 1-year-old rooted grapevines with *E. armeniacae* ascospores and observed typical symptoms the following growing season. The first researcher to use the rooted cuttings method was Chapuis (1995). Canes were collected during the dormant season; two-internode cuttings were rooted for 2 months before inoculation and grown in a greenhouse. The temperature was controlled between 20 and 24°C. Plants were watered for 5 min, twice each day with 1.89 liters/h via a drip system. They received 18 h of light per day from both ambient and supplemental lighting. Plants were inoculated following the method described by Chapuis (1995): a wound (4 to 5 mm in diameter, $\pm$ 5 mm deep up to the pith) was drilled 1 to 2 cm below the upper bud. Inoculum can be mycelial plugs taken from the margin of 7-day-old

cultures and inserted into the wound, a mycelial pellet collected by scraping the surface of the fungus growing on agar medium (Jung *et al.*, 2010), or mycelial or spore suspensions in water introduced by injection with a pipet into the hole in the cutting (different volumes can be used) (Fig. 2.4).

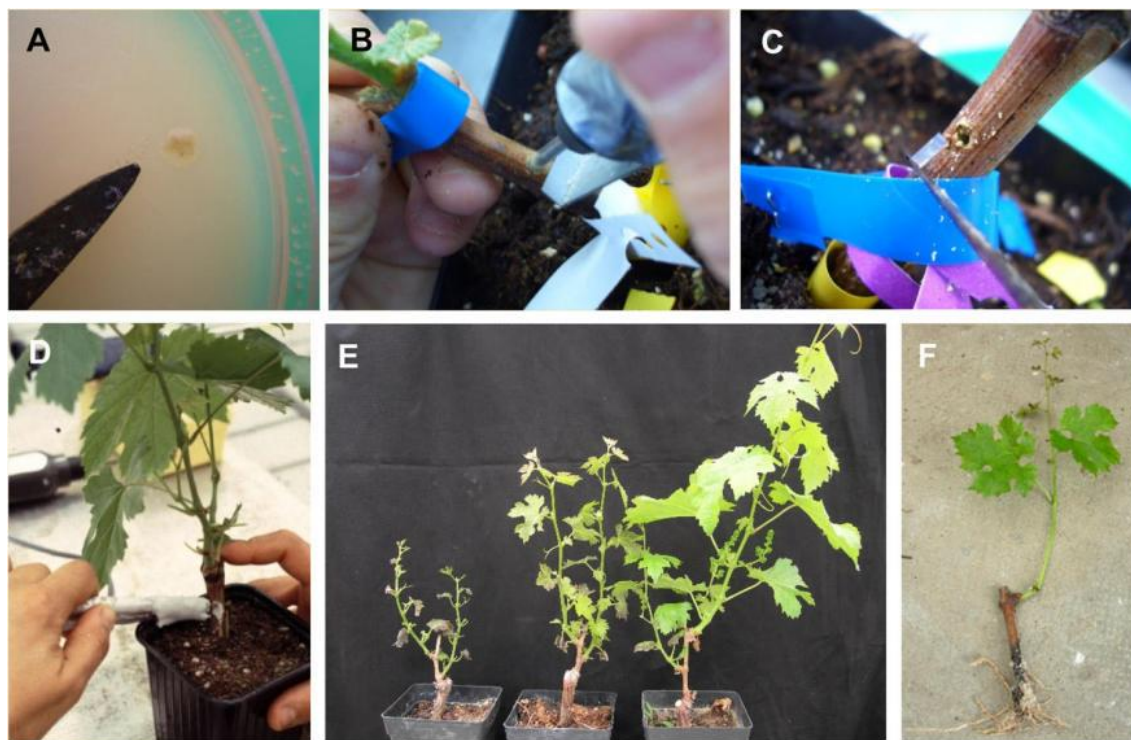


Figure 2. 4 – Process developed to reproduce *Eutypa lata* symptoms with unrooted or rooted cuttings of *Vitis vinifera*, as described by Péros and Berger (1994) and Chapuis (1995). **A**, Inoculation preparation. A pellet of *E. lata* sampled on a 1-week-old mycelium culture in petri dish. **B**, Perforation of the woody stem. On the 1-year-old lignified stem, a hole (4 to 5 mm in diameter) under the upper bud produced with a drill. **C** and **D**, Artificial inoculation of rooted cutting. The pellet, spores or a plug of *E. lata* is put in the stem hole. **E** and **F**, Foliar symptoms of *E. lata* on 1-year-old rooted cuttings.

A cultivar known to be susceptible to *Eutypa* dieback (Cabernet Sauvignon or Sauvignon Blanc) is generally preferred, especially to compare the virulence of GTD fungal strains. The use of aggressive strains is recommended when the purpose is to test cultivar susceptibility to GTD (Péros and Berger, 1999). To ensure that an artificial niche is not created, inoculation sites are not covered or wrapped with any protective plastic film but are sealed with warm wax only. Non-inoculated control vines treated with an agar plug or sterile water are generally included in the experimental designs. Cuttings are maintained in a greenhouse or under a shade house until typical *Eutypa* foliar symptoms appear the following year in spring after bud break. To date, this method has been routinely used to examine the susceptibility of cultivars visually and at the molecular level (Camps *et al.*, 2010, 2014) and to test the efficiency of control products (P.

Lecomte, personal communication). M. J. Creaser, a researcher from the South Australian Research and Development Institute in Adelaide, imported this method into Australia after her visit in 2001 to Bordeaux (France) to test cultivar susceptibility (Sosnowski *et al.*, 2007) and also to test the effect of water and temperature stress on disease severity. Since then, many authors have used similar or derived methods with GTD pathogens and for diverse epidemiological studies (Laveau *et al.*, 2009).

The method based on using unrooted cuttings is similar to the method based on rooted cuttings, except that there is rapid expression of foliar symptoms, which appear in less than 2 months (Péros and Berger, 1994). The one-internode cuttings are placed and maintained in moist rock-wool rolls or in sand terrines for approximately 3 months. Inoculations are made in the same way as described above. Symptoms appear 4 to 8 weeks after inoculation and their development is generally recorded twice at intervals of 2 and 4 weeks. Mur (1988) was probably the first researcher to obtain a high incidence of foliar symptoms using this method. Péros and Berger (1994) were the first to use it to compare the aggressiveness of isolates and the susceptibility of cultivars before they used it to study the diversity of natural *E. lata* progenies (Péros and Berger, 1999). This rapid method was also used in Australia by Sosnowski *et al.* (2007).

A method available for esca complex diseases with *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora*. Two methods to infect plants with *P. minimum* have been developed and these are based on nodal or internodal inoculation described by Pouzoulet *et al.* (2013) (Fig. 2.5). Wounds were treated with fungal mycelium or spores and the infection allowed to develop for 10 h to 12 weeks post inoculation. This model was completed with the design of a quantitative PCR (qPCR) plexor analysis to detect and follow fungal development (Pouzoulet *et al.* 2013); green fluorescent protein (*gfp*) transformants of *P. minimum* (Pierron *et al.*, 2015a) and *P. chlamydospora* were used for confocal microscopy observations to study gene expression in grapevine woody material (Pierron *et al.*, 2016).

In the above studies, 1-year-old canes of Cabernet Sauvignon clone 15 were used and the general procedure was as follows. Canes were surface sterilized in a fungicide bath containing 0.05% Cryptonol (8-hydroxyquinoline sulfate at 140 g/liter; Syngenta) for 1 h and stored at 4°C until further processing. Canes were divided into cuttings with three dormant buds (nodal treatment) or cuttings with two dormant buds (internodal

treatment). Cuttings were soaked in a water bath containing bleach and were stored at 4°C overnight in a solution of 0.05% Cryptonol, then given successive washes before planting in plastic trays filled with moistened autoclaved glass-wool. The cuttings were kept in a growth chamber (photoperiod of 16 and 8 h, 25°C, 90% humidity) and watered as necessary. Budding and rooting takes 4 to 6 weeks before the cuttings are potted in 75 cl pots containing a sterile mixture of perlite, sand, and turf (1:1:1 [vol/vol]). Cuttings were then transferred to a growth chamber (photoperiod of 16 and 8 h, 25°C, 45% humidity) and plants remained there for at least 1 week before treatments to avoid potting stress (Fig. 2.5I). Cuttings were inoculated when at least six leaves were fully developed (Fig. 2.5II). First, cuttings were partially surface sterilized by wiping with a tissue sprayed with 70% ethanol. For internodal inoculation, a wound was made at the internode by drilling with an ethanol-flamed 3-mm drill bit and inoculum was introduced into the wound. After inoculation, the wound was covered with cellophane. Inoculum was either spores or hyphae taken from cultures growing on agar plates (Fig. 2.5III). For nodal inoculation, the branch formed from the middle node was cut with an ethanol-flamed blade (Fig. 2.5I). A wound was then made at this nodal level with an ethanol-flamed 3-mm drill bit and inoculated as described for the internodal inoculation. Following inoculation at both internodal and nodal levels, plants were maintained in the growing chamber under the same conditions and watered every second day with autoclaved tap water. When screening for biological or chemical agents to control the development of esca-associated fungi, the wound can be treated prior to or following inoculation with the pathogen. Thanks to this model, understanding of the early stages of colonization by esca-associated fungi in woody tissue has increased. First of all, the type of woody tissue greatly influenced fungal colonization. *P. minimum* colonized a wound at the internode 6 weeks post inoculation, whereas it was restricted at the nodal inoculation site. Interestingly, nodal tissues presented an intense fluorescent barrier that may be involved in slowing down *P. minimum* infection. However, the fungus was able to overcome plant responses 12 weeks post inoculation.

These results orientated our investigation on the internodal response to pathogen colonization and suggested that grapevine wood may respond to *P. minimum* (Pierron *et al.*, 2015a). We could describe the healing modification caused by *P. chlamydospora* by using the internodal model (Pouzoulet *et al.*, 2013). A strong reduction of healing

tissues in plants infected with *P. chlamydospora* compared with damaged non-inoculated plants was observed. This result, associated with the fact that we could describe the fungal colonization via microscopic analysis of calcofluor-stained samples, revealed the capabilities of *P. chlamydospora* to modify plant tissue response within weeks after inoculation. Healing was also analyzed in *P. minimum*-inoculated plants, but the response was opposite to *P. chlamydospora*, because *P. minimum* did not modify tissue responses compared with untreated wounds (Pierron *et al.*, 2016).

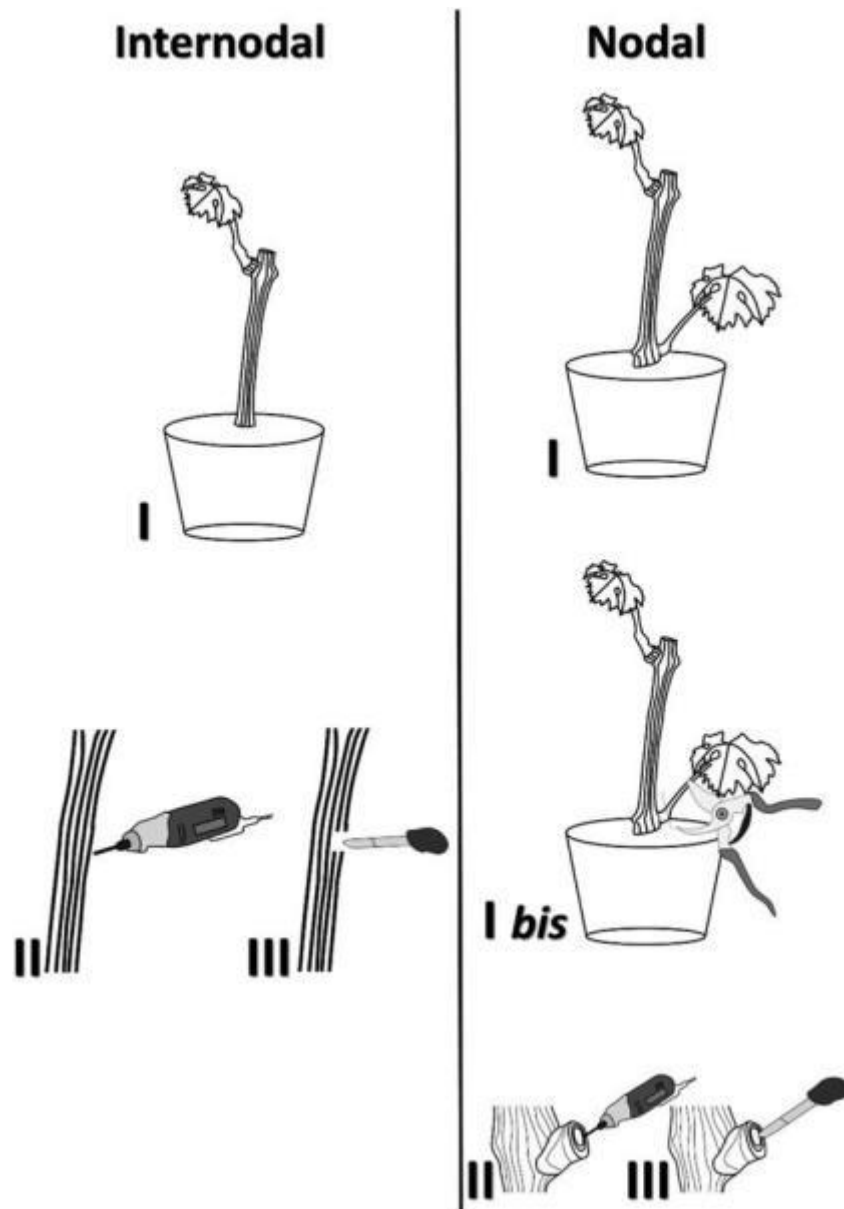


Figure 2. 5 - Cutting inoculation procedure for *Vitis vinifera* wood with esca-associated fungi. The left side describes the internodal model and the right side the nodal model inoculation methods. **I**, Plants carrying one or two branches are maintained in a growth chamber for 1 week. The internodal infection consists of wounding damage at the middle of an internode. **I bis**, For the nodal model, the intermediate branch is cut with a blade. **II**, Wounding damage is done using a drill bit. **III**, Esca associated fungi are deposited in the wound.

In the case of co-inoculation of *P. minimum* and *P. chlamydospora*, we observed an intermediate phenotype of healing tissues at the wounding site. To detail early niches of colonization of *P. minimum* via internodal sites of inoculation, we developed a *gfp*-tagged strain of *P. minimum*. Xylem vessels were colonized by the fungus within 12 weeks post inoculation via xylem fibers (Pierron *et al.*, 2015a). The colonization niches observed for the *P. chlamydospora gfp*-tagged strain were consistent with the previous study by Fleurat-Lessard *et al.* (2010). This *gfp*-transformed strain was later used in a transcriptomic study in the presence of *V. vinifera* callus (Fischer *et al.*, 2016). The particular barrier formation observed after nodal inoculation, together with the different healing response at the internode according to pathogen species, suggested a certain level of perception by the woody tissues. Induced defense and pathogen perception in the trunk were investigated by a molecular method. This model allowed gene expression to be studied in wood in response to *P. minimum* or *P. chlamydospora* inoculation in the internodal region. Despite the strong background noise induced by plant response to the wounding stress, our data revealed that (i) woody tissue perceived and reacted to *P. minimum* or *P. chlamydospora*, (ii) molecular responses differed slightly between *P. minimum* and *P. chlamydospora* treatments, and (iii) molecular responses differed between co-inoculation with the two pathogens compared with inoculation with a single pathogen (Pierron *et al.*, 2016).

The method was also developed as an easy tool to test the ability of compounds to restrict fungal growth inside the wood. In that sense, the nodal model was used to analyze the potential effect of elicitors (Pierron *et al.*, 2016). We showed the capabilities of ozone dissolved into water to limit *P. minimum* growth with the nodal model (Pierron *et al.*, 2015b). Fungal growth was monitored by qPCR. This tool can also detect the number of DNA copies present in wood samples and, thus, can be used as a diagnostic tool. Overall development of this model gave the communities investigating GTD a tool to study wood responses at different levels to esca associated fungi.

To conclude, with this laboratory method, the microbial ecology within the trunk of young grapevines can be studied. In this way, it focuses on early events involved in the esca pathosystem. It has been used to confirm that trunk-induced responses exist and may be specific to the fungal species colonizing the plant. The response was also associated with a difference in wound healing according to the fungal species. The

model also allows for a rapid screening of candidate compounds for control of esca-associated fungi.

A method for some species associated with *Botryosphaeria dieback*. According to Úrbez-Torres (2011), *Botryosphaeriaceae* species that infect grapevines can be divided into three different groups based on their aggressiveness (high, moderate, and low), where *Lasiodiplodia spp.* and *Neofusicoccum spp.* belong to the highly aggressive group, *Botryosphaeria dothidea* and *Diplodia spp.* to the moderately aggressive group, and *Dothiorella spp.* and *Spenceriopsis viticola* to the slightly aggressive (low) group. For *Botryosphaeria dieback*, pathogenicity trials have been conducted, showing lesions at the initial inoculation point (Taylor *et al.*, 2005; Úrbez-Torres and Gubler, 2009). On leaves, such inoculation could induce sudden dry shoots (Úrbez-Torres and Gubler, 2009; Mohammadi *et al.*, 2013; Larignon *et al.*, 2015) or discoloration (Reis *et al.* 2016). Reis *et al.* (2016) presented an assay where inoculation of 1-year-old grafted Tempranillo with *Neofusicoccum parvum* and *D. seriata* induced lesions on the stem and discoloration on leaves. For that model, 1-year-old grafted cuttings were potted individually in 1-liter, free-draining bags containing a sandy soil mixture and arranged as a complete randomized design in a ventilated greenhouse at 24°C under natural light. After 1 month of growth, plants were inoculated with *N. parvum* and *D. seriata* by removing a 3-mm diameter area of the bark with a cork borer from the base of the primary stems (approximately 1.5 cm in diameter) between the second and third nodes (Fig. 2.6). Wounds were inoculated with 3-mm diameter mycelial plugs taken from the actively growing margin of 8-day-old colonies grown on potato dextrose agar (PDA) at 24°C in darkness. Each inoculation point was covered with moist cottonwool and sealed with parafilm (Fig. 2.6). Positive and negative controls were inoculated using the same method, but with 3-mm-diameter mycelial plugs of other grapevine pathogens and sterile 3-mm-diameter PDA plugs to confirm that lesions were due to infection by *Botryosphaeria dieback* pathogens. The experiment was done in a greenhouse and emergence of foliar symptoms was observed 8 months after inoculation. The dimension of external lesions was evaluated 3 months after inoculation on green shoots, before lignification occurs, by measuring the width and the length of the lesion. The percentage of grapevines that showed symptoms was recorded when one or more leaves had chlorotic areas (the plant was considered positive for symptom expression). To fulfil

Koch's postulates, small pieces of necrotic tissue were taken from the edge of each lesion and incubated on PDA amended with chloramphenicol at 250 mg liter⁻¹ to recover the inoculated fungi.

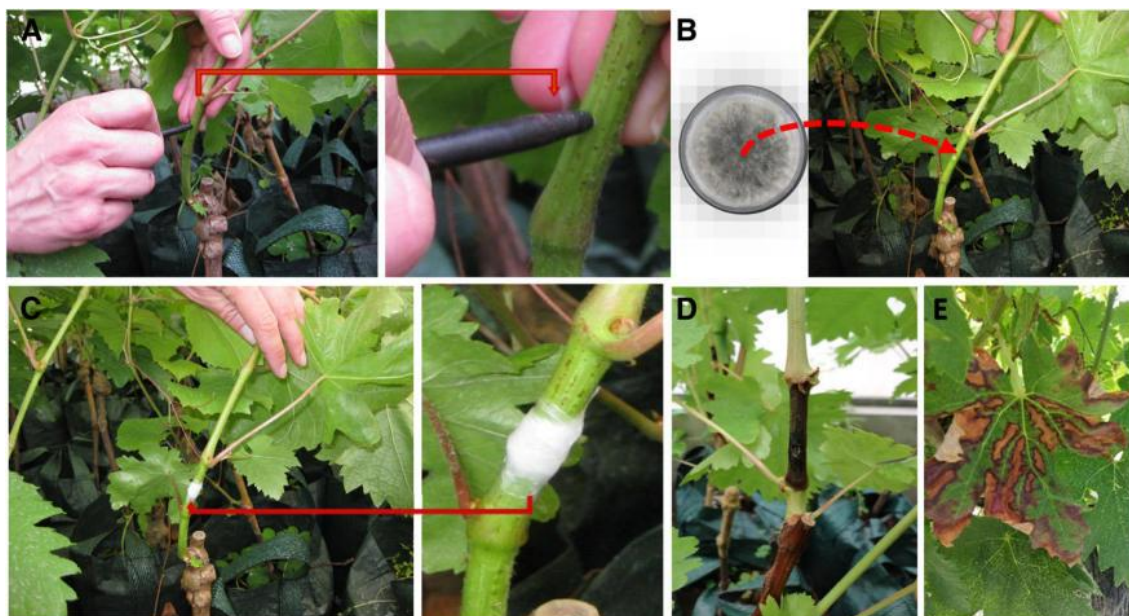


Figure 2. 6 – Inoculation of grapevine plants under controlled conditions with *Neofusicoccum parvum* and *Diplodia seriata*, as described by Reis *et al.* (2016). **A**, Perforation of the green stem. A wound is created by removing 3 mm of epidermis with a corkborer at the third internode of the green shoot. **B**, Artificial inoculation. A 3-mm mycelial plug is cut from the actively growing margin of *N. parvum* or *D. seriata* colonies and placed on the wound. **C**, The plug is covered with moist cotton wool and sealed with Parafilm for 2 weeks. **D**, External lesions observed in the green shoots of inoculated plants. **E**, Foliar symptoms expressed on inoculated plants.

Thanks to this model, it was possible to show that *N. parvum* isolates produced longer lesions with greater surface area than *D. seriata* isolates in Tempranillo (Reis *et al.*, 2016). In regard to foliar symptoms, it has been demonstrated that *N. parvum* and *D. seriata* can produce cell-wall-degrading enzymes and phytotoxic metabolites. Their synergistic action could play an important role in the development of foliar symptoms (Andolfi *et al.*, 2011; Bellée *et al.*, 2017). Both production and identification of phytotoxic metabolites have been reported (Martos *et al.*, 2008; Andolfi *et al.*, 2011; Abou-Mansour *et al.*, 2015) but their exact roles remain unclear. Their accumulation in plant tissues suggests a potential effect during the appearance of foliar symptoms (Bellée *et al.*, 2017). Nevertheless, further work needs to be carried out to elucidate the entire mechanism that modulates cultivar susceptibility toward phytotoxic fungi and to better understand the ability for *N. parvum* and *D. seriata* to synthesize and secrete more or less potent phytotoxic compounds (Abou-Mansour *et al.*, 2015). These studies will allow a better understanding of the molecular dialogue between fungi and plants (Reis *et al.*,

2016; Trotel-Aziz *et al.*, 2019). The main advantage of this assay is that the pathogens are rarely isolated other than in the wood, and artificially infected plants respond similarly to plants naturally infected in the vineyard. Thus, it can provide useful data to better explore the aggressiveness of species of *Botryosphaeriaceae* toward cultivars at morphological, physiological, and molecular levels and in relationship to the quantitative and qualitative production of fungal metabolites.

2.5 Field validation by artificial inoculation of pruning wounds and stems, the last experimental step

Among the methods available in vineyards, artificial inoculation of both pruning wounds and stem have been developed and used according to the goals of the study (Table 2.2).

Artificial inoculation of pruning wounds, the main route for GTD pathogen infection.

The first publications that reported this method were with *E. armeniacae* on apricot in the 1970s (Carter and Moller, 1971; Ramos *et al.*, 1975). These studies were the origin of methods developed from the 1980s for inoculation of grapevines either using ascospores or mycelium (Carter, 1991). Inoculum with ascospores is generally preferred and methods differ mostly in the inoculum dosages, length of incubation periods, or isolation procedures (Elena *et al.*, 2015). One of the first rapid methods for screening compounds for disease control, or to study the susceptibility of cultivars, was carried out in France and was based on inoculation of pruning wounds (Paillassa, 1992). The concept is always the same: a cane is pruned, the wound is artificially inoculated, and the fungus is recovered by isolation after incubation (Fig. 2.7). Different procedures are used in the vineyard, under greenhouse conditions, or in a climatic chamber. Only two of these methods used with *E. lata* infections are presented here: the first one is used worldwide and is presently an official method for the regulation of disease control products in European countries (EPPO, 2017) and the second method enables rapid results and is included in an official French regulation method (Method CEB 155, third edition) aimed at the efficacy evaluation of disease control compounds. The same rationale was used by others for the rapid screening of pruning wound protectants (Lecomte *et al.*, 2003; Rolshausen *et al.*, 2010; Ayres *et al.*, 2011) or for epidemiology (Chapuis *et al.*, 1998; Lecomte and Bailey, 2011). The field trial should be set up preferably in a young vineyard during winter dormancy.

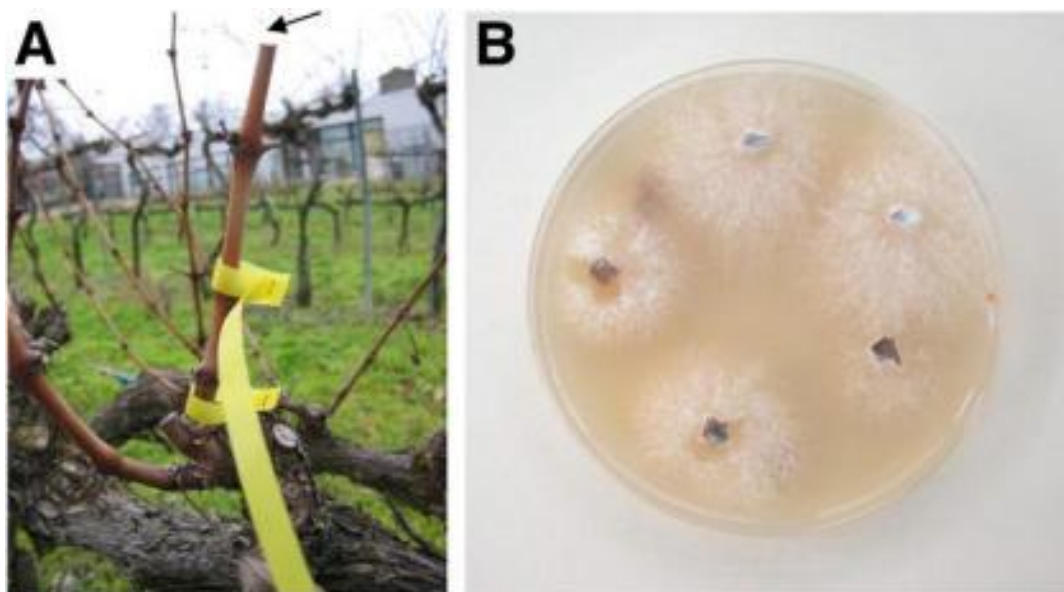


Figure 2.7 – Pruning wound methods in the vineyard, as described by Lecomte *et al.* (2003). **A**, In the vineyard, inoculation with 50 to 250 spores of *Eutypa lata* at the pruning of the canes composed of 2 to 3 internodes. **B**, In the laboratory, 5 pieces of wood from the pruning wound inoculation site were deposited onto culture medium and grown for 4 to 7 days.

A preliminary disease assessment, done the year before the trial, can help ensure that the selected vineyard is free from *Eutypa* dieback. Although the infection of dormant wood from a tolerant grapevine cultivar may also be successful, a standard susceptible cultivar is generally used for these field trials. An experimental design with four blocks is recommended with at least eight marked canes per block. Canes were pruned, leaving from 1 to 3 internodes from the base and more or less 2 cm above the nearest bud. Because *E. lata* conidia do not germinate and cannot be used for experiments, artificial inoculations should be made with ascospores. These were collected from perithecia previously harvested from local vineyards. Different methods can be used to collect ascospores from stromata: (i) perithecia collected from stromata with a scalpel are immersed in sterile water for approximately 1 h, (ii) a piece of moistened stromata is attached to a Petri dish lid with modelling clay and ascospores eject into water in the base of the dish (Carter, 1991), or (iii) perithecia are cut open with a scalpel and a droplet of sterile water is applied to the cut surface (Trouillas *et al.*, 2010). The concentration of ascospores in the suspension was adjusted to 5 to 10 spores/ μl . Then, 20 to 25 μl of the selected suspension was deposited with a micropipette onto the center of each pruning wound, representing 100 to 250 spores/wound. It is possible to reduce the inoculum pressure for trials in the vineyard but a minimum of 50 spores inoculated per wound is recommended. After a minimum incubation period of 8 months in the field, isolations

were made from each cane sample by culturing wood chips on a suitable culture medium. Canes were collected with one to two internodes, and new young shoots were removed before transfer to the lab. The upper parts of the canes were lightly cleaned, and surface disinfested by wiping with a piece of cotton soaked with 90% ethanol, then quickly passed through a flame. The bark was removed with a sterilized scalpel which was regularly disinfested, and the canes were again briefly flamed. At the pruning wood surface, a necrosis of 2 cm developed and could reach the nearest bud zone. The first 1 to 2 mm of cane was cut using pruning shears to remove tissues where many saprophytic organisms are often present. Then, 25 small wood chips of approximately 20 to 30 mm³ (3 by 3 by 3 to 4 mm) were cut from the necrotic wood, surface disinfested by immersion in a 3% solution of calcium hypochlorite for a few seconds, then placed on malt extract agar or PDA in Petri dishes (5 wood chips per dish). The observation of *E. lata* mycelium derived from a single wood chip was sufficient to confirm that the respective cane is infected. The results were expressed in the number of canes infected with *E. lata* relative to the number of canes analyzed and presented as infection percentages.

Before the tests for efficacy of disease control compounds on pruning wounds in the vineyard, a rapid trial in a climatic chamber was conducted initially to screen candidate compounds. For that, canes were collected during the winter, wrapped in a semi closed plastic bag, and kept in cold storage until use. They were cut into 32 lengths of approximately 20 cm (two to three internodes) and placed as cuttings in pots filled with sterilized wet sand. The bark of the canes was surface disinfested with a swab soaked in alcohol before pruning. The pruning cut was made 2 to 3 cm above the nearest bud. Canes were treated according to the trial objectives and inoculated as described above for the trial in the vineyard. Pots were then placed in a climatic chamber at 10 to 15°C. Mycelial development was enhanced by reproducing one or two morning dews by lightly spraying the wounds with sterile water. After an incubation period of from 15 days to 1 month, the ends of canes were cleaned, and surface disinfested by swabbing with cotton wool soaked with alcohol and then quickly flamed. The bark was peeled off with a sterilized scalpel, which was regularly disinfested by dipping in alcohol and flaming. Debarked tops of canes were rapidly heated over a flame, then cut with a cutter into at least eight slices, each approximately 1 mm thick. The cutter should be kept sterile to avoid cross-contamination of the slices. The slices were then deposited, in the order

they were cut on an agar medium in Petri dishes (4 slices/dish) that was previously marked so that it was possible to identify the order of the slices at the time of scoring. To conclude, although rapid methods are useful to obtain preliminary results, the classical method of inoculations in the vineyard after a sufficiently long period of incubation is generally considered more reliable to allow an easier recovery of the inoculated pathogen after mycelial development. This is a key aspect, especially for the evaluation of organic control agents, to avoid false-negative results with fungal antagonists such as *Trichoderma* spp. or other fast-growing microorganisms that may overgrow the colonies of the slow-growing pathogens. Artificial pruning wound infection has been widely applied worldwide in studies on the control or epidemiology of *Eutypa dieback* (Moller and Kasimatis, 1980; Chapuis *et al.*, 1998; Sosnowski *et al.*, 2008; Rolshausen *et al.*, 2010; Lecomte and Bailey, 2011).

Artificial inoculations of green stems. In the vineyard, this model was first used to identify the periods of cultivar susceptibility to species of *Botryosphaeriaceae* (Spagnolo *et al.*, 2017). The concept was to inoculate the pathogen into a shoot at different phenological stages (separated clusters, flowering, or veraison) to evaluate its behavior at each targeted stage by measuring the length of the necrosis and to determine plant physiological changes by proteomic or transcriptomic methods. With this method, it was also possible to identify the influence of the inoculum load on the host–pathogen interaction.

The trial must be conducted on a susceptible cultivar. At the phenological stage chosen, stems were wounded longitudinally with a sterile scalpel at the middle of the third internode. The wounds (8 mm long, 1 mm deep, and 5 mm wide) were inoculated, by inserting into the wound a 5-mm-diameter plug of agar taken from the edge of a 5-day-old actively growing fungal culture. Controls were inoculated with sterile plugs of the agar medium. The inoculation sites were then covered with Parafilm. Each condition was replicated eight times, with one repetition per plant. Both injured and inoculated shoots and controls were selected on different vines. Observations on lesion development and re-isolations were performed after harvest for each treatment from all phenological stages, as described by Larignon and Dubos (1997). The shoot at the level of the third internode was cut longitudinally and the length of necrosis was measured on both sides from the point of inoculation. Ten sections cut from the necrosis were collected,

disinfested with 3% calcium hypochlorite and placed in Petri dishes of malt extract agar (15 g of malt and 20 g of agar per 1,000 ml of water) and incubated at room temperature (20 to 25°C).

Observation of mycelium typical of *Botryosphaeriaceae* spp. was needed to confirm the infection of the shoot. Samples for RNA or protein extraction were collected 20 days after inoculation from the portion of the inoculated internode (the corresponding wounded internode, for controls). Immediately after collection, samples were frozen in the field with liquid nitrogen and subsequently stored at -80°C.

Thanks to this model, the relationship between the pathogen and the plant has been investigated in various conditions of the vineyard (Spagnolo *et al.*, 2017). It can be used to (i) compare the aggressiveness of different strains of the same species, (ii) compare the behavior of different grape varieties planted under the same pedoclimatic conditions and challenged by the same strain of the pathogen, or (iii) evaluate the influence of cultural practices or the effectiveness of plant protection products on disease development. Contrary to the methods using grapevine cuttings cultivated under greenhouse conditions, the weather conditions are not controlled under field conditions and, thus, the experiments must be carried out over several years.

2.6 Discussion of the strengths, weaknesses, and complementarities of the methods

Due to the diversity of GTD fungi, together with the fact that grapevine is a perennial plant grown in widely different climate and cultural conditions around the world, there are great difficulties in developing a model to decipher GTDs. It is essential to understand the mechanisms deployed in woody tissues against pathogens, because the molecular dialog between plant cells and GTD pathogens remains poorly understood. There is, currently, strong overall knowledge of the symptomatology and characteristics of GTD fungi but, although foliar symptoms are visible, the pathogens are rarely found elsewhere than in the wood. It was hypothesized and subsequently demonstrated that GTD fungi are able to produce several metabolites such as cell-wall-degrading enzymes and phytotoxic metabolites that are translocated from the infected woody tissues in the trunk to the aerial part of the affected vines, and their synergistic action may play a role in the emergence of foliar symptoms (Mugnai *et al.*, 1999). Meanwhile, studies on

defense responses induced by GTD fungi have been conducted on plants in the vineyard showing differential expression of foliar symptoms (Bertsch *et al.*, 2013). Nevertheless, host–pathogen field trials are complex because various pathogens are involved. This imposes additional problems such as (i) field results can be misleading, and they frequently differ significantly from one year to another because of different environmental conditions and (ii) they do not separate the effect of the pathogen from the effects induced by other biotic or abiotic agents present in the environment. The methods developed and described in this review aim to provide useful tools to acquire new and more profound knowledge of the interactions between grapevines and GTD pathogens. It is expected that this will lead to the development of new and improved management strategies.

***In vitro* methods.** By using *in vitro* methods, several studies of the interaction between the plant and the molecules delivered by pathogens have shown that the use of callus tissue can be of interest, especially to rapidly test a large number of GTD fungal metabolites associated with aggressiveness. They can also be used to prescreen and pre-evaluate cultivars so that those that show promising resistance to GTDs can be included in new breeding programs. Moreover, callus tissues can be used to acquire greater knowledge about early plant defense reactions, which could be induced in the fast senescence symptoms in response to pathogen infection. Callus tissue can be an interesting first tool to study host–aggressive molecule interactions because they are grown in controlled conditions. For this reason, callus has been used previously for studies of several different plant species such as elm trees (Pijut *et al.*, 1990) and potato (Behnke, 1979). Grapevine calli have been used to understand the effect of toxins and to assess the virulence of esca-associated fungi (Sparapano *et al.*, 2001; Santos *et al.*, 2005). They have also been used to study the response of *P. minimum* and *P. chlamydospora* to the *V. vinifera* environment by analyzing the transcriptomes of both fungi in axenic or mixed cultures with plant cells (Fischer *et al.*, 2016).

For *Botryosphaeria dieback*, these *in vitro* cellular assays have been used by Ramírez-Suero *et al.* (2014), in which extracellular compounds produced by several isolates of *N. parvum* and *D. seriata* induced various degrees of necrosis and triggered defense gene expression in subcultured calli of Chardonnay. Bénard-Gellon *et al.* (2015) reported for the first time the expression of defense genes induced by *Botryosphaeriaceae* protein

fractions on grapevine cells. They emphasized the importance of characterizing the functions of these extracellular proteins, in terms of localization and distribution of these secreted metabolites in infected plants, to initiate the *in planta* investigations of the toxic effects of these proteins. These effects had been previously observed by using callus methods but, by integrating and combining several methods, these important ideas should be validated. For *Botryosphaeria dieback*, cell suspensions can also be used to unveil plant–pathogen interactions, with emphasis on plant cell defense responses (Stempien *et al.*, 2017). They demonstrated that the rapid response of *Vitis* cells to secreted *Botryosphaeriaceae* proteins is in accordance with other studies using artificially inoculated plants as a model. Methods based on cell cultures are very rapid for screening large numbers of samples under highly controlled conditions and can also be very useful to help understand the specific interactions between GTD pathogens and grapevines on a molecular level. Nevertheless, they do not sufficiently mimic plant metabolism because factors such as physical restriction of fungal growth, water stress, and other microorganisms that are frequently found in grapevine are not present and not taken into consideration. Once extracellular compounds, excreted metabolites, and defense genes of GTD pathogens are characterized and analyzed, it is important to perform *in planta* assays in the greenhouse. One of the biggest questions, when using calli and cell culture methods, is to understand to what extent the results obtained can be transferred to the whole plant, even if the molecular responses studied are shown to occur.

Plants and foliar explants. The best methods to comprehend and decipher molecular and physiological effects of compounds that mediate aggressiveness excreted by GTD pathogens probably involve the use of *in vitro*-obtained plants and foliar explants. Leaf assays with either discs or full leaves can be made to reduce the time for phytotoxin identification at the early screening stage. Abou-Mansour *et al.* (2015) used this methodology to characterize phytotoxins produced by *N. parvum* and to evaluate their phytotoxicity. They demonstrated that *N. parvum* can produce several phytotoxins that confer a high adaptability and flexibility to this fungus although, as stated by the authors, the putative role of these toxins in disease expression and their mode of action still needs to be clarified. These assays can also be used in early screening for susceptibility of cultivars but, like the cell suspension and calli assays, they do not encompass the plant

as a whole. Therefore, *in vitro* plant assays are the first approach in which the whole plant is integrated and can help to deepen our understanding of the physiological and molecular effect that compounds produced by GTD pathogens have on grapevines. The basal immunity of grapevines or induced relationships with different diseases or elicitors can be studied and discriminated, as described for *Botrytis cinerea* or *Plasmopara viticola* (Trotel-Aziz *et al.*, 2006), and can also be used to characterize the detoxification capacity of grapevines toward GTD toxins. In 2005, Santos *et al.* (2005) inoculated plantlets by applying 10 µl of a spore suspension at their base, for two Portuguese cultivars (Baga and Maria Gomes) and one rootstock (R3309) with *P. chlamydospora*. They obtained symptoms of senescence close to those described in greenhouse inoculations. The results were in accordance with those of Sparapano *et al.* (2001), in which plantlets from Italian cultivars infected with *P. chlamydospora* and *P. minimum* showed typical esca foliar symptoms. Plantlets can also be used for histopathological studies, which are currently scarce. By growing *in vitro* plants and by maintaining them under controlled conditions, upon inoculation, there is a guarantee that only the fungi inoculated will be observed progressing through the plant. Although the progression of the fungi in mature wood will be different compared with that in younger plants, these young plantlets allow for studies on young tissue (for example, of the progression of *Botryosphaeriaceae* fungi causing cankers on young green stems) while *in vitro* plants, maintained under controlled conditions (in a growth chamber, for example) until they lignify, will allow for studies with more mature tissues. Because one of the main routes for GTD pathogen infection is through pruning wounds, this could unveil new information on how they penetrate and progress inside the plant, allowing further studies on the plant defense responses during the colonization.

2.6.1 The use of *in vivo* nonsterile plants

Laboratory tests are carried out under highly controlled conditions and cannot take into consideration all pathosystem components (detrimental and beneficial). Therefore, the next step is to perform *in vivo* experiments on inoculated rooted cuttings or grafted plants in the greenhouse. Such trials will allow a study of trunk microbial ecology and interactions within the trunk, interactions on cellular and molecular levels between GTD

agents and the host, and tests of new compounds for their ability to limit the impact of GTD pathogens.

Greenhouse methods. The first greenhouse methods to be developed were for Eutypa dieback to demonstrate differences in aggressiveness among isolates of *E. lata* as well as differences in the response of cultivars (Péros and Berger, 1994). Péros and Berger (1994) observed that the shoots of rooted cuttings infected with the fungus showed smaller, necrotic leaves, similar to the symptoms found in the vineyard. They also showed that the expression of symptoms depended greatly on the isolate of *E. lata* used, and that the isolates may differ in their ability to produce toxic compounds which, in turn, depended on the cultivar (Péros and Berger, 1994). Cultivars had various responses to inoculation with the same isolate, indicating their susceptibility profile to *E. lata*. Similarly, Sosnowski *et al.* (2007) demonstrated, with cuttings artificially infected by *E. lata*, that the intensity of foliar symptoms appeared to be related to the origin of the isolates, which may reflect variation in aggressiveness. The observation of cultivar tolerance was not in accordance with previous results on mature grapevines of the same cultivar. Consequently, the authors pointed out that one of the possible disadvantages of this model may be the immaturity of the cutting tissues compared with mature grapevines in the field. Greenhouse inoculation tests to assess isolate aggressiveness levels and susceptibility of cultivars to GTD may, therefore, be strongly influenced by stem maturity, whether herbaceous or more or less lignified. This has to be validated by inoculations of young stems or pruning wounded canes in the field. These methods have also been used to characterize molecular interactions between *E. lata* and grapevines (Camps *et al.*, 2010, 2014) and the effect of water and temperature stress, and for epidemiological studies (Sosnowski *et al.*, 2011). Further studies on the detection of fungal enzymes and host phenolic compounds in the cuttings provided information on the respective importance of several pathogenicity components and host resistance (Péros and Berger, 1994). Recently, Moisy *et al.* (2017) improved a rapid quantitative method, based on real-time PCR, for the assessment of wood colonization by *E. lata* after inoculation of cuttings in controlled conditions.

A similar model for esca disease with *P. chlamydospora* and *P. minimum* is available. It contains two methods to infect plants (nodal and internodal inoculations) and has been complemented by diverse techniques to better understand defense mechanisms in

woody tissues of grapevines. GFP-transformed *P. minimum* was used to investigate early events in wound colonization (Pierron *et al.*, 2015a), confirming that the niches colonized were in accordance with previous work of Fleurat-Lessard *et al.* (2010). Pierron *et al.* (2016) also developed a protocol to study gene expression of grapevine wood infected with *P. minimum* or *P. chlamydospora* where, despite the background noise created by wounding, they observed that woody tissues reacted differently according to the pathogens. Because wounding damage (pruning wounds, mechanical wounds caused by cultural practices, and so on) is considered to be the main gateway for GTD fungi, unveiling which plant responses are to the wound and which are the early responses to infection by pathogens might be a valuable asset to develop new, early diagnostic methodologies. Other tools developed with this model were the monitoring of fungal growth and quantification of DNA copies in the wood, which may be used as a diagnostic test (Pierron *et al.*, 2015a). Finally, for the development of ecofriendly control strategies, a rapid screening in greenhouse conditions could be done even if it probably does not reflect exactly the effect of a chemical or biocontrol agent under vineyard conditions (Mondello *et al.*, 2018). With infected cuttings, the capability of ozone dissolved in water to limit *P. minimum* development was demonstrated (Pierron *et al.*, 2015b) and, more recently, Pierron *et al.* (2016) used the method to analyze the potential effect of elicitors. The potential efficiency of *Pythium oligandrum*, a biocontrol agent, was also evaluated with this model (Yacoub *et al.*, 2016).

A method for reproducing necrosis and foliar symptoms with some species associated with *Botryosphaeria* dieback has been developed. This method permits the characterization of the aggressiveness of *Botryosphaeria* spp. (Úrbez-Torres and Gubler, 2009) by measuring the size of external lesions and also by evaluating the percentage of vines developing foliar symptoms (Reis *et al.*, 2016). Pathogenicity experiments revealed that external lesion sizes and foliar symptoms differed according to the species of fungi inoculated. Plants inoculated with *N. parvum* isolates showed foliar symptom expression that reached 77%, which was always higher than the percentages found for *D. seriata*, which is considered to be a less aggressive pathogen than *N. parvum* (Reis *et al.*, 2016). The plant responses to infection and emergence of foliar symptoms were studied and, among them, some genes such as superoxide dismutase, stilbene synthase, fasciclin-like arabinogalactan, and flavonoids were proposed as potential candidates to be selected

as markers for the emergence of the disease. Because the responses of the artificially infected plants show some similarities to those observed in naturally infected plants, including the ability to reproduce foliar symptoms and also the vascular brown stripe, this assay could be applied for future studies. For example, plant defense stimulators to combat *Botryosphaeriaceae* infection as well as studies aimed at unveiling the relationship between fungi and the appearance of foliar symptoms could be conducted. The greenhouse methods discussed are appropriate to further comprehend the intricate interactions between GTD-associated fungi and grapevines with molecular, physiological, and epidemiological approaches. Although cuttings and grafted plant methods already take into consideration the grapevine as a whole, these plants are growing under controlled conditions and exclude the reproductive stage, climatic events, and other abiotic or biotic factors to which field-grown grapevines are subjected. Therefore, methods that allow researchers to focus on these parameters under natural conditions (i.e., field-grown grapevines) are also needed.

Pruning wound methods. Pruning wound methods were developed to test cultivar susceptibility and to screen disease control methods, primarily against *E. lata* (Paillassa, 1992) but also against most other GTD fungi (Elena *et al.*, 2015). The most widely used approach worldwide, where the incubation period after pruning wound inoculation is at least 8 months, became the official method for regulation of disease control products in Europe (EPPO, 2017). Pruning is one of the most significant cultural practices in viticulture worldwide and the pruning wound model can be considered the best simple model to evaluate pruning wound susceptibility, especially for ecofriendly protectants (Mondello *et al.*, 2018). In light of an increasing number of biocontrol agents tested, the classical method, which is performed in the vineyard and with a long incubation period, is now considered to be the most reliable. This method can also avoid false-negative results caused by fast growing organisms which may overrun slow-growing pathogens such as *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (Elena *et al.*, 2015). Elena *et al.* (2015) demonstrated that the susceptibility of the wounds and subsequent cane colonization may show differential features according to GTD pathogens, suggesting that a new possible global strategy could be optimized by pruning on dates when wound susceptibility to pathogens could be lower. This assay was recently used to validate the new method on detection and quantification of *E. lata* and

D. seriata complex DNA in pruning wounds (Pouzoulet *et al.*, 2017). A set of primers was developed for detection via qPCR, which allows a sensitive and accurate detection of these pathogens, reinforcing the importance that this model has in aiding the development of new early diagnostic tools.

Grapevines are subjected to a series of cultural practices or environmental conditions during the vegetative growth period which may cause one or more wounds on several organs (e.g., desuckering, climatic events such as hail, or removal of lateral shoots). Because levels of airborne inoculum increase when associated with periods of rainfall and high relative humidity (Úrbez-Torres *et al.*, 2010; van Niekerk *et al.*, 2010), and because the interaction of grapevine and GTD pathogens may be influenced by the amount of inoculum present but also by weather conditions, the last method presented in this review was developed by Spagnolo *et al.* (2017) to evaluate the period of higher grapevine susceptibility to *Botryosphaeriaceae*. In this work, the authors demonstrated that the greatest sensitivity to *Botryosphaeria* dieback agents was during the flowering stage, possibly as a consequence of the high metabolic activity oriented toward the development of the inflorescence. This is correlated with a less-effective plant defense response to the attack. The relationship between grapevine phenological stage, primary metabolism, and GTD pathogen infection could not be studied in the calli and cell suspension methods and has never been investigated with greenhouse methods. Therefore, this model is adapted to focus on plant–microbe interactions; namely, expression of defense genes and physiological impact on the host under the variable conditions of a vineyard. The aggressiveness of different strains of GTD pathogens as well as the susceptibility of cultivars to them, under specific regional conditions, could be determined. Weather conditions could represent a key factor that modulates the interactions of the plant with GTD pathogens. Trials need to be repeated for at least 3 years to be conclusive.

Advantages and disadvantages. All these methods have advantages and disadvantages, as reported in this discussion but, together, they provide the scientific community with access to a set of tools that will enhance our understanding of the complex interactions between GTD pathogens and grapevines. One of the main advantages is that they can be used in combinations to deepen our knowledge about the interactions of interest. For example, by using the *Botryosphaeriaceae* model to reproduce foliar symptoms and

brown stripe, the metabolites potentially involved in symptom expression could be clarified. Calli and cell suspension methods can help to test the most interesting metabolites and toxins found with the plant model. The combined methods allow a complementary approach such as determining early grapevine responses to wood infection and characterization of microbial equilibrium in grapevines. This may contribute to a better understanding of the defense response against pathogen infection and an understanding of the molecular and physiological impact of GTDs on grapevine and unveil the interactions between these fungi and the appearance of foliar symptoms. The deeper comprehension of all of these interactions will enable the development of new strategies to diagnose, manage, and control both infection and spread of GTDs. Each of these strategies can be tested and perfected thanks to these simple methods.

2.7 Author contributions

F.F. and C.R. conceptualized the review; Each model was written by a different author. F.F. wrote the introduction; P.R. wrote the method for *Botryosphaeria dieback*, the discussion and compiled the original draft. F.F. and C.R. reviewed the final document.

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chlamydospora, a pathogen involved in Esca, by inducing plant resistance.

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CHAPTER 3

NEW INSIGHTS ON *BOTRYOSPHERACEAE* SYMPTOMS IN GRAPEVINE

Early Season Symptoms on Stem, Inflorescences and Flowers of Grapevine Associated with *Botryosphaeriaceae* Species

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3. Early Season Symptoms on Stem, Inflorescences and Flowers of Grapevine Associated with *Botryosphaeriaceae* Species

Abstract

Botryosphaeria dieback caused by several *Botryosphaeriaceae* species is one of the most important grapevine trunk diseases affecting vineyards worldwide. These fungi cause wedge-shaped perennial cankers and black streaking of the wood and have also been associated with intervein leaf chlorosis, dried or mummified berries, and eventually, the death of the plant. Early season symptoms may sometimes be disregarded by growers, being mistaken with symptoms from other diseases such as downy mildew or botrytis rot. Currently, few studies are available to determine what species may be causing these early season symptoms in grapevines. During the 2018 season, during the flowering period, grapevine samples showing necrosis on green shoots, dried inflorescences, and flowers, were collected in vineyards throughout the central regions of Portugal. Isolations were performed from symptomatic organs, and twenty-three isolates of *Botryosphaeriaceae* were selected. An analysis of the ITS and part of the translation elongation factor 1- α sequences was performed, revealing that the two main species apparently responsible for these symptoms were *Diplodia seriata* and *Neofusicoccum parvum*. In pathogenicity tests conducted on 1-year-old plants grown under controlled conditions in a greenhouse and on field-grown clusters, symptoms were reproduced, confirming the pathogenic behavior of the selection of isolates.

Keywords: grapevine; *Diplodia seriata*; *Neofusicoccum parvum*; pathogenicity; diagnostic

3.1 Introduction

Grapevine trunk diseases (GTDs) are one of the most critical problems affecting viticulture worldwide, causing yield reduction and increased production costs adding to the existing cost of the annual vineyard operations. In fact, these diseases pose a significant threat to sustainable viticulture worldwide since the cost associated with GTD losses has been increasing during recent years. For example, in Spain, there was an increase of 1.8% of incidence in vineyards in 2003 up to 10.5% in 2007 (Rubio and Garzón, 2011). In France, it is estimated that the equivalent of 1 billion euros is lost every year due to these diseases (De la Fuente *et al.*, 2016), while in Australia, the economic impact can reach up to 8.3 billion AUD (Ridgway *et al.*, 2014). GTDs include three main fungal diseases—eutypa dieback, esca disease and botryosphaeria dieback—that involve one or several species of fungi (Bertsch *et al.*, 2013; Bruez *et al.*, 2013; Mondello *et al.*, 2018). In Portugal, botryosphaeria dieback and Esca are considered the major diseases affecting adult grapevines, causing considerable economic losses, in all the country's vine growing regions (De la Fuente *et al.*, 2016).

Botryosphaeria dieback is, currently, one of the most important GTDs worldwide (Bertsch *et al.*, 2013), caused by fungi in the *Botryosphaeriaceae* family. Fungi belonging to this family have been found worldwide as endophytes, saprophytes or pathogenic in many perennial and ornamental plants (Smith *et al.*, 1996a; Smith *et al.*, 1996b; van Niekerk *et al.*, 2006; Bellé *et al.*, 2017).

Twenty-six species in different *Botryosphaeriaceae* genera have been associated with botryosphaeria dieback in grapevines (Rolshausen *et al.*, 2011; Úrbez-Torres *et al.*, 2011; Pitt *et al.*, 2013, 2015; Yang *et al.*, 2017; Gramaje *et al.*, 2018). The most common species isolated from grapevines are *Diplodia seriata* (Cristinzo, 1978; Rovesti and Motermini, 1987; Castillo-Pando *et al.*, 2001; Larignon *et al.*, 2001; Phillips *et al.*, 2007; Savocchia *et al.*, 2007; Úrbez-Torres *et al.*, 2008), *Diplodia mutila* (Lehoczky, 1974; Taylor *et al.*, 2005), *Neofusicoccum parvum* (Crous *et al.*, 2006), and *Lasiodiplodia theobromae* (Luque *et al.*, 2009; Úrbez-Torres, 2011; Rodríguez-Gálvez, *et al.*, 2015).

In different countries, species occurring on grapevines have been shown to differ in pathogenicity, in their epidemiology, and in the symptoms produced (van Niekerk, *et al.*, 2004). Therefore, the symptomatology of botryosphaeria dieback is somewhat complex.

However, the most common symptoms include no or limited burst, perennial cankers, trunk dieback, wood necrosis, vascular streaking, and plant death (Larignon and Dubos, 2001; Phillips, 2002; van Niekerk, *et al.*, 2004; Úrbez-Torres *et al.*, 2006; Úrbez-Torres, 2011; Linaldeddu *et al.*, 2015;). Infection of grapevines by *Botryosphaeriaceae* can also lead to leaf and berry symptoms, where yellowish-orange or wine-red spots on leaf margins and blades can appear, depending on the cultivar (white or red) (Larignon *et al.*, 2001; Bertsch *et al.*, 2013; Spagnolo *et al.*, 2014; Reis *et al.*, 2016, 2019).

During the 2018 season, growers and field technicians in the central region of Portugal, started to observe symptoms characterized by necrosis on the base of green shoots, which could lead to the complete detachment of the shoot later in the season, wilting of the apex of the shoot, wilting of leaves, and more important, necrosis on the peduncle and rachis of the developing clusters, drying of inflorescences and flowers. These symptoms appeared even after the planned fungicide applications against other grapevine diseases, which was puzzling for the growers. Samples were sent for analysis in our laboratory and revealed the presence of *Botryosphaeriaceae* on the tissues analyzed. Although the epidemiology and distribution of *Botryosphaeria* canker in the wood of grapevines as well as other hosts have been widely studied, the relationship between *Botryosphaeriaceae* and these symptoms have not yet been investigated and no studies were carried out to understand which species may be causing these early season symptoms. The objective of this study was thus to identify the pathogens of these early season symptoms in vineyards of Portuguese central regions by means of morphological features, DNA analysis, and pathogenicity tests.

3.2 Material and methods

3.2.1 Sampling and Fungal Isolation

During the early spring of the 2018 season, at the phenological stages E-L 23–25 (Dry and Coombe, 2004), samples from 17 vineyards spread throughout the central and southern regions of Portugal, namely Lisboa, Tejo, and Alentejo, were received at Instituto Superior de Agronomia (ISA). These samples showed necrosis on shoots and dried inflorescences and flowers, and isolations were made by cutting several pieces from symptomatic organs. Pieces collected were surface disinfected with a 7% sodium

hypochlorite solution, rinsed in sterile distilled water (SDW) and plated onto 9 mm Petri dishes containing Potato Dextrose Agar (PDA, BD Difco, Sparks, MD, USA) amended with chloramphenicol (PanReac AppliChem, Darmstadt, Germany) at 250 mg/L. After incubation at 25°C for one week, Petri dishes were assessed for the presence of *Botryosphaeriaceae* colonies which were sub-cultured onto fresh PDA dishes and, again incubated at 25°C for one week, in darkness. All isolates obtained were stored in the collection of the ISA, Lisbon, Portugal, and were afterwards characterized morphologically, as well as properly identified by DNA sequence analyses.

3.2.2 Morphological Characterization and DNA Analysis for Fungal Identification

3.2.2.1 Morphological Characterization

Isolates under study were, plated onto 6 mm Petri dishes containing 2% water agar with autoclaved pine needles (*Pinus pinea*) and incubated at 25°C under fluorescent light, in order to promote sporulation (Phillips *et al.*, 2002; Crous *et al.*, 2006; Úrbez-Torres *et al.*, 2006). Pycnidia were mounted on microscope slides in a solution of lactophenol blue, and digital images were recorded with a Leica DFC295 camera on a Leica DM 2500 microscope at a 400× amplification. Twenty conidia were measured with the Leica Suite v3.16 program, for each isolate under study. Dimensions of the conidia are given as the range of dimensions (length and width) with minimum and maximum dimensions in parentheses followed by mean and standard deviation.

3.2.2.2 DNA Analysis

DNeasy™ Plant Mini Kit by Qiagen® (Venlo, The Netherlands) was used to extract genomic DNA from 8-day-old cultures grown in PDA and incubated at 25°C, in the darkness. The ITS region was amplified using primers ITS5 and ITS4 (White *et al.*, 1990), while the primers EF1-688F and EF1-1251R (Alves *et al.*, 2008) were used to amplify part of the elongation factor 1 α gene. The PCR mixtures contained 1 × PCR buffer REDTaq Ready Mix (Sigma-Aldrich, Saint Louis, MO, USA), 3 mM MgCl₂, 0.4 mM dNTP mix, 12.5 pmol of each primer, 0.06 unit/μl of Taq Polymerase and 25–50 ng of template DNA. Each reaction volume was made up to 25 μl with sterile ultrapure water. Negative controls with sterile ultrapure water instead of the template DNA were used in every

reaction. The amplification conditions for ITS were as follows: initial denaturation of 5 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, 1 min and 40 s at 72°C and a final extension period of 10 min at 72°C. For the amplification of part of the *tef1-α* gene, the conditions were, an initial denaturation of 2 min at 94 °C, followed by 35 cycles of 30 s at 94°C, 45 s at 55°C, 1 min at 72°C and a final extension period of 10 min at 72°C. Each amplicon was separated by electrophoresis at 120 V for 30 min in a 1% agarose gel in 0,5 × TBE buffer. Gels were stained with 3 µl of GreenSafe Premium (Nzytech, Lisbon, Portugal), and were visualized using a UV transilluminator to assess PCR amplification.

The amplified PCR fragments were purified using an Illustra ExoProStar Enzymatic PCR and Sequencing Clean-up Kit (GE Life Sciences, Buckinghamshire, UK) and both strands of the PCR products were sent for sequencing at STABVIDA (Lisbon, Portugal). Sequences obtained were edited and aligned using MEGA7 (Kumar *et al.*, 2016) to find a consensus sequence. These sequences were then compared with sequences from GenBank in BLAST searches, and species identification was obtained when at least 98% of similarity was found.

3.2.3. Pathogenicity Tests

Five representative isolates were selected from the fungal collection under study for pathogenicity tests, with the attention of selecting at least one isolate from the three different species of *Botryosphaeriaceae* identified three isolates of *Diplodia seriata* (Bt201, Bt204 and Bt212), one isolate of *Neofusicoccum parvum* (Bt216) and one isolate of *Diplodia mutila* (Bt218). To reproduce all the symptoms found in the field, three different pathogenicity tests were designed, being two performed on grapevines kept in a greenhouse and one on grapevines established in the field. Greenhouse pathogenicity tests were conducted on 1-year-old grafted Aragonez (= Tempranillo) plants, since is the most planted cultivar in Portugal and field tests were conducted on clusters of Cabernet Sauvignon which is currently the most planted cultivar worldwide.

3.2.3.1. Pathogenicity tests on Tendrils and Leaves

Isolates were plated in Petri dishes containing 2% water agar with autoclaved pine needles (*Pinus pinea*) and incubated at 25 °C under fluorescent light, to promote sporulation (Phillips *et al.*, 2002; Crous *et al.*, 2006; Úrbez-Torres *et al.*, 2006). Conidia were harvested from these plates by collecting the pycnidia formed on the pine needles into a 1.5 ml Eppendorf tube containing sterile distilled water and crushing them with the help of a pestle. These spore suspensions were filtered through cheesecloth and the concentrations were adjusted to 10^5 spores/ml. To ensure full coverage of the tissues to be inoculated, 2 ml of each spore suspension was sprayed on the green tissues (leaves and tendrils) of 1-year-old grafted cuttings of cultivar Aragonez (= Tempranillo), individually potted in 1 L free draining bags containing a sandy soil mixture kept in a ventilated greenhouse at 24 °C under natural light. The aerial part of the plants was covered with a plastic bag for 3 days to promote infection, and the plants were assessed for the development of symptoms, namely necrosis on any of the inoculated organs or drying of leaves and tendrils, one week after inoculation. Ten plants were used for each isolate, while control plants were sprayed with sterile distilled water. The percentage of plants showing symptoms for each isolate was recorded.

3.2.3.2. Pathogenicity Tests on Green Stems

One-year old grafted cuttings of cultivar Aragonez (= Tempranillo), individually potted in 1 L free draining bags containing a sandy soil mixture, kept in a ventilated greenhouse at 24°C under natural light, were inoculated following the method described by Reis *et al.* (2016). Ten plants were used for each isolate, and the assessment for symptoms development, including external lesions or cankers, was performed one week after inoculation, by measuring the width and length of the lesions and calculating the elliptical area of the lesion. All statistical analysis was performed using the R program (www.r-project.org). Assumptions for variance analysis was assessed and when all the assumptions were not accomplished, the influence of distinct levels of one factor was assessed using the non-parametrical test of Kruskal-Wallis. In this case, when the significant differences were found ($P < 0.05$), the comparison between the distinct level was made using the ranks.

3.2.3.3. Field Pathogenicity Tests on Clusters

Clusters of cultivar Cabernet Sauvignon on EL –25 to 27 stage, were selected from several field-grown grapevines in a vineyard located at ISA, Lisbon, Portugal. Artificial inoculations were performed with spore suspensions obtained as referred earlier for the pathogenicity tests on green tissues. Again, to ensure full coverage of the tissues to be inoculated, 1 ml of each spore suspension was sprayed on each cluster, which were covered individually with plastic bags for 3 days. Ten replicates were used for each isolate, and control clusters were sprayed with sterile distilled water. After one week, clusters were assessed for the development of symptoms such as dried or necrotic berries and inflorescences. These symptoms were quantified by using an adaptation of the EPPO protocol for evaluation of fungicides against *Botryotinia fuckeliana* on grapevine (EPPO, 2004), where the percentage of the cluster area infected was assessed according to the following scale: 1 = no symptoms; 2 = 1–5%; 3 = 5–25%; 4 = 25–50%; 5 = >50%. The total percentage of clusters showing symptoms for each isolate was also recorded. The virulence of the different isolates was assessed through a multiple proportion test, comparing the proportion of symptomless samples of each modality towards the control. Boxplots for the infection rank of each isolate were defined to compare aggressiveness of the different isolates. All statistical analysis was performed using the R program (www.r-project.org).

In order to fulfil Koch's postulates, samples were collected from all the different types of symptoms/lesions observed, and placed on PDA (Difco, USA, BD) amended with chloramphenicol (PanReac AppliChem, Darmstadt, Germany) at 250 mg/L to recover the inoculated fungi.

3.3 Results

3.3.1 Sampling and Fungal Isolation

A total of 42 *Botryosphaeriaceae* were isolated from the samples received from the 17 vineyards, and from several different symptomatic organs, including apex and base of the shoots, clusters, and leaves. Other fungi isolated from these samples were *Alternaria alternata*, *Phomopsis* spp. and *Penicillium* spp. The majority of *Botryosphaeriaceae* isolates were obtained from the symptomatic tissue collected at the apex of the shoot

(36%), followed by the base of the shoots (33%). Isolates obtained from the rachis of the clusters represented 17% while 9% were from the clusters (flowers and inflorescences) and only 5% from the leaves. From this set of isolates, a total of twenty-three isolates (Table 3.1) were selected based on their cultural characteristics, with the attention of keeping at least one isolated obtained from each vineyard. These isolates were characterized and identified based on morpho-cultural characters, DNA sequencing, and pathogenicity tests.

Table 3.1 - *Botryosphaeriaceae* isolates obtained during the study, respective GenBank accession numbers and percent identity when compared to reference sequences.

Host			Origin	GenBank Accession Number			Percent Identity *	
Isolate Number	<i>(V. vinifera cvs.)</i>	Isolation Region		Species	ITS	Tef1- α	ITS	Tef1- α
Bt201	Seara Nova	Apex of the shoot	Vineyard 1	<i>Diplodia seriata</i>	MT786219	MW018672	100%	99%
Bt213	Seara Nova	Apex of the shoot	Vineyard 1	<i>Diplodia seriata</i>	MT786231	MW018684	100%	99%
Bt220	Seara Nova	Base of the shoot	Vineyard 1	<i>Diplodia seriata</i>	MT786238	MW018691	99%	99%
Bt221	Seara Nova	Apex of the shoot	Vineyard 1	<i>Diplodia seriata</i>	MT786239	MW018692	100%	99%
Bt202	Alicante	Base of the shoot	Vineyard 2	<i>Diplodia seriata</i>	MT786220	MW018673	99%	99%
Bt219	Alicante	Cluster	Vineyard 2	<i>Neofusicoccum parvum</i>	MT786237	MW018690	99%	100%
Bt203	Castelão	Rachis	Vineyard 3	<i>Diplodia seriata</i>	MT786221	MW018674	99%	100%
Bt204	Syrah	Base of the shoot	Vineyard 4	<i>Diplodia seriata</i>	MT786222	MW018675	100%	99%
Bt205	Castelão	Cluster	Vineyard 5	<i>Neofusicoccum parvum</i>	MT786223	MW018676	100%	99%
Bt206	Castelão	Leaf stem	Vineyard 5	<i>Diplodia seriata</i>	MT786224	MW018677	98%	99%
Bt210	Castelão	Apex of the shoot	Vineyard 5	<i>Diplodia seriata</i>	MT786228	MW018681	99%	99%
Bt207	Castelão	Base of the shoot	Vineyard 6	<i>Diplodia seriata</i>	MT786225	MW018678	100%	100%
Bt208	Aragonez	Apex of the shoot	Vineyard 7	<i>Diplodia seriata</i>	MT786226	MW018679	100%	99%
Bt209	Aragonez	Rachis	Vineyard 8	<i>Diplodia seriata</i>	MT786227	MW018680	100	100
Bt211	Arinto	Base of the shoot	Vineyard 9	<i>Neofusicoccum parvum</i>	MT786229	MW018682	99%	99%
Bt212	Castelão	Base of the shoot	Vineyard 10	<i>Diplodia seriata</i>	MT786230	MW018683	100%	100%
Bt214	Castelão	Apex of the shoot	Vineyard 11	<i>Diplodia seriata</i>	MT786232	MW018685	100%	100%
Bt215	Seara Nova	Rachis	Vineyard 12	<i>Diplodia seriata</i>	MT786233	MW018686	98%	100%
Bt216	Alicante	Apex of the shoot	Vineyard 13	<i>Neofusicoccum parvum</i>	MT786234	MW018687	100%	98%
Bt217	Aragonez	Apex of the shoot	Vineyard 14	<i>Neofusicoccum parvum</i>	MT786235	MW018688	100%	98%
Bt218	Aragonez	Base of the shoot	Vineyard 15	<i>Diplodia mutila</i>	MT786236	MW018689	100%	100%
Bt222	Castelão	Apex of the shoot	Vineyard 16	<i>Diplodia seriata</i>	MT786240	MW018693	100%	100%
Bt223	Alicante	Rachis	Vineyard 17	<i>Diplodia seriata</i>	MT786241	MW018694	99%	99%

* Reference sequences used—*D. seriata* ITS—AY259094, tef1- α —AY573220 [38]; *N. parvum* ITS—AY259098, tef1- α —AY573221 [38]; *D. mutila* ITS—KJ361837, tef1- α —KJ361829 [39].

3.3.2 Morphological Characterization and DNA Analysis for Fungal Identification

Isolates of *Botryosphaeriaceae* could be split into six groups based on colony morphology, after 8 days of growth on PDA. After promoting sporulation of the isolates under study on 2% water agar medium with autoclaved pine needles, three groups were distinguished based on the morphology of conidia. One group containing 12 isolates produced conidia initially hyaline, becoming dark brown, aseptate and ovoid, with average dimensions of (20.9–) 23.6 ± 1.1 (–26.5) $\times$ (7.8–) 9.2 ± 0.5 (10.6), which were determined to be *D. seriata* based on conidia morphology and DNA analysis (Table 3.2). A second group with 3 isolates produced ellipsoidal conidia, with flat base, unicellular and hyaline, which could develop 1 to 2 septa over time, with average dimensions of (14.2–) 16.4 ± 1.9 (–19.5) $\times$ (4.5–) 5.2 ± 0.1 (–6.0) which were determined to be *N. parvum*, based on conidial morphology and DNA analysis (Table 3.2). Finally, there was one isolate that formed hyaline and aseptate conidia, oblong to ovoid with both ends broadly rounded and with dimensions of (20.8–) 23.5 ± 1.4 (–25.0), which was determined to be *D. mutila* based on above-mentioned parameters (Table 3.2). There were seven isolates that did not sporulate until the end of this work, but their species identification was determined through DNA analysis.

Table 3. 2 - Conidial dimension of the *Botryosphaeriaceae* species under study.

Species/Isolate	Conidial dimensions	
	Length (µm)	Width (µm)
<i>D. seriata</i>		
Bt201	(21.3–) 25.2 ± 1.9 (–28.9)	(8.3–) 9.9 ± 0.9 (–11.7)
Bt202	*	*
Bt203	(20.7–) 22.5 ± 1.1 (–24.2)	(6.7–) 8.2 ± 0.6 (–9.2)
Bt204	(20.4–) 22.6 ± 1.6 (–26.2)	(7.8–) 9.1 ± 0.8 (–10.9)
Bt206	(21.0–) 23.8 ± 1.5 (–26.4)	(7.7–) 9.2 ± 0.9 (–10.8)
Bt207	(19.6–) 23.6 ± 1.9 (–27.3)	(7.5–) 9.3 ± 0.8 (–11.3)
Bt208	(21.7–) 25.3 ± 1.4 (–28.7)	(9.5–) 11.1 ± 1.0 (–13.4)
Bt209	(19.6–) 22.2 ± 1.5 (24.5)	(7.2–) 9.0 ± 0.8 (–10.5)
Bt210	*	*
Bt212	(21.3–) 23.9 ± 1.4 (–26.2)	(7.3–) 8.3 ± 0.6 (–9.4)
Bt213	(23.3–) 26.0 ± 1.6 (–29.2)	(7.7–) 9.0 ± 0.6 (–9.8)
Bt214	*	*
Bt215	(21.0–) 24.6 ± 1.5 (–27.0)	(10.1–) 11.2 ± 0.7 (–12.8)
Bt220	*	*
Bt221	(20.8–) 23.3 ± 1.3 (–25.5)	(8.6–) 10.0 ± 0.6 (–11.1)
Bt222		
Bt223	(21.4–) 22.9 ± 1.3 (–26.4)	(9.0–) 9.7 ± 0.7 (–11.4)
<i>N. parvum</i>		
Bt205	(15.5–) 17.2 ± 1.0 (–19.9)	(4.6–) 5.2 ± 0.4 (–6.1)
Bt211	(15.2–) 18.3 ± 1.7 (–22.4)	(4.7–) 5.3 ± 0.3 (–5.9)

* Isolate without sporulation.

Table 3. 2 - Conidial dimension of the *Botryosphaeriaceae* species under study (cont.).

Species/Isolate	Conidial dimensions	
	Length (µm)	Width (µm)
Bt216	(11.4–) 13.7 ± 1.3 (–16.1)	(4.2–) 5.0 ± 0.4 (–6.0)
Bt217	*	*
Bt219	*	*
<i>D. mutila</i>	(20.8–) 23.5 ± 1.4 (–25.0)	(9.5–) 11.1 ± 1.6 (–16.2)
Bt218	(21.3–) 25.2 ± 1.9 (–28.7)	(8.3–) 9.9 ± 0.9 (–11.7)

* Isolate without sporulation.

3.3.3 Pathogenicity Tests

3.3.3.1 Pathogenicity Tests on Tendrils and Leaves

All the isolates tested were pathogenic towards the Aragonez 1-year-old grafted cuttings used in the greenhouse experiment, and they were able to, in some extent, reproduce symptoms closely resembling the ones observed on the field survey, namely necrotic tendrils, apex of the shoots and leaves (Fig. 3.1 A-C). Isolate Bt204 identified as *D. seriata*, was the isolate where the highest percentage (50%) of plants showed the above-described symptoms, followed by isolate Bt216 identified as *N. parvum* (40%) (Fig. 3.2). The isolate with the lowest percentage of infected plants (20%) was Bt218 which was identified as *D. mutila*. No control plants showed any symptoms of disease, growing normally during the experimental period.

3.3.3.2 Pathogenicity Tests on Green Stems

All isolates under study proved to be pathogenic towards 1-year-old grafted Aragonez grapevines, by being able to produce lesions in the inoculated tissues (Fig. 3.1 D-F). All the isolates showed significant statistical differences when compared to the control plants (Fig. 3.3), being the largest average lesions recorded for isolate Bt216 (*N. parvum*) which showed significant differences towards the remaining isolates under study. The smallest average lesions were recorded for three isolates identified as *D. seriata* (Bt201, Bt204 and Bt212), while isolate Bt218 (*D. mutila*) showed lesions on average between those produced by *N. parvum* and *D. seriata* (Fig. 3.3).

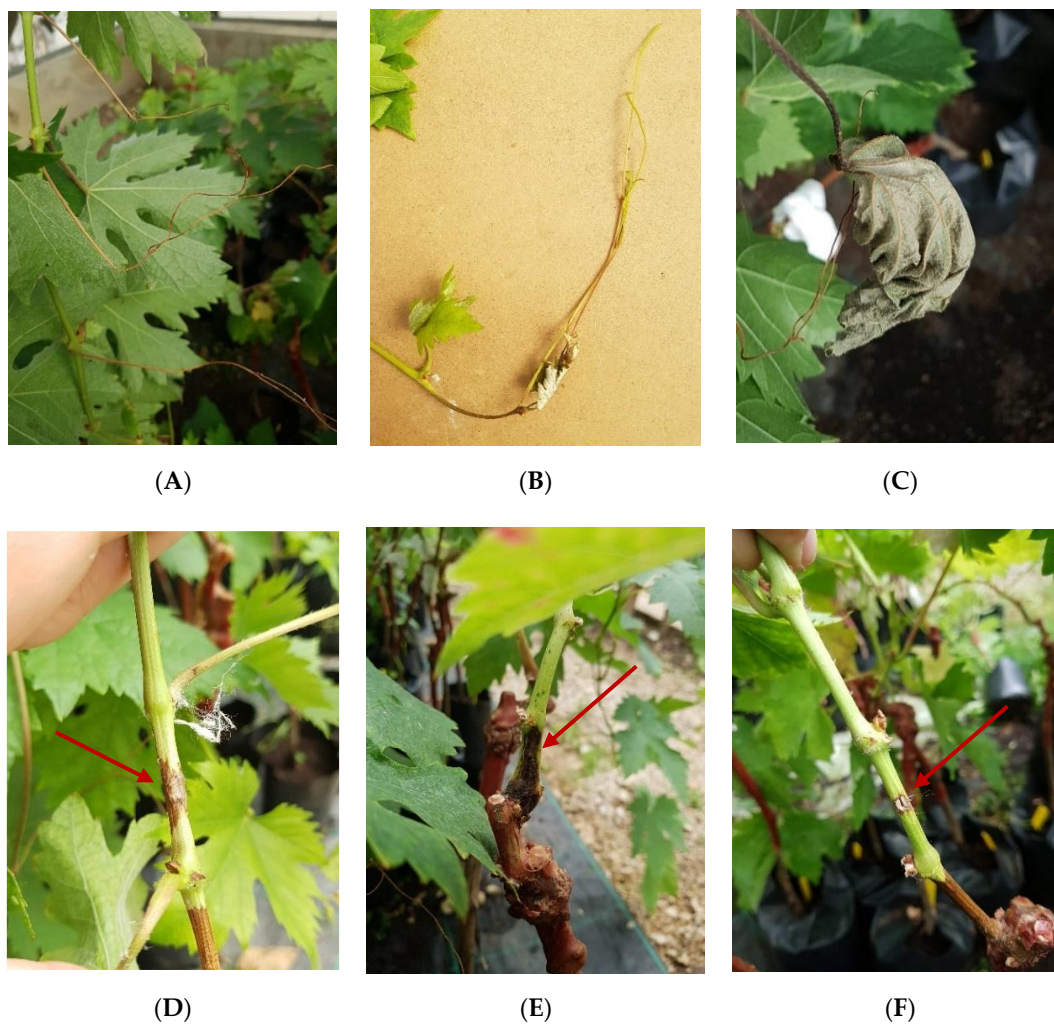


Figure 3.1 - Symptoms observed on tendril and green shoots: **(A)** necrotic tendrils obtained after inoculation with *D. mutila* (Bt218); **(B)** necrotic apex of the shoot after inoculation with *D. seriata* (Bt204); **(C)** necrotic leaf after inoculation with *N. parvum* (Bt216); **(D)** lesion obtained after inoculation with *D. seriata* (Bt204); **(E)** lesion obtained after inoculation with *N. parvum* (Bt216); **(F)** control inoculation.

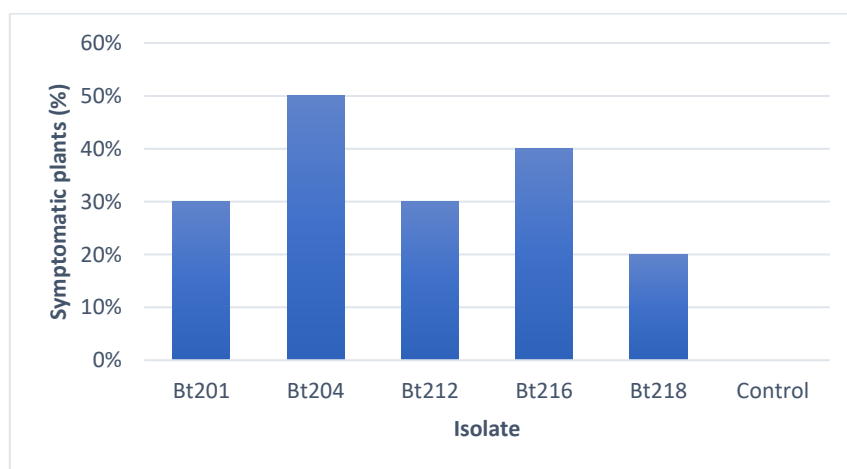


Figure 3.2 - Percentage of plants showing symptoms on tendrils and leaves, after inoculation with the *Botryosphaeriaceae* isolates under study. Ten plants were used per isolate while control plants were sprayed with sterile distilled water.

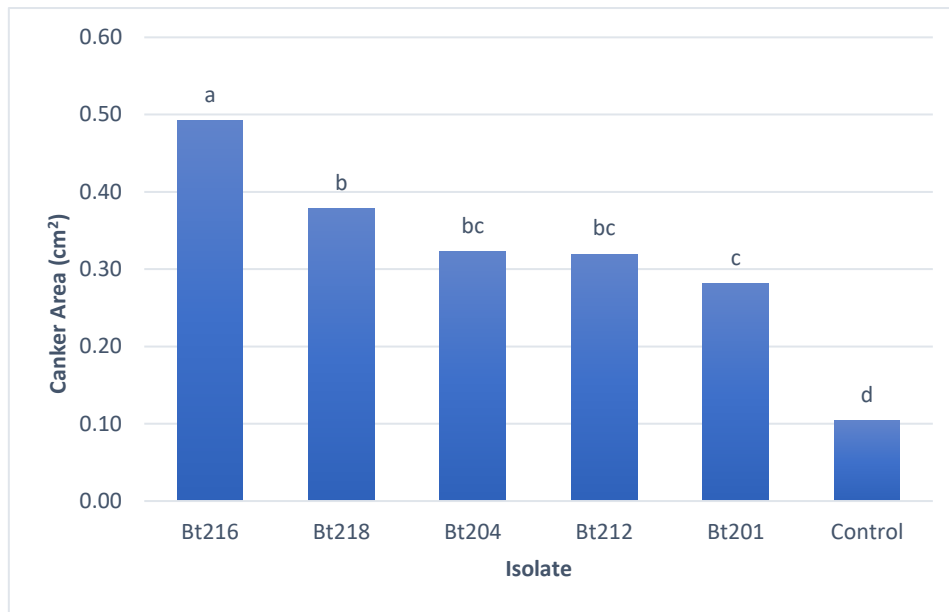


Figure 3.3 - Mean canker areas (cm²) in green stem caused by artificial inoculation with *Botryosphaeriaceae* isolates under study. Different letters in column correspond to significant differences ($P \leq 0.05$) based on ranks assessed by Kruskal-Wallis analysis. Ten plants were used per isolate while control plants were sprayed with sterile distilled water.

3.3.3.3 Field Pathogenicity Tests on Clusters

All isolates used in this study were able to cause symptoms of dried berries and inflorescences on inoculated clusters (phenological stage EL 25 to 27) of Cabernet Sauvignon (Fig. 3.4 A, B). Although only an exploratory statistical analysis was possible based on the infection scale established, due to the low number of repetitions (n), all isolates showed a significant different proportion of symptomless (1-ranked) samples ($p < 0.05$) towards the control, therefore confirming the pathogenicity of the isolates under study when artificially infecting field-grown clusters. Lower p -values ($p = 0.0049$) for isolates Bt201 and Bt218 may suggest a stronger ability of these two isolates in causing symptoms on clusters when comparing to the remaining isolates which recorded a higher p value ($p = 0.0182$). In order to address more in detail this topic, a boxplot analysis was performed (Fig. 4.5) and results showed that among the different isolates, Bt216 showed the lowest average infected area, as nearly all the samples were ranked as a 2-class, and no sample ranked more than 3-class, corresponding to less than 25% of affected area. For the isolates Bt212, Bt218 and Bt204, half of the samples, showed more that 25% of the cluster area with infection symptoms. Within these three isolates,

Bt218 seems to be less aggressive, as no samples were quoted as 3-class or more severe. Bt201 suggested once more to showed tendentially higher infected area values.

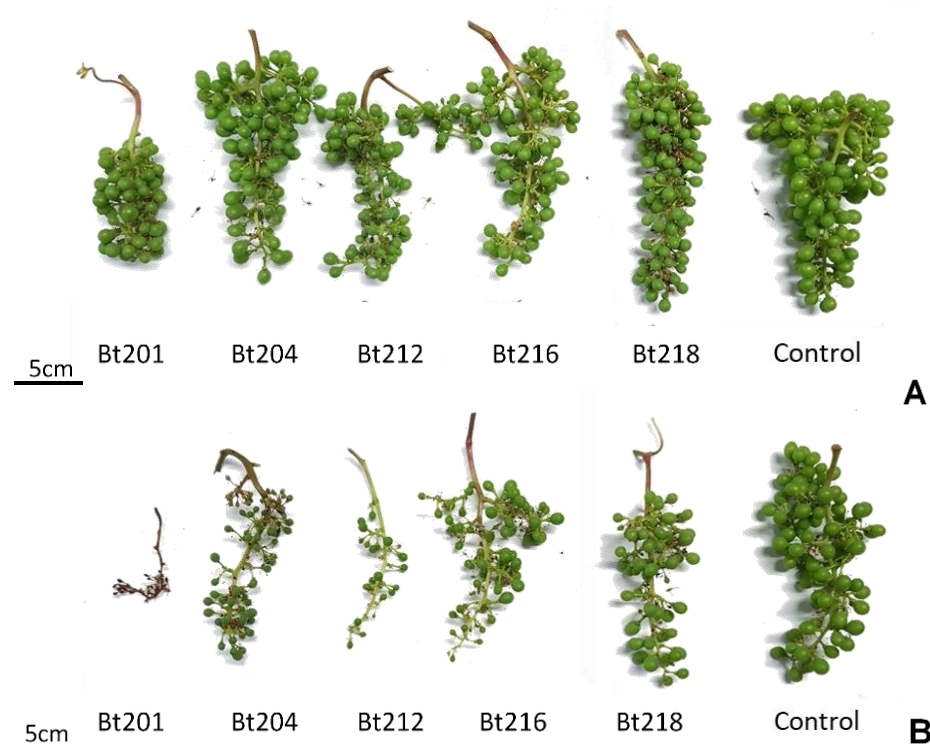


Figure 3.4 - (A) best cluster from each inoculation/isolate; (B) worst cluster from each inoculation/isolate.

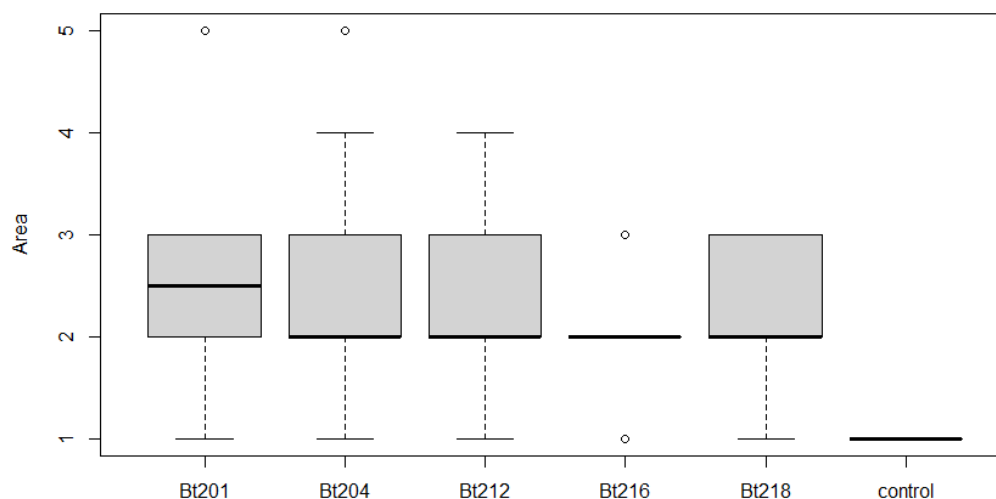


Figure 3.5 - Boxplot of the percentage of infected area on clusters artificially inoculated with *Botryosphaeriaceae* isolates under study. Scale of evaluation of area infected—1 = no symptoms; 2 = 1–5%; 3 = 5–25%; 4 = 25–50%; 5 = >50%. The median is represented by the solid line. Top and bottom lines of the box correspond to the 25th and 75th percentiles of the data, respectively. Circles represent outliers.

For all the pathogenicity experiments, pathogens were recovered from symptomatic tissues of all infected plants, while no *Botryosphaeriaceae* isolates were re-isolated from control plants.

3.4 Discussion

This is the first study aimed at describing grapevine early season symptoms associated with botryosphaeriaceous fungi in Portugal, including necrosis and wilting of the apex of the green shoots, wilting of leaves, necrosis on the peduncle and rachis of the developing clusters and, drying of inflorescences and flowers. Morphological studies and DNA sequence analysis allowed to identify the presence of three different species of *Botryosphaeriaceae*: *D. seriata*, *N. parvum* and *D. mutila*. The most common species found in our study causing the described symptoms was *D. seriata*, since 74% of the isolates belonged to this species, which agrees with previous studies such as Auger *et al.* (2004) in Chile, Luque *et al.* (2009) in Spain and Carlucci *et al.* (2015) in Italy, this being one of the most common species associated with botryosphaeria dieback in vineyards. All the species identified in the present study have been previously identified in other grape-growing regions worldwide, and they have been linked with a broad range of symptoms, including leaf spots, fruit rot, shoot dieback, bud necrosis, vascular discoloration of the wood and perennial cankers (Úrbez-Torres, 2011). All isolates tested showed the ability to cause symptoms to some extent, similar to those observed in the field. Regarding the ability for causing necrosis on tendrils and leaves, all the isolates showed few differences on the percentage of infected plants, being the only exception, *D. mutila* which was able to only cause symptoms on 20% of the plants. Considering the potential of causing lesions on green stems, the isolate belonging to the species *N. parvum* (Bt216) was able to induce the largest average lesions, whereas the lowest average lesions were recorded for the isolates belonging to *D. seriata*. These results are in accordance with previously described results regarding aggressiveness of *Botryosphaeriaceae* species (Úrbez-Torres *et al.*, 2011), in which *N. parvum* is considered to be highly aggressive towards grapevine, while *D. seriata* is considered to be only mildly aggressive. In this case, *D. mutila* showed intermediate average lesions values contrary to what was observed on the pathogenicity tests on both tendrils and

leaves, where this isolate appeared to be the least aggressive. More epidemiology studies should be performed on the behavior of these species to understand if there is a difference of aggressiveness towards different types of grapevine tissues, or if the differences observed were due to experimental design. Considering the ability to cause symptoms on clusters, such as dried berries and inflorescences, the highest average infected area was recorded for *D. seriata* and *D. mutila*. These results are contrary to the ones obtained for the other pathogenicity tests. This may be not due to the aggressiveness of the isolates/species, but to the differences on experimental conditions. These pathogenicity tests were conducted on field-grown grapevines while the other tests were conducted on grapevines growing in a greenhouse-controlled environment, not to mention the differences in plant age.

Although the main economic impact fungi of the *Botryosphaeriaceae* family are associated with the trunk and cane symptoms, damage by these fungi showing up so early in the growing season should not be overlooked, since they could perform an important role in their epidemiology and become a source of inoculum for wound infections leading to trunk diseases. *Botryosphaeriaceae* fungi occur in most parts of the world and are found as endophytes or parasites and saprophytes on a vast number of both annual and perennial plants (Úrbez-Torres, 2011). Infection by *Botryosphaeriaceae* is considered to occur mainly through pruning wounds (von Arx and Muller, 1954; Smith *et al.*, 1994; Úrbez-Torres, 2011;), since cankers start to develop from wounds on leaves, branches or stems. However, several studies have shown that these fungi can infect through lenticels, stomata, or other openings on healthy plants (Michailides, 1991; Kim *et al.*, 1999; Slippers *et al.*, 2007; Smith *et al.*, 2010; Shafi *et al.*, 2019). Nowadays, pruning wounds are considered as the main door of infection for *Botryosphaeriaceae* on grapevine. Nonetheless, Shafi *et al.* (2019) recently showed by fluorescence microscopy that these pathogens can remain latent on the grapevine bark, and even without any type of wounding, germinating conidia and mycelium could be observed near lenticels, as well as mycelia in the underlying wood, demonstrating that the pathogens had entered through the lenticels. Therefore, our observation suggests that *Botryosphaeriaceae* fungi may thus have the ability to colonize and infect healthy grapevine tissues, even without wounding, which may be one of the reasons for the symptoms observed on these vineyards during the spring of 2018.

Pycnidia of *Botryosphaeriaceae* associated with dieback disease can be detected in old pruning wounds, infected spurs, embedded in the bark of the cordons or trunk of infected grapevines, and also on pruning debris left in the vineyard (Hewitt, 1988; van Niekerk, *et al.*, 2010; Úrbez-Torres, 2011; Elena *et al.*, 2016). In France, Kuntzmann *et al.* (2009) reported that conidia of *Botryosphaeriaceae* were released during the whole vegetative period, but *D. mutila* released its spores later than *D. seriata*, indicating that either these fungi differ in their ability to grow and sporulate or that they merely differ in their response to meteorological conditions. These authors also reported that in their study 50% of the spores of *D. mutila* were captured during late summer and autumn while most of the conidia of *D. seriata* were captured during the spring months. This is in accordance with our findings, especially when taking into consideration that our sampling took place between the months of March and July, since most of the species found during our study, belonged to *D. seriata*, while only one isolate of *D. mutila* was recorded. As stated before, our study was conducted when grapevines were at the E-L 23–25 phenological stage which corresponds exactly to the flowering period which has been reported previously by Spagnolo *et al.* (2017) to be the most sensitive period to infection by botryosphaeria dieback agents, as a consequence of the high metabolic activity leaning towards the development of flowers. Therefore, we believe that the conjunction of all these factors, presence of inoculum due to precipitation during the spring months, associated with the phenological stage of the grapevines and the ability of these fungi in infecting through other pathways other than wounds, may have a strong influence on the manifestation of early season symptoms caused by *Botryosphaeriaceae*. Thus, planning further research is strongly advised on this subject with special attention for epidemiology and pathogenicity studies to determine the infection pathway and infection moments regarding environmental conditions.

To the best of our knowledge, this is the first study aimed at describing these *Botryosphaeriaceae* symptoms in grapevines. In conclusion, our work has demonstrated that Botryosphaeria dieback fungi, mostly associated with wood cankers, appear to have the potential to cause serious early season symptoms, since the pathogenicity tests conducted with all the species found were able to reproduce these symptoms on several different grapevine organs. Nevertheless, we strongly believe that further research is needed on this subject by collecting more samples from different regions of Portugal

and by testing a wider range of isolates, to try to understand what are the most common species involved in the expression of these symptoms, and what is or if there is an influence of geographical location and climate conditions (Fischer *et al.*, 2019; Songy *et al.*, 2019). The knowledge and clarification of the symptoms caused by these fungi and the development of proper diagnostic may help growers not to confuse them with symptoms of other diseases, and to set up a proper management plan.

3.5 Author Contributions

P.R., C.R., A.A and F.F. designed the experiments; P.R. and A.G. implemented the methodology; I.L. and J.S. collected the samples on the field; P.R., M.M. and A.G. did the formal analysis; P.R. did the original draft preparation; A.A., F.F. and C.R. did the review and editing; A.A., F.F. and C.R. did the supervision; A.A. and C.R. did the funding acquisition. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 4

GRAPEVINE-PATHOGEN INTERACTION: RESPONSE TO *LASIODIPLODIA* SPP. INFECTION

Response of different grapevine cultivars to infection by *Lasiodiplodia theobromae* and *Lasiodiplodia mediterranea*

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4. Response of different grapevine cultivars to infection by *Lasiodiplodia theobromae* and *Lasiodiplodia mediterranea*

Abstract

Botryosphaeria dieback is a grapevine trunk disease that affects all viticulture regions of the world. Species of the genus *Lasiodiplodia* have been reported as pathogenic towards grapevine in several growing regions and have also been previously reported from Portuguese vineyards. Species in this genus, particularly *Lasiodiplodia theobromae*, have been reported on previous studies to be more aggressive than other *Botryosphaeriaceae* species most commonly associated with Botryosphaeria dieback. The aim of this study was to assess the response of some of the more representative cultivars planted throughout Portuguese vineyards, Touriga Nacional, Touriga Franca, Alvarinho, Aragonez (=Tempranillo) and Cabernet Sauvignon, by performing artificial inoculations with *Lasiodiplodia* spp. collected in different geographic locations worldwide. Two experiments, one by inoculating two-year-old grapevines kept on a greenhouse-controlled conditions with six isolates of *L. theobromae* and one isolate of *L. mediterranea* and other by inoculating seven-year-old field grown grapevines with two isolates of *L. theobromae*, were conducted twice. Response of the cultivars was assessed by evaluating the lesion length caused by the isolates under study, five months after inoculation. The results showed that all isolates studied were able to infect the annual shoots since they were always re-isolated and produced internal wood discoloration. Significant differences were found for all isolate/cultivar combinations. For both experiments, Touriga Nacional showed the largest lesions while Aragonez recorded the smallest lesions amongst the whole lot of cultivars inoculated with *Lasiodiplodia* spp. In general, Portuguese isolates were more aggressive than those from Peru, which demonstrated to be mildly aggressive. These results give a first insight on the response of selected Portuguese cultivars to *Lasiodiplodia* species, which are present in Portugal, but not commonly associated with Botryosphaeria dieback. This contributes to improve knowledge of the impact that Botryosphaeria dieback causal agents have on crucial national cultivars, which may help winegrowers not only to manage current cultural practices, but also to optimize decision making when planning the establishment of new vineyards.

Keywords: *Vitis vinifera*, Botryosphaeria dieback, Lasiodiplodia spp., cultivar response, aggressiveness

4.1 Introduction

Grapevine trunk diseases (GTDs) currently represent one of the most important problems in viticulture worldwide. They impact all vineyard production systems by reducing yield, longevity and increasing production costs. These diseases including esca, Eutypa dieback, young vine decline and Botryosphaeria dieback are caused by fungal pathogens that invade plants mainly through pruning wounds (Úrbez-Torres *et al.* 2006). Botryosphaeria dieback is one of the most impactful GTDs worldwide being caused by several fungi from *Botryosphaeriaceae*. The most common symptoms on grapevine associated with infection by these fungi are limited or no bud burst, leaf chlorosis, dried or mummified berries, perennial cankers, wood necrosis, vascular discoloration and ultimately plant death (Cristinzo 1978; Larignon and Dubos 2001; Phillips 2002; van Niekerk *et al.* 2004; Úrbez-Torres *et al.* 2006; Larignon *et al.* 2009; Bertsch *et al.* 2013; Spagnolo *et al.* 2014; Linaldeddu *et al.* 2015; Reis *et al.* 2020). Many species from this family, namely from the genera *Diplodia*, *Lasiodiplodia* and *Neofusicoccum*, have been previously associated with Botryosphaeria dieback symptoms (Larignon and Dubos 1997; Graniti *et al.* 2000; Fischer, 2006; Phillips *et al.* 2002; Úrbez-Torres *et al.* 2006; Larignon *et al.* 2009, 2015; Luque *et al.* 2009; Úrbez-Torres 2011; Bertsch *et al.* 2013; Spagnolo *et al.* 2014). Currently, the genus *Lasiodiplodia* comprises more than 20 species (Phillips *et al.* 2013; Linaldeddu *et al.* 2015) from which eight of them have been reported from grapevine, namely *L. crassispora*, *L. mediterranea*, *L. missouriana*, *L. parva*, *L. plurivora*, *L. pseudotheobromae*, *L. theobromae* and *L. viticola* (Úrbez-Torres *et al.* 2012; Correia *et al.* 2013; Yan *et al.* 2013; Linaldeddu *et al.* 2015). *Lasiodiplodia theobromae*, the most commonly isolated from grapevine, is a plurivorous fungus, usually found in tropical and subtropical climate regions (Punithalingam 1976; Úrbez-Torres *et al.* 2008), that occurs mainly on fruit and forest trees (Alves *et al.* 2008; Liu *et al.* 2012; Linaldeddu *et al.* 2015). *Lasiodiplodia* species have been increasingly recorded from grapevines not only in tropical and subtropical regions but also in regions with temperate climates such as Australia (Taylor *et al.* 2005), Brazil (Correia *et al.* 2013),

Bolivia (Kaiser *et al.* 2009), China (Yan *et al.* 2013), Italy (Burruano *et al.* 2008), Mexico (Úrbez-Torres *et al.* 2008), Peru (Rodríguez-Gálvez *et al.* 2015), Portugal (Rego *et al.* 2010), Spain (Aroca *et al.* 2008), Turkey (Akgul *et al.* 2014) and USA (Úrbez-Torres *et al.* 2008).

The Portuguese viticulture sector has seen a vast increase during the last decades, in terms of production, exports and revenues (IVV.IP, 2021a; 2021b). Botryosphaeria dieback is reported in vineyards throughout all the wine appellations of the country, being reported as frequently occurring in 60% of the established vineyards in Portugal (Guérin-Dubrana *et al.*, 2019). Botryosphaeria dieback alongside with Esca are considered to be the major GTDs affecting Portuguese vineyards causing considerable damages and economic losses (De la Fuente *et al.*, 2016). Nevertheless, the status of *Lasiodiplodia* species on grapevine in Portugal is still unclear, being this disease usually associated mainly with *Diplodia seriata* and *Neofusicoccum parvum* (Reis *et al.* 2020). *Lasiodiplodia theobromae* has been formerly reported in Portugal (Rego *et al.* 2010), and according to previous studies in other countries, this species was frequently the most aggressive species (Úrbez-Torres *et al.* 2008, 2009; Taylor *et al.* 2015), although a high variability of aggressiveness could be found amongst isolates from grapevine (Úrbez-Torres *et al.* 2009; Rodríguez-Gálvez *et al.* 2015). Considering the ability of species from this genus to cause serious damage, combined with the lack of knowledge about the impact of *Lasiodiplodia* spp. on the most representative cultivars planted in Portugal, it is of the utmost importance to evaluate the response and possible impact of these species on the Portuguese vineyards. Therefore, the main objective of this study was to get a first insight on the response of some of the most important cultivars in Portuguese viticulture to *Lasiodiplodia* isolates, by performing artificial inoculations on plants grown in both greenhouse and vineyard. This will improve knowledge on the possible impact it may have in the expanding Portuguese viticulture sector and may therefore help improving the planning and management of current cultural practices, and in optimizing decision making when planning the implementation of new vineyards contributing to a more sustainable and productive viticulture.

4.2 Material and methods

4.2.1 Greenhouse experiment:

Plant material: The inoculations were performed on two-year-old grafted grapevine cuttings of five different cultivars, Aragonez (= Tempranillo), Touriga Nacional, Touriga Franca, Alvarinho and Cabernet Sauvignon. The rootstock used to graft all the cultivars was 1103 *Paulsen*. Plants were obtained from a commercial nursery where the plants underwent hot water treatment and potted individually in a sandy soil mixture (1/3 sand, 1/3 soil, 1/3 organic matter) and placed randomly in a ventilated greenhouse at 24°C under natural light and watered as required. The experiment was conducted twice, in 2017 and 2019.

Fungal isolates and inoculation method: Six *L. theobromae* isolates and one *L. mediterranea* isolate (Table 4.1) were used for this experiment. Isolates Bt102, Bt103, Bt105 and Bt106 were stored at the culture collection of Instituto Superior de Agronomia, Lisbon, Portugal. These isolates have been previously obtained from infected grapevines, showing brown cankers along the shoots, dieback and marginal chlorosis of the leaves, throughout different wine regions of Portugal, while isolates LA-SOL3 and LA-SV1 were obtained from symptomatic grapevine plants in Peru. These isolates were collected in a desert to semi-desert climate and had been previously tested for pathogenicity towards grapevine by Rodríguez-Gálvez *et al.* (2015) and they are currently stored at Universidade de Aveiro, Portugal. They were therefore used as a comparative reference to the remaining isolates used in the assays. *Lasiodiplodia mediterranea* is currently not reported in Portugal, nonetheless it has been previously reported in other European countries and, therefore, one isolate of this species was also included in this study, not only for comparison, but also to investigate how the set of five cultivars would respond to different species of *Lasiodiplodia*. The isolate of *L. mediterranea* CBS 124060 used in the greenhouse experiment, belongs to the CBS-KNAW culture collection from the Westerdijk Fungal Biodiversity Institute, in Utrecht, Netherlands, and was previously collected in a Sicilian vineyard. Isolates were maintained on potato dextrose agar (PDA, Difco, Sparks, MD, USA) slants and transferred to Petri dishes with PDA to promote colony growth.

As described in Reis *et al.* (2019), and similar to the inoculation method used previously by several other authors (Úrbez-Torres *et al.* 2008, Linaldeddu *et al.* 2015, Guan *et al.* 2016, Spagnolo *et al.* 2017, Rezgui *et al.* 2018 and Ramírez *et al.* 2018, Chacón *et al.* 2020). A 3 mm area of the bark was removed, with a cork borer, from shoots with around 1 cm of diameter. Inoculation was carried out between the second and third nodes, which corresponds to a height of around 10 cm from the base of the shoot. Wounds were inoculated by placing 3 mm mycelium plugs sampled from the actively growing margin *L. theobromae* and *L. mediterranea* colonies with eight days growth in PDA (Difco, BD, Sparks, MD, USA) at 25°C in the darkness, on the fresh wound. Each inoculation point was covered with moist cotton wool and sealed with Parafilm, for a week, to prevent drying. For control inoculations 3 mm PDA plugs were used. A total of 25 plants were used for each inoculation combination of isolate/cultivar, making a total of 200 plants used for each cultivar and a total of 1000 plants per trial. For both years (2017 and 2019) inoculations were made in the first week of May.

Table 4. 1 - *Lasiodiplodia* spp. isolates used for inoculation of the grapevine cultivars

Species	Isolates	Geographic origin	Cultivar	Year of collection
<i>Lasiodiplodia theobromae</i>	Bt102	Algarve, Portugal	Red Globe	2008
	Bt103	Estremadura, Portugal	Aragonez	2009
	Bt105	Alentejo, Portugal	Castelão	2009
	Bt106	Setúbal Peninsula, Portugal	Touriga Nacional	2009
	LA-SOL3	Sol Sol, Piura, Peru	Red Globe	2013
	LA-SV1	San Vicente, Piura, Peru	Red Globe	2013
<i>Lasiodiplodia mediterranea</i>	CBS 124060	Sicily, Italy	N/A	N/A

Determination of internal lesions and pathogen recovery: Five months after inoculation, internal lesions were measured by longitudinal cutting the shoots from the base until the end of the necrosis was found. Internal lesions were measured upward and downward from the inoculation point using an electronic calliper. Four pieces of necrotic tissue from the margin of the lesion were collected from all the inoculated plants, and surface disinfected with a 7% sodium hypochlorite solution, rinsed in sterile distilled water (SDW) and plated onto 9 mm Petri dishes containing PDA amended with chloramphenicol (PanReac, AppliChem, Darmstadt, Germany) at 250 mg/l. After

incubation at 25°C for one week, Petri dishes were assessed for the presence of *Botryosphaeriaceae* colonies which were sub-cultured onto fresh PDA dishes and, again incubated at 25°C for one week, in the darkness. These cultures were then plated on 60 mm Petri dishes containing 2% water agar with autoclaved pine needles and incubated at 25°C under fluorescent light, to promote sporulation (Crous *et al.* 2006; Phillips 2002; Úrbez-Torres *et al.* 2006). Pycnidia were mounted on microscope slides in a solution of lactophenol and digital images were recorded with a Leica DFC295 camera on a Leica DM 2500 microscope at a 400 X amplification, in order to confirm identification as *Lasiodiplodia* spp. A representative set of *Lasiodiplodia* spp. isolates was selected from each of the different inoculation combinations (isolate/cultivar), to undergo molecular identification and confirm the identity of the species. DNeasy™ Plant Mini Kit by Qiagen® (Venlo, The Netherlands) was used to extract genomic DNA from 8-day-old cultures grown in PDA and incubated at 25 °C, in the darkness, following manufacturer's instructions. Part of the translation elongation factor 1 α gene was amplified by using the primers EF1-688F and EF1-1251R (Alves *et al.* 2008). Amplified DNA was visualized on agarose gels stained with GreenSafe Premium (Nzytech, Lisbon, Portugal), and was visualized using with a UV transilluminator to assess PCR amplification. PCR products were purified using an Illustra ExoProStar Enzymatic PCR and Sequencing Clean-up Kit (GE Life Sciences, Buckinghamshire, UK) and both strands of the PCR products were sent for sequencing at STABVIDA (Lisbon, Portugal). Sequences obtained were then compared with sequences from GenBank in BLAST searches.

4.2.2 Field experiment:

Plant material: Inoculations were performed on field grown grapevine plants of the same five cultivars, Aragonez, Touriga Nacional, Touriga Franca, Alvarinho and Cabernet Sauvignon used for the greenhouse experiment. Plants from all the cultivars are grafted onto SO4 rootstocks. These plants belong to the ampelographic collection of the Instituto Nacional de Investigação Agrária - Estação Vitivinícola Nacional (EVN), located at Dois Portos, Torres Vedras, Portugal (Coordinates: 39° 2' 28" N, 9° 10' 55" W; Altitude: 79 m; Köppen climate classification of Csb). This vineyard was selected due to having all the five cultivars under study in the same area, thus limiting the soil and climatic effects.

Plants were 7-year-old, trained in bilateral cordons, spur-pruned and monitored for GTD symptoms. The experiment was conducted twice, during the years 2018 and 2019.

Fungal isolates and inoculation method: For this trial, two isolates of *L. theobromae* were selected from the batch described on Table 1, namely isolates Bt105 and LA-SOL3, originating from Portugal and Peru, respectively. Both isolates were chosen based on the results found in the first year of the greenhouse assay, where Bt105 appeared to be the most aggressive isolate among the Portuguese isolates, and LA-SOL3 appeared to be the most aggressive of the two Peruvian isolates. This allowed for a comparative reference for the behaviour of isolates with two different geographic origins. The inoculation method used was the same as described for the greenhouse experiments, being the inoculations made during the flowering period, which was previously considered the most sensitive period to infection by *Botryosphaeriaceae* (Spagnolo *et al.* 2014; Mondello *et al.* 2018). For each cultivar (5) and each isolate (2), a total of eight shoots were inoculated, with only one shoot inoculated per plant. For control inoculations only PDA plugs were used. This makes a total of 120 samples for this field assay.

Internal lesions length and pathogen recovery: Internal lesions were measured at the end of the experiment, five months after inoculation, immediately after harvest, using the same method as described for the greenhouse experiments. Four wood samples were again collected from each shoot, using the same methodology as described above.

4.2.3 Statistical analysis

For both experiments (greenhouse and field) statistical analysis was performed using the R-program (www.r-project.org). Before doing an analysis of variance, homogeneity of variance was tested using a Levene's test. When necessary and, in order to verify the assumption of homogeneity of variance, a log10 transformation to the variable lesion length was performed. Within each of the two assays an ANOVA was used to compare the differences of lesion length and re-isolation percentage between isolates. Before the statistical analysis, data for re-isolation percentage (RP) was transformed into the arcsine of the square root of the proportion $(RP/100)^{1/2}$. Means were compared using Tukey's test at the 5% significance level ($P < 0.05$). For the greenhouse assay, in order to

allow a better and easier way to look at the full range of the results, the isolate variables (a total of 8, including control) were subjected to a principal component analysis (PCA) in order to group the different varieties tested and to reduce the observed variables into a smaller number of principal components, which account for the majority of the variance observed. This analysis was also preformed using R-program with the Devtools package (Wickham and Chang 2016). This analysis was not performed for the field assay, due to the lower number of variables to consider.

4.3 Results

The response of the different cultivars to the inoculation with *Lasiodiplodia* spp. and aggressiveness of the isolates was evaluated through the assessment of internal lesion, or wood discoloration length. For both experiments, five months after inoculation, all isolates were able to produce internal wood discoloration in all the grapevine cultivars under study (Fig. 4.1). Through the duration of the experiment, no foliar and/or berries symptoms were observed. Significant differences in lesion length data could be found between both years, so data from each year was analysed separately. Significant differences were observed when comparing all the isolate/cultivar combinations with the non-inoculated control plants, revealing that all the isolates were aggressive towards grapevine (Table 4.2). Pathogen's re-isolation rate was recorded for all the isolates/cultivars under study (Table 4.3). Moreover, the spores obtained from the recovered cultures showed a morphology typical of *Lasiodiplodia* spp. (Phillips *et al.* 2013) and the representative isolates subjected to molecular analysis were confirmed as *L. theobromae* and *L. mediterranea*.



Figure 4.1 – Necrosis on shoots inoculated for the greenhouse (A, B, C) and field (D, E, F) experiments: A – Isolate Bt103 inoculated on Touriga Nacional (2017); B – Isolate CBS124060 inoculated on Aragonez (2019); C- Mock inoculation (Control) on Alvarinho (2019); D – Isolate Bt105 inoculated on Touriga Nacional (2018); E – Isolate LA-SOL3 inoculated on Touriga Franca (2019); F – Mock inoculation (Control) on Aragonez (2018).

4.3.1 Greenhouse experiments

Mean lesion lengths of wood discoloration caused by the *Lasiodiplodia* spp. isolates inoculated onto five cultivars in the greenhouse are shown in Table 4.2. In the first year of the experiment (2017), the mean lesion length caused by the inoculation of several isolates ranged from 1.3 cm in Cabernet Sauvignon, caused by isolate LA-SV1, to 14.7 cm caused by isolate CBS 124060 in Alvarinho, while during the second year (2019) this parameter varied from 1.7 cm caused by isolate LA-SV1 in Aragonez to 12.1 cm in Touriga Nacional, caused by isolate Bt106. During the duration of the experiment, some cultivars showed different responses to the inoculation with the isolates under study. This was particularly evident for cultivar Alvarinho that showed mean lesion length values substantially lower in 2019 than the ones recorded in 2017, with maximum mean length values ranging from 14.7 cm to only 6.5 cm, respectively. Similar behaviour could be observed for cultivar Touriga Franca where maximum values varied from 9.7 cm in 2017 to 5.0 cm in 2019. The remaining three cultivars showed similar mean lesion length values for both years of the experiment, being Touriga Nacional the one that recorded the overall largest values for both years.

Table 4.2 – Mean internal lesion length (cm) obtained five months after inoculations of five different cultivars with six isolates of *L. theobromae* and one isolate of *L. mediterranea* for the greenhouse and two *L. theobromae* isolates for the field experiments.

Mean Internal Lesion (cm) ^a											
Greenhouse experiment											
Cultivar	Aragonez		Touriga Nacional		Touriga Franca		Alvarinho		Caberbet Sauvignon		
Isolate	2017	2019	2017	2019	2017	2019	2017	2019	2017	2019	2019
CBS124060	3.0 bc	5.4 ab	9.8 b	7.4 c	5.6 bc	4.5 a	14.7 a	5.2 ab	2.8 c	4.4 ab	
Bt102	2.6 c	6.0 ab	10.3 b	10.2 ab	4.8 cd	5.0 a	11.4 ab	6.8 ab	5.0 a	3.9 ab	
Bt103	4.0 ab	6.8 ab	11.7 b	10.5 ab	6.2 bc	5.0 a	10.4 b	5.8 ab	3.2 bc	4.9 ab	
Bt105	4.3 ab	6.3 ab	14.0 a	8.5 bc	9.7 a	4.1 ab	13.3 ab	2.6 c	4.0 ab	4.2 ab	
Bt106	3.7 abc	4.9 b	10.8 b	12.1 a	6.6 b	4.4 a	5.1 c	4.4 b	3.7 b	5.2 a	
LA-SOL3	4.7 a	1.8 c	9.7 b	2.7 d	3.4 d	2.8 bc	5.8 c	2.1 c	1.9 d	3.1 bc	
LA-SV1	3.9 ab	1.7 c	3.0 c	2.2 d	2.0 e	2.8 c	2.0 d	4.2 b	1.3 e	2.1 c	
Control	0.4 d	0.6 d	0.6 d	0.6 e	0.8 f	0.5 d	0.8 e	0.6 d	0.6 f	0.6 d	
Field experiment											
Isolate	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019	2019
Bt105	4.5 a	5.2 a	9.5 a	15.4 a	2.0 a	8.5 a	2.0 a	16.2 a	7.9 a	11.1 a	
LA-SOL3	4.2 a	4.7 a	5.4 a	6.7 b	1.4 ab	7.2 a	2.2 a	13.5 a	7.0 a	7.9 a	
Control	0.5 b	0.5 b	0.5 b	0.5 c	0.6 b	0.6 b	0.6 b	0.6 b	0.7 b	0.7 b	

^a Mean internal lesion obtained from inoculation with *Lasiodiplodia* spp. calculated as the mean of the lengths measured upward and downward from the inoculation point, of all the replicates. Columns with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

The PCA analysis allowed a better visualization of the data, of both years, through the ordination of the different cultivars according to the mean lesion length caused by the several inoculated isolates (Fig. 4.2). For the 2017 results, the first principal component (PC1) explained 66.4% of the variation of the data and the second principal component (PC2) explained 12.7% of the variation (Fig. 4.2 A). In the biplot of the two principal component analyses, the axis corresponding to PC1 is clearly associated with size of the lesion caused by the different isolates. The size of the lesions caused by all isolates, with the exception of isolate LA-SV1 increase negatively, clearly separating cultivars Touriga Nacional and Alvarinho as the cultivars with the largest lesion lengths from the remaining three, during this year of the experiment. The length of the variable vectors representing each isolate inoculated, shows that isolates CBS 124060, Bt102, Bt103 and Bt105 were the main contributors to PC1 and therefore, the ones capable of causing the largest lesion length, while LA-SV1 was the isolate that caused the smallest lesion length, confirming the evidence shown on the ANOVA analysis (Table 4.2). The biplot for the PCA analysis of the data obtained during 2019 shows that PC1 explained 60.7% of the variation of the data and PC2 represented 11.7% of the variation of the data (Fig. 4.2 B). In this case, the size of all lesion length caused by all isolates also showed a positive increase with the exception of isolate LA-SV1, clearly separating cultivar Touriga Nacional from all the others. This consistency of results was already observed through the ANOVA analysis (Table 4.2) and reveals that Touriga Nacional was the cultivar under study, with the highest response to infection with *Lasiodiplodia* spp. The length of the isolate vectors, shows that all Portuguese isolates (Bt102, Bt103, Bt105 and Bt106) were the ones capable of causing largest lesion length, while, as was observed in 2017, the Peruvian isolates recorded the smallest values of lesion length. Regarding pathogen's re-isolation rate significant differences could be found amongst the several isolates, with the exception of Cabernet Sauvignon in 2017 where no differences could be observed. During the 2017 experiment, varied from 51.6% for isolate LA-SOL3 in Alvarinho to 98.4% for isolate Bt102 in Touriga Nacional (Table 4.3), while in 2019 re-isolation percentage values varied from 62.5% caused by isolate LA-SOL3 in Touriga Franca and 100% for isolate Bt106 in Touriga Nacional. Although significant differences were observed between the isolates studied, no correlation could be found between the length of the lesion caused by each isolate and the re-isolation rate of the respective

pathogen. Nevertheless, the Peruvian isolates which caused lower mean lesion lengths than the Portuguese *L. theobromae* and the Italian *L. mediterranea* isolate, also recorded lower re-isolation percentages than the isolates above mentioned (Table 4.3). No *Lasiodiplodia* spp. were re-isolated from the control (non-inoculated) plants.

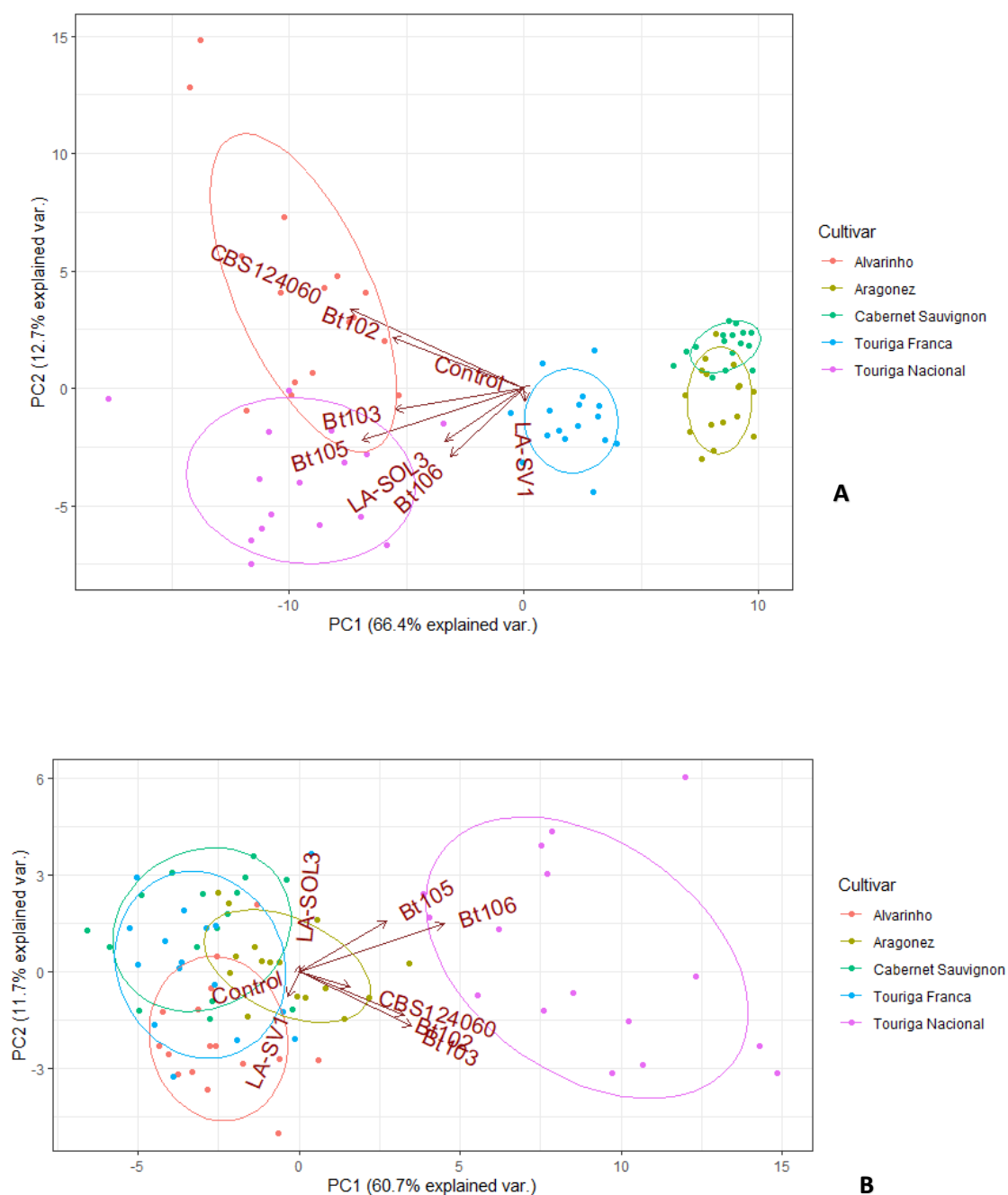


Figure 4.2 – Principal component analysis (PCA) of the response of five different grapevine varieties inoculated with eight *Lasiodiplodia* sp. isolates, during the two years of greenhouse experiment (A – 2017; B - 2019). Percentage value within parentheses represent the variance explained by each principal component.

Table 4.3 – Mean re-isolation percentage obtained five months after inoculations of five different cultivars with six isolates of *L. theobromae* and one isolate of *L. mediterranea* for the greenhouse and two *L. theobromae* isolates for the field experiments.

Re-isolation percentage (%) ^b																				
Green house experiment																				
Cultivar	Aragonez				Touriga Nacional				Touriga Franca				Alvarinho				Caberbet Sauvignon			
Isolate	2017		2019		2017		2019		2017		2019		2017		2019		2017		2019	
CBS 124060	84.3	ab	85.6	ab	96.9	a	96.9	ab	73.4	ab	82.8	ab	81.2	a	93.7	ab	59.4	a	90.6	ab
Bt102	71.9	ab	98.4	a	90.6	ab	90.1	ab	71.9	ab	92.2	a	59.4	ab	95.3	ab	78.1	a	95.3	a
Bt103	59.4	b	96.9	a	98.4	a	93.7	ab	67.2	ab	87.5	ab	85.9	a	95.3	ab	73.4	a	92.2	a
Bt105	82.9	ab	98.4	a	95.3	ab	95.3	ab	84.4	a	90.6	a	84.4	a	85.9	ab	57.8	a	90.1	ab
Bt106	93.3	a	95.3	a	89.1	ab	100.0	a	76.6	ab	84.3	ab	78.1	ab	96.9	ab	71.9	a	89.1	ab
LA-SOL3	68.7	ab	64.1	c	76.6	b	92.2	ab	53.1	b	62.5	b	51.6	b	73.5	b	54.7	a	76.6	ab
LA-SV1	89.1	a	75.0	bc	92.2	ab	81.2	b	53.1	b	76.6	ab	56.2	ab	82.8	ab	60.9	a	71.9	b
Field experiment																				
Isolate	2018		2019		2018		2019		2018		2019		2018		2019		2018		2019	
Bt105	59.4	a	87.5	a	96.9	a	93.7	a	53.1	a	100.0	a	75.0	a	87.5	a	71.9	a	87.5	a
LA-SOL3	75.0	a	78.1	a	65.6	b	81.2	a	31.2	a	96.9	a	84.4	a	90.6	a	37.5	ab	78.1	a

^b Percent of re-isolation of *Lasiodiplodia* spp. from inoculated shoots calculated as the mean frequency of positive growth samples from each shoot over the total number of shoots inoculated. Columns with the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

4.3.2 Field experiment

Mean lesion lengths of wood discoloration caused by the *Lasiodiplodia* spp. isolates inoculated onto five cultivars are shown in Table 4.2. Significant differences were found when comparing all the isolate/cultivar combinations with the non-inoculated control plants, except for isolate LA-SOL3 inoculated in Touriga Franca which did not reveal significant differences compared to the control inoculation. For the remaining combinations of isolate and cultivar, no significant differences were determined between the two isolates studied, being the only exception cultivar Touriga Nacional, during the 2019 assay, for which differences between the isolates Bt105 and LA-SOL3 were recorded. For the first year of the experiment (2018) the mean lesion length recorded varied from 1.4 cm for isolate LA-SOL3 in Touriga Franca and 9.5 cm for isolate Bt105 in Touriga Nacional. During the second year (2019), mean lesion length values were substantially higher than the ones found in 2018, being the only exception the cultivar Aragonez, in which only a slight increase on mean lesion length is observed. In this case mean lesion length ranged from 4.7 cm for isolate LA-SOL3 in Aragonez and 16.5 cm for isolate Bt105 in Alvarinho.

Concerning pathogen's re-isolation rate, similarly to the results observed for mean lesion length, higher re-isolation percentages are found for the second year of the experiment, especially for the cultivars where mean lesion length values were substantially higher than the ones observed during 2018. With the exception of Touriga Nacional in 2018, no significant differences between both isolates could be found for all cultivars, on both years of the experiment. In 2018, re-isolation percentage ranged from 31.2% for isolate LA-SOL3 in Touriga Franca and 96.9% for isolate Bt105 in Touriga Nacional, while during 2019 values recorded were higher, varying from 78.1% for isolate LA-SOL3 on both Aragonez and Cabernet Sauvignon cultivars, and 100% for isolate Bt105 in Touriga Franca. No *Lasiodiplodia* spp. strains were recovered from the non-inoculated shoots.

4.4 Discussion

This study presents the first comprehensive assessment of the response to *L. theobromae* and *L. mediterranea* of five of the most representative grapevine cultivars,

currently planted in Portugal. For both greenhouse and field experiments, all cultivars showed internal wood symptoms when inoculated with the seven *Lasiodiplodia* spp. isolates studied, but mean lesion lengths varied significantly amongst them. For the controlled condition experiment conducted in greenhouse, differences of aggressiveness between isolates of *L. theobromae* were also observed, with a prevalence of higher aggressiveness registered for the isolates collected in Portugal, comparing to the isolates originating from Peru. For most of the cultivars, the *L. mediterranea* isolate, showed an intermediate aggressiveness with mean lesion length values usually between the Portuguese and Peruvian isolates. The pathogenicity of *L. theobromae* and *L. mediterranea* on grapevines has been previously reported by several authors (Leavitt 1990; van Niekerk *et al.* 2004; Taylor *et al.* 2005; Aroca *et al.* 2008; Úrbez-Torres *et al.* 2008; Úrbez-Torres and Gubler 2009; Travadon *et al.* 2013; Carlucci *et al.* 2015; Linaldeddu *et al.* 2015) and *L. theobromae* has in fact been characterized as one of the most aggressive *Botryosphaeriaceae* species (Úrbez-Torres 2011). Nonetheless, in the present study a high variability in aggressiveness of the several isolates was observed. In fact, isolates originating from Peru were able to cause a maximum mean lesion length of only 4.7cm for isolate LA-SOL3 on cultivar Aragonez in 2017, whereas isolate LA-SV1 was able to reach a maximum of 3.9 cm for the same cultivar and year. Portuguese isolates, on the other hand, were able to cause mean lesion lengths as high as 14.0 cm for isolate Bt105 on Touriga Nacional, in 2017. This variability in aggressiveness between different isolates of the same species is in agreement with previous studies by Taylor *et al.* (2005), in Western Australia, where a high variability in virulence was observed for the *L. theobromae* isolates under study. Moreover, a variability in aggressiveness has been verified in several different geographic regions, since high aggressiveness was observed for isolates from Western Australia (Taylor *et al.* 2005), Spain (Aroca *et al.* 2008), Mexico (Úrbez-Torres *et al.* 2008), California in USA (Úrbez-Torres and Gubler 2009), Italy (Burruano *et al.* 2008) and Peru (Rodríguez-Gálvez *et al.* 2015), but only little differences could be found between the lesions caused by isolates of *L. theobromae* and control plants in South Africa (van Niekerk *et al.* 2004). This difference in aggressiveness of isolates from distinct geographic regions was previously hypothesized by Travadon *et al.* (2013) after observing that *L. theobromae* isolates from the USA were more aggressive towards

Thompson Seedless compared to Chardonnay. Úrbez-Torres *et al.* (2008) reported opposite results when studying Mexican isolates, suggesting that isolates from different locations may differ in their aggressiveness towards different cultivars. This seems to be the case with the pathogenic behaviour of the isolates used in our greenhouse trial, where there was an apparent strong relationship between geographic origin and aggressiveness for all inoculated cultivars, revealed by the ability of Portuguese isolates to cause longer lesions than the Peruvian isolates as well as slightly higher re-isolation rates. Moreover, the two Peruvian isolates had been previously tested for pathogenicity against cultivar Red Globe (Rodríguez-Gálvez *et al.* 2015). In this study isolate LA-SOL3 was considered to be one of the most aggressive, while LA-SV1 was the less aggressive from the whole set of isolates studied. Therefore, it is somewhat unexpected that isolate LA-SOL3, previously reported as a highly aggressive, was found to be so weak against the cultivars tested in our study, especially in the greenhouse experiment. These specific isolates have been obtained from grapevines growing in a desert to semi-desert climate without marked seasons (Rodríguez-Gálvez *et al.* 2015). Therefore, one possible explanation for the differences found may be, not only the different response of the cultivars tested in both our and the study by Rodríguez-Gálvez *et al.* (2015), but the environmental conditions from which these isolates were initially isolated, compared to the controlled conditions used on the greenhouse experiment. The difference in apparent aggressiveness for the several isolates, based on geographic provenance, is in fact the only pattern that could be observed in our study. During the two years assay conducted under greenhouse-controlled conditions, there was no isolate that seemed to constantly be the most virulent for one or more cultivars. The results obtained from the field experiment, although with only two isolates studied, revealed that despite the differences in the mean length of the lesions between cultivars, no statistical differences between both isolates could be observed, with the exception of cultivar Touriga Nacional for which significant differences were found but only in the second year of the experiment. This is in accordance with the study by Úrbez-Torres and Gubler (2009), which details that variability can depend on the inoculated tissue, origin of the isolate, timing of inoculation period, type of inoculum used, age of the host, and different cultivar response. In our case, the difference in age of the plants (2 and 7 years), and especially the weather events influence of the field assay may be relevant for a more

homogenous ability by the two isolates in colonizing the wood of field grown grapevines. Plant age is indeed considered a key factor in biotic and abiotic stress responses, as the effect of the combination of stress on plants is largely dependent on their age (Pandey *et al.* 2015), existing a positive correlation between grapevine age and GTD incidence (Brown *et al.* 2016; Fischer and Peighami-Ashnaei, 2019). Moreover, climatic and edaphic factors have been reported to influence the expression of esca and other GTDs (Graniti *et al.* 2000, Sosnowski *et al.* 2011; van Niekerk *et al.* 2011; Calzarano *et al.* 2018), indicating that the pathogen is usually not the sole responsible for the disease symptoms emergence (Fischer and Peghami-Ashnaei, 2019). Abiotic stresses have an impact on the defence mechanisms that plants trigger upon a pathogen attack, strongly influencing resistance or susceptibility (Kulday and Yates 2000; Amtmann *et al.* 2008; Mittler and Blumwald 2010). The impact of abiotic stresses, such as drought and/or thermal stress, have been previously reported to influence grapevine response to *Botryosphaeria* dieback (for review see Songy *et al.* 2019), which may account for the differences found in the behaviour of both isolates, Bt105 and LA-SOL3, in our experiments. The controlled conditions in the greenhouse such as temperature, humidity, and no water deficit, may suggest that these conditions are more favourable for the proliferation of the Portuguese isolates, whereas field conditions where plants are subjected to abiotic stress, may favour also a more effective colonization by the Peruvian isolate.

Regarding cultivar response, the most consistently higher mean lesion length was achieved for Touriga Nacional, which suggests that it might be the cultivar most impacted by infection with *Lasiodiplodia* spp. in our study. Although cultivar Alvarinho showed slightly larger values during the 2017 experiment in the greenhouse and during the 2019 assay in the field, when compared with Touriga Nacional, only the latest maintained significant highest levels of mean lesion length for both years and experiments. In fact, mean lesion length values during the 2019 greenhouse experiment were reduced by half for Touriga Nacional, with 6.5 cm *versus* 12.1 cm recorded for isolate Bt102, respectively. In the field experiment Touriga Nacional showed a minor increase from year 2018 to 2019, especially for isolate Bt105 (9.5 cm to 15.4 cm), but the largest increase was verified for Alvarinho and isolate LA-SOL3 (2.0 cm to 16.5 cm). The only other cultivar that also showed some variations between both years of the experiment was Touriga Nacional especially in the field assay, where mean lesion

lengths varied between 1.9 cm and 8.5 cm for isolate Bt105 and between 1.4 cm and 7.2 cm for isolate LA-SOL3. The two remaining cultivars, Aragonez and Cabernet Sauvignon showed consistent values for both years and experiments suggesting lower response from all the cultivars from the lot under study, for the field assay Aragonez appeared to show an even lower response than Cabernet Sauvignon. These differences in mean lesion length may be explained by the several factors referred previously and detailed by Úrbez-Torres and Gubler (2009). The differences reported in mean lesion lengths in the greenhouse, especially for cultivar Alvarinho, may be due to differences in plant material, since new sets of 2-year-old plants were used for each year. For the field assay, the differences found are most likely due to weather and edaphic factors, which vary from year to year. As referred by Fischer and Peighami-Ashnaei (2019), these factors influence the expression of disease symptoms, as abiotic stress impacts plant defence responses and therefore susceptibility. Our results lead us to conclude that, although no cultivar showed sign of tolerance towards infection by *L. theobromae* and *L. mediterranea*, differences in response to inoculation among cultivars were observed, with Touriga Nacional being the cultivar that showed the highest lesions and Aragonez the lowest ones. These differences in response could be related with the vessel diameter since Pouzoulet *et al.* (2014) showed that the vessels of the Merlot cultivar were smaller than those of Thompson Seedless, confirmed by similar observations for Thompson Seedless that appeared to be the most susceptible cultivar to *N. parvum* while Merlot was the least susceptible (Travadon *et al.* 2013). More recently, Bellée *et al.* (2017) showed significant differences in internal necrosis, amongst cultivars Cabernet Sauvignon, Merlot and Ugni Blanc, when inoculated with *D. mutila* and *D. seriata*. Although no research on vessel dimension and histological structure was conducted for any of the Portuguese cultivars used in our study, this relationship between vessel diameter and response to the progression of GTD pathogens may be one of the factors responsible for the differences observed between cultivars. Therefore, further research on the morphological traits of the targeted cultivars is advisable in order to better clarify the impact that intrinsic characteristics to each cultivar may have on plant pathogen colonization. In addition, several other factors may contribute to the difference in response of these cultivars. Chemical barriers are thought to restrain fungal growth (Shigo 1984), being known that cultivar susceptibility to pruning wound infections by

Eutypa lata are dependent on suberin and lignin deposition during the healing process of the wounds (Munkvold and Marois 1995). Overall, the plant defence is provided by many genes, which are involved in physiological responses by providing necessary structures and material (Niks *et al.* 2015). For GTDs, the plant responses may vary amongst cultivars (for review Fontaine *et al.* 2015; Mondello *et al.* 2018), or even clones (for review see Fischer and Peighami-Ashnaei 2019), and also with a vintage effect (Moret *et al.* 2019, 2020). Actually, Berlanas *et al.* (2017) showed that there were variations of response between clones of Tempranillo to GTDs, which was considered to be very susceptible to them (Sosnowski *et al.* 2013). Our study shows that Aragonez, which is the Portuguese name for cultivar Tempranillo, showed the milder response to inoculation with *L. theobromae* and *L. mediterranea*, with the smallest average lesion lengths recorded. Therefore, the mechanisms underlying the response of different varieties are complex, not only because of the many factors that play a role on this process, but also due to the large number of cultivars, planted worldwide, that may present only minor differences from each other. Consequently, extensive research will be needed, on all these factors as reported in the GTD model summarized by Claverie *et al.* (2020) and to try to create a better understanding of grapevine response to these pathogens.

In conclusion, our study shows that *L. theobromae* and *L. mediterranea* have the potential to cause severe lesions and cankers on some of the most important grapevine cultivars planted in Portugal, although different responses to *Lasiodiplodia* spp. infection could also be found amongst the referred cultivars. This study reported that infection with *Lasiodiplodia* spp. may highly impact vineyards of several cultivars, but specially Touriga Nacional. Moreover, this cultivar is currently considered as one of the most promising for high quality wines in Portugal, and therefore, infection with these pathogens may represent a threat to the expanding Portuguese viticulture sector.

4.5 Author contributions

P.R., C.R., A.A. and F.F. designed the experiments; P.R. and A.G. implemented the methodology; P.R. formal analysis; P.R. original draft preparation; A.A., F.F. and C.R.

review of the original draft; A.A., F.F. and C.R. supervision; A.A. and C.R. funding acquisition.

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CHAPTER 5

DISEASE MANAGEMENT:

SCREENING NEW CONTROL STRATEGIES FOR *LASIODIPLODIA* SPP. INFECTION IN GRAPEVINE

**Combining an HA + Cu (II) Site-Targeted Copper-Based Product with a
Pruning Wound Protection Program to Prevent Infection with
Lasiodiplodia spp. in Grapevine**

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5.1 Combining an HA + Cu (II) Site-Targeted Copper-Based Product with a Pruning Wound Protection Program to Prevent Infection with *Lasiodiplodia* spp. in Grapevine

Abstract

The genus *Lasiodiplodia* has been reported from several grape growing regions and is considered as one of the fastest wood colonizers, causing Botryosphaeria dieback. The aim of this study was to i) evaluate the efficacy of Esquive®, a biocontrol agent, on vineyard pruning wound protection, applied single or, in a combined protection strategy with a new site-targeted copper-based treatment (LC2017), and ii) compare their efficacy with chemical protection provided by the commercially available product, Tessior®. For two seasons, protectants were applied onto pruning wounds, while LC2017 was applied throughout the season according to the manufacturer's instructions. Pruning wounds of two different cultivars were inoculated with three isolates of *Lasiodiplodia* spp. Efficacy of the wound protectants, varied between both years of the assay and according to the cultivar studied but were able to control the pathogen to some extent. The application of LC2017 did not show clear evidence of improving the control obtained by the sole application of the other products tested. Nevertheless, LC2017 showed a fungistatic effect against *Lasiodiplodia* spp., *in vitro*, and has previously shown an elicitor effect against grapevine trunk diseases. Therefore, this combination of two protection strategies may constitute a promising long-term approach to mitigate the impact of Botryosphaeria dieback.

Keywords: *Botryosphaeriaceae*; *Trichoderma atroviride* strain I-1237; Hydroxyapatite-Copper; *Vitis vinifera*; combined strategy

5.1.1 Introduction

Botryosphaeria dieback is currently among the most significant grapevine trunk diseases (GTDs) in all the grape-growing regions of the world (Gramaje *et al.*, 2018). It represents one of the major threats to sustainable and economically viable viticulture due to the reduction in yield, increased crop management costs, and shortened life span of vines and vineyards (Bertsch *et al.*, 2013; Kaplan *et al.*, 2016; Gramaje *et al.*, 2018). This disease is caused by fungi of the family *Botryosphaeriaceae* and more than 26 taxa of this family have been associated with Botryosphaeria dieback in grapevine (Úrbez-Torres, 2011; Pitt *et al.*, 2013a; Rolshausen *et al.*, 2013; Yang *et al.*, 2017). *Lasiodiplodia* spp. and *Neofusicoccum* spp. were previously proven as being amongst the fastest wood colonizing genera, and therefore considered as some of the most virulent GTD fungi (van Niekerk *et al.*, 2004; Úrbez-Torres *et al.*, 2008, 2009; Pitt *et al.*, 2013b). The genus *Lasiodiplodia* comprises 34 species (Phillips *et al.*, 2013; Linaldeddu *et al.*, 2016; Rangel-Montoya *et al.*, 2021; Zhang *et al.*, 2021), from which ten have been reported from the grapevine (Úrbez-Torres *et al.*, 2012; Correia *et al.*, 2013; Phillips *et al.*, 2013; Yan *et al.*, 2013; Linaldeddu *et al.*, 2016; Rangel-Montoya *et al.*, 2021; Zhang *et al.*, 2021). *Lasiodiplodia theobromae* is the most commonly isolated species in grapevine and, although it is most common in tropical and sub-tropical regions, it can be found in vineyards around the world, such as Australia (Taylor *et al.*, 2005), Algeria (Arkam *et al.*, 2021), Brazil (Correia *et al.*, 2013), Bolivia (Kaiser *et al.*, 2009), China (Yan *et al.*, 2013), Italy (Burruano *et al.*, 2008), Mexico (Úrbez-Torres *et al.*, 2008), Peru (Galvez *et al.*, 2015), Portugal (Rego *et al.*, 2010), Spain (Aroca *et al.*, 2008), Turkey (Akgül *et al.*, 2014), and the USA (Úrbez-Torres *et al.*, 2008). Common external symptoms caused by infection with botryosphaeriaceous fungi on grapevine, include leaf spots and wilting, dry fruit rots, bud necrosis and perennial cankers, cordon dieback, and eventually the sudden death of the plant (Úrbez-Torres, 2011; Mondello *et al.*, 2018; Reis *et al.*, 2020). The internal wood symptoms usually consist of wedge-shaped necrotic sectors and brown streaking below the bark, sometimes beginning in the pruning wounds (Úrbez-Torres *et al.*, 2010). These fungi are air-borne and infect grapevine through any type of wound, but primarily the infection occurs through the pruning ones (Gramaje *et al.*, 2018). Pycnidia of different *Botryosphaeriaceae* species associated with Botryosphaeria

dieback can be found within old pruning wounds, in dead or cankered wood, embedded on the bark of cordons and trunks of infected grapevines. They can also be found on pruning debris left in the vineyard, constituting a potential source of inoculum for new infections (van Niekerk *et al.*, 2010; Úrbez-Torres, 2011; Elena *et al.*, 2016). Up to now, there are no curative methods to mitigate infection by *Botryosphaeriaceae* species. Preventive control methods such as pruning wound protection are currently the practice proving to be more efficient (Gramaje *et al.*, 2018; Mondello *et al.*, 2018), especially if carried out from the early stages of the vineyard lifespan (Kaplan *et al.*, 2016; Sosnowski *et al.*, 2017). The field efficacy of chemical wound protectants against botryosphaeriaceous fungi has been demonstrated in several grape-growing regions of the world, namely Australia (Pitt *et al.*, 2012), Chile (Díaz *et al.*, 2013), New Zealand (Amposah *et al.*, 2012; Sosnowski and Mundi, 2019), Portugal (Rego *et al.*, 2014), South Africa (Bester *et al.*, 2007; Mutawila *et al.*, 2015), Spain (Martínez-Diz *et al.*, 2021), and the USA (Rolshausen *et al.*, 2010). Another method considered to be the most effective strategy for controlling infection by GTD pathogens is the application of pastes and paints amended, or not, with fungicides. This latter can provide a physical barrier, preventing spore germination to occur in the wound, but if this barrier is altered by any external factor, the supplementation with a fungicide will act on the pathogen, inhibiting its growth (Gramaje *et al.*, 2018). Their efficacy has also been shown, specifically against *Botryosphaeriaceae* (Epstein *et al.*, 2008; Rolshausen *et al.*, 2010; Pitt *et al.*, 2012; Díaz *et al.*, 2013; Martínez-Diz *et al.*, 2021). The currently existing active ingredients (AIs) are effective in protecting pruning wounds but have limited systemic activity. These AIs usually do not penetrate well enough into the grapevine tissues to effectively control pathogens inside the vascular system (Mondello *et al.*, 2018; Wu *et al.*, 2020) and limit the colonization by pathogens. Therefore, new methods to efficiently deliver fungicides to specific targeted areas of the plant are considered to have great potential to improve GTD control. The application of site-targeted fungicides to protect vascular tissues against GTD pathogen colonization has been recently investigated by several authors (Fischer *et al.*, 2019; Wu *et al.*, 2020; Battiston *et al.*, 2021). Lignin nanocarriers loaded with pyraclostrobin, as a targeted drug delivery system (Fischer *et al.*, 2019), phloem mobile derivative of fenpiclonil in combination with beneficial endophyte (Wu *et al.*, 2020), and copper-based treatments, formulated with hydroxyapatite (HA) as co-

adjuvant with innovative delivery properties (Battiston *et al.*, 2018; Mondello *et al.*, 2021) have shown promising results in controlling both esca and *Botryosphaeria* dieback pathogens. This last formulation has also shown an elicitation ability towards several genes related to plant defense (Mondello *et al.*, 2021). However, increasing consumer demands for reduced chemical use and growing re-strictions on the use of synthetic pesticides have increased interest in the use of natural active ingredients such as biocontrol agents (BCAs). Research on BCAs has greatly increased over the last years, being *Trichoderma* species the most studied as biopesticides (Úrbez-Torres *et al.*, 2020). Several *Trichoderma*-based products are currently used as pruning wound protectants against GTDs (for review see Gramaje *et al.*, 2018 and Mondello *et al.*, 2018) and several studies have been conducted using these products, showing encouraging results, against *Botryosphaeriaceae* species (Mutawila *et al.*, 2011, 2015, 2016; Mounier *et al.*, 2016; Reis *et al.*, 2017; Mondello *et al.*, 2018; Berbegal *et al.*, 2020; Rusin *et al.*, 2020). However, although several promising solutions currently exist, it seems impossible to manage *Botryosphaeria* dieback using only a single approach. An integrated pest management (IPM) strategy has been recommended including cultural practices, organic products, BCAs, responsible use of chemical fungicides, and control management that may combine both chemical and biological products (Sosnowski *et al.*, 2017; Mondello *et al.*, 2018).

To the best of our knowledge, no studies on strategies integrating pruning wound protection with chemical or biological products, and the application of site-targeted fungicides, with elicitor properties, have been conducted against *Botryosphaeria* dieback pathogens, on established vineyards. The main objectives of this work were thus to (i) test the efficacy of one *Trichoderma* based formulation, namely Esquive, single or in combination with LC2017, a new site-targeted copper-based treatment, formulated with hydroxyapatite loaded with copper (II) sulphate pentahydrate (CuSPHy + HA) which has a fungistatic and elicitor effect and, (ii) to compare their efficacy with a strategy of combining the application of commercially available chemical pruning wound protectant, Tessior®, a liquid polymer containing boscalid and pyraclostrobin alone and with LC2017, to prevent both infection and colonization by *Lasiodiplodia* spp. in field trials.

5.1.2 Materials and Methods

5.1.2.1 In vitro Assays

5.1.2.1.1 Mycelial Growth Inhibition Assay

Prior to field application of the LC2017 (CuSPHy+HA) product on the field, mycelial growth assays were conducted using three *Lasiodiplodia* spp. isolates, Bt105, LA-SOL3, and CBS124060 (Table 5.1.1). An assay was also performed using the *T. atroviride* strain I-1237 (Esquive®, product developed by Agrauxine S.A. and commercialized by Idai Nature S. L.), to test the compatibility between both products. To obtain the *T. atroviride* strain I-1237, a solution was made by directly suspending the Esquive® product, on 100 mL of sterile distilled water. A 1.5 mL aliquot was transferred to a 90 mm Petri dish containing 20 mL of Potato-Dextro-Agar (PDA, Difco, Sparks, MD, USA), and spread onto the surface using a sterile plastic loop. Petri dishes were then incubated at 25°C for 7 days in absolute darkness. For the mycelial growth assays, a stock solution of product LC2017 was made by suspending the product at the recommended field concentration (250 L/ha) to be applied immediately after pruning, in 1000 mL of sterile distilled water (SDW). Six different concentrations were made in SDW and added to 50°C molten PDA, and 20 mL was poured into each 90 mm Petri dish, with six replicate plates allowed for each combination of LC2017 concentration and isolate (both *Lasiodiplodia* spp. and *T. atroviride* strain I-1237). The test range of LC2017 product concentration ranged from 0.025 to 12.5 mL L⁻¹ and the six concentrations tested were evenly distributed across that range. Four hours after pre-paring the plates, 3 mm diameter discs were cut from the actively growing margin of one-week-old colonies all the isolates and placed on the center of each plate. Control plates contained only PDA. Plates were incubated at 25°C for 48 h, in complete darkness, after which the two perpendicular diameters of the colonies were measured using a digital caliper. Mycelial growth inhibition (GI) was calculated according to Battiston *et al.* (2021): $GI = [(DC-DO)/DC] \times 100$, where DC is the diameter of mycelial growth in the control plates and DO is the diameter of mycelial growth in treated plates. To establish if the effect of LC2017 on the tested fungi was only fungistatic, inhibited fungal disks were reinoculated onto fresh PDA plates and their growth revival was observed after 48 h.

Table 5.1.1 - *Lasiodiplodia* spp. isolates used for pruning wound inoculation.

Species	Isolates	Geographic Origin
<i>L. theobromae</i>	Bt105	Alentejo, Portugal
	LA-SOL3	Sol Sol, Piura, Peru
<i>L. mediterranea</i>	CBS 124060	Sicily, Italy

5.1.2.1.2 Dual Culture Antagonism Assay

Dual culture antagonism assays were also performed to evaluate the antagonistic capability of the *T. atroviride* strain I-1237 (Esquive) against the three *Lasiodiplodia* spp. isolates targeted for study (Bt105, LA-SOL3 and CBS124060), using dual culture assays (Úrbez-Torres *et al.*, 2020; Rahman *et al.*, 2009). *Trichoderma atroviride* strain I-1237 cultures used for this assay were obtained using the same method described for the mycelial growth inhibition assays. Mycelium plugs with 5 mm diameter of *T. atroviride* strain I-1237 and each *Lasiodiplodia* sp. isolate were cut from the actively growing margin of 3-day-old colonies, growing on PDA. Plugs were placed on opposite edges of 90 mm Petri dishes containing 15 mL of PDA. Plates were then incubated for 5 days in the dark at 22°C. Each *Lasiodiplodia* sp. isolate was grown in-dividually under the same conditions as control plates. Each combination *Trichoderma/Lasiodiplodia* spp. was replicated four times and the assay was performed twice. The percentage of mycelium growth inhibition was calculated using the formula, percent inhibition (PI) = $[(B-A)/B] \times 100$ [42], where A is the radius of pathogen mycelium growth on the dual culture plates, and B is the radius of *Lasiodiplodia* spp. growth on the control plates.

5.1.2.2 Field Assays

5.1.2.2.1 Experimental Field

The assay was conducted between 2019 and 2020, on an experimental vineyard located at Instituto Superior de Agronomia (ISA) (38°42'33.5"N 9°11'15.8"W) Lisbon, Portugal, planted in 1998. Two cultivars were used for this assay, Touriga Nacional (TN) and Cabernet Sauvignon (CS), both grafted onto 140 Ruggeri. Vines were trained as bilateral cordons. Traditional cultural practices in the vineyard were kept throughout the whole assay, and disease management followed an IPM. The products applied were selected

with the care of not containing any substance that could casually interfere with the assay.

5.1.2.2.2. Fungal Isolates Used and Inoculum Preparation

Two *L. theobromae* and one *L. mediterranea* isolates were used for this assay (Table 5.1.1). *Lasiodiplodia theobromae* isolates Bt105 and LA-SOL3, were collected in Portugal and Peru, respectively, and were stored at the culture collection of Instituto Superior de Agronomia. Both were isolated from grapevine wood showing symptoms of cankers and wood necrosis. The *L. mediterranea* isolate used is from the CBS culture collection from the Westerdijk Fungal Biodiversity Institute, in Utrecht, Netherlands, with the accession CBS 124060. Although *L. mediterranea* is currently not reported in Portugal, it has been previously reported in other European countries and so, one isolate of this species was also included in this study, not only for comparison but also to investigate the efficacy of the studied products towards this species. Isolates were maintained in PDA and transferred to Petri dishes with PDA to promote colony growth. Cultures were incubated at 25°C in complete darkness for 8 days. After incubation, cultures were plated onto 6 mm Petri dishes containing 2% water agar with autoclaved pine needles (*Pinus pinea*) and incubated at 25°C under fluorescent light for a 12 h photoperiod, to pycnidia sporulation (Crous *et al.*, 2006; Phillips, 2002; Úrbez-Torres *et al.*, 2006). On the day of the inoculation, conidia were harvested by collecting pycnidia formed on the pine needles to a 1.5 mL Eppendorf tube containing sterile distilled water (SDW), crushing them with the help of a pestle, followed by shaking the tube in a vortex for one minute. Spore suspensions obtained were filtered through cheesecloth and the concentration was adjusted to 1×10^5 spores/mL with the use of a hemocytometer (Brand, Wertheim, Germany).

5.1.2.2.3. Experimental Design and Treatment Plan

Products used in this assay can be found in Table 5.1.2. Esquive was applied according to label dosage and manufacturer's instructions and Tessior is a formulation ready for application. LC2017 was applied with the timing and dosage recommended by the manufacturer (Table 5.1.2). A total of 15 treatments were set up on grapevines of both cultivars, Touriga Nacional and Cabernet Sauvignon in a completely randomized design, with 10 repetitions per treatment. One shoot was inoculated per grapevine making a

total of 150 plants used from each cultivar. The combination of treatments and isolates inoculated can be found in Table 5.1.3.

Table 5.1.2 - Treatments tested for control of *Lasiodiplodia theobromae* and *Lasiodiplodia mediterranea* under field conditions.

Product name	Manufacturer	Application time	Application rate	Active ingredient
Esquive®	Idai Nature	After pruning	4kg/ha	<i>Trichoderma atroviride</i> strain I-1237 (1 x10 ⁸ CFU g ⁻¹)
Tessior®	BASF Agricultural Solutions Portugal	After pruning	n/a	Pyraclostrobin 0.48% + boscalid 0.95%
LC2017	Natural development Group®	Immediately after harvest	400l/ha	Hydroxyapatite (HA) loaded with cooper (II) sulphate pentahydrate (CuSPHy+HA)
		After pruning (Winter)	250l/ha	
		4 leaves developed	250l/ha	
		Summer pruning	400l/ha	
		Veraisson	400l/ha	

Table 5.1.3 - Treatment plan designed to assess the efficacy of three products against pruning wound infection by *Lasiodiplodia theobromae* and *Lasiodiplodia mediterranea*. Combinations of products used and inoculations spore solution volume.

Treatment	Product	Inoculation	Spore Solution Volume (µl)
1	Esquive + LC2017	<i>L. theobromae</i> (Bt105)	20
2	Esquive + LC2017	<i>L. theobromae</i> (LA-SOL3)	20
3	Esquive + LC2017	<i>L. mediterranea</i> (CBS124060)	20
4	Tessior + LC2017	<i>L. theobromae</i> (Bt105)	20
5	Tessior + LC2017	<i>L. theobromae</i> (LA-SOL3)	20
6	Tessior + LC2017	<i>L. mediterranea</i> (CBS124060)	20
7	Tessior	<i>L. theobromae</i> (Bt105)	20
8	Tessior	<i>L. theobromae</i> (LA-SOL3)	20
9	Tessior	<i>L. mediterranea</i> (CBS124060)	20
10	Esquive	<i>L. theobromae</i> (Bt105)	20
11	Esquive	<i>L. theobromae</i> (LA-SOL3)	20
12	Esquive	<i>L. mediterranea</i> (CBS124060)	20
13	Inoculated non treated Control	<i>L. theobromae</i> (Bt105)	20
14	Inoculated non treated Control	<i>L. theobromae</i> (LA-SOL3)	20
15	Inoculated non treated Control	<i>L. mediterranea</i> (CBS124060)	20

5.1.2.2.4 Product Application and Pathogen Inoculation

For both seasons, immediately after harvest, one application of LC2017 was carried out as recommended by the manufacturer (Table 5.1.2; Fig. 5.1.1). Harvest occurred during

the month of September for both years of the assay. For both cultivars, one-year-old canes with a similar appearance, namely length, were selected for treatment followed by inoculation and were pruned at 2 cm above the third bud. After pruning, both products were prepared according to the label's rate. Esquive was weighted and mixed with water in a 200 mL spray bottle with a concentration of 4 kg/ha (Fig. 5.1.1). Tessior (ready to apply solution) and was applied using the equipment specially designed for this product application (Fig. 5.1.1). Untreated controls were mock treated with SDW, and the wound protectants were allowed to dry for a few hours. This was followed by application with LC2017, as indicated by the manufacturer (Table 5.1.3, Fig. 5.1.1), using a backpack sprayer. One day after the treatment, inoculation with the selected was performed, by applying 20 μ L of the spore suspension ($\approx$ 2000 spores) on each wound using a micropipette (Fig. 5.1.1). After inoculation, the pruning wounds were protected for one week using Parafilm M[®] (Bemis, Sheboygan Falls, WI, USA) to prevent dehydration and promote spore germination. Pruning and artificial inoculation were performed on the 14th and 15th of February 2019, and on the 20th and 21st of February 2020, during the winter dormancy, taking into consideration that all the procedures were made during favorable meteorological conditions, namely cloudy and humid, but avoiding rain periods. The same precautions were taken for the remaining applications with LC2017, with the consideration of also avoiding days with strong winds to minimize spray drift.

5.1.2.2.5 Pathogen Recovery and Identification

For both years, canes were recovered after harvest, during the month of October, and stored in a cold chamber (4 °C) until further processing. For pathogen re-isolation, the bark of each cane was removed, and a sample was collected from about 1 cm below the pruning wound (Fig. 5.1.1). Four pieces of wood were collected from the border of necrotic in-ternal tissue, surface disinfected with a 7% sodium hypochlorite solution, rinsed in SDW, and plated onto 9 mm Petri dishes containing PDA amended with chloramphenicol (PanReac, AppliChem, Darmstadt, Germany) at 250 mg/l. Plates were incubated at 25°C, in the dark, and assessed for counting *Botryosphaeriaceae* and *Trichoderma* spp. colonies (Fig. 5.1.1). A representative set of *Lasiodiplodia* spp. and *Trichoderma* spp. isolates was selected for identity confirmation. A DNeasy Plant Mini

Kit from Qiagen® (Venlo, The Netherlands) was used to extract genomic DNA from 8-day-old cultures grown in PDA and incubated at 25°C, in the dark, following the manufacturer's instructions. The identity of *Lasiodiplodia theobromae* and *L. mediterranea* was confirmed by sequencing part of the translation elongation factor 1 α gene (*tef1- α*) by using the primers EF1-688F and EF1-1251R (Alves *et al.*, 2008), while *T. atroviride* strain I-1237 was confirmed by sequencing the internal transcribed spacer region (ITS) using the universal primers ITS5 and ITS4 (White *et al.*, 1990). Amplified DNA was visualized on agarose gels stained with GreenSafe Premium (Nzytech, Lisbon, Portugal), and was visualized using a UV transilluminator to assess PCR amplification. PCR products were purified using an Illustra ExoProStar Enzymatic PCR and Sequencing Clean-up Kit (GE Life Sciences, Buckinghamshire, UK). PCR products were sequenced both ways at STABVIDA (Lisbon, Portugal) and compared with sequences from GenBank in BLAST searches.

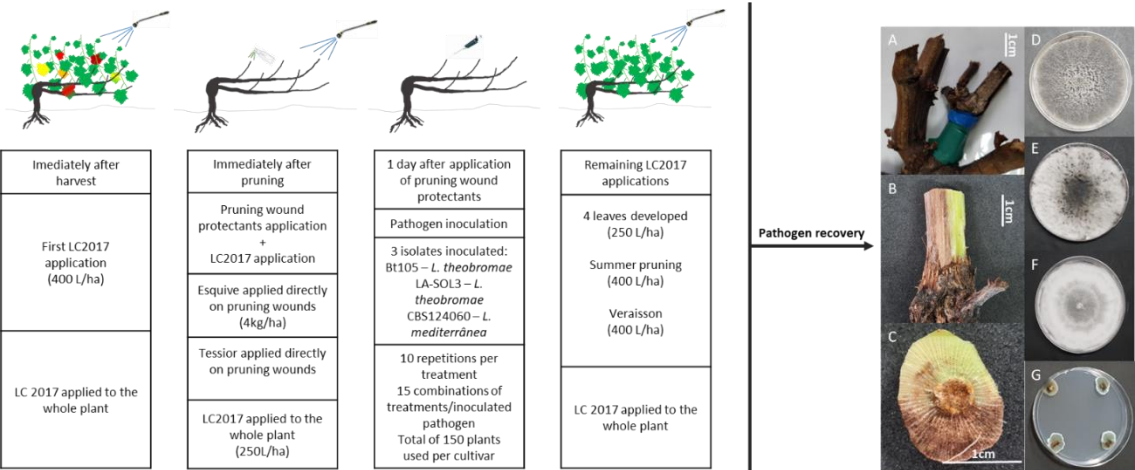


Figure 5.1.1 - Diagram showing the several steps of the field assay. Information on the products used can be found in Table 5.1.2, and a description of the different treatments can be found in Table 5.1.3. A: Marked sample collected from the field prior to analysis; B: Sample with the bark removed showing sign of necrosis; C: Sample collected about 1 cm below the pruning wound, to be divided into four pieces and plated onto PDA; D: Isolate Bt105 (*Lasiodiplodia theobromae*) recovered from infected pruning wounds; E: Isolate LA-SOL3 (*Lasiodiplodia theobromae*) recovered from infected pruning wounds; F: Isolate CBS124060 (*Lasiodiplodia mediterranea*) recovered from infected pruning wounds; G: Petri dish containing wood obtained from non-inoculated control plants showing no signs of pathogen growth.

5.1.2.2.6 Meteorological Data

For both years of the experiment, daily temperature and rainfall were obtained from the Portuguese Institute for Sea and Atmosphere (IPMA—Instituto do Mar e da Atmosfera). These data were collected on the Lisbon reference meteorological station, which is located approximately 7 km from the vineyard tested.

5.1.2.2.7 Statistical Analysis

All statistical analyses were performed using the R program (www.r-project.org). The experimental data for both in vitro assays (mycelial growth inhibition and dual culture antagonism) were compared using an analysis of variance (ANOVA) followed by a Tukey's test ($p = 0.05$). Prior to this analysis, Levene's test was performed in order to verify the homogeneity of variance. Results of the dual culture antagonism assay were plotted using the R package ggplot2. The efficacy of the wound protectants was calculated as the mean percentage recovery (MPR) of the isolates under study. Normality and homogeneity of variance were tested using Levene's test and when necessary, data were transformed into the arcsine of the square root of the proportion to verify the assumption of homogeneity of variance. For both years and cultivars, an ANOVA was used to compare the differences in the mean percentage of recovery (MPR). The means were compared using Tukey's test at the 5% significance level ($p = 0.05$). The mean percentage of disease control was also calculated according to Sosnowski *et al.* (2008, 2013) and Martínez-Diz *et al.* (2021), using the formula $MPDC = 100 \times [1 - (MPR_{\text{treatment}}/MPR_{\text{inoculated control}})]$. To better visualize the results for all the treatments (15 variables, including all the product/isolate combinations, as well as inoculated controls) on both cultivars and their interaction with the meteorological variables (temperature and rainfall), a principal component analysis (PCA) was performed on the results of all the variables. For this analysis, data obtained from the treatments and meteorological variables were considered as two individual data sets or quantitative blocks, and cultivar was considered as a qualitative variable. This analysis was also performed using the R program with the Factoshiny v2.4 package (Vaissie *et al.*, 2021).

5.1.3 Results

5.1.3.1 In vitro Assays

5.1.3.1.1 Mycelial Growth Inhibition Assays

The inhibition of mycelial growth for all fungi under study ranged from 3.7%–100% (Table 5.1.4). Significant differences were observed amongst several concentrations of fungicide used. Independently of the fungal species, the 12.5 mL/L concentration was

able to completely inhibit mycelial growth. For all *Lasiodiplodia* spp. Isolates, the remaining concentrations were able to cause a similar growth inhibition rate. It is noteworthy that a significant decrease in mycelial inhibition percentage was verified from the highest concentration of fungicide (12.5 mL/L) to the second-highest concentration (2.5 mL/L) with inhibition percentage values decreasing from 100% to 14–15%. Higher inhibition percentages could be observed for *Trichoderma atroviride* strain I-1237, with a lower decrease between the two referred concentrations, from 100% to 44.6%.

Table 5.1.4 - Mycelial growth inhibition rate (%) of product LC2017 on all the *Botryosphaeriaceae* isolates under study (Bt105, LA-SOL3, CBS124060) and the *Trichoderma atroviride* strain I-1237 (Esquivel).

LC 2017 Concentration	Mycelial Growth Inhibition (%) ^z			
	Bt105	LA-SOL3	CBS124060	<i>T. atroviride</i> (I-1237)
A (12.5 mL/L)	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a
B (2.5 mL/L)	14.5 ^b	13.9 ^b	15.0 ^b	44.6 ^b
C (1.25 mL/L)	10.4 ^c	9.4 ^c	10.6 ^c	26.0 ^c
D (0.25 mL/L)	8.0 ^{cd}	6.2 ^d	9.3 ^c	9.9 ^d
E (0.125 mL/L)	6.1 ^d	5.1 ^d	8.1 ^c	9.2 ^d
F (0.025 mL/L)	5.4 ^d	3.7 ^d	4.5 ^c	3.9 ^e

^z-Values in the same column followed by the same letter (a, b, c, d, e) do not significantly differ according to Tukey's test ($p \leq 0.05$).

For the *Lasiodiplodia* spp. isolates, similar values were observed for the remaining concentrations. Moreover, for the three isolates, no significant differences were reported for the two lowest concentrations tested of 0.125 mL/L and 0.025 mL/L. *T. atroviride* strain I-1237 showed a slightly different behavior for the 1.25 mL/L concentration compared to the *Lasiodiplodia* spp. isolates, with a higher inhibition percentage, but showed similar behavior for the lowest concentration tested. In this case, *T. atroviride* strain I-1237 was the only fungi under study that showed significant differences for the inhibition percentage at the two lowest fungicide concentrations recording the second-lowest inhibition percentage for the 0.025 mL/L concentration. To verify if the effect observed on plates with 100% mycelial growth inhibition was fungistatic and not fungicidal, mycelium disks were transferred to fresh PDA and allowed to grow for 48 h. For all fungi, mycelial growth was observed with average values of 7.0 cm for isolate Bt105, 5.36 cm for isolate LA-SOL3, 4.54 cm for CBS124060, and finally 5.08 cm for *T. atroviride* strain I-1237. The renewed growth for all fungi is an apparent indicator that, during these in vitro assays, product LC2017 merely showed a fungistatic instead of a fungicidal effect.

5.1.3.1.2 Dual culture antagonism assays

The levels of antagonism of *T. atroviride* strain I-1237 against the *Lasiodiplodia* spp. isolates evaluated, ranged from 14.0% to 51.2% (Figure 5.1.2). The lowest mean percentage of inhibition of radial mycelial growth was observed for isolate LA-SOL3 (19.8%) (Fig. 5.1.3 B) while the highest mean value of inhibition was recorded for isolate CBS124060 (30.2%) (Fig. 5.1.3 C). Significant statistical differences were observed between the mean inhibition percentage of radial growth of these two isolates. Isolate Bt105 showed values between the remaining two isolates with a mean inhibition percentage of radial growth of 27.3%, not significantly different from any of the referred isolates. Overall, the results show an apparent higher antagonism by *T. atroviride* strain I-1237 against the mycelial growth of the *L. mediterranea* isolate than for *L. theobromae* isolates (Fig. 5.1.3).

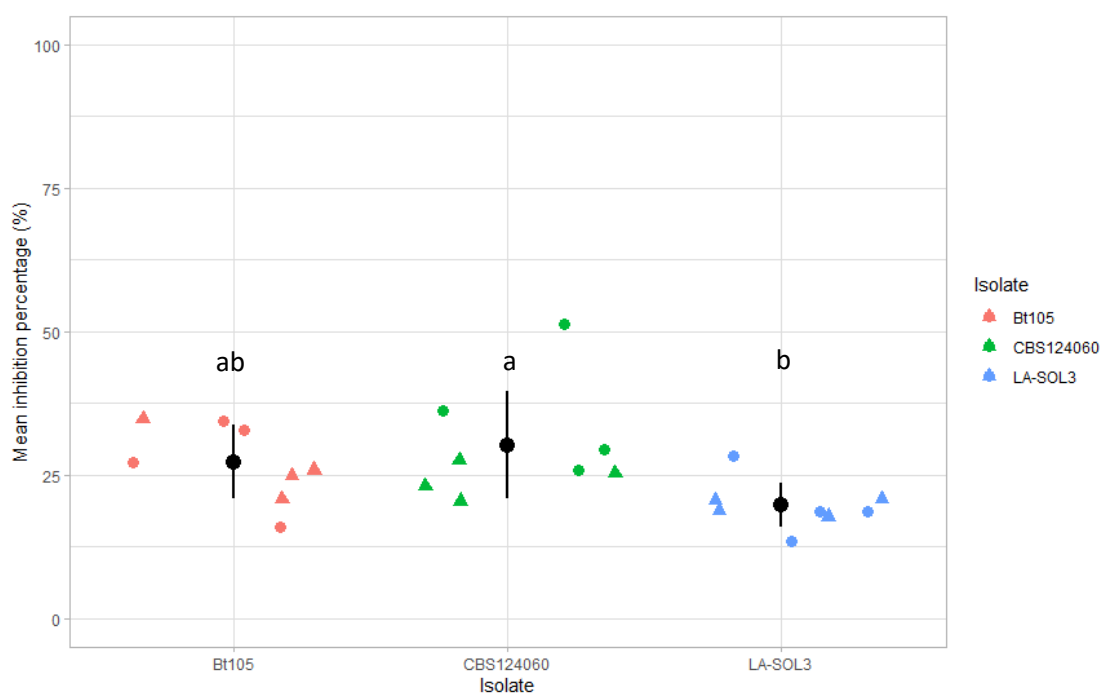


Figure 5.1.2 - Mean inhibition percentage of growth radius for *Trichoderma atroviride* strain I-1237 against *Lasiodiplodia theobromae* isolates Bt105 and LA-SOL3 and *Lasiodiplodia mediterranea* isolate CBS124060. Dots and triangles represent data from both experiments, black dots represent the mean percentage of growth inhibition for the total replicates of both experiments and black bars represent the standard error of means. Columns with the same letter (a, b) are not significantly different according to Tukey's test ($P \leq 0.05$).

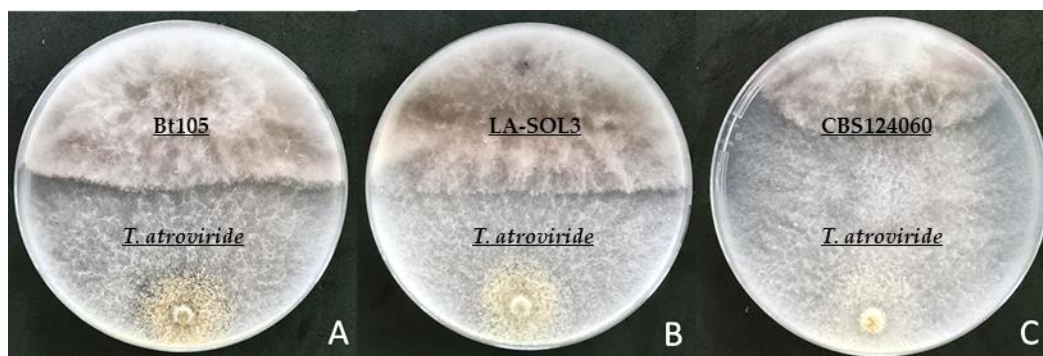


Figure 5.1.3 - Dual culture antagonism assay of *T. atroviride* strain I-1237 and the three *Lasiodiplodia* spp. isolates under study. A: isolate Bt105; B: isolate LA-SOL3; C: isolate CBS124060.

5.1.3.2 Field assays

The efficacy of the treatments was assessed based on the mean percentage of pathogen recovery (MPR) and, the mean percentage of disease control (MPDC) was calculated according to Sosnowski *et al.* (2008, 2013) and Martínez-Diz, *et al.* (2021). Statistically significant differences were found amongst the treatments for both cultivars and years of the study (Table 5.1.5) when compared to the inoculated controls. For cultivar Cabernet Sauvignon, during the 2019 assay, treatments with Esquive + LC2017 reduced the MPR of isolate CBS124060 showing an MPDC of 70.8%, while Tessior + LC2017 was able to significantly reduce the MPR for both isolates Bt105 and CBS124060, with MPDCs of 81.5% and 75.0%, respectively. Tessior alone significantly reduced the MPR of isolate LA-SOL3 with an MPDC of 78.3%, and although an MPDC of 34.8% for isolate LA-SOL3 was recorded for the treatment with Esquive alone, no significant reduction was observed for any isolate.

During 2020, a significant reduction in MPR of isolate Bt105 was verified for all the treatments, being the highest MPDC of 67.7% for the treatment with Tessior + LC2017. The only remaining significant reduction in MPR was on the pruning wound treatment with Tessior challenged with the isolate CBS124060 (MPDC 55%). Regarding cultivar Touriga Nacional, in 2019, no significant reduction in MPR was found for any treatment, when compared with the inoculated control, with the sole exception of Esquive inoculated with isolate CBS124060 (MPDC 53.3%). The 2020 assay showed a significant reduction in the MPR on treatment Esquive + LC2017 for isolates LA-SOL3 and CBS124060, with MPDCs of 48.4% and 58.6%, respectively. Isolate Bt105 provided a

significant reduction in MPR with an MPDC of 52.5% on treatment with Tessior + LC2017, while treatment with Tessior was able to provide an MPDC of 45.2%.

Table 5.1.5 - Efficacy of treatments used solely as pruning wound protectants and in a combination with the copper-based product, LC2017, to reduce *Lasiodiplodia* sp. as part of an integrated disease management.

Treatment	Product	Isolate	Cabernet Sauvignon				Touriga Nacional			
			2019		2020		2019		2020	
			MPR ^y	MPDC ^z	MPR	MPDC	MPR	MPDC	MPR	MPDC
1	Esquive + LC2017	Bt105	55.0 ^{abc}	18.5	40.0 ^{bc}	48.4	52.5 ^{abc}	25.0	57.5 ^{abc}	9.0
2	Esquive + LC2017	LA-SOL3	42.5 ^{abcde}	26.1	30.0 ^{bc}	53.8	57.5 ^{abc}	8.0	30.0 ^c	58.6
3	Esquive + LC2017	CBS124060	17.5 ^{de}	70.8	20.0 ^c	60.0	60.0 ^{abc}	20.0	40.0 ^{bc}	48.4
4	Tessior + LC2017	Bt105	12.5 ^e	81.5	25.0 ^c	67.7	42.5 ^{bc}	39.3	30.0 ^c	52.5
5	Tessior + LC2017	LA-SOL3	22.5 ^{bcde}	60.9	45.0 ^{bc}	30.8	60.0 ^{abc}	4.0	60.0 ^{abc}	17.2
6	Tessior + LC2017	CBS124060	15.0 ^{de}	75.0	35.0 ^{bc}	30.0	57.5 ^{abc}	23.3	62.5 ^{ab}	19.4
7	Tessior	Bt105	42.5 ^{abcde}	37.0	37.5 ^{bc}	51.6	52.5 ^{abc}	25.0	42.5 ^{bc}	32.8
8	Tessior	LA-SOL3	12.5 ^e	78.3	40.0 ^{bc}	38.5	50.0 ^{abc}	20.0	55.0 ^{abc}	24.1
9	Tessior	CBS124060	20.0 ^{cde}	66.7	22.5 ^c	55.0	65.0 ^{abc}	13.3	42.5 ^{bc}	45.2
10	Esquive	Bt105	62.5 ^{ab}	7.4	35.0 ^{bc}	54.8	50.0 ^{abc}	28.6	40.0 ^{bc}	36.7
11	Esquive	LA-SOL3	37.5 ^{abcde}	34.8	40.0 ^{bc}	38.5	32.5 ^c	48.0	52.5 ^{abc}	27.6
12	Esquive	CBS124060	50.0 ^{abcd}	16.7	20.0 ^c	60.0	35.0 ^c	53.3	65.0 ^{ab}	15.0
13	Inoculated Control	Bt105	67.5 ^a	-	75.0 ^a	-	70.0 ^{ab}	-	65.0 ^{ab}	-
14	Inoculated Control	LA-SOL3	57.5 ^{abc}	-	65.0 ^{ab}	-	62.5 ^{abc}	-	72.5 ^a	-
15	Inoculated Control	CBS124060	60.0 ^{abc}	-	50.0 ^{abc}	-	75.0 ^a	-	77.5 ^a	-

^y-Efficacy of all the treatments based on the mean percentage of recovery (MPR) of all the isolates used in this study, from the inoculated pruning wounds. Values in the same column with the same letter (a, b, c, d, e) do not significantly differ according to Tukey's test ($p = 0.05$). ^z-Mean percentage of disease control (MPDC) of all the treatments calculated according to the formula $MPDC = 100 \times [1 - (MPR \text{ treatment} / MPR \text{ inoculated control})]$.

No significant reduction in MPR was verified for none of the remaining treatments when compared to the inoculated controls. *Trichoderma* spp. was only isolated from samples treated with Esquive. In 2019, recovery rates on Cabernet Sauvignon ranged from 24% to 16% for Esquive + LC2017 and Esquive treatments, respectively. For Touriga Nacional, the recovery rate for Esquive + LC2017 treatment was 20%, while a 30% recovery rate was recorded for Esquive applied alone. During 2020, *Trichoderma* spp. recovery rates

for treatments with Esquive + LC2017 and Esquive alone were 38% and 28% for Cabernet Sauvignon and 25% and 22% for Touriga Nacional.

Meteorological data recorded during the time of pruning treatments and pathogen inoculation for both years are presented in Figure 5.1.4. In 2019, the average daily temperature in the week of treatment and inoculation (11th to the 15th of February) was 10.05°C with no rain events recorded. In 2020, treatments and pathogens inoculations were performed during the week from the 16th to the 20th of February, with an average temperature of 13.3°C and only one rain event recorded on the 17th with 1.1 mm of precipitation. Regarding the total rainfall and average temperature for the month of February of both years, 2019 recorded an average temperature of 12.2°C and a total precipitation of 12.7 mm spread throughout five rain events. In 2020, the average temperature was 14.0 °C, and a total precipitation of 8.4 mm with eight rain events.

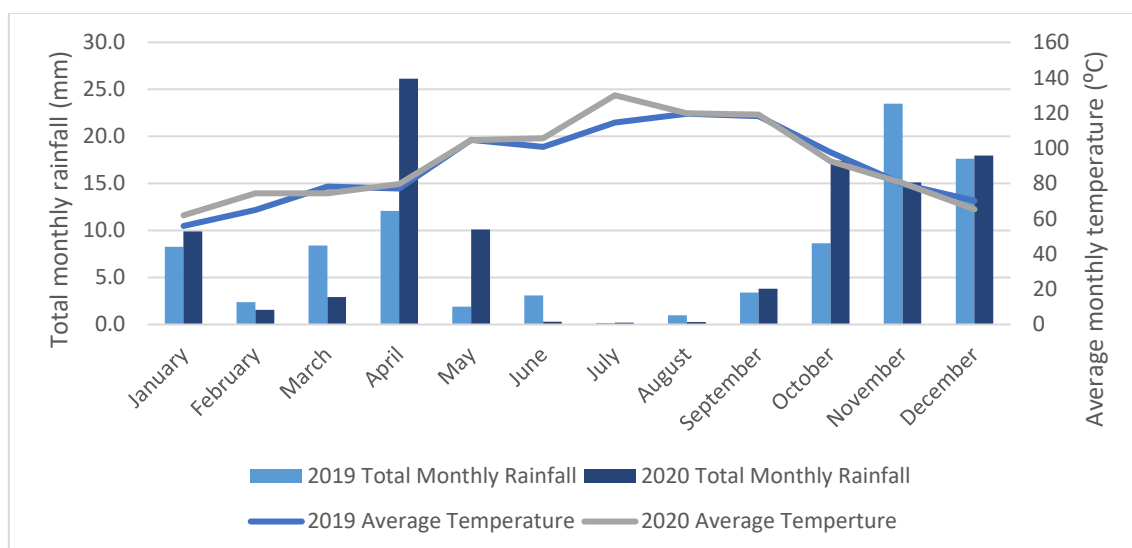


Figure 5.1.4 - Monthly rainfall and average temperature for the two years of the trial.

The principal component analysis (PCA) was performed to better visualize the relationship between all the variables (Figs. 5.1.5 A, 5.1.5 B). For 2019 (Fig. 5.1.5 A), the two first principal components, accounted for 59.9% of the total variability of the data (PC1—41.78%; PC2—18.12%), while for 2020 (Fig. 5.1.5 B) the two first principal components represented 53.98% of the total variability of the data (PC1—35.34%; PC2—18.64%). For both years, we could also observe a clear distribution of the RP of the treatments according to the cultivar, which probably results from the influence of the different susceptibility that both cultivars may have towards the different pathogens

studied. The relationship between the treatments and the meteorological variables could be observed in the vector plots of Figure 5.1.5 A and 5.1.5 B. For both years, meteorological variables showed similar behavior with precipitation being more related to component 1, while temperature showed a higher relation with component 2. In the vector plots, it was observed that the two meteorological variables were very weakly correlated, especially in 2020, where almost no correlation occurred between the variables. Since the vineyard was in an area with a Köppen climatic classification of Csa (hot-summer Mediterranean climate), it makes sense that these variables are negatively correlated since higher temperatures are equivalent to less precipitation. This is again evident for the lower correlation verified in 2020, as less rainfall was recorded during the summer months compared to 2019 (Figure 5.1.4).

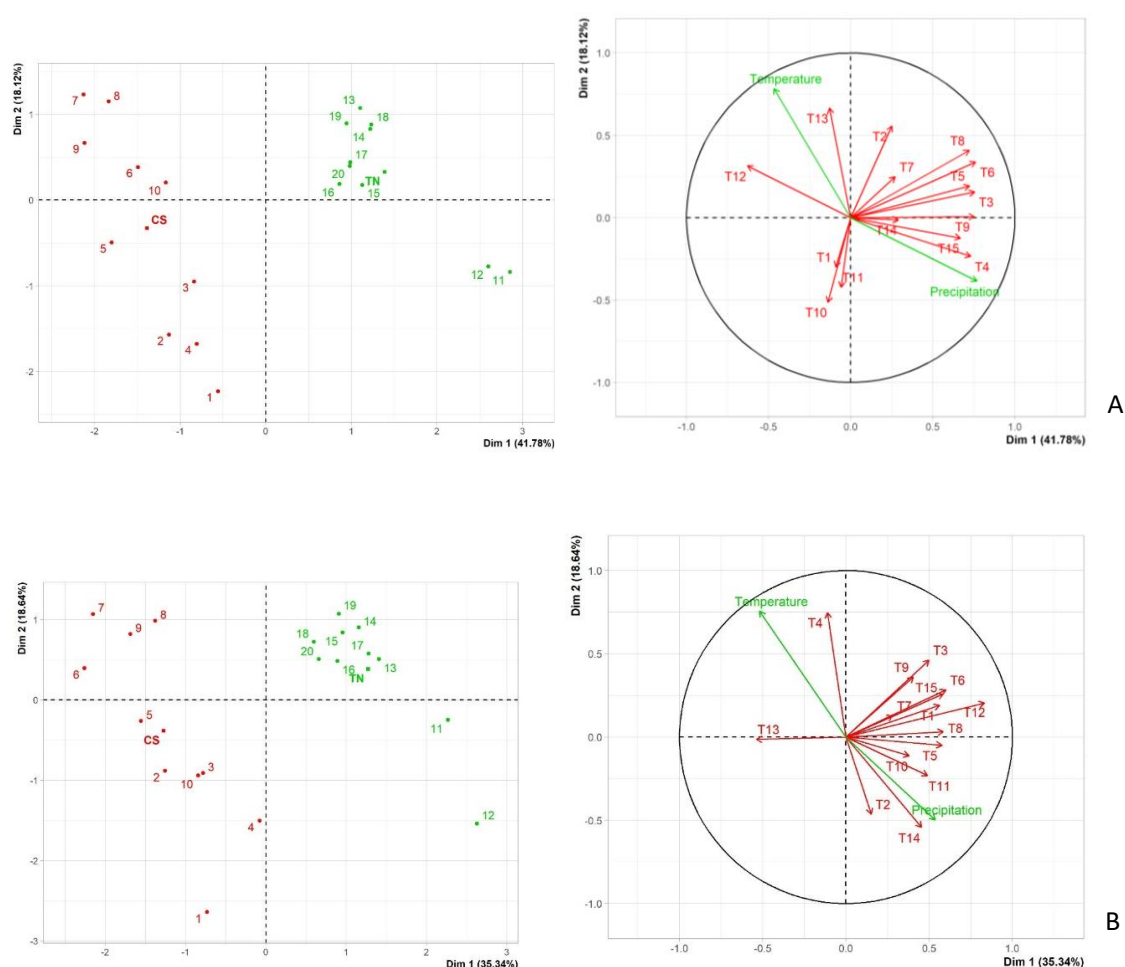


Figure 5.1.5 - Principal component analysis (PCA) for the interactions of all treatments, 15 variables including all the product/isolate combinations (T represents the treatment numbers which are stated in Table 5.1.5), as well as inoculated controls on both variables and the meteorological variables (Figure 5.1.4). Red marks represent the observations for Cabernet Sauvignon (CS) and green marks represent the Touriga Nacional (TN) observations. A-PCA analysis for 2019; B-PCA analysis for 2020.

For 2019 (Fig. 5.1.5 A), most of the treatments showed a much higher correlation with precipitation than with the average temperature recorded throughout the duration of the assay. The only exception was for treatments 2, 12, and 13 which showed a higher correlation to the average temperature recorded. A negative correlation was verified between some treatments, such as T4 and T12 and, T2 and T10. T4 was a treatment with Tessior + LC2017 inoculated with isolate Bt105 while T12 was a treatment with Esquive inoculated with isolate CBS124060. This negative correlation may be due to not only these treatments having very different modes of action (chemical combination vs BCA), but also because the behavior of the two cultivars was completely opposite. T4 appeared to have higher efficiency than T12 on Cabernet Sauvignon, with MPDC values of 81.5% and 20% respectively, while the exact opposite was verified for Touriga Nacional, with 39.3% and 53.3% (Table 5.1.5). Treatments T2 and T10 also showed a negative correlation with each other, and although they are both treatments with Esquive (T2 is Esquive + LC2017 inoculated with isolate LA-SOL3 and T10 is Esquive applied alone inoculated with isolate Bt105) again the different efficacy of the treatments in both cultivars seems to have significance, since T2 seems to be more effective in Cabernet Sauvignon with higher MPDC values than T10, but the opposite was recorded for Touriga Nacional (Table 5.1.5). This trend is also verified for treatments T2 and T1, which also show a negative correlation. Both treatments are Esquive + LC2017 but inoculated with different isolates (T1-Bt105 and T2-LA-SOL3). MPDC values of T2 (26.1%) appear to show once again a higher efficacy in Cabernet Sauvignon than in Touriga Nacional (18.5%), while T1 appears to have a better impact in protecting Touriga Nacional (25%) than Cabernet Sauvignon (18%). This behavior of treatments with Esquive (alone or in combination) where T1 and T10 showed higher MPDC levels for Touriga Nacional comparatively to Cabernet Sauvignon was also verified for T11 and was confirmed by the high correlation showed by these three variables (Fig. 5.1.5 A). It is also noteworthy that all the treatments with chemical formulations, namely Tessior + LC2017 (T4, T5, T6) and Tessior applied alone (T7, T8, T9) showed high correlation amongst themselves, all being positively correlated with PC1. The only treatment with a BCA (Esquive +LC2017) showing a high correlation with the chemical-based ones was T3. The other *Trichoderma* treatments (Esquive + LC2017 and Esquive applied alone) showed mixed behavior, while the treatments T1, T10, and T11 were highly correlated

with each other, but negatively correlated with T2 and almost uncorrelated with T12. For 2020 (Fig. 5.1.5 B), a similar behavior was observed for most of the treatments. Almost all the treatments showed a high correlation with precipitation, except for treatment 4, which revealed again a high correlation with precipitation during the previous year and for treatment 13 again. In this case, treatments 3 and 9 exhibited a similar correlation with both precipitation and temperature. Therefore, for both years of the assay, almost all isolates showed a higher correlation with precipitation rather than temperature, except for treatment T13 apparently more strongly related to temperature. This treatment also showed a negative correlation with T8, and very low to almost no correlation to the remaining treatments, which could be related to the fact that T13 corresponds to the inoculated control of isolate Bt105. During this year, treatments T2 and T4 showed a negative correlation. These are again treatments with different modes of action since T2 is a treatment of Esquive + LC 2017 inoculated with isolate LA-SOL3 and T4 is a treatment of Tessior + LC2017 inoculated with isolate Bt105. As was observed for the 2019 analysis, this negative correlation may be attributed to T2 being apparently slightly more effective in Touriga Nacional (MPDC = 58.6%) than in Cabernet Sauvignon (MPDC = 53.8%) while the opposite result is found for T4, with an MPDC of 67.7% in Cabernet Sauvignon opposed to 52.5% in Touriga Nacional. On this 2020 analysis, it is also noteworthy that all the treatments inoculated with isolate LA-SOL3 (T2, T5, T8, T11, T14) appear to have a high correlation amongst themselves, showing a low correlation with the remaining treatments. The exception was T5 which also showed a high correlation with the referred treatments, but nonetheless, this correlation shows that not only the variety and the type of product applied but also the isolate inoculated during this assay, may have some influence on the efficacy of the treatments tested.

5.1.4 Discussion

The present study reports the first field assessment on the efficacy of using management strategies, integrating biological and chemical pruning wound protectants with a copper-based site-targeted formulation, against *Botryosphaeria* dieback pathogens. Higher demand for more sustainable management practices and increasing restrictions

on pesticide use has led to an expansion of IPM programs, involving organic products, BCAs, improved cultural practices, and responsible pesticide use (Aroca *et al.*, 2008; Bertsch *et al.*, 2013). To date, and to the best of our knowledge, no field assays have been performed using the two selected pruning wound protectants against *Lasiodiplodia* spp., in combination with copper-based protection with an elicitor effect. Our results show that the efficacy of the wound protectants, when applied alone or in combination with the copper-based product, varied between both years and according to the cultivar under study.

Efficacy of Pruning Wound Protection Products Applied Alone

For 2019, the highest MPDC values observed for Cabernet Sauvignon were found for the treatment with Tessior, while the lowest values were recorded for the application of Esquive alone. Nevertheless, the same pattern was not observed in 2020 when, in general, both treatments were able to reduce MPR to a similar extent. Pitt *et al.* (2013b) reported similar results in Australia, where liquid and paste formulations showed better efficacy against *Diplodia seriata* and *Diplodia mutila* than *Trichoderma*-based products. More recently, Martínez-Diz *et al.* (2021) also described a higher efficacy of Tessior compared to Esquive, on pruning wounds infected with *D. seriata* and *Phaeomoniella chlamydospora*. A slightly different trend could be observed for cultivar Touriga Nacional in 2019 with the highest MPDC values for grapevines treated with Esquive alone. In 2020, this cultivar showed similar efficacy of the Esquive treatment comparing with the Tessior treatment, the only exception being the MDPC values found for isolate CBS124060 which were significantly lower than those reported for Tessior. In fact, all the 2020 treatments induced a slightly higher efficacy than observed in 2019, except for the Esquive treatment applied alone. Similarly, chemical fungicides, namely benomyl, were less effective than *Trichoderma* spp. treatments on wounds inoculated with *Diaporthe ampelina*, *D. seriata*, *E. lata*, *Neofusicoccum australe*, *Neofusicoccum parvum*, *L. theobromae* and *P. chlamydospora* (Kotze *et al.*, 2011; Gramaje *et al.*, 2018). This specific *T. atroviride* strain I-1237 has shown efficacy in reducing GTD incidence and severity in preliminary assays carried out in both Portuguese (Reis *et al.*, 2017) and French (Mounier *et al.*, 2014) vineyards. Nevertheless, Martínez-Diz *et al.* (2021) did not recently find significant differences between pathogen re-isolation from pruning wounds treated with *T. atroviride* strain I-1237 and inoculated non-treated controls.

The variable effectiveness of *Trichoderma*-based treatments has been previously reported by authors (John *et al.*, 2005; Mutawila *et al.*, 2011; Pitt *et al.*, 2012; Halleen *et al.*, 2016; Martínez-Diz *et al.*, 2021) and could be attributed to several reasons. The main advantage of using *Trichoderma*-based products as pruning wound protectants is the long-term protection conferred by the fungus growing in the wood. Therefore, the success of the protection provided by these products is dependent on the establishment of *Trichoderma* spp. within the wound. The influence of grapevine cultivar to wound colonization by *Trichoderma* spp. has also been highlighted by Mutawila *et al.* (2016), probably related to the plant defense responses that differ between cultivars. These different defense responses to *T. atroviride* have been recently reported by Leal *et al.* (2021), where the authors observed that *T. atroviride* may act as a priming stimulus for Tempranillo plantlets, while no stimulus could be verified for Chardonnay. This is in agreement with our PCA analysis which showed a negative correlation among treatments with *T. atroviride* (Esquive), likely due to a difference of efficacy amongst cultivars, which reinforces the importance of the cultivar in wound colonization by *Trichoderma* spp. It is known that not only cultivar but also meteorological conditions, such as temperature and precipitation, as well as the time of application influence the establishment and persistence of *Trichoderma* spp. (Pertot *et al.*, 2017). Both cultivar and meteorological conditions factors could explain the differences of *T. atroviride* strain I-1237 colonization in our study. According to Esquive manufacturer, this specific *T. atroviride* strain I-1237 can grow at temperatures above 5°C. In our study, the average temperatures recorded during pruning and treatments performed were 12.2°C and 14°C in 2019 and 2020, respectively. This may explain the apparent higher success on the colonization of the pruning wounds, especially verified for all the treatments with Esquive on Cabernet Sauvignon. Martínez-Diz *et al.* (2021), while using the same commercial formulation of *T. atroviride* strain I-1237 (Esquive), found significantly lower colonization in Spanish vineyard trials. Nevertheless, this study was conducted on a different cultivar (Godello) and under different meteorological conditions. In this latter study, temperatures recorded during pruning and treatment application were significantly lower than those recorded during our study (6.5°C and 9.8°C in Spain to 12.2°C and 14.0°C in Portugal). The PCA analysis performed during this study also showed that most of the treatments where *T. atroviride* strain I-1237 was used were

more correlated with precipitation than with temperature, being the only exceptions of treatments 12 and 13 in 2019. Nevertheless, in 2020, there were higher precipitation levels during the months of April and May, and on the PCA analysis performed for this year, all treatments with *T. atroviride* showed a high correlation with precipitation. This seems to reinforce the hypothesis that cultivar and meteorological conditions, especially rainfall, may apparently impact both the establishment and persistence of *T. atroviride* strain I-1237 in pruning wounds. As expected, for both treatments with Esquive, the highest percentages of colonization by *T. atroviride* strain I-1237 led to the highest mean percentage of disease control values. Further research is still needed to prove this hypothesis in GTDs which is characterized by extremely high complexity.

During our study, the application of Tessior (pyraclostrobin + boscalid + liquid polymer) was able to provide some reduction in infection by *Lasiodiplodia* spp. The highest mean percentages of disease control were obtained for Cabernet Sauvignon during 2019, with values as high as 78.3%, while during the next growing season, MPDC values only reached values as high as 55.5%. For Touriga Nacional, as referred to earlier, Tessior application was apparently not as effective as that of *T. atroviride*. The maximum level of MPDC was 45.2% for 2020, and MPDC values as low as 13.3% could be found during the previous year. Application of pyraclostrobin alone was effective in reducing infection by *Botryosphaeria* dieback fungi under field conditions (Rolshausen *et al.*, 2010; Díaz e and Latorre, 2013), and a mixture of pyraclostrobin with metiram in the nursery when diluted in the soaking water prior to grafting (Rego *et al.*, 2009). Prior to this work, only preliminary studies and one in-depth study have been conducted by applying Tessior to pruning wounds, but all of them for controlling *Diplodia* spp. and *P. chlamydospora*. Preliminary field studies were conducted in Greece (Kühn *et al.*, 2017; Samaras *et al.*, 2019), Germany (Kühn *et al.*, 2017; Lengyel *et al.*, 2019), and Spain (Kühn *et al.*, 2017) where Tessior was effective in reducing infection by the referred fungi. Recently, a more extensive study was conducted in Spanish vineyards by Martínez-Diz *et al.* (2021) where Tessior showed a high MPDC for both *D. seriata* and *P. chlamydospora* compared to several other commercially available fungicides and BCAs. No previous studies have been conducted using Tessior as a pruning wound protectant against *Lasiodiplodia* spp., and although on the referred study, a botryosphaeriaceous fungi was used, *D. seriata* is considered a less aggressive species in comparison to *Lasiodiplodia* spp. (Úrbez-Torres,

2011). In our study, we used two isolates of *L. theobromae* and one isolate of *L. mediterranea*. The establishment of the pathogens within grapevines depends on several factors, including not only meteorological variables and cultural practices, but also pathogen intrinsic properties such as aggressiveness (Rolshausen *et al.*, 2010). Van Niekerk *et al.* (2011) attributed the higher pathogen infection level on inoculated wounds to higher percentages of rainfall in the Stellenbosch area. This is in agreement with the PCA analysis of most of the treatments in our field study, which were strongly correlated with precipitation. The only treatment that for both years had a higher correlation with temperature was T13 corresponding to the inoculated control of isolate Bt105 (*L. theobromae*). It has been suggested that the expression of virulence factors in *L. theobromae* can be modulated by temperature (Félix *et al.*, 2016; Yang *et al.*, 2017). This isolate was collected in Portugal, and it was previously considered to be highly virulent against both cultivars under study (Reis *et al.*, 2021), suggesting that it may be better adapted to the local conditions than the other isolates under study. Moreover, our PCA analysis showed a high correlation between all the treatments in which plants were inoculated with isolate LA-SOL3 and between plants inoculated with isolate CBS124060 in 2020. Consequently, the difference in MPDC values found herein, comparatively to previous studies, may be due to the meteorological variables but also related to the pathogen used, and the difference in aggressiveness between them. Another factor that may be noteworthy is the difference in susceptibility between the two cultivars used. In fact, on average higher MPDC values were obtained for Cabernet Sauvignon during both growing seasons of the field assay, compared to the values found for Touriga Nacional. This tendency is verified, not only for the treatments with Tessior, but for most of the treatments. Previous studies conducted on the same cultivars using the same isolates, proved their high aggressiveness towards grapevine in both field and greenhouse assays (Reis *et al.*, 2021), suggesting that the difference in efficacy in reducing infection by *Lasiodiplodia* spp. may also depend on cultivar susceptibility. Moreover, Sofia *et al.* (2019) showed that from a set of four different cultivars, Touriga Nacional was one of the most susceptible to *P. chlamydospora*, suggesting that this particular cultivar may be highly susceptible to GTDs in general. Therefore, and given the differences found between both growing seasons and both cultivars, further research is recommended to evaluate each of the components of these products to

understand how their efficacy may be affected by factors such as time of application, pathogen species aggressiveness and cultivar susceptibility, pruning wound size, and vineyard terroir.

Efficacy of a Strategy Combining the Application of Pruning Wound Protectants with LC2017

The great diversity of species currently associated with *Botryosphaeria dieback*, but also with all GTDs, combined with the intrinsic differences in hundreds of cultivars planted worldwide under different terroir conditions, makes the research for an effective management strategy to reduce the impact of GTDs an extremely difficult challenge. To this extent, in this work, we tried to integrate currently used products for pruning wound protection with the application of a site-targeted product based on a copper (II) compound (copper sulfate) and synthetic nanostructured particles of hydroxyapatite (HA) which have already shown interesting drug delivery properties *in planta* (Mondello *et al.*, 2018; Battiston *et al.*, 2018, 2019, 2021). In our work, the application of this new site-targeted copper (II) formulation (LC2017), does not seem to greatly increase the efficacy of the pruning wound protection strategy. In some cases, the combination of the two strategies actually increased the mean percentage of disease control, but in others, the opposite was also verified. For example, in 2019, and for the inoculation of isolate CBS124060 on Cabernet Sauvignon, an MPDC of 70.8% was obtained for the treatment with Esquive + LC2017 while only 16.7% was recorded for the treatment with Esquive alone. During the next year (2020) the same value of 60% was found for both treatments. In 2019, on Cabernet Sauvignon, the application of Tessior + LC2017 caused also an MPDC of 81.5% for isolate Bt105, while only 37% was found for the treatment with Tessior alone. For the two other isolates under study, LA-SOL3 showed an increase in MPDC when Tessior was applied alone, while the isolate CBS124060 showed an opposite trend. Although the highest concentration of LC2017 used in the *in vitro* assay for the inhibition of mycelial growth showed fungistatic effect for all the isolates and *T. atroviride* strain I-1237, there is no apparent pattern on the influence of the LC2017 application as part of an IPM for any of the cultivars, or specific isolate. Moreover, the fungistatic effect verified for *T. atroviride* strain I-1237 did not seem to impact the colonization of pruning wounds by this BCA, since the highest values of re-isolation

percentage could be found for the treatments combining Esquive and LC2017 except for Touriga Nacional during 2019. Moreover, no relevant correlation between the treatments using LC2017 was observed for both years by the PCA analysis, indicating a low influence of LC2017 application on the MPDC values found in this study.

The copper formulation used (LC2017) has already shown both fungistatic and fungicidal effects *in vitro* and *in planta* against *Phaeoacremonium minimum* (Battiston et al, 2021). Mondello *et al.* (2021) have also reported that LC2017 showed the same *in vitro* fungistatic effect on *D. seriata* and *N. parvum* as it was verified for our *L. theobromae* isolates. The same authors described as well, the ability of this formulation to activate GTD-related plant defense reactions (Mondello *et al.*, 2021). Di Marco *et al.* (2011) also tested a copper formulation against *P. chlamydospora* and *P. minimum*, which revealed the ability to reduce conidial germination in *in vitro* assays but was not able to reduce *P. chlamydospora* colonization on young potted grapevines. Amposah *et al.* (2012) tested a copper hydroxide formulation against *Botryosphaeria dieback* fungi and were not able to obtain any significant control both in *in vitro* and in plant tests. More recently, Mondello *et al.* (2021) performed greenhouse *in planta* assays that did not show significant differences in stem necrosis length of plants treated with LC2017 and inoculated with *D. seriata* and *N. parvum*. This agrees with our results since the same copper formulation used does not appear to provide a significant impact when combined with pruning wound protectants in controlling these specific *Botryosphaeria dieback* fungi. Therefore, further research is needed on testing all the components of this formulation, since HA applied alone has shown a non-fungitoxic and stimulant activity on *P. minimum* (Battiston *et al.*, 2021) and *Botrytis cinerea* (Battiston *et al.*, 2019). However, the efficacy of copper (II) products has been previously related not only as a fungicide but also as an elicitor of some plant defense responses. Aziz *et al.* (2006) observed this eliciting effect for CuSO₄ sprayed on leaves, and Battiston *et al.* (2021) have also shown that this formulation of CuSPHy was able to strongly induce several plant defense genes. Mondello *et al.* (2021) also showed that LC2017 (HA + Cu (II)) had the same elicitation potential as BTH (S-methyl benzo (1,2,3) thiadizole-7-carbothioate), a commonly marketed elicitor (BION®, Syngenta, France) by inducing genes related with chitinase and glucanase synthesis and also genes related to the biosynthesis pathways of several phenolic compounds.

Therefore, new methods to efficiently deliver fungicides to specific targeted areas of the plant are considered to have great potential to improve GTD control, and further research is needed to investigate not only the impact that these formulations may have on several GTD pathogens but also on the plant microbiome. Further long-term field trials should also be undertaken to investigate the effectiveness that a prolonged management control combining pruning wound protection and site-targeted fungicides might provide in controlling GTDs.

During this work, to ensure a proper establishment of infection with the fungi under study, approximately 2000 spores of each of the *L. theobromae* and *L. mediterranea* isolates were used to challenge each pruning wound. This represents a high inoculum pressure compared to the levels that pruning wounds are usually exposed to under natural field conditions. This suggests that due to the higher inoculum applied to the pruning wounds, the real efficacy of all the treatments tested against *Lasiodiplodia* spp. may have been underestimated (Mondello *et al.*, 2018).

In conclusion, this study demonstrated the field potential of pruning wound protection formulations to control *Lasiodiplodia* spp., applied alone or in combination with a novel site-targeted copper (II) formulation. The efficacy of the studied products differed between the two cultivars used and between the two growing seasons of the duration of the assay. Nevertheless, some measure of control was achieved for all treatments studied, despite the application of the copper-based product LC2017 not showing clear evidence of improving the control obtained by the pruning wound protection products applied alone. Still, this new site target copper-based product may prove to be a viable way of reducing the amount of copper applied for diseases management on vineyards, especially with further European Union restrictions on copper use in agriculture. Furthermore, the combination of effective pruning wound protection management by using a combination of a BCA with a more sustainable copper-based product has already proven to have interesting results not only as an elicitor by strongly inducing plant defense genes but also in the control of *P. minimum*, as well as *Plasmopora viticola*, may lead, in the long term, to healthier grapevines, which may reduce the expression of GTD symptoms in vineyards. However, this hypothesis still needs to be clarified by conducting studies using the same management strategy over a longer period of time, targeting not only other grapevine cultivars but also other *Botryosphaeriaceae* species.

Thus, good pruning practices and wound protection combined with sustainable management strategies, such as bio stimulants and host resistance inducers, can reduce the impact of *Botryosphaeria* dieback, not only by controlling pathogens already common in a certain wine region but also by making it difficult for the potential establishment of new species.

5.1.5 Author contributions

P.R., C.R., A.A. and F.F. designed the experiments; P.R. and A.G. implemented the methodology; P.R. formal analysis; P.R. original draft preparation; A.A., F.F. and C.R. review of the original draft; A.A., F.F. and C.R. supervision; A.A. and C.R. funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Effect of the Combined Treatments with LC2017 and *Trichoderma atroviride* Strain I-1237 on Disease Development and Defense Responses in Vines Infected by *Lasiodiplodia theobromae*

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5.2 Effect of the Combined Treatments with LC2017 and *Trichoderma atroviride* Strain I-1237 on Disease Development and Defense Responses in Vines Infected by *Lasiodiplodia theobromae*

Abstract

Grapevine trunk diseases constitute one of the major problems for viticulture worldwide, with *Botryosphaeria dieback* considered as one of the most important of these diseases. In this work, we aimed to (i) evaluate the effect of the combination of two products, Esquive® (a *Trichoderma*-based product) and LC2017 (a low-copper-based product), in the control of *Lasiodiplodia theobromae*, by evaluating the internal lesion length caused by inoculation of this pathogen on greenhouse kept grapevines of cvs Cabernet Sauvignon and Touriga Nacional and, (ii) investigating their elicitor effect on plant defense responses, through the analysis of the expression of a set of genes. The pathogen was always re-isolated from the infected tissues and was able to cause wood discoloration. Touriga Nacional exhibited longer lesions than Cabernet Sauvignon, and the application of both products did not appear to reduce lesion length when compared to LC2017 applied alone. The elicitor effect of LC2017 on plant defense was confirmed by gene expression analysis, and no significant differences were found between plants treated with LC2017 and with both products. Moreover, a specific response related to the cultivar was verified, but this apparently unique interaction between product, cultivar and pathogen remains to be further investigated.

Keywords: *Botryosphaeria dieback*, internal necrosis length, gene expression, cuttings, Touriga Nacional, Cabernet Sauvignon, sustainable control

5.2.1 Introduction

Grapevine trunk diseases (GTD) are a group of fungal diseases currently affecting grape-growing regions throughout the entire world. These diseases attack the wood of perennial organs of grapevine resulting in yield reduction, progressive decline and, finally in increasing costs for winegrowers (Bertsch *et al.* 2013; Gramaje *et al.* 2018; De la Fuente *et al.*, 2016). GTD control in vineyard is extremely complex, especially after the prohibition of several active ingredients, namely sodium arsenite and benzimidazoles, efficient but dangerous for humans and environment (for review see Gramaje *et al.* 2018; Mondello *et al.*, 2018). The increasing consumer demands for healthier and sustainable agriculture have also led in viticulture the need of new strategies and Plant Protection Products (PPPs) to control diseases.

Among disease control strategies, the Integrated Pest Management (IPM) is currently one of the most promising. With the harmonic and coordinated use of natural products as well as biocontrol agents (BCA), cultural practices and PPPs, the IPM could yield rapid results in terms of increasing and protecting biodiversity and health in the agroecosystem (FAO, 1998; Kogan, 1998). For these reasons, and to reduce the high number of treatments in vineyard required to control the main fungal diseases such as downy- and powdery-mildew, gray mold and GTDs, the research on BCAs in viticulture has strongly developed. Among the BCA tested in viticulture, the fungal genus *Trichoderma* has shown promising results towards several GTD pathogens, such as black-foot and Petri diseases, Botryosphaeria-dieback and Esca (Berbegal *et al.*, 2020; Leal *et al.*, 2021; Martínez-Diz *et al.*, 2021; Mutawila *et al.*, 2011, 2015, 2016; Mounier *et al.*, 2014, 2016; Reis *et al.*, 2017, 2021a, Úrbez-Torres *et al.*, 2020). Currently, several *Trichoderma*-based products are available for winegrowers as wound protectants, since the main entry for GTD pathogens are the wounds produced by pruning practices in vineyard. *Trichoderma*-based products could act against GTD pathogens both directly by competition for space and nutrients, mycoparasitism, antibiosis, production of cell-wall degrading enzymes (Harman, 2006; Pieterse *et al.*, 2014; Van Wees *et al.*, 2008; Vinale *et al.*, 2008; Waghunde *et al.*, 2016) and indirectly, as plant growth promoter and defense elicitors (Harman, 2006; Leal *et al.*, 2021; Pieterse *et al.*, 2014; Van Wees *et al.*, 2008; Vinale *et al.*, 2008; Waghunde *et al.*, 2016). One of the main problems for a

treatment with *Trichoderma* spp. and, more generally with BCAs, is their optimal integration in IPM strategies. The use of PPP with biocide action can strongly interfere with the BCA efficacy, even leading up to their elimination. For these reasons, Mutawila *et al.* (2015) attempted to facilitate the introduction of *Trichoderma* spp.-based treatments into IPM strategies using Benzimidazole-resistant strains of *Trichoderma*.

In viticulture, copper has been used for over 130 years as active ingredient to control the most important grapevine fungal diseases, the downy mildew (*Plasmopara viticola*) (De La Fuente *et al.*, 2021). The continuous use of Cu based-fungicides in vineyards has led to an increase of its concentrations in soils due to the tendency to accumulate on the upper layers (Perez-Rodriguez *et al.*, 2016). This has caused severe negative impacts on soil microbial biodiversity and on the surrounding ecosystems (De La Fuente *et al.*, 2021). The environmental problems have led the European Union to limit the use of copper-based products in viticulture to a maximum of 28 kg ha⁻¹ of metallic copper in 7 years, i.e., 4 kg ha⁻¹ per year (1981/2018 EC regulation, 1107/2009 EC regulation) (De La Fuente *et al.*, 2021). This limitation is a major concern especially for organic viticulture (Berkelmann-Löhnertz *et al.*, 2012), where there is no alternative to cupric fungicides for the control of downy mildew. Since copper-based products are not easily replaceable in organic viticulture, the agrochemical producing industry changed its focus, synthesizing new copper formulations with lower ecotoxicological profiles. Among these innovative formulations, the HA+Cu(II), namely LC2017, containing low copper amount (3.5%) with hydroxyapatite (HA) has recently revealed promising results in the control of GTD pathogens (Battiston *et al.*, 2018, 2021; Mondello *et al.*, 2021; Reis *et al.*, 2021a). Moreover, LC2017 showed interesting elicitation properties towards several genes linked with plant defense system when tested in controlled conditions (Battiston *et al.*, 2021; Mondello *et al.*, 2021, 2022). Recently, Reis *et al.* (2021a) successfully demonstrated the compatibility between *Trichoderma atroviride* strain I-1237 and LC2017 treatments to control GTDs in vineyard. Due to the ascertained abilities of both LC2017 (Mondello *et al.*, 2021) and *Trichoderma* spp. (Waghunde *et al.*, 2016) in eliciting the host plant defense system, it seems interesting to further characterize this combination of treatments in grapevines.

Therefore, the main objective of this study was to evaluate the efficacy of the combination of LC2017 and *T. atroviride* strain I-1237 applications for the control of *Lasiodiplodia theobromae*, agent of the Botryosphaeria-dieback (Úrbez-Torres 2011), under controlled conditions. A greenhouse bioassay was thus conducted on Cabernet Sauvignon and Touriga Nacional cuttings to evaluate the effects of both products on the control of *L. theobromae* infection by assessing (i) the internal lesion length analyses and the pathogen re-isolation, and (ii) plant defense responses by studying the induction of the expression of targeted genes.

5.2.2 Material and methods

5.2.2.1 Fungal isolates and inoculum preparation

was isolated in 2009 from internal wood necrosis of grapevine cv Castelão, in Portugal, and is currently stored at the culture collection of Instituto Superior de Agronomia, Lisbon, Portugal. This particular isolate was selected due its previously shown aggressiveness towards the two cultivars under study (Reis *et al.*, 2021a, 2021b). The isolate maintained on Potato Dextrose Agar (PDA, Difco, Sparks, MD, USA) slants, was firstly transferred to Petri dishes containing PDA to promote colony growth and then incubated in the darkness, for 8 days at 25°C. To promote the production of pycnidia, the fungus was thus transferred into Petri dishes containing 2% water agar (Difco, Sparks, MD, USA) with sterilized pine needles (*Pinus pinea*) and incubated at 25°C with a 12h photoperiod (fluorescent light) (Crous *et al.*, 2006; Phillips, 2002; Úrbez-Torres *et al.*, 2006). Prior to inoculation, conidia were harvested by removing the pycnidia from the pine needles surface to a 1.5 mL Eppendorf tube containing sterile distilled water (SDW). Pycnidia were crushed with the use of a pestle, and the tubes were shaken on a vortex to release the conidia. The spore suspension obtained was filtered through cheesecloth and final concentration was adjusted to 1×10^5 spore mL⁻¹ with the help of a hemocytometer (Brand, Wartheim, Germany).

5.2.2.2 Plant material, experimental design and treatments

5.2.2.2.1 Plant material

Inoculations were performed on cuttings of two different cultivars, Touriga Nacional and Cabernet Sauvignon coming from a commercial nursery. Cuttings were rooted at 24°C under natural light on a rooting table containing perlite and a month after rooted cuttings were individually potted in a sandy soil mixture (1/3 sand, 1/3 soil, 1/3 organic matter). At the end, rooted cuttings were placed in a ventilated greenhouse at 24°C under natural light and watered as needed.

5.2.2.2.2 Tested products

The products and treatments plan used in this study are reported in table 1. We tested two products: (i) Esquive® (product developed by Agrauxine S.A., Quimper, France, and commercialized by Idai Nature S. L., Valencia, Spain), a commercially available *Trichoderma*-based wound protectant, containing *Trichoderma atroviride* strain I-1237 and (ii) LC2017, a copper sulphate pentahydrate salt (Cu(II) 35 g L⁻¹), transported throughout the plant by hydroxyapatite molecules. Both products were applied according to label and/or manufacturer recommendation or previous works (Mondello *et al.* 2021). A total of 6 conditions were set up for both cultivars in a completely randomized design with 8 replicates for each treatment, for a total of 48 plants per cultivar.

Table 5.2.1 – Treatments tested on cultivars Cabernet Sauvignon and Touriga Nacional.

Treatment	Product	Inoculation
1	LC2017	Water (control)
2	LC2017	<i>Lasiodiplodia theobromae</i> (Bt105)
3	Esquive	Water (control)
4	Esquive	<i>Lasiodiplodia theobromae</i> (Bt105)
5	LC2017 + Esquive	Water (control)
6	LC2017 + Esquive	<i>Lasiodiplodia theobromae</i> (Bt105)

5.2.2.3 Product application and pathogen inoculation

The scheme for product application and inoculation are reported in Figure 1. After three months from pot individualization a pruning wound was made with scissors in the upper part of the cuttings, immediately treated with Esquive/water suspension (27 g L^{-1}).

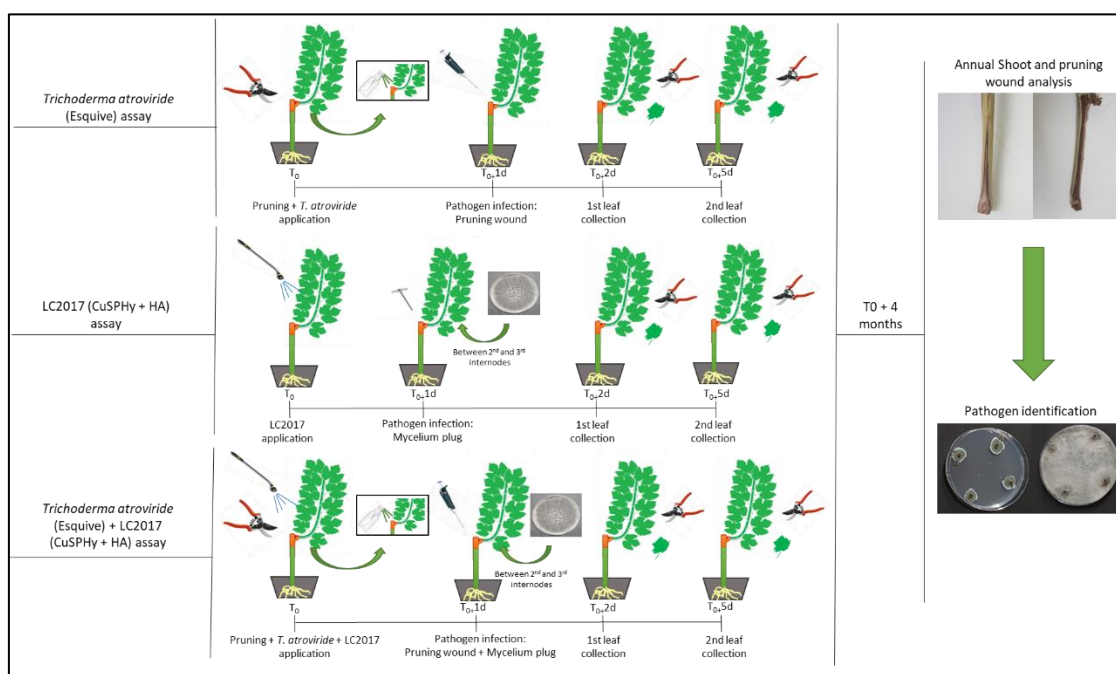


Figure 5.2.1 - Diagram showing the several steps of treatment and inoculation of greenhouse kept grapevine of cultivars Cabernet Sauvignon and Touriga Nacional. Information on the treatments and products used can be found on table 5.2.1.

For the condition with only LC2017 treatment, no pruning wound of the upper part of the cuttings was made, and the product was applied to the whole plant (cutting + annual shoot), using a backpack sprayer. For the condition with both treatments, cuttings were pruned in its upper part, the wounds treated with Esquive and after letting the product dry, a LC2017 application to the whole plant (cutting + annual shoot) was carried out. Twenty-four hours after products applications, two different kinds of inoculations with *L. theobromae* were performed. The first one, with 20 μL of *L. theobromae* spore suspension (≈ 2000 spores) i) directly put on pruning wounds of cuttings treated with Esquive only. The second one, using mycelial plugs put in wounds created between the 2nd and 3rd internodes using a cork-borer (Reis *et al.* 2019) in the annual shoot of cuttings treated with LC2017 only. For cuttings treated with both Esquive and LC2017, the two kinds of inoculation were performed in the same plant. For the controls, STW and sterile plugs of PDA were inoculated. Being not in the aim of the study, no untreated

plants were inoculated with the pathogen. After inoculation, to prevent dehydration and promote spore germination, the pruning wounds were protected for one week using Parafilm M® (Bemis, Sheboygan Falls, WI, USA). The site of inoculations on the annual shoots was covered with moist wool cotton and sealed with Parafilm M® (Bemis, Sheboygan Falls, WI, USA) for one week, to prevent dehydration.

5.2.2.4 Gene expression analysis

To study the effects of the treatments (Esquive, LC2017, Esquive + LC2017) and of the GTD pathogen *L. theobromae* on plant defense response in both cultivars, a targeted gene expression analysis was set up. The leaf sampling for this transcript analysis was done at T0+2d and at T0+5d (T0 = Esquive or LC2017 application; d for days). Each sample consisted of the two leaves immediately above and below the inoculation point, with three cuttings per condition being used as replicates for each time-point. Leaves collected were immediately frozen in liquid nitrogen and stored at -80°C. Following the RNA extractions, quantitative real-time RT-PCR analysis was carried out according to Magnin-Robert *et al.* (2014) and Spagnolo *et al.* (2017), respectively. Results were expressed as the values of relative expression levels ($\Delta\Delta Ct$), corresponding to the mean of three independent biological replicates. *39SRP*, *60SRP* and *EF-1 α* genes were used as housekeeping genes. Since our objective was to evaluate the interaction between the two treatments (Esquive and LC2017), both analyses were compared to the Esquive T0+2d condition, which represented our control herein.

The specific primers used in the gene expression study are listed in Supplementary Table S5.2.1. These genes were selected based on similar previous studies (Spagnolo *et al.* 2014, 2017; Magnin-Robert *et al.* 2011, 2014, 2016; Trotel-Aziz *et al.* 2019, Mondello *et al.* 2021, 2022). Specifically, 11 genes were chosen to evaluate the grapevine cultivars response to the above-mentioned treatments and are related to: the phenylpropanoid pathway (*PAL* and *STS*) and other defense protein markers (*CHIT4C*, *GLUC*, *PR1*, and *PR10*), detoxification processes (*GTS1*) and the “recovered” health-status markers highlighted in leaves of GTD-infected vines treated with sodium arsenite (*PME25*, *MSR*, *WRKY* and *HYD2*; unpublished data, Fontaine F.). The genes analyzed were considered to be up- or down-regulated when changes in their expression were either >2-fold or <0.5-fold.

5.2.2.5 Determination of internal lesions and pathogen recovery

Four months after pathogen inoculations, internal lesions were measured longitudinally both on the woody part of the rooted cuttings and the annual shoots. Lesions were measured using an electronic caliper, and four fragments of necrotic tissue from the margin of the lesion were collected. These fragments were surface disinfected with a 7% sodium hypochlorite solution, rinsed in SDW and plated onto 9 mm Petri dishes containing PDA amended with 250 mg L⁻¹ of chloramphenicol (PanReac, AppliChem, Darmstadt, Germany). After incubation at 25°C for one week, Petri dishes were assessed for the presence of *Botryosphaeriaceae* colonies which were sub-cultured onto fresh PDA dishes and, again incubated at 25°C for one week, in the darkness. A representative set of *L. theobromae* isolates was selected from each of the treatments to undergo molecular confirmation of species identity.

5.2.2.6 Statistical analysis

Statistical analysis was performed using the R-program v. 4.0.5 (www.r-project.org). Assumptions for variance analysis were assessed. For data normality and homogeneity of variance Shapiro-Wilk and Levene's tests were performed, respectively. According to the results, the influence of distinct levels was assessed using the non-parametrical Kruskal-Wallis test. For significant differences ($p < 0.05$), a comparison between the distinct levels was performed using the ranks. The re-isolation percentage data (RP) were transformed into the arcsine of the square root of the proportion $(RP/100)^{1/2}$. Statistical significance ($p \leq 0.05$) for the data collected for gene expression was determined with Mann–Whitney non-parametric tests using GraphPad Prism v.5.0 (GraphPad Software, San Diego, CA, USA).

5.2.3 Results

5.2.3.1 Relationship between the lesion length induced by *L. theobromae* and treatments

The mean lesion lengths of wood discoloration caused by *L. theobromae* on both cultivars are presented on Figure 5.2.2. *Lasiodiplodia theobromae* Bt105 was able to cause wood discoloration in both cultivars independently to the treatments. For Cabernet Sauvignon (CS) cuttings, the mean lesion length values were significantly lower

than for Touriga Nacional (TN). For TN, the pathogen determined larger lesions in pruning wounds than in annual shoots. The mean lesion length for the pruning wounds ranged from 0.63 cm for the control cuttings inoculated with PDA and treated with Esquive or LC2017 and 2.24 cm for those inoculated with the pathogen and treated with LC2017 + Esquive. In this case no significant differences are observed between the inoculated cuttings treated with Esquive and the respective controls, while the cuttings treated with both Esquive + LC2017 showed differences compared to the control cuttings (Figure 5.2.2).

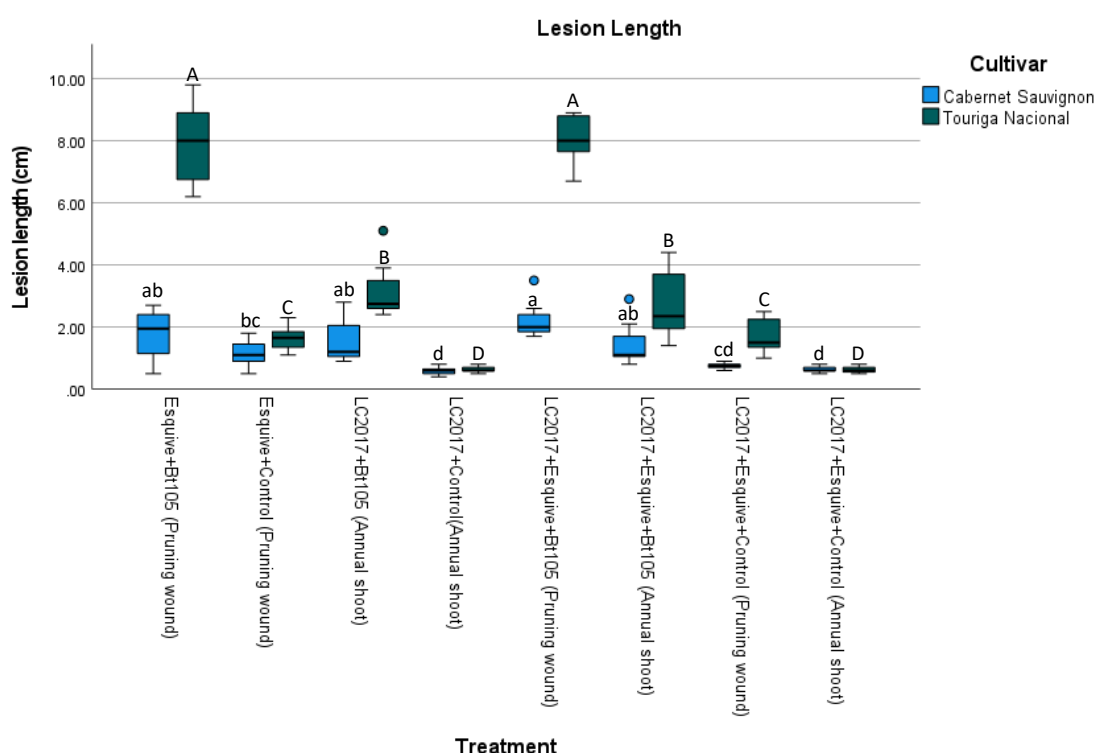


Figure 5.2.2 - Boxplot representing the average lesion lengths recorded for the inoculation of cuttings from two different grapevine cultivars Cabernet Sauvignon and Touriga Nacional with an isolate of *L. theobromae*, under greenhouse conditions. The median is represented by the solid line. Mean lesion lengths of isolates with different letter are significantly different at $P < 0.05$, Tukey's test. Lower-case letters for Cabernet Sauvignon and upper-case letters for Touriga Nacional. Dots represent outliers.

Regarding annual shoot inoculation, the mean lesion length varied between 0.59 cm for the LC2017 control cuttings and 1.53 cm for cuttings treated with LC2017. Significant differences are observed for all the treatments with inoculated cuttings compared with their respective controls. Regarding the TN cuttings, the mean lesion length on the pruning wounds varied between 1.6 cm for the control cuttings inoculated with PDA and treated with Esquive and 8.1 cm on the cuttings inoculated with the pathogen and treated with LC2017 + Esquive. Although the mean lesion length value recorded for the

control cuttings is apparently high, as in the pruning wounds of cuttings treated with LC2017 + Esquive (1.7 cm), this may be attributed to the healing and a phenolic compounds accumulation due to the process of wounding through pruning. Moreover, significant differences were found among all of the inoculated and the control plants that underwent pruning wound inoculation (Figure 5.2.2). Mean lesion length for the annual shoot inoculations were also on average shorter than the ones recorded for the pruning wounds with values ranging from 0.62 cm for controls treated with both LC2017 and Esquive and 3.15 cm on cuttings treated only with LC2017. Significant differences are observed for most of the isolates inoculated compared to the controls (Figure 5.2.2). On average, mean lesion lengths recorded were significantly higher in TN than in CS. Moreover, the mean lesion lengths were longer on the inoculated pruning wounds than on the inoculated annual shoots for both cultivars.

5.2.3.2 Pathogen recovery

Re-isolation of *L. theobromae* was higher from the inoculated annual shoots than from the pruning wounds for both cultivars. Moreover, no pathogen was recovered from the control plants inoculated with PDA plugs or water for both cultivars. Cabernet Sauvignon inoculations recorded a maximum re-isolation percentage ranging from 18.7% for the inoculated pruning wounds of LC2017+Esquive treated cuttings, to 81.2% for annual shoots treated with LC2017 + Esquive. For TN, re-isolation percentages from inoculated annual shoots showed the highest minimum value of 43.7% on cuttings treated with both products but also the lowest maximum value of re-isolation percentage of 65.6% on cuttings treated with LC2017 (Figure 5.2.3). Re-isolation percentages at pruning wounds were the same (21.8%) for the cuttings treated with only Esquive and for cuttings treated with both products. For both cultivars, significant differences were only found between the inoculated annual shoots and the inoculated pruning wounds. On average, slightly higher percentages of re-isolation are reported for CS than for TN, in contrast to what was observed for the lesion length where TN showed the highest values. Also, no product applied conjointly or alone appears to be more efficient in reducing the presence of *L. theobromae*.

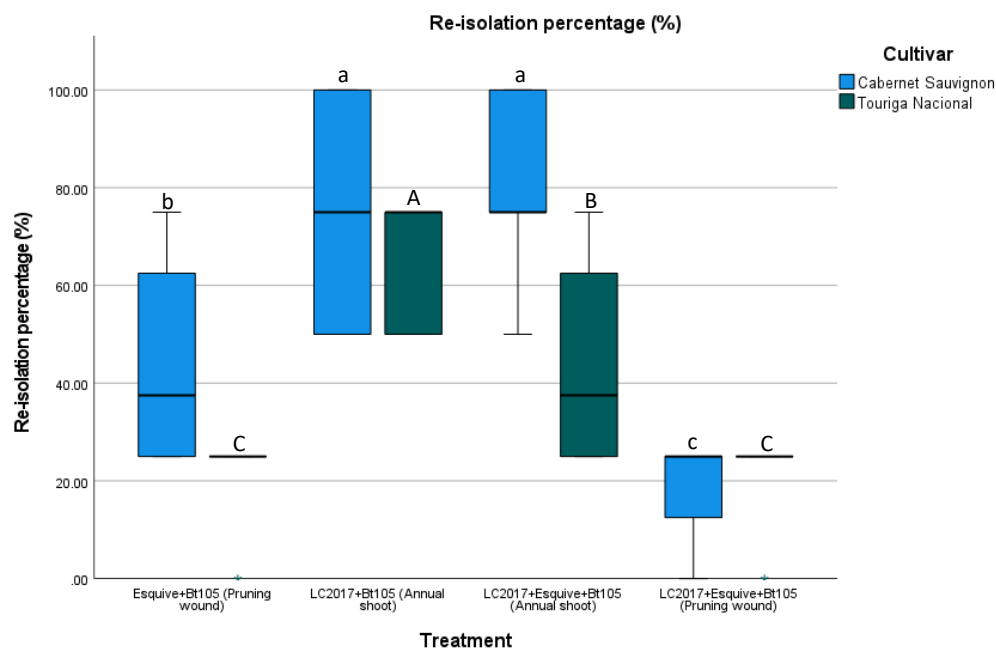


Figure 5.2.3 - Boxplot representing the re-isolation percentage of *L. theobromae* recorded from the cuttings of two cultivars, Cabernet Sauvignon and Touriga Nacional. The median is represented by the solid line. Mean lesion lengths of isolates with different letter are significantly different at $P < 0.05$, Tukey's test. Lower-case letters for Cabernet Sauvignon and upper-case letters for Touriga Nacional. Dots represent outliers.

Trichoderma atroviride was only recovered from cuttings treated with Esquive. Lower *T. atroviride* recovery rates were obtained from treated cuttings of CS than TN. Moreover, although no application of Esquive was performed on the annual shoots of cuttings, still small percentages of recovery were reported from this part of the cuttings treated with both Esquive and LC2017. Although extreme caution was done when applying Esquive to pruning wounds, this presence may be due to some spray drift to adjacent plant organs. For CS, recovery rates ranged from 3.1% to 59.4% for annual shoots of cuttings treated with LC2017 + Esquive and, pruning wounds of cuttings treated with Esquive alone, respectively. Concerning *T. atroviride* recovery rates from TN cuttings, as referred before, higher values were obtained with 12.5% for the annual shoots of cuttings treated with both products and a maximum of 81.2% for pruning wounds treated with Esquive alone.

5.2.3.3 Plant responses at gene expression levels according to the treatments

The results of the gene expression studies on cv Cabernet Sauvignon and Touriga Nacional submitted to the different treatment conditions are reported in Figures 5.2.4 and 5.2.5, respectively.

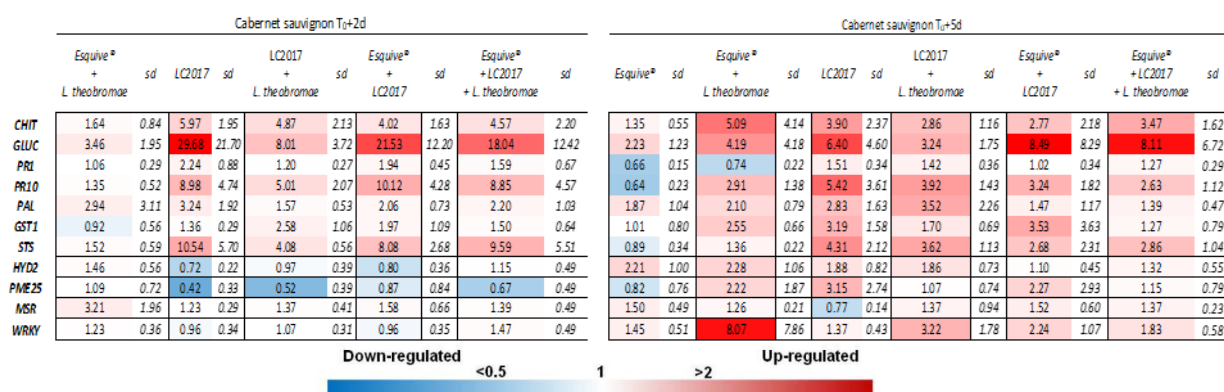


Figure 5.2.4 - Expression levels of selected genes (determined with quantitative reverse-transcription polymerase chain reactions) and relative standard deviation values (sd) recorded on leaves cv Cabernet Sauvignon at T₀ + 2d and T₀ + 5d. The values (each the mean of three technical replicates) represent expression levels ($\Delta\Delta\text{Ct}$) of treatments relative to the control (condition Esquiv® T₀ + 2d). The expression of each gene was considered up-regulated when the value was >2-fold compared to the controls or down-regulated when the value was <0.5-fold compared to the controls.

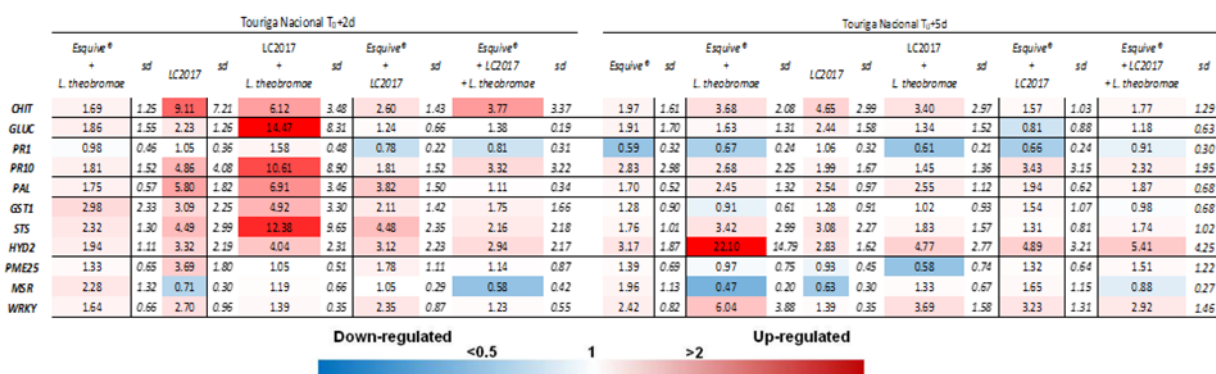


Figure 5.2.5 - Expression levels of selected genes (determined with quantitative reverse-transcription polymerase chain reactions) and relative standard deviation values (sd) recorded on leaves cv Touriga Nacional at T₀ + 2d and T₀ + 5d. The values (each the mean of three technical replicates) represent expression levels ($\Delta\Delta\text{Ct}$) of treatments relative to the control (condition Esquiv® T₀ + 2d). The expression of each gene was considered up-regulated when the value was >2-fold compared to the controls or down-regulated when the value was <0.5-fold compared to the controls.

5.2.3.3.1 Effect of LC2017 treatment alone

In both cultivars, treatments with LC2017 alone and in absence of the pathogen, determined the induction of several genes, already at T₀+2d, such as *CHIT*, *GLUC*, *PR*-

proteins, PAL, STS and GST1. These genes were found induced also at T0+5d for Cabernet Sauvignon and, in a lesser extent, for Touriga Nacional. A similar behaviour was recorded in both cultivars upon *L. theobromae* infection. In LC2017 treated vines and according to the considered cultivar, some genes are either induced (*GST1* in Cabernet Sauvignon - Figure 5.2.4; *GLUC* in Touriga Nacional - Figure 5.2.5) or repressed (*PAL, PME25* and *WRKY* in Touriga Nacional - Figure 5.2.5) upon *L. theobromae* challenge.

5.2.3.3.2 Effect of the double treatment Esquive and LC2017

The expression values recorded at T0+2d in rooted cuttings treated with both *T. atroviride* and LC2017 did not statistically differ from those recorded in the LC2017 condition, indicating a non-synergic effect of the double treatment on the targeted genes for both cultivars (Figure 5.2.6 and 5.2.7). The non-synergic effect between the two treatments could be supported by the expression level recorded at T0+5d in vines treated with *T. atroviride* only. These values were very similar to the control, except for few genes (*GLUC* and *HYD2* in Cabernet Sauvignon and *PR10, HYD2* and *WRKY* for Touriga Nacional) that were slightly overexpressed with values ranging from 2.42 to 3.17 (Figure 5.2.5). The presence of *L. theobromae* showed globally no effect in vines treated with both *T. atroviride* and LC2017, with induction values similar to the related no-infected condition, except for *PAL* significantly repressed in Touriga Nacional (Figure 5.2.7).

5.2.3.3.3 Influence of cultivar on defense responses

The gene expression study highlighted also differences in the expression levels according to the cultivars towards the tested treatments. For Cabernet Sauvignon, *CHIT, GLUC, PR10, PAL*, and *STS* were induced at both T0+2d and T0+5d when treated with LC2017 as well as on the double treatment (Figure 5.2.4). At T0+5d, an induction of several other genes such as *GST1* and *PME25*, was also verified for both LC2017 alone treatment and double treatment with Esquive. On the contrary, several genes were only overexpressed at T0+2d in Touriga Nacional such as *PR10, GST1, PME* and *WRKY* in LC2017 treatment and *CHIT, PAL, GST1* and *STS* in the double treatment (Figure 5.2.4).

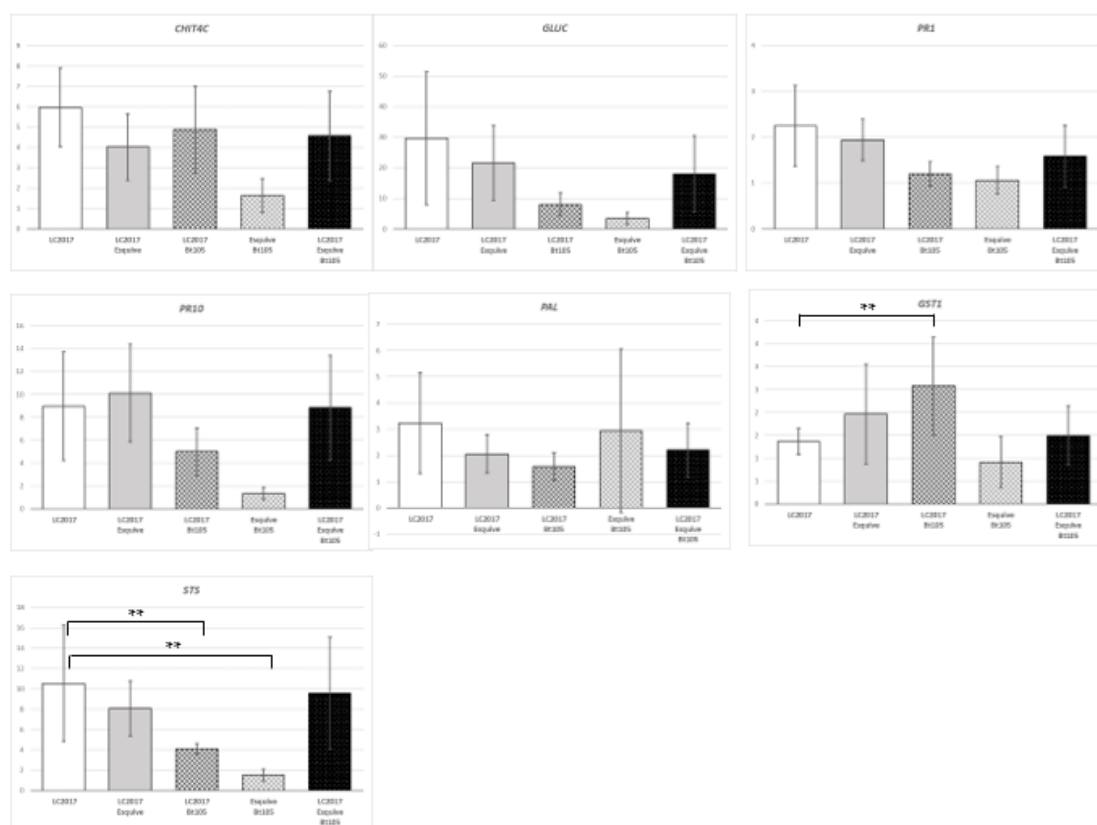


Figure 5.2.6 - Overexpressed genes (values > 2) at $T_0 + 2d$ in Cabernet Sauvignon cuttings with single (LC2017) or double (LC2017 + Esquire®) treatment, not inoculated or inoculated with *L. theobromae* strain Bt105. Statistical analysis with the Mann-Whitney U test showed no significant differences between medians of LC2017 and LC2017 + Esquire® and between LC2017 + Bt105 and LC2017 + Esquire® + Bt105. Only a significant effect of pathogen presence was observed in some genes in LC2017-treated vines. The asterisk indicates a statistical significance (Mann-Whitney U test) between the medians of the two considered conditions. * = $p < 0.05$; ** = $p < 0.01$.

For this latter treatment, other genes namely *PR10*, *HYD2* and *WRKY*, showed induction only at $T_0 + 5d$ in Touriga Nacional. Significant differences in the gene expression between both cultivars were observed (Supplementary Figure S5.2.1), with values higher for Cabernet Sauvignon than for Touriga Nacional, except for *HYD2* and *WRKY*.

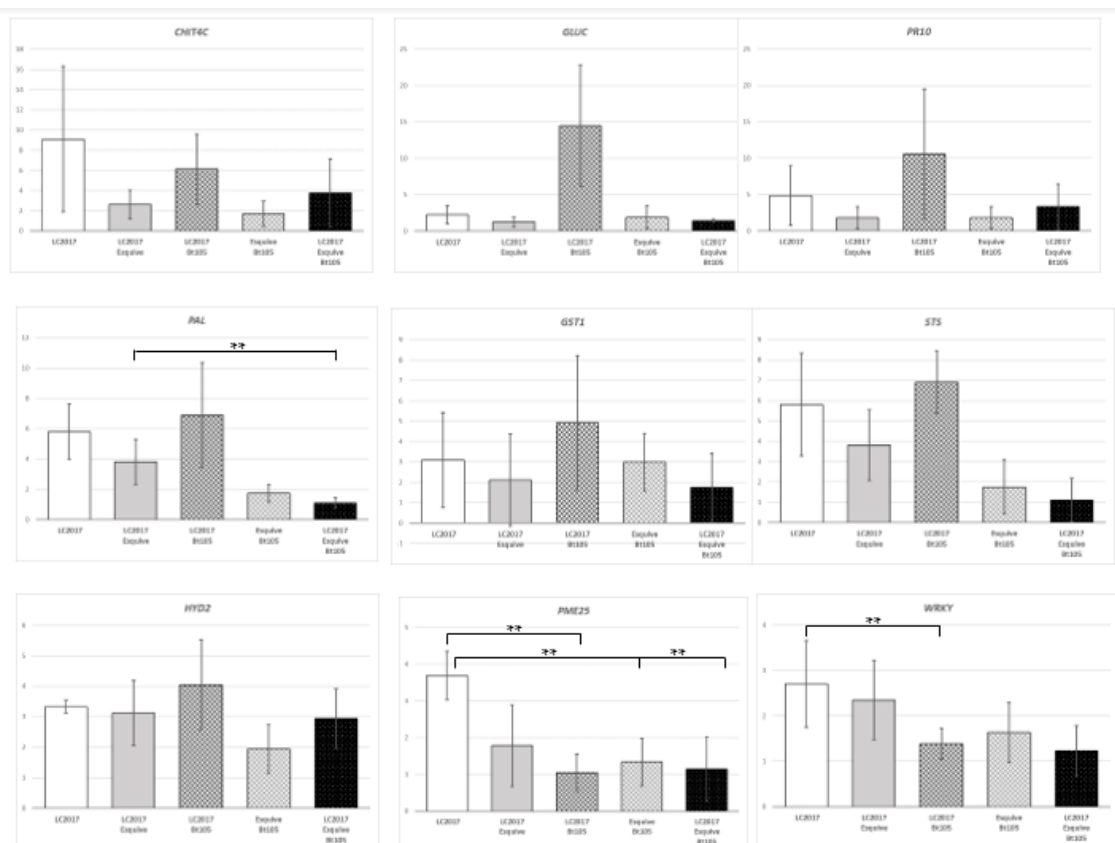


Figure 5.2.7 - Overexpressed genes (values > 2) at T₀ + 2d in Touriga Nacional cuttings with single (LC2017) or double (LC2017 + Esquive®) treatment, not inoculated or inoculated with *L. theobromae* strain Bt105. Statistical analysis with the Mann-Whitney U test showed no significant differences between the medians of LC2017 and LC2017 + Esquive® and between LC2017 + Bt105 and LC2017 + Esquive® + Bt105. Only a significant effect of pathogen presence was observed in some genes in LC2017-treated vines and in double-treated vines. The asterisk indicates a statistical significance (Mann-Whitney U test) between the medians of the two considered conditions. * = $p < 0.05$; ** = $p < 0.01$.

5.2.4 Discussion

The aim of this study was to further test the strategy combining *T. atroviride* (Esquive) and HA+Cu(II) (LC2017), previously assessed as pruning wound protectant for the control of the Botryosphaeria-dieback associated pathogen *L. theobromae* (Reis *et al.*, 2021a). These products have been individually tested against several Botryosphaeria-dieback pathogens (Mounier *et al.*, 2014; Reis *et al.*, 2017; 2021a; Martínez-Diz *et al.*, 2021 for Esquive®; Mondello *et al.*, 2021; Reis *et al.*, 2021a for LC2017). Nevertheless, no studies on the impact of the application strategy of the two formulations on plant defense responses at gene level have been previously investigated. To achieve this goal, we studied the interaction of these two formulations on two artificially inoculated

cultivars with an isolate of *L. theobromae* (Bt105) under controlled conditions. The efficiency of this combination was assessed on both the pathogen and the disease progression, by studying pathogen's presence and internal lesions development, respectively. Furthermore, we studied the possible synergic elicitor effects of the two combined PPPs treatments on genes related to the grapevine defense system.

Effect of treatments on lesion length induced by *L. theobromae*

Despite the treatment and the type of inoculation used, *L. theobromae* isolate Bt105 was always re-isolated and able to cause wood discoloration on both cultivars, though cuttings from CS always showed lower mean lesion length values than TN. These results are similar to those obtained in our previous study, carried out in the field with the same cultivars and *L. theobromae* isolate (Reis *et al.* 2021a). Also, for both cultivars, lesions length recorded on inoculated pruning wounds were longer than those on inoculated shoots, being the shortest lesion length found on the inoculated shoots treated with both formulations. Our results agree with Úrbez-Torres and Gubler (2009), which reported that the variability on aggressiveness of *Botryosphaeriaceae* observed in inoculation tests could depend on the isolate, type of inoculum used, inoculation tissue (e.g., mature canes vs green shoots), as well as on cultivar, age of the host and length of the inoculation period. Moreover, our previous study (Reis *et al.*, 2021b) showed that TN had a higher susceptibility towards *L. theobromae* than CS, both in controlled condition and in the field. Thus, the higher susceptibility towards *L. theobromae* could explain the higher lesions on TN. The significant differences between cultivars on lesion length in pruning wounds treated with Esquive (alone and in combination with LC2017), cannot be related with the different colonization ability of *T. atroviride* on the two cultivars. In fact, higher re-isolation percentages values of *T. atroviride* were observed on TN rather than CS. This seems in contrast with what reported by Mutawila *et al.* (2016) about the correlation between the protection to grapevine conferred by *Trichoderma* sp. and its colonization abilities, influenced by intrinsic differences among cultivars. Thus, even if higher *T. atroviride* re-isolation percentages were found in TN, the susceptibility of this cultivar previously mentioned to *L. theobromae* may have masked the influence of the BCA on pathogen colonization. Similarly, the application of LC2017, did not show a significant effect on internal lesion length as well as the double

combined treatment. This concurs with previous works in which vines treated with LC2017 in controlled conditions and inoculated with *Botryosphaeriaceae* did not evidence a significant reduction of internal lesion (Mondello *et al.* 2021), nor increased the efficacy of pruning wound protection, when combined with Esquive (Reis *et al.* 2021a).

The combination of Esquive with LC2017 does not appear to improve plant defense response

Our study has confirmed the elicitation abilities of LC2017 on the grapevine defense systems, especially on the genes linked to molecules directly addressed to contrast fungal pathogen attack (*CHIT*, *GLUC* and *PR*-proteins) as well as the Phenylpropanoid metabolism (*PAL*, *STS*) and detoxification process (*GST1*). Our results agree with previous studies, which reported that LC2017 is able to induce the host plant defense system in a similar manner to the commercial product (Bion) in healthy and GTD pathogens -infected vines (Battiston *et al.*, 2021; Mondello *et al.*, 2021; 2022). Our transcript analysis results confirm those of Mondello *et al.* (2021) in which *CHIT*, *GLUC*, *PAL* and *STS*, exerting a role in grapevine tolerance to biotic and abiotic stresses (Chong *et al.*, 2009; Trotel-Aziz *et al.*, 2019), underwent elicitation.

Even if *Trichoderma*-based formulations have been reported to activate defense genes in planta (Stempien *et al.*, 2020; Leal *et al.*, 2021), no significant differences in gene expression levels were found between cuttings treated with LC2017 alone and with Esquive and LC2017, for both time points. Our results may be related to a host-plant effect on *Trichoderma* spp. induction abilities, as observed by Stempien *et al.* (2020) on rootstock R110 and by Leal *et al.* (2021) on Chardonnay. The low elicitation by *T. atroviride* could be explained also by both the short period of colonization (5 days) and the inoculation strategy (spores) adopted in this study, rather than to a negative impact of LC2017 treatment on the BCA. In fact, despite the LC2017 ascertained fungistatic effect (Mondello *et al.*, 2021), *T. atroviride* was not affected by the LC2017 treatment (Reis *et al.*, 2021a). It is to highlight the induction of genes linked to the “arsenite recovery effect” (Mondello *et al.*, 2021, 2022) in both cultivars, despite the considered treatment. These genes seem to play a role in the suppression of GTD foliar symptoms expression, observed in Esca-affected vineyards treated with LC2017 (Mondello *et al.*,

2022). It will therefore be extremely interesting to carry out further research if this symptom suppression is to be verified also for field grown *Botryosphaeria* dieback infected vines.

Several other authors have shown that LC2017 has not only an eliciting effect on plant defense genes but also stimulates plant growth and photosynthesis (Battiston *et al.*, 2021; Mondello *et al.*, 2021, 2022). These features in addition to a capability of delivering a fungicide to a specific targeted area in the plant may have potential on blocking and/or limiting infection by *Botryosphaeria*-dieback pathogens. Therefore, extended trials, especially on field-grown vines, are also recommended to assess the long-term impact of LC2017 on *Botryosphaeria*-dieback pathogens, and to validate combination/s with other PPPs may be of great interest in establishing an integrated management strategy against GTDs.

In conclusion, the use of the commercial product Esquive, based on *Trichoderma atroviride*, is known to limit the annual GTD pathogens infection at pruning wounds in vineyard and contamination in nursery (Berbegal *et al.*, 2020; Leal *et al.*, 2021; Martínez-Diz *et al.*, 2021; Mounier *et al.*, 2016; Reis *et al.*, 2017, 2021, Úrbez-Torres *et al.*, 2020). For LC2017 which is still under development, recent studies reported its fungistatic effect against GTD pathogens and an elicitor plant effect (Battiston *et al.*, 2021; Mondello *et al.*, 2021, 2022). Firstly, the results confirmed the compatibility of Esquive and LC2017 as reported by Reis *et al.* (2021a). Then, the use of this combination to improve the control of *L. theobromae* associated to *Botryosphaeria*-dieback in our experimental conditions and according to the parameters targeted (length necrosis, pathogen re-isolation, plant responses) does not appear to be relevant. Finally, the elicitor effect of LC2017 is confirmed as well as a specific response related to the cultivar, as previously reported by Mondello *et al.* (2021).

5.2.5 Author Contributions

P.R, C.R., A.A and F.F. designed the experiments; P.R., I.D. and V.M., implemented the methodology; PR., I.D. and V.M. did the formal analysis; P.R. and V.M. did the original

draft preparation; A.A., F.F. and C.R. did the review of the original draft; A.A., F.F. and C.R. did the supervision; A.A., C.R. and F.F. did the funding acquisition.

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CHAPTER 6

FINAL CONSIDERATIONS

6. Final considerations

Grapevine is considered as the host compiling the broadest range of challenging pathogens, of all woody crops with agronomic interest. From these diseases, grapevine trunk diseases (GTDs) are the most relevant threat to the modern wine industry. The high number of ascomycetous and basidiomycetous fungi causing these diseases, associated with the sheer number of different grapevine cultivars planted worldwide, makes research and control of GTDs a subject of extreme complexity. The GTD complex comprises six main different pathologies which are black-foot, Petri disease, Esca complex and Eutypa, Phomopsis and Botryosphaeria diebacks.

The complexity of doing research on GTDs lead us to compile the information available on the several methods and protocols used for laboratory and field work regarding these diseases. This allowed us to plan, optimize and standardize all the methods that could be used throughout the development of this PhD program. This compilation might also be useful for other researchers in having a quick reference of what methods are currently being used in GTD research and finding out if they are useful to their approaches on this subject.

Botryosphaeria dieback is currently among the most significant GTDs and it is caused by fungi from the *Botryosphaeriaceae* family. Its infection can lead to leaf and berry symptoms, but the most commonly described and accepted symptoms are perennial cankers, trunk dieback, vascular streaking and wood necrosis. However, symptoms such as necrosis on the base of green shoots, which could lead to the complete detachment of the shoot later in the season, wilting of the apex of the shoot, wilting of leaves, and more notably, necrosis on the peduncle and rachis of the developing clusters and drying of inflorescences and flowers, were sometimes observed by Portuguese growers and field technicians, in several vineyards across the central region of Portugal. Our approach allowed the association of these symptoms with the most currently isolated *Botryosphaeriaceae* species from grapevine in Portugal, *Neofusicoccum parvum*, *Diplodia seriata* and *Diplodia mutila*. Morphological and molecular studies were carried out and Koch's postulates were fulfilled by replicating the symptoms on field and greenhouse experiments. Although the main economic impact of Botryosphaeria dieback is associated with trunk and cane symptoms, the damages that these fungi could

cause so early in the growing season should not be overlooked, since its presence may also become a source of inoculum, especially on after cultural practices such as de-suckering and summer pruning. The somewhat erratic occurrence of these symptoms on Portuguese vineyards still raises some questions, such as if the multiple fungicide application against other disease, like powdery and downy mildews could have some impacts on the expression of these early season symptoms, or if this expression could be more related to weather and abiotic factors that could vary from season to season. Further and more in-depth research is thus highly recommended to better characterize the cause and expression of these symptoms.

Even if the main *Botryosphaeriaceae* species isolated in Portugal were *N. parvum* and *D. seriata*, other species have been associated with Botryosphaeria dieback in our country, such as *D. mutila*, *Botryosphaeria dothidea* and *Lasiodiplodia* spp. These last species are more frequently found in warmer, even tropical climates like in Mexican or Algerian vineyards, but have been increasingly isolated from grapevines established in more temperate climates. *Lasiodiplodia theobromae* is considered as one of the most aggressive species of *Botryosphaeriaceae* affecting grapevine and, until now, no studies had been conducted on the impact that the hypothetical establishment of this species may have on Portuguese viticulture. For this reason, the last two chapters of this PhD program focused on assessing the response that the most cultivated grapevine cultivars have against this pathogen, and on testing new control methods to mitigate its impact on grapevine. Our results showed that all the cultivars tested showed internal wood symptoms caused by *L. theobromae*, both in greenhouse and field assays, despite the geographical origin of the isolates inoculated. There were significant aggressiveness differences among the isolates tested, with Portuguese isolates causing longer lesion than the Peruvian isolates used on this work. Difference of aggressiveness amongst different isolates has been previously reported in several studies, so it was not surprising that the same pattern was verified on our essays. More importantly, however, were the differences verified among the different cultivars. We used five different cultivars and Touriga Nacional emerged as the cultivar with the highest response for both the greenhouse and field assays, while Aragonez was the least impacted by *L. theobromae* inoculation. Several factors can be contributing to this difference in response to this

pathogen, such as differences in vessel dimension and histological structure and plant defense responses, to the gene level, which can vary among cultivars or even clones. The cultivars Aragonez and Cabernet Sauvignon showed the lowest response to inoculation with *L. theobromae*. According to the Portuguese Instituto da Vinha e do Vinho (IVV), Touriga Nacional is now one of the most recognizable Portuguese cultivars, with a high demand for high quality wines, especially for exportation. Thus, the infection with *L. theobromae* may represent a serious impact on our viticulture. The information gathered on this study, can be extremely useful not only for researchers but also for growers so that they can put in place an appropriate management plan.

On the light of the impact that these pathogens may have on viticulture, it is of the utmost importance to find out new, improved and more sustainable control solutions against *Botryosphaeria* dieback pathogens. Until the current date, no curative methods are available to control infection by *Botryosphaeriaceae*, so preventive methods, like proper pruning wound protection, are recommended as the best measure to mitigate the impacts of this disease. The increasing consumer demands for more sustainable and environmentally friendly agriculture has led to the search for new plant protection products to control diseases. As part of this new approach to grapevine disease management, new biocontrol agents (BCA) and chemical products are now available on the market. Also, due to the increasing restrictions for the use of copper-based products by the European Union, new low copper concentration products with innovative delivery properties, have also created great interest. Therefore, the last chapter of this PhD program was to test new solutions to try to mitigate the impact of *L. theobromae* in grapevine. Starting by set up field trials to assess the efficacy of combining a pruning wound protection strategy based on a BCA with the application of a new copper-based formulation based on hydroxyapatite particles (HA), namely LC2017, on pruning wound challenged with *L. theobromae* and *L. mediterranea*. Additionally, a combination of a pruning wound strategy with Tessior and LC2017 were also tested and compared. The efficacy of these treatments varied between the two years of the study and the two cultivars (Touriga Nacional and Cabernet Sauvignon). Several factors can be at play to explain these differences on the efficacy of the pruning wound protectants applied alone, such as cultivar and weather conditions that differed between both years of the

trial and are known to influence the establishment of Trichoderma-based BCA onto the grapevine. In fact, the statistical analysis performed (PCA) did indeed reinforce this hypothesis since all the treatments with Esquive showed high correlation with precipitation. Our study also pointed out that factors such as cultivar susceptibility and pathogen aggressiveness may likewise be of great importance when planning a control strategy. The application of LC2017 did not appear to greatly increase the efficacy of the pruning wound protection strategy with Esquive or Tessior. This copper-based product showed *in vitro* fungistatic abilities against *L. theobromae* but also against *T. atroviride* strain I-1237, which is the strain present on Esquive. This fungistatic influence did not seem to deeply interfere with the establishment of *T. atroviride* on the grapevines tested, since high re-isolation rates were obtained from the wood. Although the addition of LC2017 did not show clear evidence that it could increase the efficacy of the tested pruning wound strategies, this new product has potential for further long-term research. This formulation has shown interesting results regarding other GTDs and grapevine pathogens, and moreover, it has also presented an elicitor effect of some plant defense responses. This elicitor effect is of great interest since a treated plant can show an improved capability of resisting pathogen infection. To evaluate this elicitation effect, we conducted a greenhouse trial to assess not only the influence of LC2017 and Esquive on *L. theobromae* ability to cause internal wood symptoms on green shoots of young grapevine cuttings and if there was a plant defense response through the induction of the expression of a set of genes. Our results shown that LC2017 does indeed have an elicitation capability towards the two cultivars studied (Touriga Nacional and Cabernet Sauvignon). However, the induction of genes did, varied between the cultivars, and Cabernet Sauvignon does appear to maintain this elicitation effect for a longer period than Touriga Nacional. This feature of LC2017 added to its innovative delivering properties of a fungicide may play a potential role in aiding the grapevine ability to react to GTDs. Nevertheless, this can be considered as a first approach to the use as this product against Botryosphaeria dieback, and so, new extended research opportunities are still to be undertaken specially on the long-term effect, when using LC2017 in combination with other plant protection products. In fact, it could be interesting to assess its influence on other diseases such as downy mildew. This may allow new

information on possible new strategies to help wine growers achieving a profitable and at the same time, more sustainable viticulture.

In conclusion, the data obtained in this thesis provide new and useful information on: a) improving the information about the available diagnostic and protection methods against GTDs; b) increasing the knowledge on symptoms associated with *Botryosphaeria* dieback, facilitating an early diagnostic from the beginning of grapevine's growth period; c) the response of the main cultivars planted in Portugal to *Lasiodiplodia* spp. infection; d) new, improved and more sustainable control solutions against GTDs; e) the elicitor effect that selected product application may have on grapevine defense responses on the interaction product (biocontrol agent; elicitor), cultivar (Touriga Nacional and Cabernet Sauvignon) and the pathogen *L. theobromae*.

SUPPLEMENTARY MATERIAL

Supplementary material Sub-Chapter 5.2

Supplementary material

Table S5.2.1. Primers of genes analyzed by quantitative real-time reverse-transcription polymerase chain reaction.

Function	Gene	Primer Sequences	GenBank or TC TIGR* Accession Number
Reference genes	<i>EF1</i> (EF1- α elongation factor)	5'-GAACTGGGTGCTTGATAGGC-3'	GU585871
		5'-AACCAAAATATCCGGAGTAAAGA-3'	
	<i>60SRP</i> (60S ribosomal protein L18)	5'-ATCTACCTCAAGCTCCTAGTC-3'	XM_002270599
		5'-CAATCTGTCTCCTTCCT-3'	
	<i>39SRP</i> (39S ribosomal protein L41)	5'-AACCAAAATATCCGGAGTAAAGA-3'	XM_002285709
		5'-GACTGACTTCAAGCTTAAACC-3'	
Phenylpropanoid metabolism	<i>PAL</i> (Phenylalanine ammonia lyase)	5'-TCCTCCCGAAAACAGCTG-3'	X75967
		5'-TCCTCCAAATGCCTCAAATCA-3'	
	<i>STS</i> (Stilbene synthase)	5'-AGGAAGCAGCATTGAAGGCTC-3'	FJ851185
		5'-TGCACCAGGCATTCTACACC-3'	
Defense protein	<i>CHIT4C</i> (Chitinase class V)	5'-TCGAATGGGATGGTGGAAA -3'	NM_001281244
		5'-TCCCCTGTCGAAACACCAAG -3'	
	<i>GLUC</i> (β -1,3 glucanase)	5'-TCAATGGCTGCAATGGTGC-3'	DQ267748
		5'-CGGTCGATGTTGCGAGATTTA-3'	
	<i>PR1</i> (pathogenesis-related protein 1)	5'- GGAGTCCATTAGCACTCCTTTG -3'	XM_002273752
		5'- CATAATTCTGGGCGTAGGCAG -3'	
	<i>PR10</i> (pathogenesis-related protein 10)	5'- CGTTAAGGGCGGCAAGAG -3'	DQ396809
		5'- GCATCAGGGTGTGCCAAGA -3'	
Detoxification and Stress tolerance	<i>GST1</i> (Glutathione s-transferase 1)	5'- TGCAATGGAGGAGGAGTTCGT -3'	NM_001281248
		5'- CAAGGCTATATCCCATTTCTTC -3'	
Arsenite recovery genes	<i>PME25</i> (pectin esterase inhibitor)	5'-TGTCGGAGCTGAATGTGGA-3'	XM_002273391.4
		5'-CGATCCCGCTAAGCAGAGTTT-3'	
	<i>MSR</i> (Peptide methionine sulfoxide reductase))	5'-GCATTTGGGCGTGTAGATGG-3'	XM_010662104.2
		5'-GCTTCAGTGTGGCCTGTTCT -3'	
	<i>WRKY</i> (transcription factors)	5'-GGGCAGAAGGACATTCTTGA -3'	XM_002272684.3
		5'-AGGGATCTTCATCCGAACGC -3'	
	<i>HYD2</i> (<i>ABA</i> 8' hydroxylase 2)	5'-GAAGCTTGTTCTCCGAGCCT -3'	NM_001281052.1
		5'-TATTGATCGACCGCCTTCC -3'	

* see <http://www.jcvi.org/cms/research/projects/tdb/overview/>

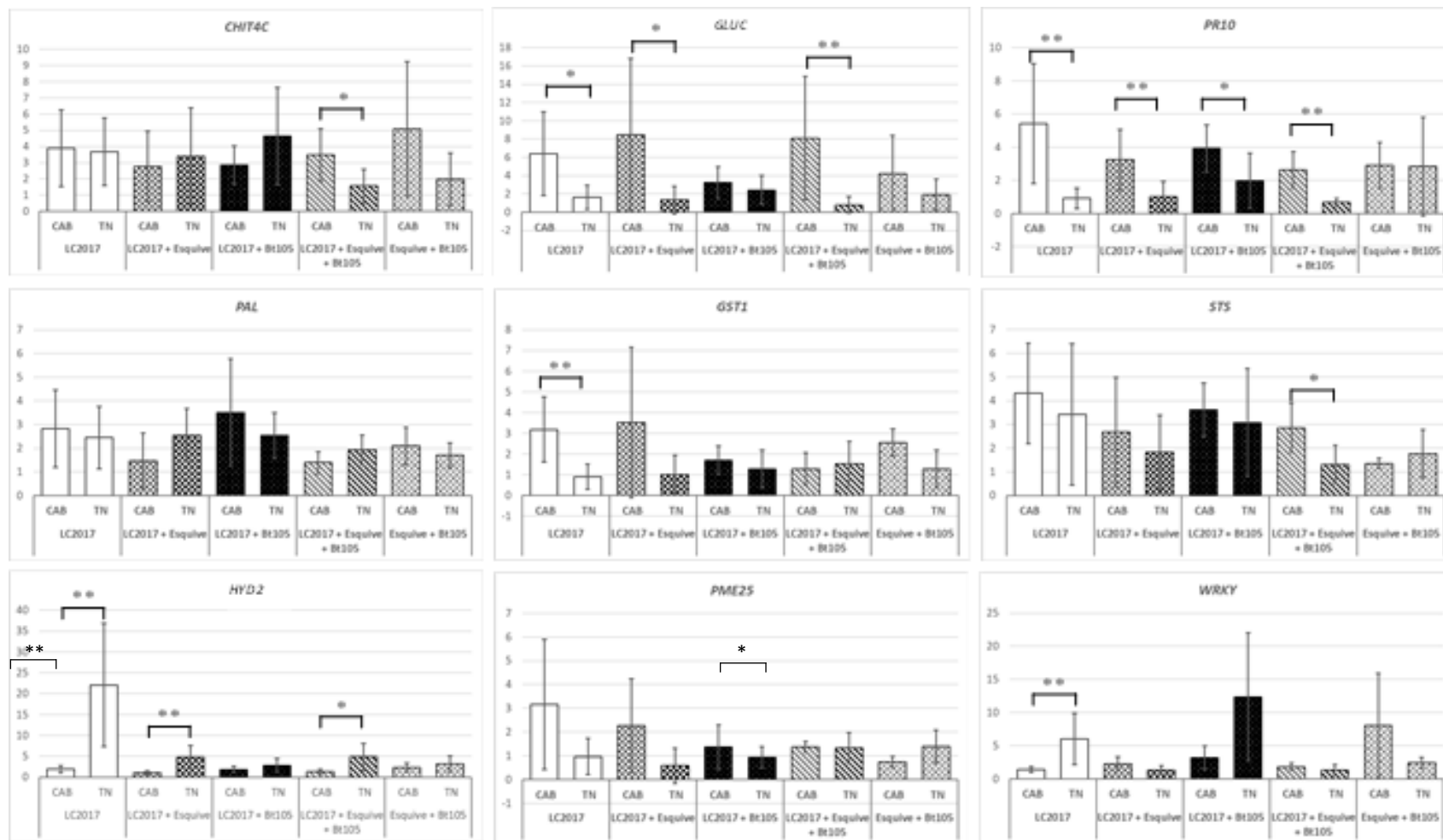


Figure S5.2.1 - Comparison of the 9 targeted genes with induction values >2 recorded at T0+5d on the different condition of treatments (LC2017 alone, LC2017 and Esquire) between Cabernet Sauvignon (CAB) and Touriga Nacional (TN) not infected or inoculated with *L. theobromae* (Bt105). The asterisk indicates a statistical significance (Mann-Whitney U test) between the medians of the two considered condition. * = p<0.05; ** = p<0.01