UNIVERSITAT DE BARCELONA

Biologia i epidemiologia de fongs patògens de la fusta de la vinya i noves perspectives en el seu control

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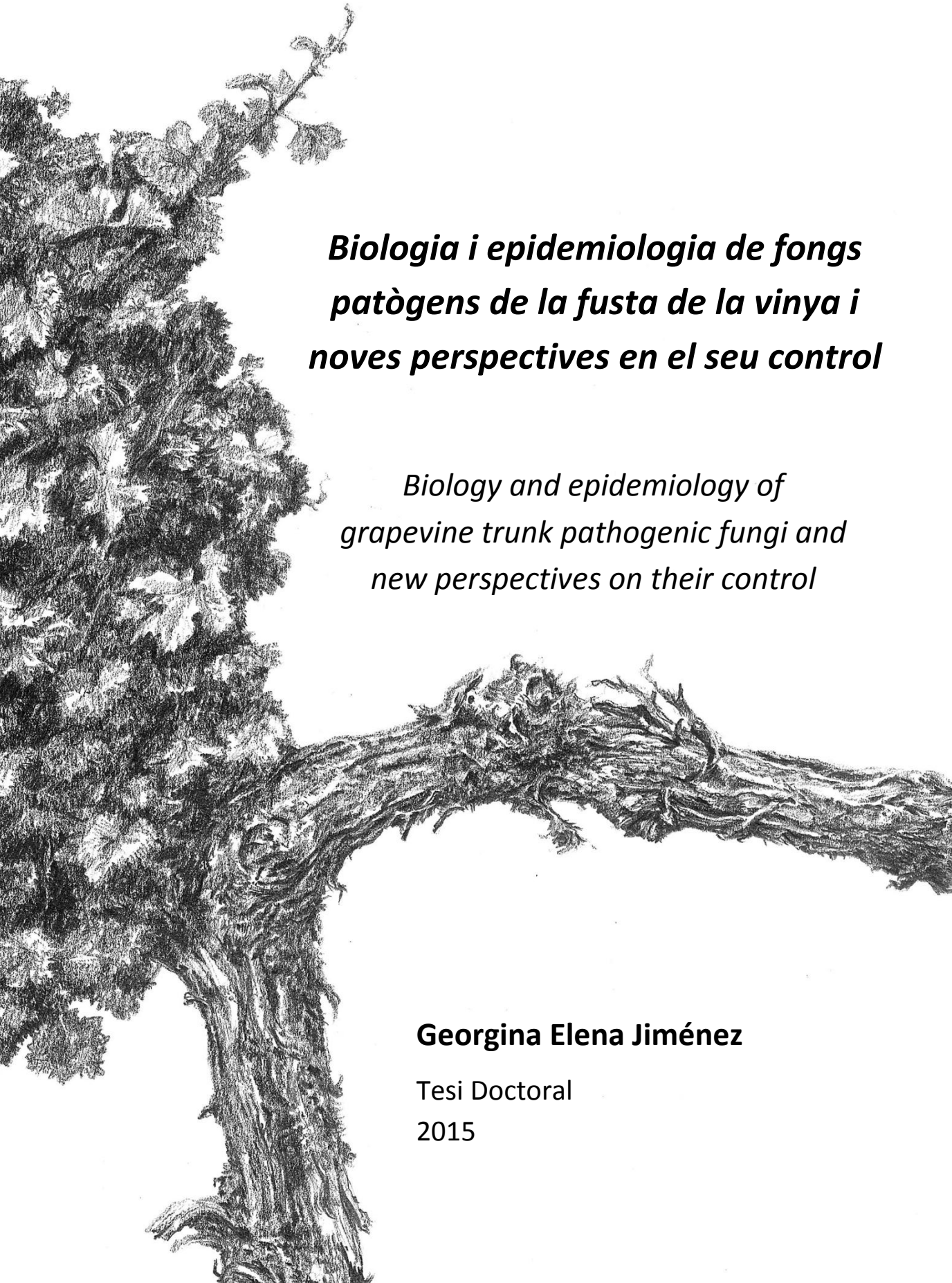
Georgina Elena Jiménez



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Georgina Elena Jiménez

Tesi Doctoral

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Biology and epidemiology of grapevine trunk pathogenic fungi and new perspectives on their control

Memòria presentada per Georgina Elena Jiménez per optar al grau de doctora per la Universitat de Barcelona

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Resum

En les últimes dècades, les malalties de la fusta de la vinya han estat motiu d'una preocupació creixent, paral·lela a l'augment de la seva incidència arreu del món. En aquest context, l'objectiu global d'aquesta tesi ha estat el d'ampliar el coneixement de la biologia i l'epidemiologia d'aquestes malalties, així com el de valorar l'aplicació d'aquests nous coneixements en seu el control. Diferents espècies de fongs de la família *Botryosphaeriaceae* -entre elles, *Diplodia seriata-*, *Eutypa lata* i *Phaeomoniella chlamydospora* són alguns dels fongs patògens més importants.

En un primer estudi es van caracteritzar 83 soques de *D. seriata*, des del punt de vista molecular, fenotípic -basat en la morfologia dels conidis, el creixement miceliar i la compatibilitat vegetativa i sexual- i patogènic. L'estudi molecular va mostrar un polimorfisme del 88 % entre les soques, que es van classificar en dos grups genètics diferenciats. La resta d'estudis realitzats no van ser congruents amb l'agrupació genètica establerta i es va posar de manifest una gran variabilitat intraespecífica a *D. seriata*. Malgrat això, es va confirmar el caràcter patogènic d'aquesta espècie.

Per a millorar algunes tècniques de treball amb els tres patògens citats anteriorment, es va determinar el rang de concentració d'espores òptim per a realitzar inoculacions artificials en sarments de vinya. Per a obtenir percentatges d'infecció d'entre 50 i 70 % van ser necessaris de 100 a 1000 conidis de *D. seriata* per ferida inoculada, de 100 a 2000 conidis de *P. chlamydospora* i de 100 a 500 ascòspores d'*E. lata*.

Es va estudiar l'alliberament de conidis de *D. seriata* en les restes de poda de la vinya. Es va observar la reducció progressiva de l'inòcul, amb disminucions significatives en el nombre de picnidis amb conidis, el nombre de conidis per picnidi i el seu percentatge de germinació . Tot i això, tres anys i mig després de la poda encara es detectaven conidis amb capacitat germinativa, fet indicatiu d'una gran persistència de la font d'inòcul.

Es va determinar la micoflora d'infeccions naturals de les ferides de poda de la vinya. Els fongs més freqüents van ser, en ordre decreixent, *D. seriata*, *P. chlamydospora* i *Cryptovalsa ampelina*. En conjunt, les infeccions van ser més freqüents després de la poda d'hivern, en comparació amb la poda primerenca, a la tardor. La pluja acumulada després de la poda i les

temperatures registrades durant aquest període van correlacionar positivament amb els percentatges d'infecció observats.

La susceptibilitat de les ferides de poda a *D. seriata* i *P. chlamydospora* va disminuir a mesura que augmentava el temps entre la poda i la infecció. Les ferides van restar més temps susceptibles a la infecció de *D. seriata* després d'una poda a l'hivern. La longitud de l'entrenús podat no va semblar interferir en la colonització del sarment de *D. seriata*; en canvi, va dificultar la de *P. chlamydospora*.

Finalment, es va estudiar l'efecte del tractament de termoteràpia amb aigua calenta sobre la viabilitat de vuit espècies de *Botryosphaeriaceae*. En un primer assaig *in vitro*, es va avaluar la supervivència i el creixement del miceli després de sotmetre els fongs a diverses combinacions de temps i temperatura en un bany d'aigua calenta. En un segon assaig *in planta*, els fongs, prèviament inoculats en sarments de vinya, es van sotmetre a un rang de 50-53 °C durant 30 minuts i se'n va determinar la supervivència. En l'assaig *in vitro*, *D. seriata*, *Spencermartinsia viticola*, *Neofusicoccum luteum* i *N. parvum* van ser les espècies més sensibles, i *N. vitifusiforme* i *Lasiodiplodia theobromae*, les més tolerants. En l'assaig *in planta*, totes les espècies van ser controlades a 51 °C, quedant demostrada l'eficàcia d'aquesta tècnica i la seva potencialitat per a ser usada en el procés de producció de planta al viver.

Abstract

Over the past few decades, the incidence of grapevine trunk diseases has increased worldwide and has become a serious concern for the scientific community and for grape and wine producers. Therefore, the main objective of this thesis was to provide new background knowledge about the biology and epidemiology of these diseases as well as to apply new insights into disease control methods. Some of the most significant pathogenic fungi involved in grapevine trunk diseases include fungi in the Botryosphaeriaceae (which include *Diplodia seriata*), *Phaeomoniella chlamydospora* and *Eutypa lata*.

Eighty-three isolates of *D. seriata* were characterized with respect to their genetic, phenotypic (conidial size, mycelial growth and vegetative and sexual compatibility) and pathogenic features. Molecular analyses showed 88 % polymorphism among isolates. These were grouped into two distinct genetic clusters. No relationships were found between the clustering results and the results of the remaining analyses, thus revealing the high intraspecific variability of *D. seriata*. However, this species was confirmed as pathogenic on grapevines.

In order to optimize the inoculum potential used in artificial inoculations with *D. seriata*, *E. lata* and *P. chlamydospora*, pruning wounds were inoculated with different spore concentrations. Infection percentages between 50-70 % were achieved when wounds were inoculated with 100-1000 conidia of *D. seriata* per wound, 100-2000 conidia of *P. chlamydospora* and 100-500 ascospores of *E. lata*.

The release of *D. seriata* conidia in pruning debris was also studied within the period 2-3.5 years after canes were pruned. A progressive reduction in the inoculum pressure was recorded, as indicated by a decrease in the number of pycnidia containing conidia, the mean amount of conidia per pycnidia, and the percentage of viable conidia. However, 3.5 years after pruning conidia which were still able to germinate were detected, thus showing that pruning debris left in the vineyards becomes an important long-lasting inoculum source.

Micoflora resulting from the natural infection of pruning wounds was evaluated in a 4-year study. The pathogenic fungi identified in this study were, in order of descending frequency, *D. seriata*, *P. chlamydospora* and *Cryptovalsa ampelina*. Infection rates were generally higher after a late pruning carried out

in winter, as compared with an early pruning done in autumn. Accumulated rainfall up to 3 months after pruning and temperatures recorded during the same period showed a positive correlation with infection rates.

The susceptibility of pruning wounds to *D. seriata* and *P. chlamydospora* decreased as the time between pruning and the infection of wounds increased. Pruning wounds remained susceptible for a longer period when infection with *D. seriata* was carried out after a late pruning in winter. However, no overall seasonal changes in susceptibility were detected for *P. chlamydospora*. The length of the pruned internodes did not make difficult cane colonization by *D. seriata*. However, cane colonization decreased for *P. chlamydospora* when pruned internodes were longer.

The effect of hot water treatment on the viability of eight Botryosphaeriaceae species was also studied. In an *in vitro* assay, survival of fungi and their mycelial growth were evaluated after various treatments where different combinations of temperature and time exposures were tested using a water bath. In the *in planta* experiment, Richter rootstock canes which had been previously colonized with the fungi were exposed to a water bath within a range 50-53 °C for 30 minutes and their survival was further assessed. In the *in vitro* assay it was observed that *D. seriata*, *Spencermartinsia viticola*, *Neofusicoccum luteum* and *N. parvum* were the most susceptible species, whereas *N. vitifusiforme* and *Lasiodiplodia theobromae* were the most tolerant. In the *in planta* assay, all the viability of isolates was dramatically reduced after 30 minutes at 51 °C and higher temperatures, thus showing the feasibility of this technique as a control method to be used in the grapevine propagation process.

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1.1. La vinya

1.1.1 Característiques generals

La vinya cultivada és una planta dicotiledònia pertanyent a la família *Vitaceae* Juss. i al gènere *Vitis* L. Aquest gènere data d'uns 90,7 milions d'anys enrere (Magallón i Castillo, 2009) i es troba dividit en dues seccions: sect. *Muscadinia* i sect. *Euvitis*. La primera d'elles està formada per espècies amb 40 cromosomes (n=20), i presenta circells simples, escorça no exfoliable i nusos sense diafragma en la medul·la. En aquesta secció s'hi troben tres espècies, *V. munsoniana* J.H. Simpson ex Planch., *V. popenoei* J.L. Fennell i *V. rotundifolia* Michx., de les quals només la darrera d'elles es cultiva i té importància econòmica. La sect. *Euvitis* està caracteritzada per espècies que presenten 38 cromosomes (n=19), circells bifurcats, escorça exfoliable i nusos amb diafragma, i està distribuïda geogràficament per Amèrica, Àsia i Europa. Les representants americanes tenen poques aptituds viníferes, amb l'excepció de *V. labrusca* L. Tot i això, hi ha vàries espècies que s'han utilitzat agronòmicament per la seva resistència a la fil·loxera -causada per l'hemípter de la família *Phylloxeridae*, *Daktulosphaira vitifoliae* (Fitch 1855)-, com ara *V. vulpina* L. (=*V. riparia* Michx.), *V. rupestris* Scheele, *V. berlandieri* Planch. i *V. cordifolia* Lam., així com també un gran nombre d'híbrids derivats dels creuaments entre aquestes i d'altres espècies. Les espècies asiàtiques no presenten interès per la producció de raïm. Dins del grup d'espècies europees, *Vitis vinifera* L. és la que presenta les millors qualitats per a la producció de raïm, panses i vi. L'aparició d'aquesta espècie es remunta a finals del Pliocè a Europa, uns 5 M d'anys abans del present (Hidalgo, 2002). En contrast amb les seves aptituds pel cultiu, *V. vinifera* és extremadament sensible a diverses plagues i malalties causades per insectes, nematodes i fongs.

Vitis vinifera es caracteritza per ser un arbust sarmentós i trepador, que es recolza i fixa sobre tutors naturals o artificials mitjançant circells, o bé que s'estén pel terreny en cas de que no hi hagi cap tutor. En alguns casos pot arribar a assolir més de 100 anys de vida. La vinya té un ampli rang d'adaptació a diverses condicions climàtiques. Tot i ser sensible a les gelades, la latència hivernal li permet suportar temperatures de fins a -15 °C. Tot i això, un cop ha començat a brotar, una exposició a tan sols -2 °C podria ocasionar la pèrdua de la collita. En l'altre extrem, pot suportar temperatures de fins a 45 °C. No

obstant això, es considera que, pel seu cultiu, les temperatures mitjanes anuals no haurien de ser inferiors als 9 °C; amb una temperatura mitjana òptima d'entre 11 °C i 18 °C (Hidalgo, 2002). Pel que fa als requeriments del sòl, la vinya és capaç de viure en diversos tipus de substrat, una característica que es veu millorada per l'ús de diferents portaempelts, sobre els que s'empelta la varietat vinífera, que es troben adaptats a diferents característiques edàfiques i/o ambientals. Respecte les necessitats hídriques, es tracta d'un cultiu que es considera, en línies generals, resistent a la sequera.

1.1.2. El cultiu de la vinya en l'actualitat

La vinya es cultiva tradicionalment en climes de tipus mediterrani, i la seva àrea general de cultiu correspon a les zones compreses entre els paral·lels 30^{o} i 50^{o} de latitud Nord, i entre 30^{o} i 40^{o} de latitud Sud. Aquests límits s'amplien a zones de latituds intermèdies, gràcies a la anomenada viticultura tropical, possibilitant el seu desenvolupament entre els tròpics de Càncer i Capricorn, on antigament era difícil el seu cultiu o només es realitzava a escala familiar (Hidalgo, 2002).

L'aparició de la plaga de la fil·loxera a Amèrica del Nord i la seva posterior propagació a Europa, durant la segona meitat del segle XIX i principi del XX, va causar la destrucció de moltes vinyes a tot el món. Algunes espècies americanes de vinya van mostrar-se resistents a la fil·loxera, però eren plantes que produïen un fruit petit i/o comercialment poc apreciat. La resistència a la fil·loxera està basada en la resistència de l'arrel de la planta al paràsit. Aquesta característica es va aprofitar per a empeltar les varietats viníferes productives (sensibles a fil·loxera) sobre els peus resistents. Des d'aleshores, l'ús de portaempelts resistents a la fil·loxera representa l'única manera de mantenir una planta resistent a la plaga i amb la qualitat vinífera desitjada. La presència de nematodes en els sòls on es cultiva la vinya també pot resoldre's actualment amb l'ús de portaempelts adequats per a aquests casos. A banda de la resistència a la fil·loxera i als nematodes, hi ha altres requisits que s'exigeixen a un bon portaempelt: bona adaptació al medi (resistència a la manca de nutrients, -com el ferro, el potassi, el magnesi, etc.-, a la sequera, a l'excés d'humitat, a la compactació del sòl i a la salinitat, així com l'adaptació a l'acidesa). Per descomptat, el portaempelt ha de presentar una afinitat

satisfactòria amb la part vinífera. Per últim, i no menys important, un bon portaempelt s'ha de caracteritzar per un estat sanitari òptim, lliure d'infeccions patògenes. A Espanya, els portaempelts més utilitzats són 161-19 Couderc (161-49C), 41B Millardet Grasset (41B Mgt), 1103 Paulsen (1103P), 110 Richter (110R) i 140 Ruggeri (140Ru), cadascun d'ells adaptats a condicions específiques de les zones de plantació (Hidalgo, 2002; Gramaje *et al.*, 2010b).

En les darreres dècades, la viticultura s'ha caracteritzat per una progressiva tecnificació del cultiu. Entre els exemples més estesos de la mecanització del cultiu es troben l'ús de màquines prepodadores per a facilitar la poda definitiva dels ceps, l'ús de màquines per a l'aplicació de productes fitosanitaris i les màquines per a la verema (Hidalgo, 2002). Tot i això, hi ha algunes feines difícils de mecanitzar, com és el cas de la poda, la fase final de la qual encara depèn de la feina de personal especialitzat. Altres exemples de la tecnificació del cultiu consisteixen en l'aplicació de sensors per a l'estudi de diferents variables del cultiu (sòl, planta i ambient), l'ús de dades i tecnologia del Sistema de Posicionament Global (GPS), així com de Sistemes d'Informació Geogràfica (SIG/GIS), en un conjunt de tècniques que s'han resumit en un únic concepte, el de la viticultura de precisió (Proffitt *et al*., 2006). La viticultura de precisió persegueix una optimització de la producció i la qualitat del raïm, minimitzant alhora els riscos i impactes ambientals -incloent-hi malalties i plagues- i basant-se en l'anàlisi de la informació proporcionada per diferents fonts (sensors, GPS, GIS...) i l'aplicació de tècniques de maneig del cultiu (conducció, fertirrigació, poda...). En contrast amb una tecnificació creixent a moltes zones vitivinícoles mundials, les zones on el cultiu es porta de forma tradicional, amb una tecnificació mínima i un component de força de treball humana encara important, estan força esteses arreu.

La superfície mundial dedicada al cultiu de la vinya l'any 2013 va assolir 7,16 Mha, corresponent a Espanya la major extensió de vinya plantada (944.200 ha), seguit de França (760.615 ha) i Itàlia (702.100 ha). Europa va representar aproximadament el 50 % del total de la superfície mundial plantada amb vinya (3,56 Mha) (FAO, 2015). La producció mundial de raïm el mateix any va ser de 77,2 Mt i l'europea de 29,0 Mt, per sota del 50 %. Pel que fa als països amb major extensió de plantacions, a Itàlia es van produir 8,0 Mt de raïm, a Espanya, 7,5 Mt, i a França, 5,5 Mt. La producció mundial de vi es va situar en

27,4 Mt en el 2013. Europa va ser el principal continent productor de vi (15,6 Mt), i França va ser el país amb major producció (4,3 Mt), seguit d'Itàlia (4,1 Mt) i finalment, Espanya, en tercera posició (3,2 Mt) (FAO, 2015). A Espanya, el rendiment de producció de la vinya va ser el més baix comparat amb França i Itàlia, per la estructura vitícola, el règim de pluges i les condicions de maneig del cultiu. Tot i això, el raïm és de gran qualitat i amb una bona maduració, cosa que fa que hi hagi una gran varietat de vins de qualitat. A Espanya es cultiven, de manera reconeguda pel *Registro de Variedades Comerciales de Vid del Ministerio de Agricultura, Pesca y Alimentación*, 233 varietats de raïm que es conreen dins un total de 69 denominacions d'origen (MAGRAMA, 2015a). A Catalunya, la superfície dedicada al cultiu de la vinya l'any 2012 era de 55.374 ha i la producció de raïm, 0,27 Mt (MAGRAMA, 2015a), amb dotze denominacions d'origen reconegudes: Alella, Cava, Conca de Barberà, Costers del Segre, Empordà, Montsant, Penedès, Pla de Bages, Priorat, Tarragona, Terra Alta i Catalunya, aquesta última sobreposant-se a les zones de producció de la resta (MAGRAMA, 2015a).

1.2. Malalties de la fusta de la vinya

S'entén per malalties de la fusta de la vinya el conjunt de malalties causades per fongs patògens que tenen en comú el deteriorament de la fusta, amb una simptomatologia prou diversa -necrosis de consistència dura, podridures toves, estries necròtiques en els vasos, etc.- i que posteriorment, en un període de temps indeterminat, condueixen a la mort de la part afectada de la planta o de la totalitat de la planta. En les últimes dècades, la incidència d'aquestes malalties i el seu impacte econòmic negatiu sobre el cultiu han augmentat considerablement a diverses regions vitivinícoles del món, i no es coneix cap varietat de vinya, cultivada o salvatge, que en sigui resistent (Surico *et al.*, 2006; Wagschal *et al.*, 2008; Larignon *et al.*, 2009). Degut a això, l'interès per aquestes malalties ha portat a desenvolupar noves línies d'investigació arreu del món per a aprofundir en el seu coneixement i per a desenvolupar noves estratègies i mesures de control (Mugnai *et al.*, 1999; Larignon i Dubos, 2001a; Eskalen *et al.*, 2007; Halleen *et al.*, 2007a; Phillips *et al.*, 2008; Sosnowski *et al.*, 2008; Luque *et al.*, 2009; Rolshausen *et al.*, 2010a; Gramaje i Armengol, 2011; Lecomte i Bailey, 2011; Úrbez-Torres, 2011; Úrbez-Torres i Gubler, 2011; van Niekerk *et al.*, 2011).

Les malalties de la fusta poden ser causades per un nombre considerable d'espècies fúngiques, que afecten tant la planta jove com l'adulta. Aquests fongs patògens estan associats a una sèrie de patologies que poden donar-se independentment, de manera simultània o, fins i tot, ser precursores d'altres malalties a la mateixa planta (Mugnai *et al*., 1999; Luque *et al*., 2009). A continuació es descriuen els trets més importants cada una d'aquestes malalties, i amb més detall, les associades a la planta adulta, l'objecte principal d'aquesta tesi.

1.2.1. Malalties de la fusta de la vinya en planta jove

Les principals malalties de la fusta de la vinya que formen part del complex del decandiment de la vinya jove (*young vine decline*, YVD) són la malaltia de Petri i la del peu negre (Gramaje i Armengol, 2011). A més, diverses espècies de la família *Botryosphaeriaceae* han estat aïllades en plantes joves afectades per decandiment a diverses regions vitivinícoles del món (Gramaje i Armengol, 2011), que també s'integrarien en aquest complex. A la vinya, els símptomes visibles de les malalties de Petri i del peu negre són molt similars i, en conseqüència, difícils de diagnosticar; s'observa principalment una disminució de la vigorositat, caracteritzada per un retard o fallida en la brotada, un creixement reduït, el marciment i/o clorosi a les fulles i, ocasionalment, el col·lapse sobtat de la planta (Scheck *et al.*, 1998a; Gramaje i Armengol, 2011).

La malaltia de Petri està causada per diverses espècies de fongs patògens, entre les que destaquen *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams i *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai) Berl. (teleomorf: *Togninia minima* (Tul. & C. Tul.)). Recentment, en una revisió del gènere *Phaeoacremonium* W. Gams, Crous & M.J. Wingf on es simplifica la nomenclatura i es proposa l'ús del nom anamòrfic (*Phaeoacremonium*) en lloc del teleomòrfic (*Togninia* Berl.), l'espècie *P. aleophilum* ha quedat reclassificada com *P*. *minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous (Gramaje *et al.*, 2015). A banda d'aquest tàxon, s'han identificat fins a 24 espècies més del gènere *Phaeoacremonium* associades a la malaltia de Petri (Crous *et al.*, 1996; Dupont *et al.*, 2000; Groenewald *et al.*, 2001; Mostert *et al.*, 2005; Mostert *et al.*, 2006; Essakhi *et al.*, 2008; Gramaje *et al.*, 2009a; Úrbez-Torres *et al.*, 2014).

Darrerament, algunes espècies del gènere *Cadophora* Lagerb & Mlein també han estat associades a aquesta malaltia (Overton *et al.*, 2005; Halleen *et al.*, 2007b). Entre elles, s'ha confirmat la presència freqüent de *Cadophora luteo-olivacea* (J.F.H. Beyma) T.C. Harr. & McNew en vinyes afectades a Califòrnia (Rooney-Latham, 2005), Sud-àfrica (Halleen *et al.*, 2007b), Uruguai (Abreo *et al.*, 2008), Nova Zelanda (Manning i Mundy, 2009), Nordest d'Amèrica (Rolshausen *et al.*, 2010b) i Espanya (Gramaje *et al.*, 2011). *Pleurostomophora richardsiae* (Nannf.) L. Mostert, W. Gams & Crous (≡*Cadophora richardsiae* Nannf.) també ha estat associada a la malaltia de Petri a Califòrnia (Rolshausen *et al.*, 2010a) i s'ha confirmat com el causant de decoloracions en la fusta de la vinya similars a les de la malaltia (Halleen *et al.*, 2007b). A Espanya, l'espècie *Cadophora melinii* Nannf. ha estat aïllada de material vegetal de vivers que mostrava símptomes de necrosis vasculars (Gramaje *et al.*, 2011) i, recentment, ha estat reclassificada com a *Cadophora viticola* D. Gramaje, L. Mostert & Armengol (Crous *et al.*, 2015). A Estats Units, un estudi ha identificat tres noves espècies de *Cadophora* (*Cadophora novi-eboraci* Travadon, Lawrence, Rooney-Latham, Gubler, Wilcox, Rolshausen & K. Baumgartner, *Cadophora orientoamericana* Travadon, Lawrence, Rooney-Latham, Gubler, Wilcox, Rolshausen & K. Baumgartner i *Cadophora spadicis* (Prodi, Sandalo, Tonti, Nipoti & A. Pisi) Travadon, Lawrence, Rooney-Latham, Gubler, Wilcox, Rolshausen & K. Baumgartner) associades al decandiment de la vinya (Travadon *et al*., 2015).

La malaltia de Petri també s'havia conegut anteriorment amb els noms de *black goo*, *young vine decline* o, fins i tot, *young esca* (Rego *et al.*, 2000; Edwards *et al.*, 2001b; Morton, 2001), per bé que molts d'aquests noms avui en dia són poc usats. El nom de la malaltia reconegut actualment honora l'investigador, Lionello Petri, que va ser el primer en descriure els símptomes típics de la malaltia (Petri, 1912). Externament, les plantes afectades per aquesta malaltia es mostren dèbils, amb una disminució del creixement clara durant l'època vegetativa, amb entrenusos curts, fulles poc desenvolupades i d'aspecte cloròtic o necròtic. Els símptomes que apareixen a la fusta consisteixen en necrosis als vasos, que es veuen com puntejades de color negre o fosc rodejant la medul·la en talls transversals, i com estries fosques en talls longitudinals. Aquests símptomes poden aparèixer al llarg de tota la fusta del portaempelt, de la zona de l'empelt, o fins i tot de la varietat vinífera, però en general són més accentuats a la zona basal del portaempelt. Algunes de les

espècies associades a la malaltia de Petri també formen part del complex de fongs de l'esca, una patologia pròpia de la planta adulta, els símptomes de la qual es manifesten en plantes de vuit o més anys d'edat (Mugnai *et al.*, 1999; Surico *et al.*, 2006). Actualment es considera que les dues malalties es troben estretament relacionades, doncs les plantes joves afectades pels fongs de la malaltia de Petri poden, amb el temps, veure's infectades per espècies de fongs basidiomicets associades a l'esca (Mugnai *et al.*, 1999; Graniti *et al.*, 2000; Serra *et al.*, 2000).

La malaltia del peu negre és una malaltia prou estesa en vivers i vinyes joves (Halleen, 2006a). La primera vegada que es va descriure sobre vinya va ser l'any 1961 (Grasso i Magnano Di San Lio, 1975). Originàriament, es creia que *Cylindrocarpon destructans* (Zinss.) Scholten n'era l'agent causal principal (Rego *et al.*, 2000; Petit i Gubler, 2005). No obstant això, posteriorment es va veure que diverses soques de *C. destructans* corresponien realment a una espècie diferent, *Cylindrocarpon liriodendri* J.D. MacDonald & E.E. Butler, basant-se en caràcters morfològics i multigènics (Halleen *et al.*, 2006b; Petit i Gubler, 2007; Chaverri *et al.*, 2011). *Cylindrocarpon obtusisporum* (Cooke & Harkness) Wollenweber va ser també identificat com l'agent causal del peu negre a Itàlia (Grasso i Magnano Di San Lio, 1975) i Califòrnia (Scheck *et al.*, 1998b), però estudis posteriors van indicar que es tractava realment de *Cylindrocarpon macrodidymum* Halleen, Schroers & Crous (Halleen *et al.*, 2004; Petit i Gubler, 2005). Chaverri *et al.* (2011) van establir el gènere *Ilyonectria* P. Chaverri & C. Salgado per acomodar millor els anamorfs del gènere *Cylindrocarpon* Wollenw., de manera que aquest darrer va quedar dividit en quatre grups basats en la presència o absència de microconidis i clamidòspores. *Cylindrocarpon liriodendri* i *C. macrodidymum* van ser redefinits com *Ilyonectria liriodendri* (Halleen, Rego & Crous) P. Chaverri & C. Salgado i *Ilyonectria macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & Salgado, respectivament (Chaverri *et al.*, 2011). Estudis moleculars posteriors van veure que el gènera *Ilyonectria* era parafilètic (Cabral *et al.*, 2012; Lombard *et al.*, 2013). Cabral *et al.* (2012) van realitzar un estudi filogenètic del complex *I. macrodidyma*, en el que van reconèixer sis noves espècies de *Ilyonectria* (*Ilyonectria* sp. 1, *Ilyonectria* sp. 2, *I. estremocensis* A. Cabral, Nascimento & Crous, *I. alcacerensis* A. Cabral, Oliveira & Crous, *I. novozelandica* A. Cabral & Crous i *I. torresensis* A. Cabral, Rego & Crous) i l'espècie *I. macrodidyma* dins d'aquest complex. Recentment, Lombard *et al.*

(2014) han introduït el nou gènere *Dactylonectria* L. Lombard & Crous, que inclou noves combinacions de membres procedents del gènere *Cylindrodendrum* Bonord, *Ilyonectria* -entre ells *I. macrodidyma*-, i *Neonectria* Wollenw, i dues espècies noves. Vuit espècies dins d'aquest nou gènere estan associades a la malaltia del peu negre de la vinya: *D. alcacerensis* (A. Cabral, Oliveira & Crous) L. Lombard & Crous (≡*lyonectria alcacerensis*), *D. estremocensis* (A. Cabral, Nascimento & Crous) L. Lombard & Crous (≡*Ilyonectria estremocensis*), *D. macrodidyma* (Halleen, Schroers & Crous) L. Lombard & Crous (≡*Ilyonectria macrodidyma*), *D. novozelandica* (A. Cabral & Crous) (≡*Ilyonectria novozelandica*), *D. pauciseptata* (Schroers & Crous) L. Lombard & Crous (≡*Cylindrocarpon pauciseptatum* Schroers & Crous), *D. torresensis* (A. Cabral, Rego & Crous) L. Lombard & Crous (≡*Ilyonectria torresensis*), *D. vitis* (A. Cabral, Rego & Crous) L. Lombard & Crous (≡*Ilyonectria vitis* A. Cabral, Rego & Crous) i una nova espècie, *D. pinicola* L. Lombard & Crous. A banda de les espècies citades, també es troben associades a aquesta malaltia les espècies fúngiques següents: *Campylocarpon fasciculare* Schroers, Halleen & Crous, *C. pseudofasciculare* Halleen, Schroers & Crous, *Cylindrocladiella parva* (P.J. Anderson) Boesewinkel, *C. peruviana* (Batista, J.L. Bezerra & M.P. Herrera) Boesewinkel, *I. europaea* A. Cabral, Rego & Crous, *I. lusitanica* A. Cabral, Rego & Crous, *I. pseudodestructans* A. Cabral, Rego & Crous, *I. robusta* (A.A. Hildebr.) A. Cabral & Crous, i dues espècies d'*Ilyonectria* encara per caracteritzar (Úrbez-Torres *et al.*, 2014).

Els agents causals del peu negre han estat aïllats freqüentment de portaempelts, d'estaques arrelades, de la zona de l'empelt i de vinyes joves empeltades (Rumbos i Rumbou, 2001; Halleen *et al.*, 2003; Fourie i Halleen, 2004a; Oliveira *et al.*, 2004; Aroca *et al.*, 2006; Halleen *et al.*, 2007a). A més, aquests patògens són relativament comuns en els camps d'arrelament dels vivers (Halleen *et al.*, 2003; Halleen *et al.*, 2007a). Els símptomes més característics d'aquesta malaltia corresponen a una reducció de la biomassa de l'arrel i dels pèls radiculars, així com a la presència de lesions necròtiques i de xancres en tota l'arrel (Rego *et al.*, 2000; Halleen *et al.*, 2006a; Alaniz *et al.*, 2007; Abreo *et al.*, 2010). Quan es retira l'escorça de les arrels infectades, s'observen necrosis de color fosc als teixits vasculars i la medul·la també es veu força enfosquida (Scheck *et al.*, 1998b; Larignon, 1999; Fourie i Halleen, 2002; Halleen *et al.*, 2006a). Els símptomes externs es caracteritzen per una reducció

del vigor de la planta, amb sarments curts, entrenusos també curts, fullatge dispers i fulles petites amb clorosis i necrosis entre els nervis del limbe foliar (Scheck *et al.*, 1998b; Rego *et al.*, 2000; Halleen *et al.*, 2006a; Alaniz *et al.*, 2007; Alaniz *et al.*, 2009; Abreo *et al.*, 2010).

En les dues darreres dècades, el decandiment de vinyes joves s'ha incrementat dràsticament a moltes zones vitícoles del món, coincidint amb la ràpida expansió de la indústria de la vinya i amb l'establiment extensiu de noves vinyes arreu (Graniti *et al.*, 2000; Gramaje i Armengol, 2011). Els fongs associats a la síndrome del YVD han estat extensament estudiats i es consideren una de les majors causes de mortalitat. La detecció d'aquests patògens en planta jove i la seva dispersió a través del material de propagació dels vivers han estat un dels principals temes d'estudi. S'ha arribat a establir que el material comercialitzat pels vivers ja es troba infectat per aquests fongs, en un grau variable, ja sigui perquè la infecció procedeix de la planta mare o bé perquè el material vegetal es contamina durant el procés de propagació (Oliveira *et al.*, 2004; Gramaje i Armengol, 2011).

1.2.2. Malalties de la fusta de la vinya en planta adulta

Les tres malalties de la planta adulta que presenten un major impacte arreu del món són l'esca, l'eutipiosi i el decandiment causat per *Botryosphaeria*. Els símptomes d'aquestes malalties poden manifestar-se en el cep a partir dels vuit anys d'edat (Larignon i Dubos, 1997; Mugnai *et al.*, 1999; Larignon *et al.*, 2009), però la seva expressió és molt variable i depèn de múltiples factors, de manera que pot ser impredictible i discontínua -erràtica- any rere any (Mugnai *et al.*, 1999; Surico, 2000; Wagschal *et al.*, 2008). L'aplicació de l'arsenit sòdic, com a protector de les ferides de poda, era l'únic tractament eficaç contra aquestes malalties, especialment contra l'esca (Fussler *et al.*, 2008; Larignon *et al.*, 2008). L'ús generalitzat d'aquest pesticida a molts països es va començar a prohibir a principis del segle actual, degut al fort impacte ambiental que generava i al seu efecte cancerigen en humans (Trouba *et al.*, 2002). A Espanya, l'ús d'aquest producte es va prohibir definitivament l'any 2003. Des d'aleshores, existeix l'opinió generalitzada al sector vitivinícola de que la incidència de les malalties de la fusta s'ha vist incrementada precisament per la prohibició d'aquest pesticida. Altres autors, però, creuen que el problema és més complex

i que s'hi han de considerar altres factors, entre els que hi destaca la pràctica forçada del cultiu per a obtenir majors rendiments agrícoles i l'ús de plantes procedents de viver que ja es troben infectades pels patògens (Gramaje i Armengol, 2011).

En les seccions següents es descriuen els trets més importants de les principals malalties de la fusta de la vinya en planta adulta.

1.3. El decandiment causat per *Botryosphaeria*

Aquesta malaltia va ser originalment descrita amb el nom de braç mort negre (*black dead arm*, BDA) l'any 1974, en la regió vitivinícola de Tokaj (Hongria), i va ser associada al fong *Diplodia mutila* (Fr.) Mont. (Lehoczky, 1974). No obstant això, en estudis posteriors es va observar que altres espècies de la mateixa família *Botryosphaeriaceae* Theiss. & H. Syd., principalment *Diplodia seriata* De Not. i *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, apareixien freqüentment associades a símptomes relacionats amb aquesta malaltia (Cristinzio, 1978; Rovesti i Montermini, 1987; Larignon i Dubos, 2001a; Larignon *et al.*, 2009). Els autors anteriors continuaven relacionant BDA amb la presència de diferents espècies de *Botryosphaeriaceae*, alhora que s'hi descrivien també nous símptomes relacionats. Degut al nombre creixent d'espècies de la família *Botryosphaeriaceae* que han estat progressivament aïllades i identificades en vinya en els darrers anys (Crous *et al.*, 2006; Úrbez-Torres, 2011), i que han estat associades a símptomes diferents dels descrits originalment pel BDA, finalment Úrbez-Torres (2011) va proposar un nom nou, el de decandiment causat per *Botryosphaeria* (*Botryosphaeria dieback*), que recollia tots els símptomes descrits en vinya i els agents causals corresponents.

1.3.1. Agents causals

Fins ara, 28 espècies de la família *Botryosphaeriaceae* han estat associades a malalties de la fusta de la vinya (Montagne, 1836; De Notaris, 1845; Cesati i De Notaris, 1863; Griffon i Maublanc, 1909; Shoemaker, 1964; Punithalingam, 1976; Pennycook i Samuels, 1985; Phillips, 2002; Alves *et al.*, 2004; Slippers *et al.*, 2004; van Niekerk *et al.*, 2004; Burgess *et al.*, 2005; Luque *et al.*, 2005; Phillips *et al.*, 2005; Burgess *et al.*, 2006; Crous *et al.*, 2006; Crous *et*

al., 2007; Phillips *et al.*, 2007; Phillips *et al.*, 2008; Úrbez-Torres *et al.*, 2012; Correia *et al.*, 2013; Pitt *et al.*, 2013b; Linaldeddu *et al.*, 2014). A Espanya s'han detectat 12 d'aquestes espècies: *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (Armengol *et al.*, 2001), *Diplodia mutila* (Martin i Cobos, 2007), *D. seriata* (Armengol *et al.*, 2001), *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves i *D. sarmentorum* (Fr.) A.J.L. Phillips, A. Alves & J. Luque (Martin i Cobos, 2007), *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Aroca *et al.*, 2008; Martín *et al.*, 2009), *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips (Aroca *et al.*, 2010), *N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Luque *et al.*, 2009), *N. mediterraneum* Crous, M.J. Wingf. & A.J.L. Phillips (Aroca *et al.*, 2010), *N. parvum* (Aroca *et al.*, 2006), *N. vitifusiforme* (Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips (Aroca *et al.*, 2010) i *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous (Phillips *et al.*, 2008).

Fins fa relativament poc, la identificació de les espècies d'aquesta família s'havia basat en les característiques morfològiques de l'anamorf, ja que els caràcters del teleomorf són molt semblants entre els diferents tàxons i són insuficients per a diferenciar-los entre sí. A més, és difícil trobar el teleomorf a la natura o fins i tot induir-lo en condicions de laboratori (Jacobs i Rehner, 1998; Denman *et al.*, 2000; Phillips, 2002), cosa que dificulta encara més el seu estudi. En conseqüència, la caracterització de les espècies ha estat força controvertida. En una classificació avui en dia obsoleta, el gènere teleomòrfic *Botryosphaeria* Ces. & De Not. estava integrat per diferents gèneres anamòrfics (Denman *et al.*, 2000; Phillips, 2002; Alves *et al.*, 2004; Slippers *et al.*, 2004): *Fusicoccum* Corda, amb conidis hialins i de paret fina (que inclou també *Macrophomopsis* Petr.); *Diplodia* Fr., amb conidis bicel·lulars, pigmentats i de paret gruixuda; *Sphaeropsis* Sacc., amb conidis unicel·lulars, pigmentats i de paret gruixuda; *Lasiodiplodia* Ellis & Everh., amb conidis bicel·lulars foscos, amb estries a les parets cel·lulars; i *Macrophoma* (Sacc.) Berl. & Vogl., amb conidis unicel·lulars grans, hialins i de paret llisa. Més endavant, Phillips *et al.* (2005) van recuperar el gènere *Dothiorella* Sacc., segregat de *Diplodia*, que inclou espècies amb conidis foscos i septats des de les primeres fases del seu desenvolupament en el picnidi.

Un dels problemes més importants que plantejava la taxonomia de *Botryosphaeria* era que no complia l'associació 'un anamorf-un teleomorf' (Seifert *et al.*, 2000). Amb la implementació de tècniques d'identificació basades en l'ADN i les anàlisis filogenètiques associades es van poder resoldre alguns d'aquests conflictes taxonòmics (Jacobs i Rehner, 1998; Denman *et al.*, 2000; Smith i Stanosz, 2001; Zhou i Stanosz, 2001; Crous *et al.*, 2006). Crous *et al.* (2006) van determinar que la família *Botryosphaeriaceae* era polifilètica, basant-se en l'estudi de seqüències de la subunitat gran de l'ADN ribosomal (rDNA 28S). Des d'aleshores, el gènere *Botryosphaeria* ha estat restringit únicament a les espècies que presenten l'anamorf *Fusicoccum*. A més, en aplicació del principi 'un anamorf-un teleomorf', Crous *et al.* (2006) van suggerir únicament l'ús del nom del gènere anamòrfic per a la resta de tàxons de la família, ja que no van introduir nous noms teleomòrfics. Tot i això, aquests autors no varen poder resoldre la segregació dels gèneres *Diplodia* i *Lasiodiplodia*. En un estudi posterior, Phillips *et al.* (2008), basant-se en un anàlisis filogenètic multigènic, van resoldre aquest problema i actualment s'accepta que *Lasiodiplodia* pertany a un llinatge diferent del de *Diplodia*.

1.3.2. Simptomatologia

El desenvolupament i la propagació d'aquesta malaltia és lent, d'anys, encara que el període no s'ha pogut establir amb exactitud. Els símptomes que presenta inclouen un creixement reduït dels brots en la represa de l'activitat vegetativa a la primavera, i fins i tot la mort d'aquests (Figura 1.1a). Això sol anar acompanyat de la presència de fulles petites, que ocasionalment tenen també aspecte cloròtic (Figura 1.1b). Però els símptomes més rellevants del decandiment causat per *Botryosphaeria* són la mort progressiva dels braços de la planta (*dieback*, en anglès) (Figura 1.1c), així com els xancres (Figura 1.1d) i les necrosis presents en el tronc i/o els braços de les vinyes infectades (Luque *et al.*, 2009; Úrbez-Torres i Gubler, 2011). Un cop la fusta és colonitzada pel fong, la part afectada mor i es torna de color fosc, tonalitat que s'estén amunt i avall des de la zona d'infecció. Aquest símptoma normalment es veu en seccions transversals de les parts afectades com una necrosi en forma de V (Figures 1.1e i 1.1f). Tot i que els símptomes en fusta són prou reconeguts per la comunitat científica, els símptomes foliars han estat matèria de discussió al llarg de les últimes dècades. Mentre que Lehockzy (1974) va observar clorosis foliars

lleugeres i difuminades, així com el marciment de la fulla, Larignon i Dubos (2000) van descriure un patró inicial de decoloració de les fulles, entre els nervis del limbe, de color vermell en el cas de les varietats de raïm negre i groc verdós en el cas de les varietats de raïm blanc. Aquestes decoloracions s'acabaven

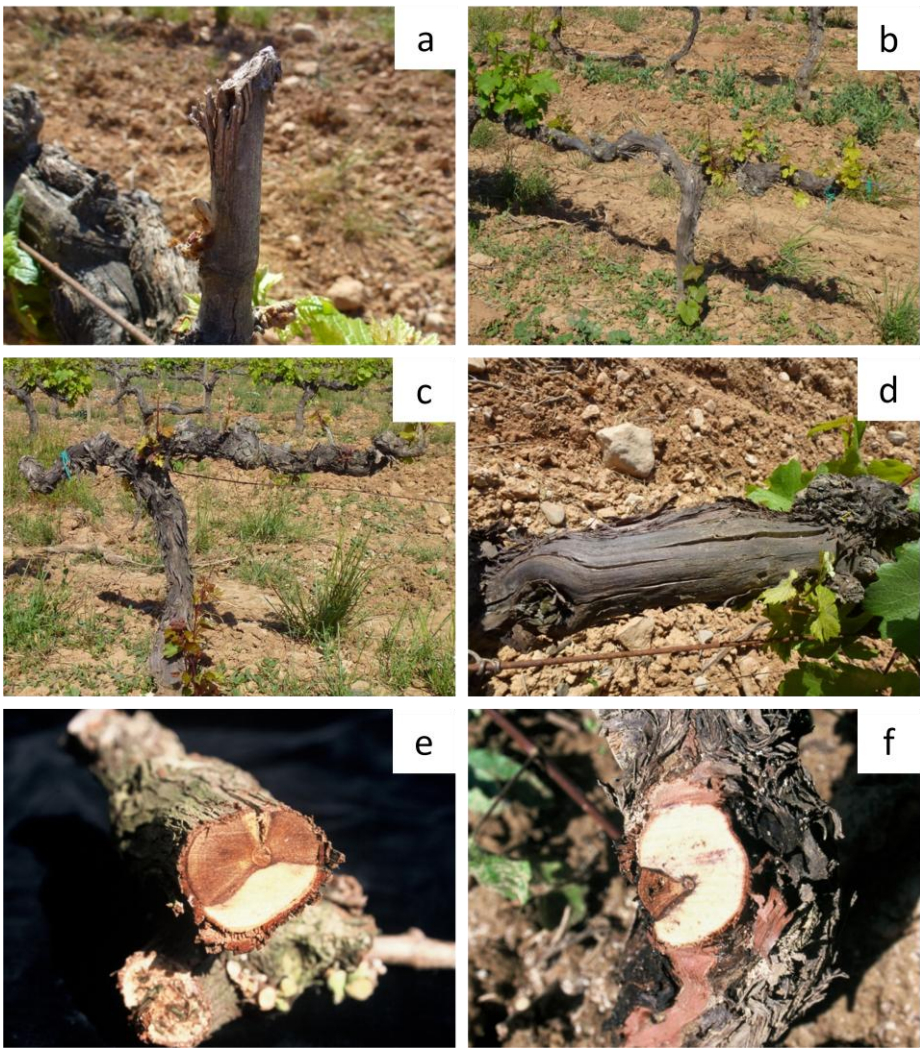

Figura 1.1. Simptomatologia pròpia del decandiment causat per *Botryosphaeria*. Símptomes externs: manca de brotada (a), fulles d'aspecte cloròtic (b) i xancres a la fusta (c i d). Símptomes interns: necrosi sectorial al tronc i als braços en forma de V, observada en talls transversals (e i f).

convertint en una necrosi contínua en fase tardana. Aquests símptomes són molt semblants als símptomes foliars que presenta la malaltia de l'esca, però Larignon *et al.* (2001a) van postular que no es podien confondre ja que els símptomes foliars del decandiment causat per *Botryosphaeria* apareixen abans que els de l'esca. Aquestes similituds van ser discutides posteriorment per Surico *et al.* (2006) i Lecomte *et al.* (2005b), que van argumentar que l'esca pot mostrar un rang molt variable de símptomes al llarg de tota l'època vegetativa. Finalment, Lecomte *et al.* (2012) van demostrar, a partir del seguiment individualitzat de fulles aparentment afectades pel decandiment causat per *Botryosphaeria*, que en realitat es tractava de manifestacions primerenques de l'esca. Així doncs, i fins que no hi hagi més evidències que aclareixin aquest aspecte, els símptomes foliars del decandiment causat per *Botryosphaeria sensu* Larignon i Dubos (2000) són considerats realment com una manifestació d'esca per molts investigadors.

1.3.3. *Biologia i epidemiologia*

Tot i que el decandiment causat per *Botryosphaeria* es considera una malaltia pròpia de la planta adulta, diverses espècies d'aquesta família també han estat detectades amb freqüència en planta jove (Crous *et al.*, 2006; Ridgway *et al.*, 2011; Úrbez-Torres, 2011). A més, també s'han detectat en el material de propagació a Espanya (Aroca *et al.*, 2006; Giménez-Jaime *et al.*, 2006; Martín i Cobos, 2007; Gramaje *et al.*, 2009c), Grècia (Rumbos i Rumbou, 2001), Itàlia (Spagnolo *et al.*, 2011), Mèxic (Úrbez-Torres *et al.*, 2008), Nova Zelanda (Billones-Baaijens *et al.*, 2013b; Billones-Baaijens *et al.*, 2013c), Portugal (Oliveira *et al.*, 2004) i Sud-àfrica (Halleen *et al.*, 2003).

No es coneixen amb exactitud les fases del cicle de vida de totes les espècies d'aquesta família. Dins de la família hi ha espècies sapròfites i paràsites (Smith *et al.*, 1996); les darreres ocasionen xancres, decandiment i altres malalties en molts hostes llenyosos a banda de la vinya (Michailides, 1991; Phillips, 2000; Denman *et al.*, 2003; Slippers *et al.*, 2004). En l'actualitat es creu que la majoria de les espècies de *Botryosphaeriaceae* podrien tenir també una fase endofítica, en la qual els fongs es desenvolupen en l'interior de l'hoste sense que s'observin els símptomes de la infecció de forma visible (Slippers i Wingfield, 2007). Els cossos fructífers dels fongs, majoritàriament corresponents

a l'anamorf, es desenvolupen en ferides de poda velles, brots infectats, sota l'escorça dels braços o del tronc i en les restes de poda abandonades a les vinyes. La disseminació dels conidis es dóna principalment per l'impacte de les gotes d'aigua, però també es pot donar immediatament després d'un episodi de pluja o reg, tot indicant que l'impacte de les gotes no és l'únic factor ambiental necessari (Úrbez-Torres *et al.*, 2010; van Niekerk *et al.*, 2010). Els fongs infecten les vinyes principalment a través de les ferides de poda, on els conidis germinen i inicien la colonització dels teixits llenyosos del cep (Úrbez-Torres i Gubler, 2009; Rolshausen *et al.*, 2010a). La colonització dels teixits de la planta porta finalment a la formació de xancres, la majoria dels quals tenen precisament el seu punt d'inici en alguna ferida de poda vella. Amb el temps, en la superfície dels xancres es desenvolupen els cossos fructífers, on s'hi formaran les espores que iniciaran un nou cicle del patogen. L'existència de cossos fructífers d'algunes espècies de *Botryosphaeriaceae* en sarments procedents de les restes de la poda (Luque *et al*., 2005; Luque *et al*., 2009), per exemple *Diplodia seriata* i *Spencermartinsia viticola*, ens indica que aquests fongs també tenen la capacitat saprofítica de créixer sobre la vinya. En el Capítol 6 d'aquesta tesi s'avalua el paper de les restes de poda com a possible font d'inòcul per a noves infeccions. En el procés viverístic de producció de planta, aquests fongs són propagats de forma similar a la dels fongs del complex YVD (Halleen *et al.*, 2003; Giménez-Jaime *et al.*, 2006; Gramaje i Armengol, 2011; Billones-Baaijens *et al.*, 2013b; Billones-Baaijens *et al.*, 2013c).

El caràcter patogènic de les espècies de *Botryosphaeriaceae* vers la vinya ha estat font de controvèrsia en les darreres dècades. Determinar la patogenicitat d'aquests fongs s'ha vist dificultat pels problemes existents en la taxonomia del grup i pels resultats, a vegades poc consistents, que s'han obtingut en proves de patogenicitat realitzades en condicions diverses. Així, per exemple, *Botryosphaeria dothidea*, *D. seriata* i *Diplodia mutila* van ser considerats patògens a França (Larignon *et al.*, 2001a), mentre que no ho van ser a Portugal (Phillips, 1998; Phillips, 2002). El millor exemple de controvèrsia pel que fa a la patogenicitat és *D. seriata*. Tot i que ha estat descrit com patogen de la vinya a Austràlia (Castillo-Pando *et al.*, 2001; Savocchia *et al.*, 2007), Xile (Auger *et al.*, 2004), França (Larignon *et al.*, 2001a), Itàlia (Rovesti i Montermini, 1987), Mèxic (Úrbez-Torres *et al.*, 2008), Sud-àfrica (van Niekerk *et al.*, 2004), Espanya (Luque *et al.*, 2009) i Estats Units (Úrbez-Torres *et al.*, 2009), ha estat

considerat un patogen feble a la regió del Sud d'Austràlia i Nova Gales del Sud (Pitt *et al.*, 2013a), Xina (Yan *et al.*, 2013), Iran (Mohammadi *et al.*, 2013), Nova Zelanda (Amponsah *et al.*, 2011) i Portugal (Phillips, 2002) i no es va considerar patogen a l'oest d'Austràlia (Taylor *et al.*, 2005). Recentment, un estudi d'Elena *et al*. (2015b), recollit en aquesta tesi doctoral (Capítol 4), ha posat de manifest el caràcter patogen de les 14 soques que es varen estudiar, però amb nivells de virulència variables entre sí.

La producció de metabòlits fitotòxics per part de les espècies de la família *Botryosphaeriaceae* ha estat extensament estudiada. Djoukeng *et al*. (2009), en un bioassaig realitzat amb *D. seriata*, van identificar quatre dihidroisocumarines: melleïna, *cis* i *trans* 4-hidroximel·leïna i 4,7-dihidroximelleïna. Martos *et al*. (2008) van informar sobre la producció de molècules hidrofíliques d'alt pes molecular amb propietats fitotòxiques en les espècies *Fusicoccum aesculi* Corda in Sturm, *D. seriata*, *Neofusicoccum parvum*, *N. luteum* i *S. viticola*. A més, *N. parvum* i *N. luteum* també produeixen fitotoxines lipofíliques de baix pes molecular, posant de manifest que aquests metabòlits podrien estar implicats en la patogenicitat d'aquestes espècies sobre la vinya. Andolfi *et al.* (2012) van observar que dues soques de *Neofusicoccum australe* produïen ciclobotryòxid, entre d'altres metabòlits, un compost fitotòxic força actiu en un bioassaig realitzat sobre fulles de vinya. Bénard-Gellon *et al.* (2014) van revelar que, sota les mateixes condicions experimentals, *N. parvum* va produir més proteïnes extracel·lulars i en major concentració que *D. seriata*. A més, Bénard-Gellon *et al.* (2014) van observar que aquestes substàncies induïen majors necrosis i activaven més gens de defensa. Abou-Mansour *et al.* (2015) van aïllar fins a 13 metabòlits diferents de 13 soques de *N. parvum*. Dues d'aquestes fitotoxines aïllades van ser trobades en estries necròtiques presents a la fusta de plantes que presentaven símptomes de decandiment causat per *Botryosphaeria*.

1.4. L'esca

L'esca és segurament una de les primeres malalties de la vinya que es van descriure, doncs està documentada des de les antigues civilitzacions grega i romana (Surico, 2000). La recerca sobre la seva etiologia va començar a finals del segle XIX a França (Ravaz, 1898), però no va ser fins a les dues darreres

dècades del segle XX quan es va intensificar el seu estudi, fruit d'un greu ressorgiment de la malaltia (Mugnai *et al.*, 1999). Malgrat ser una malaltia coneguda des de fa temps, el seu coneixement ha generat moltes controvèrsies i diferents hipòtesis per a explicar-la. Avui en dia encara persisteixen moltes opinions diverses al voltant de la identitat dels agents causals, del paper de cadascun d'ells dins de la malaltia i de l'expressió dels símptomes, entre d'altres qüestions. Les revisions més exhaustives que s'han publicat al respecte són les de Mugnai *et al*. (1999), Surico *et al*. (2006) i Berstch *et al*. (2013).

1.4.1. Agents causals

La identificació dels fongs causants de l'esca va ser, durant molt temps, motiu de debat entre els investigadors (Crous *et al.*, 1996; Larignon i Dubos, 1997; Crous i Gams, 2000; Graniti *et al.*, 2000). Una de les idees més esteses actualment és que en l'esca hi participa una successió de diferents agents patògens. En primer lloc, determinats fongs pioners causen la mort dels teixits vasculars, el que afavoreix la infecció posterior d'espècies secundàries (principalment, el basidiomicet *Fomitiporia mediterranea* M. Fisch), que causen la degradació i podridura de la fusta morta (Larignon i Dubos, 1997; Mugnai *et al.*, 1999; Graniti *et al.*, 2000; Surico, 2000). Dins dels fongs patògens pioners, es consideren fongs causants de l'esca les espècies *Phaeomoniella chlamydospora* i *Phaeoacremonium minimum*, així com 23 espècies més de *Phaeoacremonium*, associades també amb a la malaltia de Petri (Larignon i Dubos, 1997; Adalat *et al.*, 2000; Eskalen *et al.*, 2001; Sparapano *et al.*, 2001; Feliciano *et al.*, 2004; Santos *et al.*, 2005). Els basidiomicets *F. mediterranea* i, amb una freqüència i importància cada cop menor, *Stereum hirsutum* (Willd.:Fr.) S.F. Gray, són els responsables de la podridura tova de la fusta, el darrer estadi en la progressió de l'esca. No obstant això, *F. mediterranea* va ser identificat erròniament en un principi com *Phellinus igniarius* (L.:Fr.) a Itàlia, i com *Phellinus punctatus* (P. Karst.) Pilát. a França. Posteriorment se li va donar el nom de *Fomitiporia punctata* (Fr.) Murrill (Mugnai *et al.*, 1999) fins que, finalment, Fischer (2002) va actualitzar i validar el nom d'aquest fong tal com es coneix avui dia, *F. mediterranea.* Hi ha autors que sostenen que d'altres fongs patògens de la vinya, com per exemple *Eutypa lata* (Pers.: Fr.) Tul. & C. Tul., poden actuar igualment com a fongs pioners de l'esca, causant una necrosi de la fusta sobre la que posteriorment es desenvoluparà l'agent causal de la podridura tova

(Larignon i Dubos, 1997). Tot i això, en contra de la idea de que l'esca es causada per una successió de patògens, diversos autors han demostrat que les espècies de basidiomicets també poden actuar com a patògens primaris (Chiarappa, 1997; Sparapano *et al.*, 2000; Gatica *et al.*, 2004).

1.4.2. Simptomatologia

Des de fa temps, els símptomes d'aquesta malaltia s'havien diferenciat entre els símptomes interns, que afecten la fusta, i els externs, que es classificaven en dues classes diferents segons el decurs de la malaltia: lenta o crònica, que es caracteritza per un deteriorament lent del fullatge, i ràpida o apoplexia, que consisteix en una mort sobtada de la planta (Mugnai *et al.*, 1999). Actualment, i segons diferents autors (Surico, 2009; Bertsch *et al.*, 2013), el complex de l'esca consta de cinc síndromes que engloben tant els símptomes presents a la fusta com a les fulles, en planta adulta, com ara també la simptomatologia de la malaltia de Petri, en planta jove: 1) les estries necròtiques de color fosc a la fusta, 2) la malaltia de Petri, 3) l'esca jove (de l'anglès *young esca*), 4) la podridura blanca, i 5) l'esca pròpia (de l'anglès *esca proper*). Les tres primeres síndromes estan associades a *Phaeomoniella chlamydospora* i les espècies de *Phaeoacremonium*, principalment *Phaeoacremonium minimum*. Els símptomes comuns en tots tres casos consisteixen en diversos tipus de decoloració dels teixits llenyosos, dels quals, les estries necrosades en un o més vasos del xilema i les àrees necrosades envoltant la medul·la són els més característics (Figura 1.2a) (Surico *et al.*, 2006). Els símptomes externs propis de la malaltia de Petri són, com ja s'ha esmentat abans, una aturada del creixement, l'existència ocasional de clorosi en les fulles i la pèrdua de vigor global de la planta. Els símptomes externs en l'esca jove es caracteritzen per taques cloròtiques que apareixen entre els nervis i en les vores de les fulles, que s'expandeixen i acaben confluint, donant lloc a línies cloròtiques i necròtiques que deixen només una franja verda al voltant dels nervis foliars (patrons tigrats), que en el cas de les varietats de raïm blanc són de color groc verdós (Figura 1.2b) i en el cas de les varietats de raïm negre són de color vermellós (Surico *et al.*, 2008) (Figura 1.2c). A Estats Units també s'han descrit taques a l'epidermis del raïm, anomenades *black measles* (Mugnai *et al.*, 1999). Els símptomes foliars d'aquestes tres síndromes no estan associats directament amb els que s'observen a la fusta, doncs poden aparèixer anys

després de que la fusta hagi estat infectada. A més, la intensitat dels símptomes foliars pot variar d'un any per l'altre a la mateixa planta, manifestant-se de forma irregular o erràtica (Mugnai *et al.*, 1999; Surico *et al.*, 2008).

La síndrome de la podridura blanca es caracteritza per ser seca i esponjosa, de color groc clar (Figura 1.2d). Aquesta podridura pot localitzar-se tant en el tronc principal com en els braços, però generalment es troba associada a les ferides de poda o a d'altres ferides causades mecànicament (Mugnai *et al.*, 1999; Surico *et al.*, 2006). D'aquesta podridura esponjosa s'aïlla principalment el fong *Fomitiporia mediterranea* (Larignon i Dubos, 1997; Mugnai *et al.*, 1999; Graniti *et al.*, 2000), tot i que en assaigs de patogenicitat, només Sparapano et al. (2001) han aconseguit reproduir aquest símptoma. La cinquena síndrome, l'esca pròpia, té lloc quan les síndromes de l'esca jove i la podridura blanca coincideixen en una mateixa planta, de forma que en talls transversals de les parts afectades es pot veure la podridura blanca, normalment rodejada d'una línia fina fosca, i puntejades de color fosc que es veuen com a estries necròtiques en talls longitudinals (Figura 1.2e). Altres tipus de símptomes observats en l'esca pròpia són unes àrees de color marró-rosat, ubicades principalment al centre del tronc o en el marge de les àrees necrosades (Figura 1.2e) i àrees fosques, que varien en forma i textura, i que es troben normalment en parts que connecten amb ferides importants (Figura 1.2e) (Mugnai *et al.*, 1999; Luque *et al.*, 2009)

Un símptoma prou aparent de les vinyes afectades d'esca jove i d'esca pròpia és l'apoplexia, que consisteix en una mort sobtada de la planta en els moments de màxima calor a l'estiu (Surico *et al.*, 2006). Els fruits i les fulles es deshidraten de manera sobtada i s'acaba produint la mort de tota la planta en pocs dies (Figura 1.2f) (Beltrán *et al.*, 2004). Aquest fenomen té lloc normalment a mitjans d'estiu, quan hi ha un episodi de pluja després de dies secs i calorosos (Mugnai *et al.*, 1999). L'apoplexia es pot donar sense que el cep hagi manifestat abans cap tipus de símptoma. D'una altra banda, Luque *et al.* (2009) van trobar evidències que apuntaven a que l'apoplexia podria no estar relacionada necessàriament amb els danys de l'esca, en observar plantes afectades d'apoplexia en les que sols hi havia lesions causades per xancres de *Botryosphaeria*, de manera que van hipotetitzar que aquest fenomen podria

estar més relacionat amb el dany físic i la manca de funcionalitat de la fusta afectada que no pas amb el dany provocat pels agents causals d'esca.

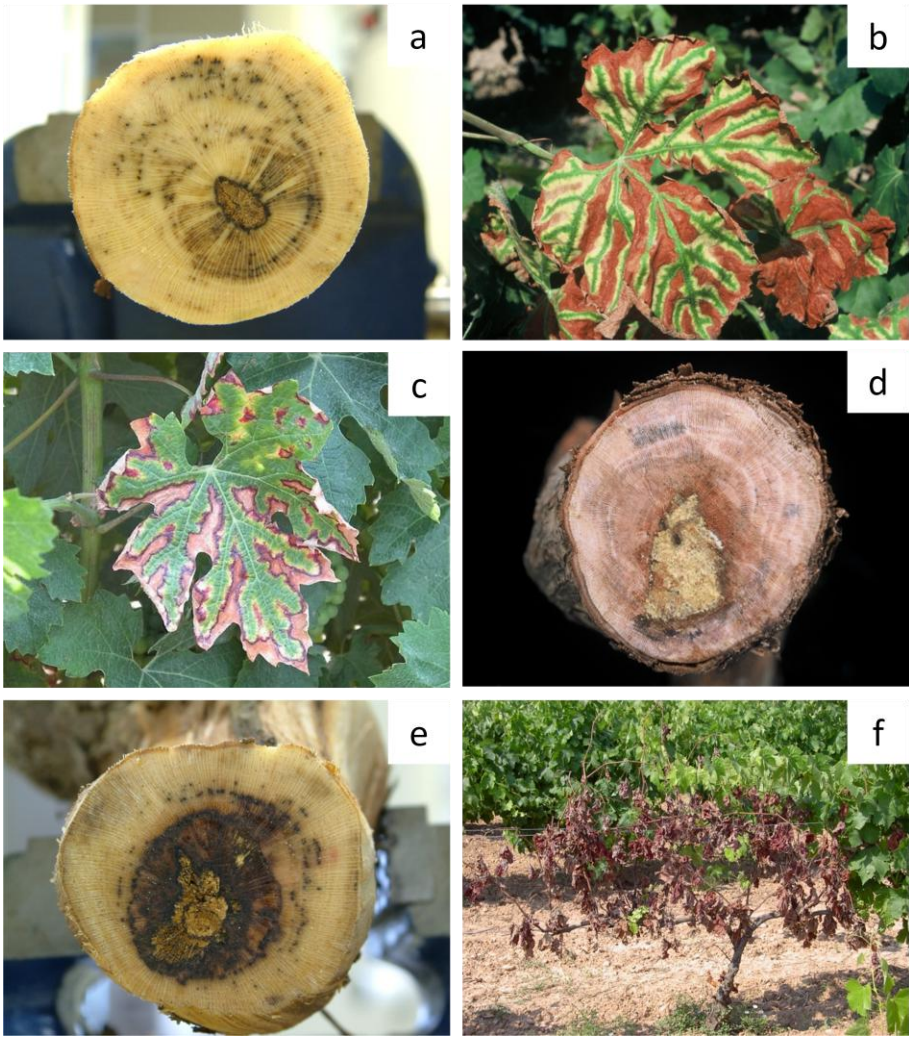

Figura 1.2. Simptomatologia de les principals síndromes que composen el complex de l'esca: estries necròtiques que es veuen com punts foscos en talls transversals (a); coloracions groguenques en el limbe foliar de varietats de raïm blanc (b) o vermelloses, en les varietats de raïm negre (c), que deriven en la necrosi de la fulla; podridura esponjosa de color groguenc (d i e), envoltada per una línia fosca (e); àrees de color marró-rosat i àrees fosques barrejades amb la resta de símptomes (e); i símptomes propis d'apoplexia o mort sobtada de tota la planta (f).

Alguns canvis en la terminologia de l'esca han estat proposats recentment, segons els quals es respecta la terminologia de la malaltia de Petri, però l'esca jove passa a anomenar-se malaltia de la fulla tigrada de la vinya (*grapevine leaf stripe disease*) i la podridura blanca i l'esca pròpia tornarien a englobar-se dins d'un únic terme, esca, pròpiament dit (Surico, 2009). Tenint en compte que la síndrome de les estries necròtiques a la fusta, la malaltia de Petri i la malaltia de la fulla tigrada de la vinya tenen els mateixos agents causals, Surico (2009) ha proposat que aquestes tres síndromes quedin agrupades en un únic nom, la *feotraqueomicosi* de la vinya (*grapevine phaeotracheomycosis complex*). Fins ara, aquesta proposta no sembla haver reeixit entre els especialistes, de forma que els noms i conceptes associats a les diferents síndromes del complex de l'esca encara resten pendents d'una consolidació definitiva.

La reproducció total o parcial dels símptomes de l'esca en condicions artificials, això és, després de la inoculació artificial dels fongs suposadament associats a l'esca, s'ha dut a terme amb molt pocs casos d'èxit. Això ha dificultat en cert grau els estudis de patogenicitat dels fongs associats a la malaltia. Sparapano *et al.* (2001) van reproduir els símptomes de la malaltia quan van inocular els tres fongs principals (*P. chlamydospora*, *P. minimum* i *F. mediterranea*, en aquell moment classificada com a *Fomitiporia punctata* (P. Karst.) Murrill) en vinyes de varietat 'Matilde' i 'Itàlia', en condicions de camp. Úrbez-Torres *et al.* (2014) van reproduir els símptomes vasculars i foliars propis de l'esca en plantes mantingudes en test quan van inocular *P. chlamydospora* i diverses espècies de *Phaeoacremonium*.

1.4.3. Biologia i epidemiologia

En termes generals, la incidència i severitat dels símptomes de l'esca augmenten amb l'edat de la planta. Degut a això, en el passat es creia que els nivells elevats d'infecció per esca només s'assolien en plantes de 25 a 35 anys, aproximadament. Quan s'observa la simptomatologia pròpia de l'esca en plantes joves, de menys de 8 anys, es creu que aquestes plantes han estat infectades en els seus estadis inicials de formació i, per tant, poden assolir més aviat els alts nivells d'afectació (Surico *et al.*, 2006). A més, la incidència d'aquesta malaltia en plantacions cada cop més joves ha estat associada també

a la producció de material ja infectat als vivers (Gramaje i Armengol, 2011). En el cas de l'apoplexia, en canvi, està força acceptat que es tracta d'un fenomen propi de la planta adulta i es dóna freqüentment en les estacions més caloroses de l'any (Surico *et al.*, 2006). Les plantes adultes que ja han mostrat símptomes foliars d'esca durant molts anys, tenen proporcionalment una part més gran del sistema vascular afectat, de manera que són més susceptibles al fenomen de l'apoplexia quan hi ha manca d'aigua.

La manifestació dels símptomes foliars de l'esca, de forma discontínua i aparentment a l'atzar en el decurs dels anys, segueix sense estar explicada de forma clara avui dia. Mugnai *et al*. (1999) van suggerir que aquests símptomes estan causats per substàncies que s'originen en les parts afectades de la fusta i que es transloquen a les fulles mitjançant la saba. Aquestes substàncies podrien produir-se, bé com una reacció de la planta a la invasió patògena, o bé pels propis fongs, tractant-se en aquest darrer cas de micotoxines (Sparapano *et al.*, 1998; Evidente *et al.*, 2000; Tabacchi *et al.*, 2000). Escilatona i isosclerona, dues naftalenones policètides, i el pul·lulà, un polisacàrid de maltotriosa, han estat detectats en cultius de *Phaeomoniella chlamydospora* i *Phaeoacremonium minimum* i s'ha vist que presenten fitotoxicitat en fulles de vinya (Bruno i Sparapano, 2006a; Bruno i Sparapano, 2006b; Bruno *et al.*, 2007). Per aquesta raó, es creu que aquests metabòlits podrien intervenir en el desenvolupament de la malaltia, però el seu mode d'acció no ha estat determinat amb exactitud fins ara (Andolfi *et al.*, 2011). Lecomte *et al*. (2005b) van postular que els símptomes externs podrien ser provocats en part per la cavitació del xilema, de forma que l'aparició d'aire en els vasos comprometria la seva funcionalitat. Aquesta teoria s'ha reservat com la possible causa de l'apoplexia, on tot el sistema hidràulic de la planta es col·lapsa, ja que sembla poc probable que la cavitació, per sí mateixa, pugui explicar la particularitat de la simptomatologia foliar de la malaltia (Surico *et al.*, 2006). Com ja s'ha dit anteriorment, s'ha vist que els símptomes foliars són afavorits per episodis de pluja durant la primavera i l'estiu (Surico, 2000; Marchi *et al.*, 2005). Tot i això, no es coneix quin és el mecanisme que fa que la pluja desencadeni l'expressió dels símptomes foliars de l'esca.

La via principal d'entrada d'aquesta i d'altres malalties de la fusta a la planta són les ferides de poda, així com d'altres ferides fetes a la planta durant

la fase de conducció del tronc, la poda en verd de primavera o fins i tot durant la realització de l'empelt (Surico *et al.*, 2006). També s'ha vist que una altra forma de transmissió possible dels agents patògens seria a través de les eines de poda, un fet confirmat per l'estudi d'Agustí-Brisach *et al*. (2015). Diversos estudis realitzats a Califòrnia, França, Austràlia i Sud-àfrica han permès saber que els propàguls de *P. chlamydospora* i de *Phaeoacremonium* spp. es formen en cossos fructífers localitzats en la superfícies dels troncs, dels sarments, així com en circells vells que queden en les estructures de suport i guia de les vinyes (Eskalen *et al.*, 2004). A Califòrnia es va observar que les espores de *P. chlamydospora* es dispersaven en períodes plujosos dels mesos d'octubre i novembre (Gubler *et al.*, 2004). A França, en canvi, les espores de *P. chlamydospora* es capturaven tot l'any, però sempre i quan hi haguessin episodis de pluja (Larignon i Dubos, 2000). Pel que fa a *P. minimum*, s'ha vist que a França la dispersió de les seves espores es pot començar a donar a principis de març, però que aquesta és més freqüent entre mitjans de maig i mitjans de juny (Larignon i Dubos, 2000). Els basidiomes de *Fomitiporia mediterranea* es formen quasi exclusivament en vinyes molt velles i afectades pel fong. També s'han trobat en altres espècies llenyoses a banda de la vinya, com ara *Olea europaea* L.*, Corylus avellana* L.*, Acer negundo* L.*, Lagerstroemia indica* L.*, Actinidia chinensis* Planch. (Fischer, 2002) i algunes espècies de *Citrus* L. (Kalomira *et al.*, 2006). Per aquesta raó, es creu que l'inòcul de *F. mediterranea* pot procedir tant de vinyes velles pròximes com d'altres espècies llenyoses properes, tot i que aquesta ultima opció requereix d'estudis més exhaustius per a que es pugui arribar a confirmar que les soques de *F. mediterranea* patogèniques en altres hostes també ho són en la vinya.

1.5. L'eutipiosi

El decandiment causat per *Eutypa* o eutipiosi, una malaltia coneguda en anglès amb els noms de *dying arm disease* o *Eutypa dieback* (Lecomte i Bailey, 2011), és una de les malalties de la fusta de la vinya més devastadores a totes les grans regions vitivinícoles del món (Carter, 1991; Munkvold *et al.*, 1994; Munkvold i Marois, 1995; Creaser i Wicks, 2000). Els danys més importants que causa aquesta malaltia corresponen a fortes reduccions en el rendiment i la longevitat de la vinya, a més de malmetre la qualitat del raïm, ja que en els ceps afectats per eutipiosi pot observar-se ocasionalment una maduració

descompensada del fruit que afecta l'aroma i les capacitats enològiques del raïm (Wicks i Davies, 1999).

1.5.1. Agents causals

En les darreres dècades, *Eutypa lata* (=*E. armeniacae* Hansf. & M.V. Carter) ha estat considerat l'agent causal de l'eutipiosi de la vinya (Carter, 1991; Péros, 1995; Dubos, 1996; Lecomte *et al.*, 2005a). La seva forma asexual correspon a *Libertella blepharis* A.L. Smith. *Eutypa lata* és un fong cosmopolita i plurívor, que afecta al voltant de 90 espècies llenyoses -pertanyents a 28 famílies- entre les que destaquen els arbres fruiters següents: l'albercoquer (*Prunus armeniaca* L.), la prunera (*Prunus domestica* L.), el cirerer (*Prunus avium* L.), l'ametller (*Prunus amygdalus* Batsch), el pistatxer (*Pistacia vera* L.), l'olivera (*Olea europaea*), el llimoner (*Citrus ×limon* (L.) Burm.f.), la pomera (*Malus domestica* Borkh.), el codonyer (*Cydonia oblonga* Mill.), la perera (*Pyrus communis* L.) i la noguera (*Juglans regia* L.), així com el riber negre (*Ribes nigrum* L.), entre d'altres (Bolay i Carter, 1985; Carter, 1991). Aquest fong va ser descrit per primera vegada sobre albercoquer a Austràlia, detectat després a Estats Units i, finalment, a Europa (Carter, 1991). En els darrers anys, però, altres espècies de la família *Diatrypaceae* han estat observades en xancres de vinyes afectades, com ara *Diatrype stigma* (Hoffm.:Fr.) Fr., *Diatrype whitmanensis* J.D. Rogers & Glawe, *Cryptosphaeria pullmanensis* Glawe, *Cryptovalsa ampelina* (Nitschke) Fuckel (Trouillas i Gubler, 2010; Trouillas *et al.*, 2010) i *Eutypella vitis* (Schwein.) Ellis & Everh. (=*E. aequilinearis* (Schwein.) Starbäck) (Jordan i Schilder, 2007). Recentment s'ha descrit la presència d'altres espècies de diatripàcies a Austràlia, en concret, *Eutypella microtheca* Trouillas, W.M. Pitt & Gubler, *E. citricola* Speg., *E. leptoplaca* (Mont.) Rappaz, *Cryptovalsa rabenhorstii* (Nitschke) Sacc., *Cryptosphaeria* sp. i *Diatrypella vulgaris* Trouillas, W.M. Pitt & Gubler (Trouillas *et al.*, 2011) i, també a Xile, *E. leprosa* (Pers.) Berl. (Díaz *et al.*, 2011). Algunes d'elles també han estat trobades a Espanya, entre les quals s'ha descrit *Anthostoma decipiens* (DC.) Nitschke per primera vegada en vinya (Luque *et al.*, 2012). Malgrat el nombre creixent d'espècies de *Diatrypaceae* que han estat detectades darrerament sobre la vinya, *E. lata* continua essent considerat l'agent causal de l'eutipiosi més estès arreu del món i el més important.

1.5.2. Simptomatologia

Els símptomes visibles de l'eutipiosi es manifesten de forma preferent a la primavera, durant la represa del creixement vegetatiu del cep. Els entrenusos de ceps afectats són més curts i les fulles presenten una mida més petita, estan deformades i tenen el marge recorbat i amb aspecte cloròtic (Figures 1.3a, 1.3b i 1.3c). Algunes vegades, les fulles presenten petites taques necròtiques que, amb el temps, poden fer-se coalescents (Figures 1.3d i 1.3e). Les inflorescències s'assequen abans d'obrir-se i els fruits que aconsegueixen prosperar en un brot infectat són més petits i sovint no arriben a madurar (Moller *et al.*, 1974). Els símptomes es van accentuant amb el pas dels anys; els sarments deixen de rebrotar i s'observa la mort progressiva dels braços. En última instància, la planta acaba morint. El tall transversal del braç o tronc que desenvolupa aquests símptomes sol presentar una necrosi en forma de V, similar a l'observada en els xancres causats per espècies de *Botryosphaeriaceae* (Figures 1.3f i 1.3g) (Péros i Berger, 1994; Chapuis *et al.*, 1998).

La degradació de la fusta es caracteritza per la mort de les cèl·lules associades als vasos (Rudelle *et al.*, 2005). Diverses anàlisis de la fusta infectada amb *Eutypa lata* han demostrat que aquest fong disminueix les reserves de glucans del parènquima i del xilema (Rudelle *et al.*, 2005; Rolshausen *et al.*, 2008; Bertsch *et al.*, 2013). Alguns estudis anatòmics en fulles de plantes infectades per *E. lata* també han revelat canvis en la estructura tissular (Philippe *et al.*, 1992; Valtaud, 2007). Aquesta infecció afecta la fotosíntesi, ja que disminueix el contingut d'aigua en la fulla i provoca una acumulació d'àcid abscísic (Koussa *et al.*, 2002). Altres estudis sobre canvis en els processos fisiològics de la planta semblen indicar que la disminució en el creixement dels nous sarments i els símptomes foliars són causats per l'acció de toxines segregades per *E. lata* (Deswarte *et al.*, 1996; Octave *et al.*, 2006). L'eutipina (1,4-hidroxi-3-(3-metilbut-3-ene-1-inil benzaldehid) ha estat identificada com la principal fitotoxina produïda per aquest fong (Tey-Rulh *et al.*, 1991). Altres metabòlits relacionats estructuralment amb l'eutipina (eutipinol, siccaïna, àcid eutipínic i els seus productes ciclats, cromanones epoxidades i eutipòxid B) també han estat detectats *in vitro* a partir de cultius d'*E. lata* i d'altres espècies del mateix gènere (Renaud *et al.*, 1989; Jiménez-Teja *et al.*, 2006). Cadascun

d'aquests compostos ha estat caracteritzat segons la seva toxicitat i les seves dianes en les estructures cel·lulars de l'hoste (Molyneux *et al.*, 2002).

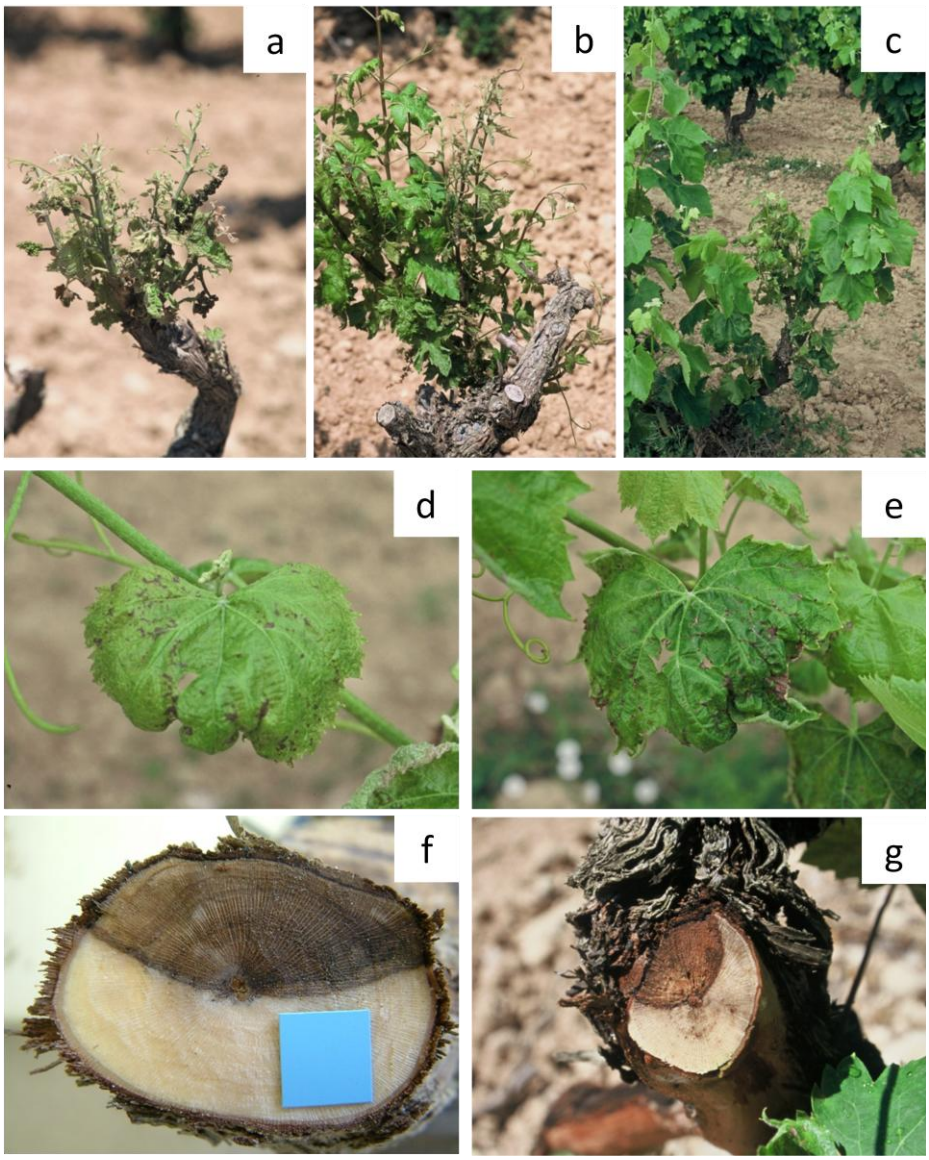

Figura 1.3. Simptomatologia pròpia de l'eutipiosi. Símptomes externs: entrenusos curts i fulles de mida petita, deformes i cloròtiques (a, b i c), que poden presentar taques necròtiques (d i e). Símptomes interns: necrosis sectorials en forma de V observades en talls transversals de tronc i braç (f i g).

1.5.3. Biologia i epidemiologia

Eutypa lata es distribueix per totes les regions vitivinícoles del món, tant en zones d'hiverns durs, com ara l'Europa central o l'est dels Estats Units, com en zones més temperades, com ara la costa de Califòrnia, el sud-est d'Austràlia, el sud de França, i diverses regions d'Espanya, Itàlia o Sud-àfrica. Tot i això, aquesta espècie està limitada per la incidència de les pluges, de manera que és molt abundant quan la mitjana anual de precipitacions excedeix els 600 mm, però és més rar trobar-la en regions on no s'excedeixen els 250 mm (Carter, 1991; Munkvold *et al.*, 1994; Creaser i Wicks, 2000).

Es creu que la forma preponderant de reproducció d'*E. lata* és la reproducció sexual, segons s'ha confirmat mitjançant un estudi de la variabilitat genètica del fong (Péros *et al.*, 1997). Les ascòspores són les responsables de propagar la malaltia, ja que es creu que els conidis de *Libertella blepharis* no són infectius (Péros *et al.*, 1997). Els peritecis es desenvolupen en estromes formats sobre els xancres de les vinyes afectades. Tot i que els peritecis maduren a l'inici de la primavera, les ascòspores s'alliberen durant tot l'any (Pearson, 1980; Trese *et al.*, 1980), sempre i quan s'enregistrin pluges que excedeixin els 0,5 mm (Ramos *et al.*, 1975; Petzoldt *et al.*, 1983; Païllassa *et al.*, 1992). L'alliberament de les ascòspores comença 2-3 h després de l'inici de la pluja i acaba unes 24 h després de la finalització de la mateixa (Pearson, 1980). La infecció de la planta té lloc per les ferides de la poda, susceptibles durant la fase de latència hivernal de la vinya (Bertsch *et al.*, 2013). Les espores infecten els ceps podats durant l'hivern, degut a una major freqüència de precipitacions en aquesta època (Ramos *et al.*, 1975; Lecomte i Bailey, 2011). Tot i això, a França també s'ha vist que la infecció es pot donar a la primavera, a través de les ferides ocasionades després d'una poda en verd, quan s'eliminen sarments laterals o secundaris (Lecomte i Bailey, 2011). Els primers símptomes de la malaltia comencen a fer-se evidents al cap de 3 o 4 anys de la infecció (Dubos, 2002).

1.6. El control de les malalties de la fusta de la vinya

L'augment de la incidència de les malalties de la fusta en les darreres dècades està relacionat amb diversos factors. Pel que fa a la planta jove, alguns autors opinen que una disminució en la cura del procés de propagació del material vegetal als vivers ha comportat una reducció en la seva qualitat

sanitària, que es tradueix en índexs d'infecció patògena considerables en el material emprat en les noves plantacions (Chiarappa, 2000; Graniti *et al.*, 2000; Gramaje i Armengol, 2011). Això fa que, tot i que aquestes vinyes infectades puguin arribar a establir-se en la nova plantació, tenen una elevada probabilitat de presentar símptomes de decandiment un cop arriben a la maduresa (Waite, 2006). És per això que molts investigadors, en un esforç per a reduir l'impacte en les plantacions noves de vinya, han centrat els seus estudis en la detecció, la identificació i el control d'aquests fongs durant el procés de propagació als vivers (Gramaje i Armengol, 2011). El tractament del material vegetal de propagació amb aigua calenta representa, en els darrers temps, una opció innovadora i molt prometedora per a millorar la qualitat sanitària de les plantes produïdes als vivers (Gramaje i Armengol, 2011). Se'n parlarà amb més detall en un apartat posterior d'aquesta secció (1.6.3. *Mètodes de sanejament*) i, a més, s'estudiarà el cas particular de la supervivència i el creixement de diverses espècies de *Botryosphaeriaceae* després del tractament de termoteràpia amb aigua calenta (TAC) (Capítol 9; Elena *et al.*, 2015a, *en premsa*).

Pel que fa a la planta adulta, es creu que la tecnificació del cultiu ocorreguda en les darreres dècades, el que inclou noves tècniques en la conducció de la planta, la pràctica de la poda, la intensificació del cultiu, entre d'altres, han augmentat la susceptibilitat general de la planta als fongs patògens (Hidalgo, 2002; Dal *et al.*, 2008). D'entre les qüestions anteriors, la manca de protecció efectiva de les ferides de poda és probablement la més important, atès al paper de les ferides de poda com a vies d'entrada de les infeccions patògenes (Larignon i Dubos, 2000; Serra *et al.*, 2008; Úrbez-Torres i Gubler, 2011; van Niekerk *et al.*, 2011). L'arsenit sòdic, un pesticida que s'havia mostrat eficaç en el control d'aquestes malalties, va ser prohibit en els primers anys d'aquest segle pels seus efectes cancerígens en els humans, així com per la seva toxicitat elevada en l'ambient (Larignon *et al.*, 2008). Aquest fet va fer pensar que la retirada d'aquest producte havia comportat l'increment en la incidència de les malalties (Larignon *et al.*, 2009). En els darrers anys, s'han estudiat diversos mètodes alternatius a l'arsenit sòdic per tal d'augmentar la protecció de les ferides de poda. S'ha treballat en el desenvolupament de matèries actives de síntesi química i d'origen biològic (agents de control biològic i productes naturals). Fins i tot, s'ha avaluat l'eficàcia de mètodes de sanejament, com ara la poda terapèutica, i d'altres pràctiques proactives en el maneig del cultiu per a

reduir l'impacte de les malalties. Com a eina per poder dur a terme aquest tipus d'avaluacions, en el Capítol 5 s'ha determinat el rang de concentració d'espores òptim per realitzar inoculacions artificials amb patògens (Elena *et al.*, 2015c, *en premsa*).

Entre els professionals del sector vitivinícola i els investigadors d'aquestes malalties, cada cop més es reforça l'opinió de que l'efectivitat d'un sol mètode de control és i serà sempre limitada, i que en el futur caldrà la integració de diferents mesures en un protocol de bones pràctiques, dirigit a mitigar l'impacte d'aquestes malalties.

1.6.1. Control químic

El principal objectiu de les mesures basades en el control químic és la protecció de les ferides de poda mitjançant l'ús de fungicides; normalment, s'utilitzen productes químics que poden contenir una o més matèries actives. Aquests productes s'apliquen a les ferides de poda en forma de pastes o substàncies líquides formulades, tot i que també es poden injectar al sòl o al tronc (Calzarano *et al.*, 2004; Sosnowski *et al.*, 2004; Rolshausen i Gubler, 2005). Les formulacions líquides s'apliquen normalment per polvorització i són el mètode més pràctic, però poden ser lixiviades fàcilment per la pluja. Aplicar els productes en forma de pastes i amb l'ajut d'un pinzell és el mètode més efectiu, però a la pràctica això requereix molt de temps i resulta molt costós (Di Marco *et al.*, 2000; Rolshausen *et al.*, 2010a). Les aplicacions mitjançant injeccions en sòl o en el tronc, reconegudes en el *Registro de Productos Fitosanitarios* (MAGRAMA, 2015b) per a determinades matèries (per exemple, el ciproconazol, amb núm. de registre 18.736) semblen també poc aplicables en la pràctica.

L'eficàcia dels fungicides és molt variable i depèn bàsicament de la susceptibilitat del patogen a la pròpia substància. Bester *et al.* (2007) van demostrar l'eficàcia dels productes tebuconazol, flusilazol, benomil i prochloraz per a reduir la viabilitat de diverses espècies de *Botryosphaeriaceae* en experiments *in vitro* i amb sarments de vinya mantinguts en hidroponia. Gramaje i Armengol (2011) van observar que els compostos carbendazima, tebuconazol, protioconazol+tebuconazol i fluazinam inhibien del creixement miceliar d'*Eutypa lata* i d'altres espècies de la família *Diatrypaceae*. Amponsah *et al.* (2012) van provar 16 fungicides per a determinar el seu efecte inhibidor en

el creixement miceliar i la germinació dels conidis de *Neofusicoccum australe*, *Neofusicoccum luteum* i *Diplodia mutila*; carbendazima, procimidona, iprodiona, flusilazol i mancozeb van ser els més efectius en tots els casos, però el flusilazol va ser el que va oferir els millors resultats, en disminuir el reaïllament dels patògens en un assaig en plantes de la varietat 'Chardonnay' de 12 anys. En el camp, el tractament amb metil-tiofanat és un dels que ofereix millors resultats contra els patògens *Botryosphaeria dothidea*, *Diplodia seriata*, *Spencermartinsia viticola*, *E. lata*, *Lasiodiplodia theobromae*, *Phaeoacremonium parasiticum*, *Pa chlamydospora*, *Pleurostomophora richardsiae* i *Phaeoacremonium minimum* (Rolshausen *et al.*, 2010a). Altres productes també han mostrat resultats positius en el control d'aquests patògens, encara que l'èxit d'aquests productes depèn de factors com ara la forma i el número d'aplicacions, la persistència del producte en el camp i les espècies objecte de control (Di Marco i Osti, 2005; Sosnowski *et al.*, 2005; Fourie i Halleen, 2006; Pitt *et al.*, 2010; Rolshausen *et al.*, 2010a). Malgrat el considerable volum de dades referents a l'eficàcia dels diferents productes, cal tenir en compte que avui dia alguns d'ells ja no estan admesos en les legislacions de molts països (per exemple, carbendazima i benomil). En el cas concret d'Espanya, en l'actualitat sols es reconeix l'ús específic en vinya de la matèria activa ciproconazol (MAGRAMA, 2015b).

1.6.2. Control biològic

Diversos estudis publicats en la darrera dècada indiquen que determinats tractaments basats en l'ús d'agents de control biològic (ACB), en especial amb fongs del gènere *Trichoderma* Pers., han estat efectius en assaigs *in vitro* i vivers, en reduir la incidència dels patògens associats a diferents malalties de la fusta de la vinya (Hunt *et al.*, 2001; Di Marco *et al.*, 2004; John *et al.*, 2004). Vinevax™, (Agrimm Technologies Ltd.), un producte formulat amb cinc soques de *Trichoderma atroviride* P. Karst., s'ha vist que és efectiu contra l'eutipiosi (John *et al.*, 2001) i que també redueix la incidència de la colonització de *Diplodia seriata* (Pitt *et al.*, 2010), així com de *Phaeomoniella chlamydospora*. Recentment, Esquive® WP (Agrauxine), ha estat enregistrat a França pel tractament de les ferides de poda i el control de les malalties de la fusta. Altres ACB, com ara *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 (Ferreira *et al.*, 1991; Halleen *et al.*, 2010), *Fusarium lateritium* Nees (Munkvold i Marois, 1993), *Pantoea agglomerans* (Ewing and Fife 1972) Gavini *et al*. 1989 (=*Erwinia*

herbicola) (Schmidt *et al.*, 2001), *Cladosporium herbarum* (Pers.) Link (Rolshausen i Gubler, 2005), *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud (Munkvold i Marois, 1993) i *Rhodotorula rubra* (Schimon) F.C. Harrison (Munkvold i Marois, 1993), així com també els anomenats 'productes naturals', com ara el quitosà i la cisteïna, han mostrat una certa eficàcia contra aquestes malalties (Octave *et al.*, 2005; Nascimento *et al.*, 2007). L'efectivitat d'aquests ACB està molt lligada a la capacitat dels microorganismes per a colonitzar les ferides de poda (John *et al.*, 2008).

1.6.3. Mètodes de sanejament

Els diferents mètodes de sanejament que s'han anat desenvolupant en els darrers anys, conjuntament amb els protectors de les ferides de poda, constitueixen l'opció més prometedora en el control de les malalties de la fusta de la vinya. Els mètodes de sanejament poden agrupar-se bàsicament en dos àmbits. En l'àmbit del viver, estarien dirigits a aconseguir un material de propagació amb un menor nivell d'infecció dels agents patògens. En l'àmbit de la vinya en explotació, el sanejament es basaria en reduir el risc d'infecció de les ferides de poda, combinant diferents tècniques que s'expliquen més endavant.

La qualitat del material de plantació, la desinfecció del material de propagació en els vivers i l'ús del tractament de termoteràpia amb agua calenta (TAC) són crucials per a obtenir plantes comercialitzables de bona qualitat i amb nivells baixos de patògens (Gramaje i Armengol, 2011). L'aplicació del TAC durant el procés viverístic, en les fases finals de preparació de la planta, és una pràctica que s'ha incorporat en alguns vivers d'arreu del món. Normalment, aquesta tècnica s'aplica submergint el material en un bany d'aigua calenta a 50 °C, durant uns 30 minuts. En determinats casos ja s'ha vist que cal afinar-la, és a dir, calibrar l'eficàcia de la combinació de diferents temperatures i temps d'exposició per a obtenir millors nivells d'efectivitat (Gramaje i Armengol, 2011). Segons diversos autors (Waite i Morton, 2007; Gramaje *et al.*, 2009b), l'efectivitat de la tècnica depèn de la susceptibilitat particular de les diferents varietats de *Vitis vinifera* i de les espècies patògenes a la temperatura.

La doble poda (de l'anglès *double pruning*; vegi's Weber *et al.*, 2007) o la l'acció combinada de prepoda i poda definitiva, com es coneix en el nostre país, ajuda als agricultors a que la poda final es faci més ràpid i, a més, redueixi la

incidència de les malalties (Weber *et al.*, 2007). Però aquesta darrera opinió ha estat molt discutida, perquè la prepoda no evita les infeccions que es puguin produir després de la segona poda. De fet, Luque *et al*. (2014), en un treball reproduït en Capítol 7 d'aquesta tesi, van observar un major nombre d'infeccions després de la segona poda, en comparació a la prepoda. A més, en el Capítol 8, en un estudi sobre la susceptibilitat de les ferides a la infecció per *Diplodia seriata* i *Phaeomoniella chlamydospora*, es va observar que les ferides de poda eren més susceptibles a la infecció per *D. seriata* després d'una poda tardana.

Retirar les parts mortes o infectades de la planta, així com les restes de la poda, són mesures que s'han recomanat de forma generalitzada per a ajudar a reduir les fonts d'inòcul patogen a la vinya (Carter, 1991; Di Marco *et al.*, 2000). Sosnowski *et al.* (2011b) van avaluar l'efecte que tenia la poda terapèutica (consistent en tallar i retirar els braços i troncs infectats) per controlar l'eutipiosi en vinyes de la varietat 'Shiraz', a Austràlia. Els autors van demostrar realment que aquesta pràctica reduïa la incidència i la severitat dels símptomes foliars; tot i això, l'eficàcia de la mesura va variar depenent del grau d'infecció del qual partien les plantes.

Capítol 2

Objectius de la tesi

L'objectiu principal d'aquesta tesi és el d'ampliar el coneixement de la biologia i l'epidemiologia d'alguns dels fongs patògens de la fusta de la vinya més importants, respecte als processos de dispersió, infecció i patogènesi en la planta adulta. A més, es pretén que els coneixements adquirits puguin ser aplicats en el desenvolupament de mesures de control innovadores en els àmbits de la vinya i del viver. Per a assolir aquest objectiu general s'han plantejat els següents objectius específics:

1. Caracteritzar el fong *Diplodia seriata* des del punt de vista molecular, morfològic i patogènic, així com de la seva compatibilitat vegetativa, amb la finalitat de determinar si la seva variabilitat intraespecífica aporta noves evidències sobre el paper d'aquest fong en el conjunt de les malalties de la fusta de la vinya.

2. Avaluar les infeccions de les ferides de poda de la vinya obtingudes a partir d'inoculacions amb suspensions d'espores dels fongs *Diplodia seriata*, *Eutypa lata* i *Phaeomoniella chlamydospora*, per a millorar les tècniques de treball basades en inoculacions artificials d'aquests patògens.

3. Fer un seguiment de l'alliberament de conidis de *Diplodia seriata* en restes de poda de la vinya abandonades al camp, per a confirmar així el rol d'aquestes restes com a font d'inòcul potencial per a noves infeccions.

4. Identificar la micoflora patògena que infecta, de forma natural, les ferides de poda de la vinya en dues èpoques diferents -després d'una poda primerenca o bé tardana-, i avaluar en ambdós casos la influència de les condicions meteorològiques sobre el grau d'infecció.

5. Determinar el període durant el qual les ferides de poda de la vinya resten susceptibles a la infecció dels fongs *Diplodia seriata* i *Phaeomoniella chlamydospora*.

6. Estudiar la colonització patògena de sarments de vinya podats a diferents llargades i inoculats amb els fongs *Diplodia seriata* i

Phaeomoniella chlamydospora, amb l'objectiu de determinar la influencia del procés de cicatrització de les ferides de poda sobre la colonització patògena.

7. Estudiar l'efecte del tractament de termoteràpia amb aigua calenta (TAC) sobre la viabilitat de vuit espècies fúngiques associades a la malaltia del decandiment causat per *Botryosphaeria*, en condicions *in vitro* i *in planta*, amb la finalitat de determinar la potencialitat d'aquesta tècnica com a mètode de control de les malalties de la fusta en el viver.

Capítol 3

Informe del director de la tesi

El sotasignant, JORDI LUQUE i FONT, amb DNI 77110528-S, Doctor en Ciències Biològiques i investigador del subprograma de Patologia Vegeta, adscrit al Programa de Protecció Vegetal Sostenible de l'Institut de Recerca i Tecnologia Agroalimentàries (IRTA)

INFORMA

Que la doctoranda, Sra. GEORGINA ELENA JIMÉNEZ, ha participat en les activitats i tasques dels articles que componen aquesta tesi segons es detalla a continuació. S'indica el capítol de la tesi al qual fa referència el treball objecte d'aquest informe i se'n detallen les activitats.

Capítol 4. Intraspecific variation in *Diplodia seriata* isolates occurring on grapevines in Spain. Article publicat a *Plant Pathology 64, 680-689 (2015).*

Impact Factor (2014): 2.121

ISI Journal Citation Reports© Ranking: 2014: 17/81 (Agronomy) 1Q; 63/200 (Plant Sciences) 2Q.

La doctoranda ha participat activament en el desenvolupament de l'estratègia de caracterització molecular, fenotípica i patogènica de les soques de *D. seriata*, així com en l'execució directa de totes les tasques experimentals. Ha recopilat les dades i les ha elaborat, resumit i analitzat estadísticament quan ha estat necessari. Ha participat en la redacció i correcció de l'article, conjuntament amb la resta de coautors. Firma el manuscrit en primer lloc.

Capítol 5. Effect of the inoculum dose of three grapevine trunk pathogens on the infection of artificially inoculated pruning wounds. Article enviat per a publicació a la revista *Phytopathologia Mediterranea* (Manuscrit núm. 16010, enviat el 3 de març de 2015; acceptat el 15 de juliol de 2015).

Impact Factor (2014): 0.970

ISI Journal Citation Reports© Ranking: 2014: 38/81 (Agronomy) Q2; 129/200 (Plant Sciences) Q3.

La doctoranda ha participat decisivament en l'elaboració de la hipòtesi de treball, conjuntament amb el Dr. Luque. Ha participat també en el desenvolupament del calendari de les activitats, així com en l'execució íntegra d'aquestes. El treball amb els fongs *Diplodia seriata* i *Phaeomoniella chlamydospora* s'ha desenvolupat a les instal·lacions de l'IRTA, a Cabrils. El treball amb *Eutypa lata* s'ha realitzat parcialment a Bordeaux (França) i Adelaide (Austràlia), en les dues estades internacionals que ha finançat l'organisme proveïdor de la beca doctoral (INIA, *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria*). La doctoranda ha recopilat les dades i les ha elaborat, resumit i analitzat estadísticament quan ha estat necessari. Ha participat portant la iniciativa en la redacció i correcció de l'article i n'és l'autora indicada per a la correspondència. Firma el manuscrit en primer lloc.

Capítol 6. Pruning debris of grapevine as a potential inoculum source of *Diplodia seriata*, causal agent of Botryosphaeria dieback. Article enviat per a publicació a la revista *European Journal of Plant Pathology* (Manuscrit EJPP-D-15-00373, enviat el 3 de juny de 2015).

Impact Factor (2014): 1.490

ISI Journal Citation Reports© Ranking: 2014: 25/81 (Agronomy) Q2; 6/33 (Horticulture) Q1; 89/200 (Plant Sciences) Q2.

La doctoranda ha participat decisivament en l'elaboració de la hipòtesi de treball, conjuntament amb el Dr. Luque. Ha participat també en el desenvolupament del calendari de les activitats, així com en l'execució íntegra d'aquestes. Ha recopilat les dades i les ha elaborat, resumit i analitzat estadísticament quan ha estat necessari. Ha participat portant la iniciativa en la redacció i correcció de l'article i n'és l'autora indicada per a la correspondència. Firma el manuscrit en primer lloc.

Capítol 7. Natural infections of pruning wounds by fungal trunk pathogens in mature grapevines in Catalonia (Northeast Spain). Article publicat a *Australian Journal of Grape and Wine Research 20, 134-143 (2014).*

Impact Factor (2014): 1.816

ISI Journal Citation Reports© Ranking: 2014: 42/123 (Food Science and Technology) Q2; 5/33 (Horticulture) Q1.

La doctoranda ha participat activament en el les tasques de recollida i anàlisi de les mostres corresponents als dos darrers períodes de mostreig (tardor de 2011 i primavera de 2012), després de la seva incorporació a l'equip investigador del departament (setembre de 2011). Ha elaborat parcialment les dades i ha participat en la redacció i correcció de l'article, conjuntament amb la resta de coautors. Firma el manuscrit en segon lloc.

Capítol 8. Seasonal susceptibility of grapevine pruning wounds and cane colonization following artificial infection with *Diplodia seriata* and *Phaeomoniella chlamydospora* in Catalonia, Spain. Article en preparació i pendent de ser enviat per a publicació a la revista *Plant Disease.*

Impact Factor (2014): 3.020

ISI Journal Citation Reports© Ranking: 2014: 40/200 (Plant Sciences) Q1.

La doctoranda ha participat activament en l'elaboració de les hipòtesis de treball, conjuntament amb el Dr. Luque. Ha participat també en el desenvolupament del calendari de les activitats, així com en l'execució íntegra d'aquestes. Ha recopilat les dades i les ha elaborat, resumit i analitzat estadísticament quan ha estat necessari. Ha participat portant la iniciativa en la redacció i correcció del manuscrit. Firma el manuscrit en primer lloc.

Capítol 9. Viability of Botryosphaeriaceae species pathogenic to grapevine after hot water treatment. Article acceptat per a publicació a la revista *Phytopathologia Mediterranea* (Manuscrit núm. 15526, enviat el 9 de febrer de 2015; acceptat el 8 d'abril de 2015).

Impact Factor (2014): 0.970

ISI Journal Citation Reports© Ranking: 2014: 38/81 (Agronomy) Q2; 129/200 (Plant Sciences) Q3.

La doctoranda ha participat activament en la recopilació de les dades i les ha elaborat, resumit i analitzat estadísticament quan ha estat necessari. Ha participat en la redacció i correcció del manuscrit. Firma el manuscrit en primer lloc.

Cabrils, 1 de juliol de 2015

Dr. Jordi Luque

Patologia Vegetal. IRTA Cabrils

Capítol 4

Intraspecific variation in *Diplodia seriata* isolates occurring on grapevines in Spain

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Plant Pathology (2015) 64, 680-689.

4.1. Resum

En aquest estudi s'ha avaluat la variabilitat intraespecífica de *Diplodia seriata*, fong associat al decandiment de la vinya causat per *Botryosphaeria*, a nivell genètic, fenotípic i patogènic. La tècnica *Inter Simple Sequence Repeat* (ISSR) es va usar per a estudiar la diversitat genètica de 83 soques de *D. seriata*. Cinc dels encebadors ISSR avaluats van mostrar empremtes genètiques caracteritzades per patrons de bandes d'ADN polimòrfics i reproduïbles. Les dades obtingudes es van analitzar amb diferents mètodes d'agrupació, mitjançant l'anàlisi Bayesià i l'anàlisi discriminant de components principals (DAPC). Les soques es van assignar a dos grups genètics diferenciats. No es va trobar cap relació entre l'origen geogràfic de les soques o de l'hoste de procedència i els grups genètics observats. Diverses soques representatives de cada grup DAPC es van usar per a estudiar les mides dels seus conidis, el seu creixement miceliar *in vitro*, la seva compatibilitat vegetativa i sexual i la seva patogenicitat en sarments podats i en plantes de vinya mantingudes en test. No es van detectar diferències significatives entre els dos grups genètics pel que respecta les mides mitjanes dels conidis. Es van observar reaccions de compatibilitat vegetativa entre les soques, però aquestes no van estar relacionades amb els grups genètics establerts. No va ser possible observar la formació de cossos fructífers sexuals entre soques que havien mostrat prèviament compatibilitat vegetativa. En l'estudi de patogenicitat, les catorze soques avaluades van mostrar-se patògenes de la vinya d'acord amb la longitud de les lesions necròtiques que van produir i la seva freqüència de recuperació de la planta inoculada. Es van detectar diferències estadísticament significatives entre les longituds de les necrosis produïdes, posant de manifest l'existència de diferents nivells de virulència dins de l'espècie.

4.2. Abstract

Variation of *Diplodia seriata*, a fungal species associated with Botryosphaeria dieback of grapevine, was investigated with respect to its genetic, phenotypic and pathogenic characteristics. The inter-simple sequence repeat (ISSR) technique was used to investigate the genetic diversity of 83 isolates of *D. seriata*. Five ISSR primers were able to provide reproducible and polymorphic DNA fingerprint patterns, thus showing a relevant genetic

variability in the species. Analyses of ISSR data by different clustering methods grouped the isolates into two distinct clusters through the Bayesian and DAPC analyses. No relationships between either geographic or host origin of isolates and genetic clusters were observed. Several representative isolates from each genetic cluster were chosen for studying their conidial dimensions, *in vitro* mycelial growth, vegetative and mating compatibility, and pathogenicity on detached grapevine canes and potted vines. No significant differences in conidial dimensions were detected among the groups. Vegetative compatibility reactions were observed among isolates but this was not related with the genetic clustering. Production of sexual fruiting bodies in vegetative compatible crossings was not observed under the experimental conditions used in the study. All 14 isolates tested for pathogenicity were confirmed to be pathogenic according to the length of the necrotic lesions that they caused and their reisolation frequencies from the infected plant tissues. Differences in the length of necrosis were detected among isolates, thus revealing the existence of different virulence levels in the species.

4.3. Introduction

Diplodia seriata is a cosmopolitan and plurivorous fungal species occurring on many plant genera and families. Punithalingam and Walker (1973) reported this fungus to be isolated from 35 different plant species but the current number of known hosts is clearly greater according to the Systematic Mycology and Microbiology Laboratory Fungal Database (Agricultural Research Service, United States Department of Agriculture), which reports more than 200 species and 130 genera as herbaceous and mainly woody hosts (Farr and Rossman, 2013). The fungus causes canker, dieback, fruit rot, and leaf spot diseases on several economically important forest and horticultural species (Farr and Rossman, 2013). On grapevines *D. seriata* is known to produce spring bud mortality, leaf chlorosis, fruit rot and trunk dieback, with brown, hard necrosis of the wood that appears as V-shaped necroses in cross sections of the affected plant parts (van Niekerk *et al.*, 2006; Úrbez-Torres, 2011). Moreover, streaking and pith necrosis of wood, failure of graft union in young vines and cane bleaching have also been reported for this fungus (Úrbez-Torres, 2011). *Diplodia seriata* is one of the most cited Botryosphaeriaceae species occurring on grapevines worldwide and is frequently associated with the 'black dead arm'

disease of grapevine (Larignon *et al.*, 2001a; Úrbez-Torres, 2011). In the USA *D. seriata* was reported as one of the causal agents of Botryosphaeria canker of grapevines (Úrbez-Torres and Gubler, 2009). Recently Úrbez-Torres (2011) proposed the name 'Botryosphaeria dieback' to include the increasing number of botryosphaeriaceous species besides *D. seriata* that have been associated with most of the symptoms and diseases above.

The pathogenicity of *D. seriata* on grapevine has been a matter of discussion. This species has been reported as pathogenic in Australia (Castillo-Pando *et al.*, 2001; Savocchia *et al.*, 2007), Chile (Auger *et al.*, 2004), France (Larignon *et al.*, 2001a), Italy (Rovesti and Montermini, 1987), Mexico (Úrbez-Torres *et al.*, 2008), South Africa (van Niekerk *et al.*, 2004), Spain (Luque *et al.*, 2009) and the USA (Úrbez-Torres *et al.*, 2009). However, it was considered as weakly pathogenic in South Australia and New South Wales (Pitt *et al.*, 2013a), China (Yan *et al.*, 2013), Iran (Mohammadi *et al.*, 2013), New Zealand (Amponsah *et al.*, 2011) and Portugal (Phillips, 2002), and nonpathogenic in Western Australia (Taylor *et al.*, 2005). Differences in virulence among *D. seriata* isolates have been reported for this species within the same grape growing areas (Larignon *et al.*, 2001a; Savocchia *et al.*, 2007; Úrbez-Torres *et al.*, 2008; Luque *et al.*, 2009). The variability in pathogenicity that has been reported for *D. seriata* may have made it difficult to determine the importance of this fungus as a grapevine pathogen.

Complementary studies of both genetic and virulence diversity made with a considerable number of grapevine fungal pathogens have been reported recently. Alaniz *et al.* (2009) studied the genetic and virulence diversity of a wide collection of 82 *Ilyonectria* spp. isolates (formerly *Cylindrocarpon liriodendri* and *Cylindrocarpon macrodidymum*) and observed different virulence levels among the isolates that could be assigned to distinct genetic groups. Baskarathevan *et al.* (2012) reported on the genetic and virulence variability of 50 isolates of *Neofusicoccum parvum*, but the authors found no relationship between the genetic groups and the virulence of isolates in this species. Billones-Baaijens *et al.* (2013a) reported that 66 isolates of *Neofusicoccum luteum*, corresponding to different pathotypes, were genetically diverse but no obvious genotype-pathotype relationships were observed. In a study of genetic and virulence diversity of *Togninia minima* in Spain, a fungus that is associated

with young vine decline, Gramaje *et al.* (2013) observed no significant differences in the virulence of isolates with regard to the different groups found in the genetic analyses. Martín *et al.* (2013) studied the genetic fingerprinting of Spanish isolates of *D. seriata* obtained from grapevine based on amplified fragment length polymorphism (AFLP) patterns but no pathogenicity data were provided. To date, the combined genetic and pathogenic characterization in *D. seriata* isolates obtained from grapevine has not been studied in detail.

The inter-simple sequence repeat (ISSR) or random amplified microsatellites (RAMS) technique (Zietkiewicz *et al.*, 1994) has proven to be a powerful tool for the analysis of genetic diversity and has also been shown to be applicable to fungi (Hantula *et al.*, 1996). This technique combines the simplicity of the random amplified polymorphic DNA approach, the reliability of bands derived from known heritable domains of the genome, and the potential to differentiate populations or recently diverged species (Zhou *et al.*, 2001). Genetic diversity of a species is highly influenced by the relative contribution of its asexual and sexual reproduction forms. Reproduction of Botryosphaeriaceae species under natural conditions is believed to be predominantly asexual (Phillips, 2002; Úrbez-Torres, 2011), and reports of sexual fruiting bodies found in the field are rare (van Niekerk *et al.*, 2004). However, it has been reported that fungal compatible genotypes can exchange genetic material through parasexual recombination, a process that involves hyphal fusion and posterior plasmogamy from two compatible mycelium types (Leslie, 1993). Characterization of fungal populations into different vegetative compatibility groups (VCG) is highly relevant to provide more information on the potential for genetic exchange in species where sexual reproduction is not prevalent. This method has been widely used for indirectly assessing the genetic variability among isolates of fungal plant pathogens (Leslie, 1993). Concerning specific studies on Botryosphaeriaceae species affecting grapevines, VCG were studied in *N. parvum* by Baskarathevan *et al.* (2012), who found that isolates belonging to distinct VCG were able to form anastomoses in compatible and partially compatible pairings and were likely to promote genetic exchange between isolates.

The main objective of this study was to obtain a better understanding of both the genetic and the virulence diversity of *D. seriata* isolates obtained from

grapevine in Spain, by evaluating their intraspecific variation with respect to genetic, phenotypic and pathogenic characteristics. According to the results obtained in the genetic study through the ISSR technique, several representative isolates of each genetic group were then chosen for the posterior phenotypic and pathogenicity studies. Conidial dimensions and *in vitro* mycelial growth rates were studied as phenotypic characters, whereas pathogenicity assays conducted on detached canes and living young vines were used to estimate the species variation in pathogenicity. All the results obtained in the assays were analysed in relation to the groups obtained in the genetic variability study. Moreover, a study of vegetative compatibility and sexual mating was also done with some selected isolates. The final goal was to expand the knowledge about *D. seriata* that could help in clarifying the pathogenic role of this fungal species on grapevine. Several *D. seriata* isolates obtained from non-grapevine hosts were also included to expand the scope of the study.

4.4. Material and Methods

4.4.1. Fungal isolates

Eighty-three isolates of *Diplodia seriata* were included in this study (Table 4.1). The isolates were obtained from eight different plant hosts but mainly grapevine (72 isolates), and from different regions in Spain (82 isolates). Most of the isolates were obtained from cankers and wood necroses of plants that were surface-sterilized (70 % ethanol, 4 minutes) then small diseased wood pieces (5 × 5 mm) were cultured on potato dextrose agar (PDA, Difco, Becton, Dickinson and Company) amended with streptomycin sulphate at 100 units·ml^{-1}. Some of the cultures were obtained from monosporic isolations (data not shown); all isolates were finally grown by hyphal tip subculturing to obtain the material for the study. Identification of *D. seriata* was confirmed by morphological characters of the fruiting structures following the description by Phillips *et al.* (2007). Tentative morphological identifications were resolved by sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA. The isolates were maintained at 4 °C as mycelia plugs contained in tubes filled with sterile distilled water. Isolates were grown for 7 days on PDA at 25 °C in the dark to obtain enough mycelial material for the experiments.

4.4.2. DNA extraction and PCR amplifications

Fungal mycelium from pure cultures was scraped from the colony and put into a 1.5 ml Eppendorf tube. Total DNA was extracted using the E.Z.N.A. Plant Miniprep kit (Omega Biotek) following the manufacturer's instructions. DNA was visualized after electrophoresis on 0.9 % agarose gels stained with ethidium bromide under UV light. DNA was stored at -20 °C until further use.

The ITS region was sequenced using the primers ITS1 and ITS4 (White *et al.*, 1990) and the results were compared to those in GenBank using the BLAST tool. The method of amplification and sequencing was described by Luque *et al.* (2005).

A total of 24 primers were evaluated for their ability to produce polymorphic, scorable and reproducible DNA fingerprint patterns from the isolates. The primers comprised 11 dinucleotide, seven trinucleotide, five tetranucleotide and one pentanucleotide with or without 50 anchors, namely: $(AG)_8G$, $(AT)_8B$, $(GA)_8C$, $(TC)_8RA$, $(TG)_8RC$, $HBH(AG)_7$, $DBDA(CA)_7$, $HVH(TG)_7$, $DVD(CT)_7C$, $YHY(GT)_7G$, $HYH(GT)_7G$, $(CAG)_5$, $BBD(AAC)_5$, $HYH(GTG)_5$, $BDB(ACA)_5$, $DHB(TCG)_5$, $DDB(CCA)_5$, $DHB(CGA)_5$, $(ACTG)_4$, $(GGAC)_4$, $HBDB(GACA)_4$, $(GGAC)_3C$, $(GGAC)_3T$, and $(GGGGT)_2G$. Each PCR mix contained 10 µl Taq PCR Master Mix (QIAGEN), 0.8 µl template DNA containing 50−100 ng DNA, and 1 µl of the corresponding primer at 10 µM. The PCR mix was adjusted to a final volume of 20 µl with RNAse-free water (QIAGEN). PCR amplifications were performed on a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer, Applied Biosystems). The amplification profile consisted of an initial step of 5 minutes at 95 °C, followed by 40 cycles of denaturation at 95 °C for 1 minute, annealing at the appropriate temperature for 1 minute (Table 4.2), and elongation at 72 °C for 2 minutes. A final extension was performed at 72 °C for 10 minutes. PCR products were separated by electrophoresis in 2 % agarose gels (agarose D-1 Low EEO; Conda) stained with ethidium bromide and visualized under UV light. Gene Ruler 100 bp DNA ladder plus (MBI Fermentas) was used as molecular weight marker. Amplifications for each primer were repeated at least three times in independent PCR amplifications from a single DNA extraction per isolate, and only clear and reproducible bands within the range 500-2000 bp were considered. Reproducibility of the technique was further assessed by

repeating the above procedure using the primers that produced informative band patterns in new independent PCR amplifications with new DNA extractions obtained from newly cultured colonies of eight randomly selected isolates (Table 4.1).

Table 4.1. Host, geographic origin, genetic groups (DAPC and STRUCTURE) and other complementary studies done with 83 isolates of *Diplodia seriata*. Numbers in the DAPC and STRUCTURE columns followed by an asterisk corresponded to isolates with percentage assignment to groups less than 80 % and 95 %, respectively.

Isolate	Host	Locality	Province	DAPC group (K=2)	STRUCTURE group (K=2)	Other studies[1]
507	*Vitis vinifera*	Sant Sadurní d'Anoia	Barcelona	1	1	1, 2, 3, 4, 5
582	*Corylus avellana*	La Fatarella	Tarragona	1	1	1, 2, 4, 5
495	*Vitis vinifera*	Batea	Tarragona	1	1*	1
354	*Vitis vinifera*	Caldes de Montbui	Barcelona	1	1	1,6
398	*Vitis vinifera*	Pacs del Penedès	Barcelona	1	1	1, 2, 3, 4, 5
417	*Vitis vinifera*	Masquefa	Barcelona	1	1	1
473	*Vitis vinifera*	Bot	Tarragona	1	1	1, 2
406	*Vitis vinifera*	Mediona	Barcelona	1	1	1, 2, 3, 4, 5,6
592	*Prunus dulcis*	Constantí	Tarragona	1	1	1, 2, 3, 4, 5
610	*Vitis vinifera*	Garriguella	Girona	1	1	1, 2, 4, 5
771	*Vitis vinifera*	Uruñuela	La Rioja	1	1	1
779	*Vitis vinifera*	Beniganim	Valencia	1	1	1
395	*Vitis vinifera*	L'Arboç del Penedès	Tarragona	1	1	1
404	*Vitis vinifera*	La Múnia	Barcelona	1	1	1
409	*Vitis vinifera*	Vimbodí	Tarragona	1	1	1
420	*Vitis vinifera*	Falset	Tarragona	1	1	1, 2
426	*Vitis vinifera*	La Vilella Baixa	Tarragona	1	1	1
433	*Vitis vinifera*	Òdena	Barcelona	1	1	1,6
460	*Vitis vinifera*	El Montmell	Tarragona	1	1	1, 2
478	*Vitis vinifera*	Batea	Tarragona	1	1	1
482	*Vitis vinifera*	Gandesa	Tarragona	1	1	1
532	*Vitis vinifera*	Albinyana	Tarragona	1	1	1

Table 4.1. (cont.)

Isolate	Host	Locality	Province	DAPC group (K=2)	STRUCTURE group (K=2)	Other studies[1]
624	*Prunus dulcis*	Roses	Girona	1	1	1
628	*Olea europaea*	Cabanelles	Girona	1	1	1
629	*Cotoneaster* sp.	Vilassar de Mar	Barcelona	1	1	1
638	*Cotoneaster* sp.	Lleida	Lleida	1	1	1
699	*Corylus avellana*	La Pobla de Mafumet	Tarragona	1	1	1
777	*Vitis vinifera*	Sisante	Cuenca	1	1	1
781	*Vitis vinifera*	Anna	Valencia	1	1	1
782	*Vitis vinifera*	Anna	Valencia	1	1	1
693	*Malus pumila*	La Tallada d'Empordà	Girona	1	1*	1, 2, 3, 4, 5
715	*Corylus avellana*	Cabra del Camp	Tarragona	1	1*	1
764	*Vitis vinifera*	Santa Marta de los Barros	Badajoz	1	1*	1
773	*Vitis vinifera*	Alcázar de San Juan	Ciudad Real	1	1*	1
351	*Vitis vinifera*	Caldes de Montbui	Barcelona	1	1*	1
421	*Vitis vinifera*	Capçanes	Tarragona	1	1*	1, 2,6
423	*Vitis vinifera*	Capçanes	Tarragona	1	1*	1
471	*Vitis vinifera*	Vilalba dels Arcs	Tarragona	1	1*	1, 2, 3, 4, 5
778	*Vitis vinifera*	Xàtiva	Valencia	1	1*	1
783	*Vitis vinifera*	Albaida	Valencia	1	1*	1
786	*Vitis vinifera*	Unknown	Valencia	1	1*	1
792	*Vitis vinifera*	Mollina	Málaga	1	1*	1
414	*Vitis vinifera*	La Granada	Barcelona	1	2*	1
464	*Vitis vinifera*	Masquefa	Barcelona	1	2*	1
529	*Vitis vinifera*	Castellet i la Gornal	Barcelona	1	2*	1
543	*Vitis vinifera*	Miravet	Tarragona	1	2*	1
623	*Juglans regia*	Constantí	Tarragona	1	2*	1

Table 4.1. (cont.)

Isolate	Host	Locality	Province	DAPC group (K=2)	STRUCTURE group (K=2)	Other studies[1]
679	*Vitis vinifera*	Barbastro	Huesca	1	2*	1
680	*Vitis vinifera*	Barbastro	Huesca	1	2*	1
780	*Vitis vinifera*	Xàtiva	Valencia	1	2*	1
785	*Vitis vinifera*	Arenales de San Gregorio	Ciudad Real	1	2*	1
457	*Vitis vinifera*	Santa Maria de Miralles	Barcelona	1*	2*	NS
769	*Vitis vinifera*	Villarobledo	Albacete	1*	2*	NS
776	*Vitis vinifera*	Socuéllamos	Ciudad Real	1*	2*	NS
486	*Vitis vinifera*	Batea	Tarragona	2	2	1
537	*Vitis vinifera*	Olèrdola	Barcelona	2	2	1
538	*Vitis vinifera*	Falset	Tarragona	2	2	1
555	*Vitis vinifera*	Tivissa	Tarragona	2	2	1
613	*Vitis vinifera*	Vilajuïga	Girona	2	2	1
765	*Vitis vinifera*	Tarazona de la Mancha	Albacete	2	2	1
766	*Vitis vinifera*	Tudela	Navarra	2	2	1
768	*Vitis vinifera*	Villafranca de los Caballeros	Toledo	2	2	1
772	*Vitis vinifera*	Pozoamargo	Cuenca	2	2	1
774	*Vitis vinifera*	Las Mesas	Cuenca	2	2	1, 2, 3, 4, 5
775	*Vitis vinifera*	Villarta de San Juan	Ciudad Real	2	2	1, 2, 4, 5
784	*Vitis vinifera*	Albaida	Valencia	2	2	1,6
787	*Vitis vinifera*	Sinarcas	Valencia	2	2	1
789	*Vitis vinifera*	Fuendejalón	Zaragoza	2	2	1
790	*Vitis vinifera*	Fuendejalón	Zaragoza	2	2	1
793	*Vitis vinifera*	Villena	Alicante	2	2	1
794	*Vitis vinifera*	Moixent	Valencia	2	2	1
795	*Vitis vinifera*	Villarubia de los Ojos	Ciudad Real	2	2	1
797	*Vitis vinifera*	Aielo de Malferit	Valencia	2	2	1

Table 4.1. (cont.)

Isolate	Host	Locality	Province	DAPC group (K=2)	STRUCTURE group (K=2)	Other studies[1]
798	*Vitis vinifera*	Aielo de Malferit	Valencia	2	2*	1,6
561	*Vitis vinifera*	Sant Esteve Sesrovires	Barcelona	2	2*	1
796	*Vitis vinifera*	Aielo de Malferit	Valencia	2	2*	1
770	*Vitis vinifera*	Malagón	Ciudad Real	2*	2	NS
788	*Vitis vinifera*	Ontiyent	Valencia	2*	2	6
379	*Vitis vinifera*	La Bisbal del Penedès	Tarragona	2*	2*	NS
435	*Vitis vinifera*	Sant Llorenç d'Hortons	Barcelona	2	2*	1, 2, 3, 4, 5
631	*Quercus suber*	Bortigiadas	Olbia, Sardinia, Italy	2	2	1, 2, 3, 4, 5
791	*Vitis vinifera*	Cózar	Ciudad Real	2	2	1, 2, 3, 4, 5,6
767	*Vitis vinifera*	Sinarcas	Valencia	2	2	1, 2, 3, 4, 5

Other studies: 1, Conidial size measurements; 2, Vegetative compatibility; 3, Sexual mating; 4, Mycelial growth rate; 5, Pathogenicity; 6, Reproducibility of the ISSR technique; NS, non selected.

Table 4.2. Summary data of the annealing temperatures and output results for five ISSR primers used in the genetic variability study of 83 isolates of *Diplodia seriata*.

ISSR Primer	Annealing Temperature (ºC)	Amplified bands (N)	Polymorphic Bands (N)	% Polymorphism	Fingerprinting patterns (N)
$(AG)_8G$	40	20	18	90.00	71
$(CAG)_5$	55	26	21	80.77	49
$DDB(CCA)_5$	45	22	18	81.82	62
$HYH(GTG)_5$	60	21	21	100.00	73
$YHY(GT)_7G$	50	16	14	87.50	60
Total	-	68	57	83.82	315

4.4.3. ISSR characterization and analysis of genetic variability

Amplified fragments were visually scored as absent (0) or present (1) depending on the approximate size of the bands and their relative position in the agarose gel. To reduce subjectivity during the visual band scoring, conflicting data were independently analysed by two researchers before making a decision on the number and arrangement of bands. An initial study of the genetic variability of isolates was performed by analysing the band patterns with a Bayesian clustering method implemented in STRUCTURE v.2.2.3 (Falush *et al.*, 2003), assuming a model with population admixture where allele frequencies were correlated within populations. Runs for each *K* value (from 1 to 12) were independently replicated 20 times with a burn-in of 100000 followed by 500000 iterations of the Monte Carlo Markov chain (MCMC). The estimate of the posterior probability of the data (Ln *P*(*D*)) was standardized using the ad hoc Δ*K* statistic, based on the rate of change between successive *K* values, and later used to infer the uppermost level of structure in the data set (Evanno *et al.*, 2005). Discriminant analysis of principal components (DAPC), a multivariate method implemented in the R package ADEGENET (Jombart *et al.*, 2010), was used to assign the isolates to well-defined clusters that apparently had mixed ancestry after the analysis performed with STRUCTURE. The first step of DAPC is based on a principal component analysis of the allelic diversity among isolates, and the posterior sequential *K*-means procedure was used to infer the optimal number of groups, thus minimizing the within-group genetic variability. The Bayesian information criterion (BIC) was finally used to assess the optimal

number of groups, and the discriminant analysis was used to summarize the genetic differentiation among the groups (Jombart *et al.*, 2010). Differences in group assignment among clusters generated after the Bayesian analyses were detected through an analysis of molecular variance (AMOVA) using the software ARLEQUIN v.3.5 (Excoffier *et al.*, 2005). After the clustering methods were done, several representative isolates were chosen from the resulting groups for the phenotypic and pathogenic characterization as explained below.

4.4.4. Phenotypic characterization

Conidial size of isolates: Only isolates that were well defined in the different genetic clusters were included in this study (Table 4.1). Isolates were grown on water agar (WA, Bacto Agar, Becton, Dickinson and Company) plates with sterile fragments of pine needles for 4 weeks at 25 °C under combined near-UV and white fluorescent light (Philips TL-D 18W BLB and Sylvania Standard F18W/ 33-640-TS cool white, respectively) in a 12/12 h photoperiod to induce pycnidia production. Length (L) and width (W) of 50 conidia were measured for each isolate, and the ratio L:W was calculated. Mean values ± standard error of the mean are used throughout the text unless specified.

Mycelial growth: Fourteen *Diplodia seriata* isolates were selected from the genetically distinct groups according to the following criteria: i) all the representative isolates should be well defined in the different genetic groups, ii) both grapevine and non-grapevine isolates should be included, and iii) the ratio of isolates in each DAPC group should be kept after the selection of isolates. Thus, eight isolates were selected from the DAPC cluster 1 and six isolates from the DAPC cluster 2 (Table 4.1). Ten isolates were obtained from grapevine and four from other hosts. All isolates were incubated on PDA plates at 22 °C in darkness. Colony radius was measured after 3 days by averaging four radii measurements taken at 90° to each other. The experiment was repeated three times using three PDA plates per isolate in each repetition.

VCG and sexual compatibility: Eighteen isolates similarly selected as above were used to determine the vegetative compatibility in non-self pairings (Table 4.1). To set up the pairings, three mycelial plugs (3 mm diameter) of different isolates were taken from the edge of 7-day-old colonies and placed 6 cm apart on a half-strength PDA plate. Plates were incubated at 25 °C in darkness. After 7,

12, 15, 18 and 21 days' incubation each pairing was visually examined and the vegetative compatibility was scored as compatible (C) when mycelia of two isolates merged together uniformly, vegetatively incompatible (I) when mycelia of two isolates grew to a meeting point on the agar but remained separated by a 'barrage-like' reaction formed along the line of contact between the paired isolates, or partially incompatible (PI) when the barrage line appeared interrupted, as described by Baskarathevan *et al.* (2012). Each pairing was replicated three times in different independent experiments. Eleven compatible pairings resulting from the previous experiment, namely 435-774, 435-631, 507-631, 507-767, 507-774, 507-791, 767-406, 767-693, 767-398, 791-471 and 791-592, were selected to determine their potential sexual compatibility as follows. Mycelial plugs of two vegetatively compatible isolates were placed 6 cm apart on a WA plate. A sterile 5-cm grapevine cane fragment was placed between the two isolates on the medium surface and plates were incubated for 8 weeks at 10 °C under combined near-UV and white fluorescent light in a 12/12 h photoperiod, as described above. The plates were observed weekly within the 4-8 weeks' incubation period to check for production of perithecia. Each pairing was replicated three times.

4.4.5. Pathogenicity tests

The same 14 isolates used for measuring mycelial growth were tested for pathogenicity on detached grapevine canes and potted vines (Table 4.1).

Detached canes assay: Fragments of grapevine dormant canes with three internodes and uniform thickness were excised from mature healthy vines cv. 'Tempranillo' using pruning shears. A 4 mm diameter hole was drilled in the mid internode. A mycelium plug from an actively growing colony was placed into the hole and the wound was wrapped with Parafilm. Control canes were treated similarly but with sterile PDA plugs instead of the mycelial inoculum. The ends of the canes were sealed with paraffin to prevent excessive water loss from canes. Ten canes per isolate were used as replicates. Inoculated canes were placed in a plastic bag and incubated at 25 °C in darkness for 5 weeks. After incubation, the bark of the canes was removed with a sterile scalpel and the necrotic lesions that developed in the vascular tissues were measured. The mean length of the necrosis extending to both sides from the inoculation site

was calculated and used in the statistical analyses. Inoculated fungi were reisolated to fulfil Koch's fourth postulate as follows. Two 1-cm fragments of the debarked canes were cut 1 cm away at both sides from the inoculation site. Two additional 1-cm fragments were obtained from both ends of the necrotic lesions. Wood pieces were surface-sterilized by dipping them into 70 % ethanol for 4 minutes, then were plated onto PDA supplemented with streptomycin sulphate at 100 units ml^{-1}. Plates were incubated at 25 °C until *D. seriata* was identified from its colony morphology, growth rate and colour characteristics.

Potted vines assay: Two-year-old vines cv. 'Tempranillo' grafted onto Richter 110 rootstock were used. Vines were maintained in 3-l pots containing a peat:sand mixture (1:1; v:v). Inoculations were performed in November 2012 on dormant canes from the current season. Inoculations were made as described in the previous assay. Control vines were treated with a sterile PDA plug instead of the mycelial inoculum. Fifteen vines were used per treatment. The vines were arranged in a completely randomized design in the greenhouse, where they grew for 1 year under controlled temperature (15-30 °C) with watering as needed to avoid water stress. Inoculated canes were removed from the vines in November 2013 and necrosis length measurements and reisolations were made as described previously.

4.4.6. Statistical analyses

Data obtained in the genetic study were analysed with STRUCTURE v.2.2.3 and DAPC as previously described. All other data in this study were analysed with SAS v.9.2 software (SAS Institute Inc.). Groups resulting from the DAPC clustering method (the DAPC groups hereafter) were used as independent variables in the analyses. In addition, groups based on the host origin of isolates were also considered as independent variables in complementary analyses. Prior to the statistical analyses, data of conidial dimensions, mycelial growth rates and necrosis lengths were checked for normality and homoscedasticity criteria and were transformed if necessary. The dependent variables were analysed by one-way ANOVA and posterior Tukey's Honestly Significant Difference test to detect differences among the means. Pearson's correlation coefficients were calculated for the pairwise comparisons of the mycelial growth rates and the length of necrosis obtained in the pathogenicity assays.

4.5. Results

4.5.1. Genetic variability

Nineteen of the 24 ISSR primers produced either no polymorphic bands or weak non-evaluable bands, and were thus uninformative. The remaining five primers produced polymorphic and reproducible bands and were selected to evaluate the genetic variability among the *Diplodia seriata* isolates (Table 4.2). Amplification products generated a total of 105 bands between 500 and 2000 bp, 92 of them (88 %) being polymorphic, and resulting in 315 different DNA fingerprinting patterns (Table 4.2). When checking for the reproducibility of the technique, all primers amplified the DNA from eight randomly selected isolates (10 % of total isolates) under the same experimental conditions used before. Identical band patterns to those obtained in the ISSR fingerprinting of isolates were observed in 16 out of the 40 total combinations (8 isolates × 5 primers). In 28 combinations the percentage of coincident bands was greater than 90 %. The overall percentage of coincident bands among all combinations was estimated to be 92 %.

Bayesian analysis carried out with STRUCTURE grouped the 83 isolates into two genetically distinct clusters (ΔK=347.54, ln $P(D)$=2764.675). Most individuals were estimated to have 80 % of their ancestry belonging to a single distinct population; however, 30 isolates apparently had mixed ancestry (Figure 4.1; Table 4.1). These two clusters obtained with STRUCTURE were later redefined by the DAPC analysis with a posterior probability for the individuals higher than 95 %: cluster 1 with 51 isolates, and cluster 2 with 26 isolates (Table 4.1). Six isolates, namely 379, 457, 769, 770, 776 and 788, showed posterior probability values within the range 71-87 % in the DAPC analysis and therefore were excluded from further studies. The two DAPC clusters were close to being significantly different (P=0.056) after the AMOVA analysis, wherein 0.5 % of the total variation was apportioned between the two clusters and the remaining 99.5 % within the clusters. Ten isolates obtained from a host other than grapevine belonged to DAPC group 1 whereas only one (631, from *Quercus suber*) was placed in DAPC group 2 (Table 4.1). No relationships between the geographic origin of isolates and the clustering classification obtained through DAPC were observed (data not shown).

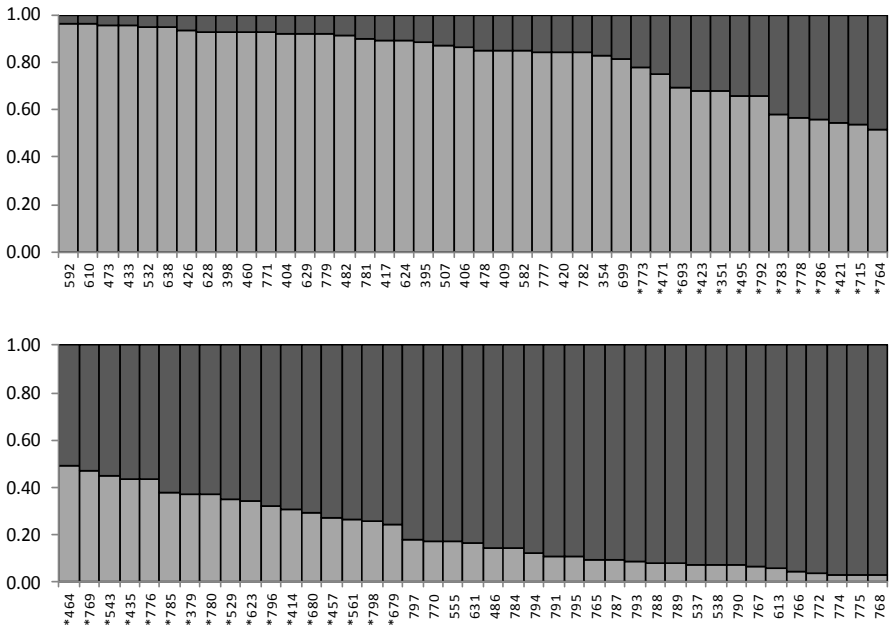

Figure 4.1. Estimated genetic structure for *K*=2 groups of 83 *Diplodia seriata* isolates inferred by Bayesian clustering of ISSR data in 20 independent runs using the software STRUCTURE v.2.2.3. Each isolate is represented by a bicoloured column according to its posterior probability. Isolate numbers followed by an asterisk correspond to isolates with mixed ancestry at <80 %.

4.5.2. Phenotypic characterization

All the isolates except isolate 767 produced conidia under the culture conditions used in the assay. The mean conidial sizes of the isolates classified into the DAPC groups did not show significant differences in length, width and L:W ratio. Absolute differences between mean values between the DAPC groups were 0.06 µm in length (group 1, 21.70±0.13 µm; group 2, 21.76±0.19 µm) and 0.23 µm in width (group 1, 10.33±0.05 µm; group 2, 10.56±0.14 µm), respectively. The mean L:W ratio was 2.11±0.02 µm for group 1 and 2.07±0.05 µm for group 2. The minimum, maximum and mean ±95 % confidence interval values for all the isolates used in the study were as follows: length (19.5) - 21.72±0.20 µm - (24.1) × (9.4) - 10.41±0.12 µm - (13.2). The L:W ratio for all the isolates in the study was 2.10±0.02. According to the host species from which isolates were obtained, mean values in length and width of the

grapevine and non-grapevine groups were not significantly different (grapevine isolates, 21.77±0.12 µm × 10.37±0.05 µm; non-grapevine isolates, 21.41±0.19 µm × 10.64±0.13 µm). However, the L:W ratio of grapevine isolates (2.12±0.02) was significantly higher (P<0.015) than the L:W ratio from other non-grapevine isolates (1.99±0.08). Mycelial growth rates were significantly different among isolates (P<0.001), with values ranging from 1.45 to 2.84 cm (Figure 4.2a). No significant differences were observed between the mean growth rates of the DAPC groups (Table 4.3).

In the VCG study, incompatibility was detected in 49.0 % of total pairings (excluding self-pairings), whereas 22.9 % showed full compatibility and 28.1 % partial incompatibility. Most of the isolates showed incompatibility when paired against each other, even if belonging to the same genetic group (Table 4.4). Isolate 791 was compatible with all isolates except 420 and 610, whereas isolate 420 did not show compatibility with any other isolate used in the study. The remaining isolates showed partial or full compatible reactions with a variable number of isolates (Table 4.4). Perithecia were never observed in any combination of the 11 selected pairings for mating assessment. Instead, pycnidia were produced on the grapevine cane fragments for all isolates and replicates.

Table 4.3. Mean *in vitro* mycelial growth rate and mean length of necrosis caused by *Diplodia seriata* isolates, grouped according to the discriminant analysis of principal components (DAPC) clusters, on detached canes and potted vines cv. 'Tempranillo'.

Clustering method	Group	Mycelial growth rate (cm)				Necrosis length canes (cm)				Necrosis length vines (cm)			
		-CI	Mean	+CI	Sig.	-CI	Mean	+CI	Sig.	-CI	Mean	+CI	Sig.
DAPC	1	0.12	2.27	0.12	a	0.11	0.79	0.13	a	0.13	2.21	0.14	a
	2	0.14	2.40	0.15	a	0.11	0.73	0.13	a	0.15	2.27	0.17	a
	Control	-	-	-		0.04	0.12	0.06	b	0.07	0.45	0.08	b

Values correspond to the detransformed means and lower (−) and upper (+) 95 % confidence interval (CI). Statistically significant (Sig.) differences (P<0.05) are shown in different letters for each variable.

Table 4.4. Vegetative compatibility reactions of selected pairings of *Diplodia seriata* isolates, indicated by compatible (C), partially incompatible (PI) and incompatible (I) reactions of all tested combinations.

DAPC groups		1										2					
Isolate		*507*	*582*	*398*	*473*	*406*	*592*	*610*	*420*	*421*	*460*	*471*	*693*	*774*	*775*	*435*	*631*	*791*	*767*
1	*507*	C																	
	582	PI	C																
	398	I	PI	C															
	473	PI	I	PI	C														
	406	I	I	I	I	C													
	592	C	I	I	I	I	C												
	610	C	I	I	I	I	I	C											
	420	I	I	I	I	I	I	I	C										
	421	C	PI	I	I	I	I	I	I	C									
	460	C	PI	PI	PI	I	I	I	I	PI	C								
	471	PI	I	I	I	PI	I	PI	I	PI	PI	C							
	693	PI	PI	I	I	I	I	I	PI	PI	PI	PI	C						
2	*774*	C	PI	I	I	I	I	I	I	PI	I	I	PI	C					
	775	PI	PI	I	I	I	I	PI	I	I	PI	I	PI	I	C				
	435	PI	PI	PI	C	I	PI	PI	PI	PI	I	PI	PI	C	C	C			
	631	C	I	PI	I	I	C	C	I	PI	I	I	PI	I	PI	C	C		
	791	C	C	C	C	C	C	PI	PI	C	C	C	C	C	C	C	C	C	
	767	C	I	C	I	C	I	I	I	I	C	I	C	C	I	C	C	C	C

4.5.3. Pathogenicity tests

No foliar symptoms potentially associated to a pathogenic behavior of the inoculated *Diplodia seriata* isolates were observed during the experimental period. Moreover, the only symptoms observed in the pathogenicity tests were the necrotic lesions in the wood extending from the inoculation sites. In the detached cane assay, statistical analysis showed significant differences (P<0.001) in lesion length among isolates. All isolates caused necrotic lesions significantly longer than the control (Figure 4.2b), with mean values ranging from 0.23 cm (isolate 610) to 2.02 cm (isolate 398).

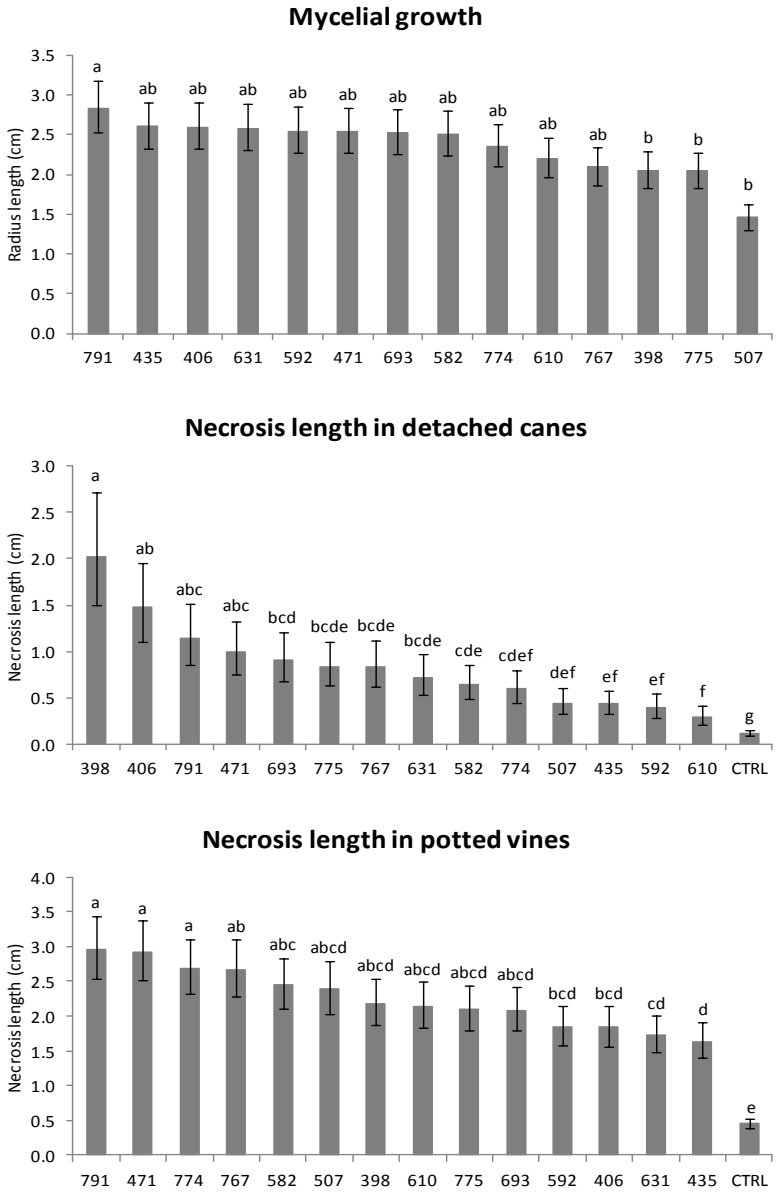

Figure 4.2. Mean mycelial growth rate (a) of *Diplodia seriata* isolates and mean necrotic lengths caused on grapevine cv. 'Tempranillo' detached canes (b) and potted vines (c). Bars correspond to the detransformed means and the upper and lower 95 % confidence intervals of the mean are shown as error bars. Statistically significant differences (P<0.05) are shown in different letters.

Both DAPC groups produced mean lesion lengths longer than the uninoculated control, with no statistically significant difference between the groups (Table 4.3). All *D. seriata* isolates were reisolated from the four 1 cm wood fragments of each cane from >85 % of inoculated canes except for isolate 767, which was reisolated from only 55 % of inoculated canes (data not shown). In the potted vine assay, all isolates and both DAPC groups produced lesions significantly longer than the control treatment (Figure 4.2c; Table 4.3). Mean necrosis values of isolates ranged from 1.61 cm (isolate 435) to 2.95 cm (isolate 791), and no differences in length necrosis were observed between the DAPC groups (Table 4.3). All isolates were reisolated from the four 1 cm fragments of each inoculated shoot from >70 % of inoculated vines except for isolate 767, which was reisolated from 68.3 % of inoculated vines (data not shown). No significant correlations were observed between the *in vitro* mycelia growth rates and the length of necrosis obtained in the pathogenicity assays and between the lesion lengths in both tests.

4.6. Discussion

The ISSR technique was useful for studying the intraspecific genetic variability among the *Diplodia seriata* isolates obtained from different hosts and geographic regions. Moreover, the pathogenicity tests performed with several isolates selected from the distinct genetic groups found in this study confirmed the existence of different levels of virulence in *D. seriata*, as demonstrated by differences in the length of necrosis produced by the isolates in artificially inoculated canes and potted vines cv. 'Tempranillo'. Bayesian clustering and multivariate analyses of ISSR markers provided a useful starting point to investigate the genetic structure of *D. seriata* isolates. The STRUCTURE and DAPC analyses identified two main groups of *D. seriata* isolates, although some isolates were not consistently adscribed to these genetic groups. Moreover, isolates from different geographic origins or from different hosts were not grouped in genetically distinct clusters. This suggests that the isolates share a similar genetic background regardless of their geographic and host origin. A similar conclusion was reported by Phillips *et al.* (2007), who found no correlation between the host origin of different *D. seriata* isolates and the clustering structure obtained from a phylogenetic study of this species based on ITS sequence data. Despite the establishment of only two groups by the

STRUCTURE and DAPC analyses, the five ISSR primers used in this study revealed a high percentage of polymorphism (88 %). This figure is consistent with the results of Martín *et al.* (2013), who found 86 % polymorphism in 13 Spanish *D. seriata* isolates using AFLP fingerprinting patterns. Similarly, Baskarathevan *et al.* (2012) observed 93 % polymorphism in 60 isolates of *Neofusicoccum parvum* by using a universally primed (UP)-PCR approach. Moreover, Billones-Baaijens *et al.* (2013a) observed 93 % polymorphism in 29 isolates of *Neofusicoccum luteum* using the same UP-PCR technique.

Morphological characters of both the sexual and asexual morphs have been used as taxonomical tools for differentiation and identification of Botryosphaeriaceae species for many years. With the increasing number of new Botryosphaeriaceae species being described in recent decades, the morphological characters used for species identification (dimensions, shape, septation and pigmentation of both conidia and ascospores) has been shown to be unreliable and has made species differentiation difficult, particularly for cryptic species (Pavlic *et al.*, 2009; Phillips *et al.*, 2013; Slippers *et al.*, 2013). Furthermore, phylogenetic inference is revealing cryptic species complexes that cannot be distinguished based on morphology alone (Pavlic *et al.*, 2009; Phillips *et al.*, 2013). However, in the current study the conidial morphology of *D. seriata* isolates corresponded well with relevant published descriptions (Punithalingam and Walker, 1973; Phillips *et al.*, 2007) and no significant differences were noticed between the DAPC groups. Only the isolates obtained from grapevine showed a significantly higher L:W ratio than isolates from other hosts, but the length and width means were not significantly different.

In the current study, typing of VCG was not supported by any clustering method as incompatibilities between isolates belonging to the same group were observed. However, compatibility reactions and partial compatibility were observed between isolates from different genetic clusters. It is suggested that the lack of correlation between genetic clustering and the vegetative compatibility observed in this study could be explained because the ISSR markers may not be designed to target the areas of the genome that regulate the mycelial compatibility. The lack of clear groups of VCG and both the compatible and partially incompatible reactions among isolates may signify weak barriers to anastomosis and genetic exchange between isolates, thereby

making gene flow possible between individuals. Baskarathevan *et al.* (2012) also reported weak barrage reactions in *N. parvum* from the observations of hyphal anastomosis between incompatible isolates. All *D. seriata* isolates tested for pathogenicity were able to induce brown necrotic lesions on grapevine detached canes and potted vines cv. 'Tempranillo', and all isolates but one (767) were reisolated from over 85 % of the inoculated canes and from over 70 % of the inoculated potted vines. All isolates were successfully reisolated from the lesions they caused and thus they should be considered as potential pathogens of grapevines. Although different symptoms such as dark vascular discolouration, perennial cankers and elongated black regions on the shoots have been associated with pathogenicity of Botryosphaeriaceae species on grapevine (Úrbez-Torres, 2011), the pathogenicity and virulence of these fungi are usually evaluated through their ability to cause brown necrotic lesions in the wood and the length of these lesions respectively, as has been previously described (Larignon *et al.*, 2001a; van Niekerk *et al.*, 2006; Úrbez-Torres, 2011). Larignon *et al.* (2001a) reported that isolates of *D. seriata* could be divided into four virulence groups depending on the lesion lengths caused in artificial inoculations on canes of grapevine cv. 'Cabernet Sauvignon'. Van Niekerk *et al.* (2004) also found that several *D. seriata* isolates showed a variable degree of grapevine wood colonization based on the length of the necrosis, which is confirmed by the present study. Similar findings regarding virulence variability in *D. seriata* have been reported by Úrbez-Torres and Gubler (2009) and Luque *et al.* (2009). Additional studies to those cited above have confirmed the pathogenicity of *D. seriata* by artificial inoculations on grapevine (Rovesti and Montermini, 1987; Auger *et al.*, 2004; van Niekerk *et al.*, 2006). However, in contrast to these findings, other authors have reported this species to be weakly pathogenic or probably saprophytic. In Portugal, isolates of *D. seriata* caused small lesions on wounded shoots of grapevine cv. 'Espadeiro' but did not infect unwounded shoots (Phillips, 1998), which suggests that this pathogen requires wounded tissue to initiate infection. Taylor *et al.* (2005) reported that *D. seriata* did not produce any lesions on grapevine cuttings cv. 'Red Globe' after artificial inoculation. Amponsah *et al.* (2011) observed that lesions in detached green shoots or attached shoots cv. 'Pinot Noir' that had been artificially inoculated with *D. seriata* did not extend significantly beyond the inoculation point. In the first report confirming the presence of *D. seriata* in Iran, the pathogenicity test showed that this fungus was pathogenic but caused small necrotic lesions in

cuttings of cv. 'Cabernet Sauvignon' (Mohammadi *et al.*, 2013). Pitt *et al.* (2013a) also considered *D. seriata* as weakly pathogenic on the variety 'Chardonnay' in a study carried out with isolates obtained from grapevines in South Australia and New South Wales. The results of the present study suggest that *D. seriata* is a weak pathogen to grapevine because only small necrotic lesions were observed, and neither foliar symptoms nor canker formation around the inoculation wounds were observed during the experiments. However, previous studies have shown that *D. seriata* is very frequently isolated from diseased grapevines in northeast Spain (Luque *et al.*, 2009) as well as from naturally infected pruned canes (Luque *et al.*, 2014). These findings would support the idea that this pathogen may cause serious infections on grapevines under natural conditions that are difficult to reproduce with artificial inoculations under laboratory or greenhouse controlled conditions.

According to various authors (van Niekerk *et al.*, 2004; Úrbez-Torres, 2011; Baskarathevan *et al.*, 2012), the differences found between pathogenicity tests could be a result of several factors including the differential susceptibility of grapevine varieties; the conditions, age and tissue type of the host plant; the inoculation method used; the geographic origin of isolates; and the different incubation periods of the experiments. Savocchia *et al.* (2007) stated that conflicting observations on the pathogenicity of *D. seriata* could be due additionally to the existence of intraspecific variability within the pathogen. In the present study, the necrotic lesions caused by the isolates were significantly longer than the control treatment and statistical differences were detected among isolates, thus demonstrating the existence of intraspecific variation in virulence. However, no difference in virulence was detected between the DAPC groups. Genetic variability and variation in pathogenicity were recently studied in other Botryosphaeriaceae species, with similar conclusions to those obtained in the present study. Thus, Baskarathevan *et al.* (2012) reported no relationship between the genetic groups and the virulence of isolates in *N. parvum*, and Billones-Baaijens *et al.* (2013a) reported no obvious genotype-pathotype relationships in *N. luteum*

The non-grapevine isolates used in the pathogenicity tests, namely 582 (from hazelnut), 592 (almond), 631 (cork oak) and 693 (apple), were irregularly distributed among all isolates tested for pathogenicity in terms of necrosis

length, thus suggesting that pathogenicity and virulence did not depend on the host origin. This was expected because of the known plurivorous character of *D. seriata* (Phillips *et al.*, 2007). In addition, the pathogenic behavior of the isolates was not correlated with their *in vitro* mycelial growth rate, as isolates that showed the highest *in vitro* growth did not necessarily produce the longest necrotic lesions in the pathogenicity assays. Martos *et al.* (2008) showed that *D. seriata* is able to produce secondary metabolites with phytotoxic properties, and it is possible that it may have other additional mechanisms apart from mycelial growth in the host that could be related to its virulence. However, this hypothesis remains to be studied in future research.

The phenotypic characterization did not show biological differences among the isolates that could potentially be related to the genetic clusters observed. In addition, pathogenicity tests showed that *D. seriata* is a weak pathogen and it is also variable in virulence, which was not related to either the geographic and host origin or their genetic variability. To the best of the authors' knowledge, this is the first study of genetic, phenotypic and pathogenicity variation made on a wide collection of *D. seriata* isolates.

4.7. Acknowledgements

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Capítol 5

Effect of the inoculum dose of three grapevine trunk pathogens on the infection of artificially inoculated pruning wounds

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5.1. Resum

En aquest estudi es van avaluar els percentatges d'infecció resultants de la inoculació artificial de ferides de poda de la vinya amb suspensions d'espores dels fongs patògens *Diplodia seriata*, *Phaeomoniella chlamydospora* i *Eutypa lata*. En el cas de *D. seriata* i *P. chlamydospora*, es varen usar plantes de la varietat 'Tempranillo' mantingudes en test, que es van inocular amb un rang d'entre 10 i 4000 conidis per ferida. El percentatge de recuperació de *D. seriata* va estar comprès entre 10 i 100 %, i el de *P. chlamydospora*, entre 16 i 94 %. *Eutypa lata* va ser inoculat en vinyes de la varietat 'Shiraz' i en sarments podats de la varietat 'Cabernet Sauvignon', en un rang de concentració d'entre 10 i 1000 ascòspores per ferida en ambdós casos. Els percentatges de recuperació del patogen en tots els assajos van estar compresos entre 17 i 95 %. En l'assaig de camp es van realitzar inoculacions úniques o dobles, aquestes darreres amb la mateixa dosi total d'espores que les úniques, i els sarments inoculats es van recol·lectar i processar per a recuperar el patogen en dos temps diferents, set o onze mesos després de la inoculació. No es van observar diferències significatives entre els dos tipus d'inoculació ni entre els dos temps d'incubació després de la inoculació. Els percentatges de recuperació obtinguts van presentar diferències significatives entre els experiments, fet que ja s'havia observat en estudis anteriors i que indica que altres factors com la virulència, els paràmetres ambientals i les condicions experimentals poden influir en el procés d'infecció. D'acord amb aquest estudi, per a obtenir un percentatge de reaïllament òptim (entre 50 i 70 %) s'han d'inocular de 100 a 1000 conidis de *D. seriata* per ferida, de 100 a 2000 conidis de *P. chlamydospora* i de 200 a 500 ascòspores d'*E. lata*.

5.2. Abstract

This study assessed the infection rates of different spore inoculum doses of the grapevine trunk pathogens *Diplodia seriata*, *Phaeomoniella chlamydospora* and *Eutypa lata* following artificial inoculation of pruning wounds. Potted vines of cv. 'Tempranillo' were inoculated with doses ranging from 10 to 4000 conidia per wound of *D. seriata* and *P. chlamydospora* and led to recovery percentages of 10-100 % for *D. seriata* and 16-94 % for *P. chlamydospora*. *Eutypa lata*, when inoculated onto wounds of vines in a mature

vineyard (cv. 'Shiraz') and on detached canes (cv. 'Cabernet Sauvignon') with a dose range of 10 to 1000 ascospores per wound, led to recovery percentages of 17-95 %. In the field assay, there was no difference in recovery from wounds that were exposed to single or double inoculations with the same total spore dose, or between canes that were harvested 7 or 11 months after inoculation. The results obtained in this study showed significant variability in pathogen recovery between trials, comparable with that reported previously, which suggests that factors such as pathogen virulence, environmental parameters and experimental conditions may influence the infection process. According to this study, in order to obtain optimal recovery percentages of 50-70 % for robust evaluation of pruning wound treatments, dose ranges of 100-1000 conidia of *D. seriata*, 100-2000 conidia of *P. chlamydospora*, and 100-500 ascospores of *E. lata* per wound would be required.

5.3. Introduction

Botryosphaeria dieback, esca and Eutypa dieback are three of the most serious diseases of grapevines (*Vitis vinifera* L.) worldwide. More than 20 Botryosphaeriaceae species have been associated with Botryosphaeria dieback (Úrbez-Torres, 2011). Grapevine disease symptoms caused by these fungi include leaf spots, fruit rots, shoot dieback, bud necrosis and perennial cankers (Luque *et al.*, 2009; Úrbez-Torres, 2011). *Diplodia seriata* (De Not.) is one of the species most frequently associated with dieback and decline symptoms in most wine regions around the world (Úrbez-Torres, 2011). *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams is considered one of the primary causal agents of esca and Petri diseases (Mugnai *et al.*, 1999; Edwards and Pascoe, 2004; Surico *et al.*, 2006). Esca is a disease complex where symptoms and their expression are highly variable (Mugnai *et al.*, 1999; Surico *et al.*, 2006). Most recognised foliar symptoms of esca are characterised by interveinal chlorosis or discolorations that coalesce in large necrotic areas (Surico *et al.*, 2006). Symptoms of Petri disease include reduced plant vigour, with retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting, and dieback (Gramaje and Armengol, 2011). *Phaeomoniella chlamydospora* is associated with necrotic lesions in the wood of esca and Petri affected vines which include brown spots and streaking in the xylem vessels. Eutypa dieback is caused primarily by the

fungus *Eutypa lata* (Pers.) Tul. & C. Tul., which reduces growth and yield in vineyards causing stunted growth of the shoots with short internodes, small, chlorotic and cupped leaves with marginal necrosis and V-shaped necrosis in cross-section of the wood (Sosnowski *et al.*, 2008).

Research studies evaluating pathogenicity, susceptibility of grapevine varieties and efficacy of control methods often rely on artificial inoculation with these pathogens. Different types of artificial inoculation have been routinely used; mycelial agar plugs placed in holes in the internode of stems, and spore suspensions which can be either vacuum-inoculated in grapevine canes, applied by soaking grapevine cuttings or seedlings or placed on wounded tissues. Mycelial agar plugs have been used as inoculum with *D. seriata* (Elena *et al.*, 2015b), other Botryosphaeriaceae species (van Niekerk *et al.*, 2004; Amponsah *et al.*, 2011), *P. chlamydospora* and *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai (Gramaje *et al.*, 2013; Mohammadi *et al.*, 2013), *E. lata* (Sosnowski *et al.*, 2007) and a wide range of other trunk pathogens (Úrbez-Torres *et al.*, 2012). Spore suspensions which are vacuum-inoculated into grapevine canes have been used with *P. chlamydospora* and *P. aleophilum* (Gramaje *et al.*, 2009b). Alternatively, spore suspensions of *Phaeoacremonium* species have been used in inoculations by soaking grapevine cuttings or seedlings (Aroca and Raposo, 2009). Other studies used spore suspensions as inocula of these fungi in inoculations on pruning wounds to better mimic natural infection by airborne fungal spores. A wide range of conidia or ascospore doses has been reported in scientific literature for inoculation of pruning wounds: lowest to highest, from 1000 to 20000 conidia per wound in studies carried out with *D. seriata* (Kotze *et al.*, 2011; Pitt *et al.*, 2012), from 4000 to 100000 conidia in the case of *P. chlamydospora* (Eskalen *et al.*, 2007; Rolshausen *et al.*, 2010a) and from 10 to 20000 ascospores for *E. lata* (Kotze *et al.*, 2011; Ayres *et al.*, 2014). Given the wide range of spore doses used in previous studies, this study aimed to determine the optimal dose range for artificial inoculation of pruning wounds by the grapevine trunk pathogens *D. seriata*, *P. chlamydospora* and *E. lata* under different experimental conditions.

5.4. Material and Methods

5.4.1. Plant material

Experiments performed with *Diplodia seriata* and *Phaeomoniella chlamydospora* were conducted on 5-year old potted vines of cv. 'Tempranillo' grafted onto Richter 110 rootstock located in Cabrils, Barcelona. Vines were maintained outdoors in 50 l pots filled with a peat: perlite mixture (1:1, v:v) and watered adequately to avoid water stress. In the case of *E. lata*, two different experiments were carried out. The first one was a field assay conducted on cv. 'Shiraz' vines grafted in 2001 onto 'Sauvignon Blanc' which was planted in 1985 at the Nuriootpa Research Centre in Barossa Valley, South Australia. The second experiment was a detached cane assay (DCA) using canes of cv. 'Cabernet Sauvignon' (clone 337) collected from a 30-year old vineyard grafted onto 101-14 and located in the experimental field of the Institute National de la Recherche Agronomique (INRA), in Bordeaux area (Château Couhins, Cadaujac, France). Canes were stored in a cool room at 5 °C before they were used in the assay.

5.4.2. Fungal isolates and inoculum production

Diplodia seriata CBS121485 and *Phaeomoniella chlamydospora* CBS121483 both collected in 2003 from diseased grapevines, cvs. 'Cabernet Sauvignon' and 'Carignan', respectively, were used for the potted vine assay. These isolates were previously maintained as mycelial plugs contained in tubes filled with sterile distilled water (SDW) kept at 4 °C. Spore suspension of *D. seriata* was prepared by modifying the method by van Niekerk *et al.* (2005) as follows. A mycelium plug of *D. seriata* was grown for 10 days on potato dextrose agar (PDA, Difco, Becton, Dickinson and Company) plates at 25 °C to generate enough mycelia for production of inocula. A mycelium plug of *D. seriata* was grown on water agar (WA, Bacto Agar, Becton, Dickinson and Company) plates with sterile 1 cm fragments of pine needles laid on the medium surface for 4 weeks at 25 °C under combined near UV and white fluorescent light (Philips TL-D 18W BLB and Sylvania Standard F18W/33-640-TS cool white, respectively) with a 12 h photoperiod. One day before inoculation, fragments of pine needles (*N*=40) with *D. seriata* pycnidia were placed in a beaker containing 30 ml SDW. The solution was kept overnight (16 h aprox.) at 4 °C to prevent early

germination of conidia and in constant agitation, with the help of a magnetic stirrer, to induce conidia release from the pycnidia. The next day, the suspension was vacuum-filtered through a 60 µm nylon mesh with a Steriflip filter (EMD Millipore Corporation) in order to remove debris and produce a cleaner conidial suspension of the fungus. *Phaeomoniella chlamydospora* was grown on PDA plates for 3 weeks at 25 °C in darkness. On the day of inoculation, 10 ml of SDW was added to each plate and the mycelia gently scraped with a sterile cotton stick in order to release conidia. The conidial suspension was recovered from the plate with a pipette.

Inocula of *Eutypa lata* were obtained from natural sources. For the field assay, dead grapevine wood with stromata of the pathogen was collected from a vineyard at the Nuriootpa Research Centre. Ascospore suspensions were obtained using a method adapted from Carter (1991) as follows: wood segments (approximately 3-4 cm^2) were soaked for 1 h in distilled water and then attached to polypropylene lids (70 mm diameter), which were screwed onto polycarbonate containers (300 cm^3) and left overnight to allow ascospores discharge. The following day, the ascospores were collected by adding approximately 5-10 ml of SDW. In the case of the DCA, *E. lata* perithecial stromata were collected from infected wood parts of vines cv. 'Cabernet Sauvignon' growing in Bordeaux area. Three or four pieces of stroma (approximately 0.5 cm^2) were immersed in tubes containing 2 ml SDW and agitated for 30 minutes with a rotary shaker to encourage ascospore release. The resulting spore suspension was collected.

In all experiments, spore suspensions were stored at 4 °C until inoculation to prevent early spore germination.

5.4.3. Inoculation procedures

Serial dilutions were performed by adding SDW and using a microscope and haemocytometer to obtain *Diplodia seriata* and *Phaeomoniella chlamydospora* conidial suspensions ranging in concentration from 2.5×10^2 conidia·ml^{-1} to 1×10^5 conidia·ml^{-1}. Vines were pruned in January 2011 leaving 4-5 buds per cane. For both pathogens, using a pipette, wounds were inoculated with 40 µl droplets of suspension corresponding to doses of 10, 100, 1000, 2000 and 4000 conidia per wound. A control treatment of 40 µl of SDW

was included. The potted vine assay was conducted as a fully randomized design with 20 vines (replications) per pathogen with six canes per vine treated with the different inoculum doses and the control treatment. To prevent natural infection, all of the wounds were sealed with Parafilm. This experiment was repeated in January 2012.

The *Eutypa lata* spore suspensions for the field experiment were prepared by serial dilution to make suspensions ranging in concentration from 5×10^2 ascospores·ml^{-1} to 5×10^4 ascospores·ml^{-1}, corresponding to 20 µl water droplets containing 10, 50, 100, 200, 500 and 1000 ascospores, which were applied to wounds with a pipette. Prior to inoculation, 0.05 % Tween 20 (BDH Laboratory Supplies) was added as a surfactant to each suspension. Additionally, double-inoculation treatments were included for 200 (100 × 2), 500 (250 × 2) and 1000 (500 × 2) ascospores per wound. On each vine, 10 canes were pruned to two buds on June 2013 with each vine assigned a treatment. The following day, wounds were moistened by spraying with SDW and inoculated. For the double-inoculation treatments, the second inoculation was performed 3 days after pruning. Non-inoculated control treatments were only sprayed with SDW. The field assay was set up as a randomised block design with 10 replications using 100 grapevines.

For the DCA, spore suspensions were prepared in a range from 5×10^2 ascospores·ml^{-1} to 5×10^4 ascospores·ml^{-1} using a microscope and haemocytometer and adding SWD, to provide 10, 50, 100, 200, 500 and 1000 ascospores per 20 µl droplet with which to inoculate the wounds. Canes of 4-5 buds were placed in pots (12.8 dm^3) with moistened sand (30 canes per pot). On the day of inoculation, canes were pruned leaving 2-3 buds as previously described (Lecomte *et al.*, 2004). Prior to cutting, the cane surface was sterilized with cotton wool soaked in 96 % ethanol. A control treatment was inoculated with SDW. After inoculation, canes were incubated at 15 °C with a 12 h photoperiod. Canes were sprayed with SDW and sand was moistened by a watering can with tap water twice a week throughout the experiment. The DCA was designed as a completely randomized design with 30 replications using seven pots. The experiment was repeated.

In all experiments, inocula viability was assessed by counting germinated spores under a microscope after plating 100 µl of spore suspensions onto PDA for *D. seriata*, *P. chlamydospora* and *E. lata* (field assay) and Malt Agar (MA, Bacto Malt Extract, Becton, Dickinson and Company) for *E. lata* (DCA) and incubating for 24 h at 25 °C.

5.4.4. Isolation procedures

In all experiments, pathogens were reisolated from inoculated canes to determine the relationship between inoculum dose and infection of wounds. In the potted vine assay, reisolations of *Diplodia seriata* and *Phaeomoniella chlamydospora* were performed four months after inoculation. Canes were cut about 20 cm below the pruning wounds and bark was removed with a sterile scalpel from the top 5 cm segment, including the pruning wound. The top 2 mm of the cane was discarded with sterile pruning shears and two further fragments of approximately 5 mm were cut. Fragments were surface-sterilized by soaking in 70 % ethanol for 4 minutes and then placed onto PDA amended with streptomycin sulphate at 50 $mg \cdot l^{-1}$. Plates were incubated at 25 °C until fungal colony growth allowed for pathogen identification (Crous and Gams, 2000; Phillips *et al.*, 2007) (3-4 days for *D. seriata* and 4-7 days for *P. chlamydospora*) and recovery percentages were calculated.

For the *Eutypa lata* field assay, treated canes from five replications were randomly selected and removed for assessment 7 months after inoculation, and the other five replications, 11 months after inoculation. In the laboratory, bark was removed from each cane using a sharp knife and then they were surface sterilized by soaking in 2.5 % sodium hypochlorite for 10 minutes and washed two times with SDW. Secateurs were sterilized by dipping blades into ethanol and flaming, and then used to cut wood chips (*ca*. 3 × 2 × 2 mm) from each side of the margin between the stained and apparently healthy wood. For each treated cane, 10 wood chips were randomly selected and plated onto PDA amended with streptomycin sulphate (25 $mg \cdot l^{-1}$). Cultures were incubated at 25 °C under fluorescent light with a 12 h photoperiod for 7 days and then assessed for the presence of *E. lata* based on culture morphology (Carter, 1991).

The DCA was assessed two weeks after inoculation. Canes were surface-sterilised by rapid flaming with 96 % ethanol before and after the bark was

removed with a sterile scalpel. Ten 1-mm-thick wood disks per cane were aseptically excised with the help of a cutter as used by Lecomte *et al.* (2004). Wood chips were plated onto MA supplemented with 50 $\mu g \cdot l^{-1}$ of chloramphenicol and placed on the medium, maintaining the order in which they were cut. Plates were incubated at 25 °C in dark conditions and assessed for the presence of *E. lata* as above.

5.4.5. Statistical analyses

Mean percentage recovery was calculated for each pathogen and inoculum dose in each experiment. All data were subjected to analysis of variance (ANOVA) using statistical procedures in SAS System v.9.2 software (SAS Institute Inc.). Prior to statistical analyses, mean percentage recovery of each pathogen was checked for normality and homoscedasticity criteria and transformed if necessary. The significance of differences among treatments was tested with ANOVA and least significant difference (LSD) test was used to detect differences among the means at the 5 % significance level. Regression equations were calculated for recovery percentages of each pathogen in relation to the inoculum doses.

5.5. Results

Germination tests of each pathogen after inoculation showed greater than 90 % germination in all cases (*data not shown*), indicating a similarly high viability of inocula in all experiments. Statistical analysis of data from potted vines and DCAs revealed significant differences ($P<0.05$) between repetitions so each experiment was analysed separately.

For the field assay with *Eutypa lata*, no significant differences ($P>0.05$) were found between canes removed at 7 and 11 months after inoculation, so all data were analysed together. In the potted vine assay, neither pathogen was recovered from the non-inoculated controls. When inoculated with doses of 10 to 4000 *Diplodia seriata* conidia per wound, pathogen recovery ranged from 44-100 % and 10-100 % for the two experiments, respectively (Figure 5.1). Recovery differed significantly ($P<0.05$) between conidia doses up to 100 in the first experiment and up to 1000 in the second experiment. In the case of *Phaeomoniella chlamydospora*, recovery ranged from 27-94 % and 16-80 % for

the series of inoculum doses in the two experiments, respectively (Figure 5.2). Recovery differed significantly (P<0.05) between conidia doses up to 1000 in the first experiment and up to 100, and then between 100 and 4000, in the second experiment.

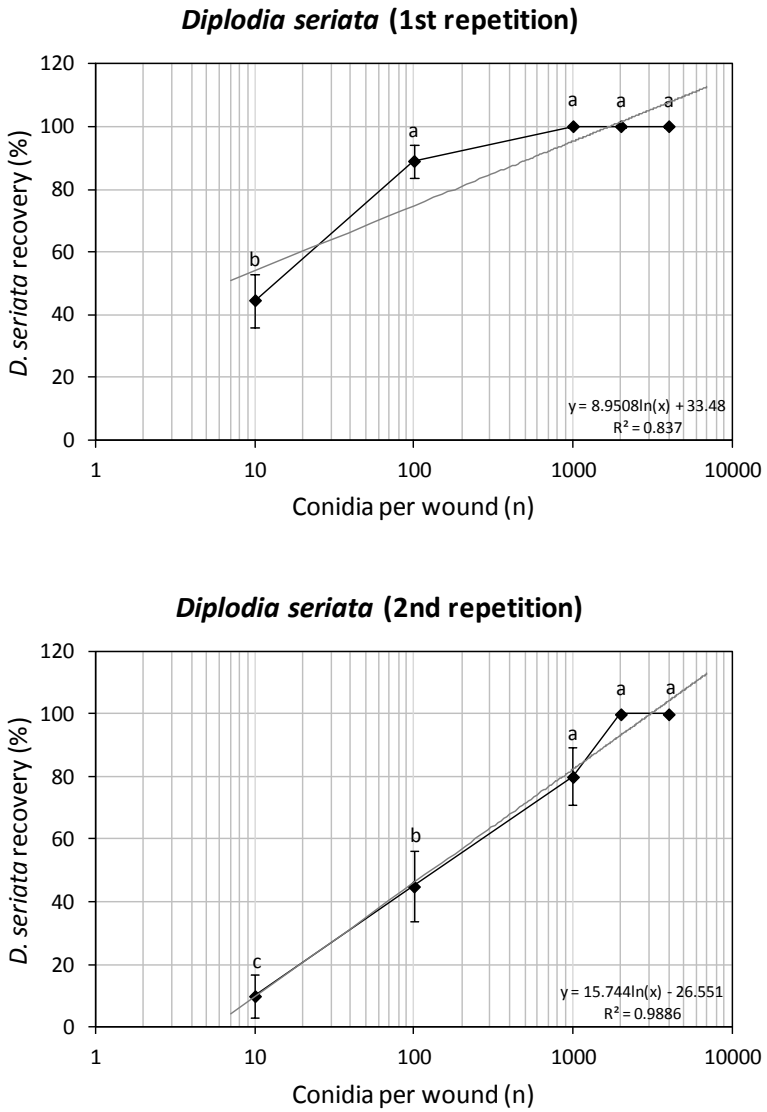

Figure 5.1. Mean percentage recovery from the two potted vine experiments (cv. 'Tempranillo') inoculated with *Diplodia seriata*. Twenty replications (canes) per pathogen were used for each experiment and two canes per vine allocated with each inoculum dosage of 10, 100, 1000, 2000 or 4000 conidia/wound. Significant differences among means (P<0.05) are indicated by different letters. Bars correspond to the standard error of the mean.

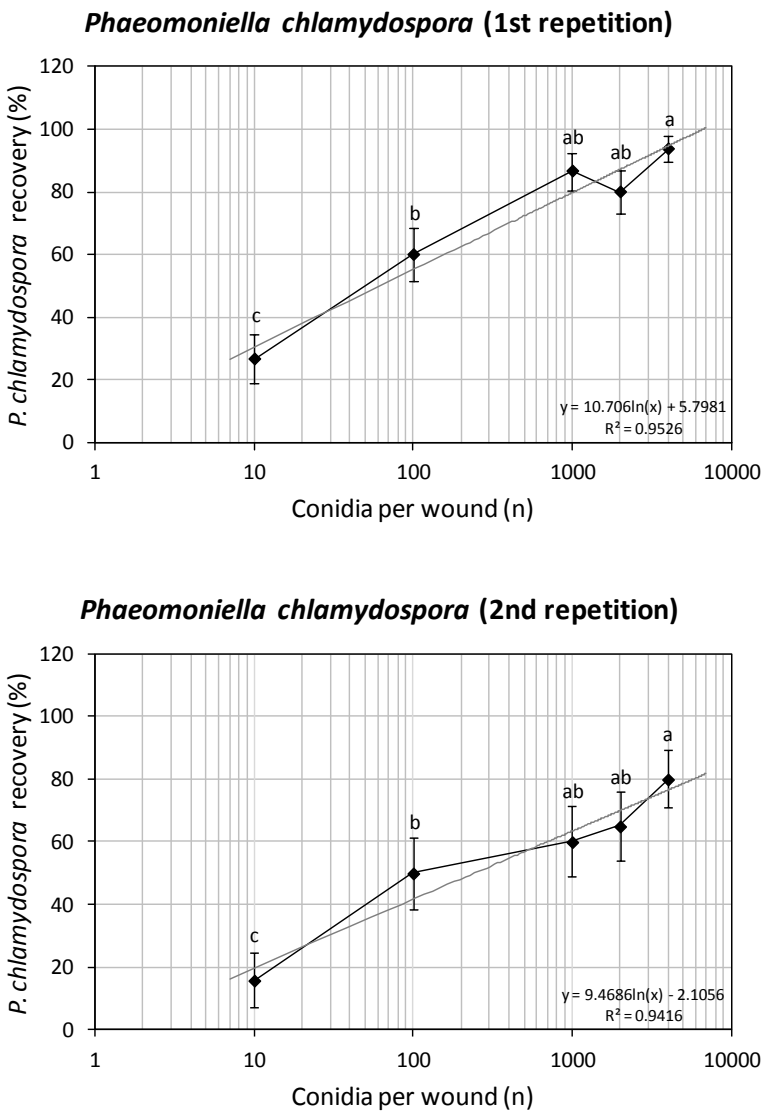

Figure 5.2. Mean percentage recovery from the two potted vine (cv. 'Tempranillo') experiments inoculated with *Phaeomoniella chlamydospora*. Twenty replications (canes) per pathogen were used for each experiment and two canes per vine allocated with each inoculum dosage of 10, 100, 1000, 2000 or 4000 conidia/wound. Significant differences among means (P<0.05) are indicated by different letters. Bars correspond to the standard error of the mean.

In the field assay, *E. lata* was recovered from 12 % of non-inoculated controls whereas pathogen recovery ranged from 27-95 % when inoculation doses ranged between 10 and 1000 ascospores (Figure 5.3). Recovery differed significantly (P<0.05) between conidia doses up to 500, apart from between 100 and 200 ascospore doses. There was no significant difference (P>0.05) in recovery between the single and double inoculations.

In the DCA, no *E. lata* was recovered from non-inoculated controls. Recovery of *E. lata* varied from 17-87 % and 23-70 % in the two DCAs, respectively (Figure 5.4). Recovery of *E. lata* differed significantly (P<0.05) between inoculum doses up to 200 ascospores in the first DCA, and up to 100 ascospores in the second DCA.

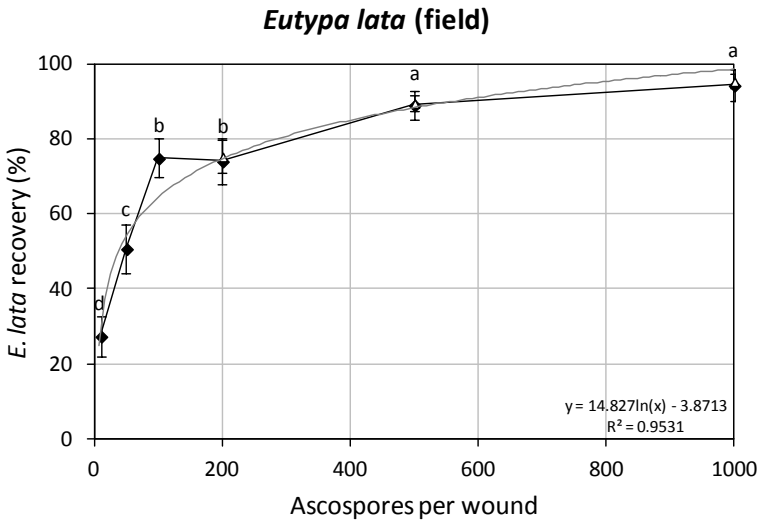

Figure 5.3. Mean percentage recovery from the field experiment (cv. 'Shiraz') inoculated with *Eutypa lata* at different inoculum dosages of 10, 50, 100, 200, 500 or 1000 ascospores/wound applied once (black rhombus) or twice (2 × 100, 2 × 250 and 2 × 500; white triangles). Ten replications (vines) were used with 10 canes/vine allocated to each inoculum dosage. Significant differences among means (P<0.05) are indicated by different letters. Bars correspond to the standard error of the mean.

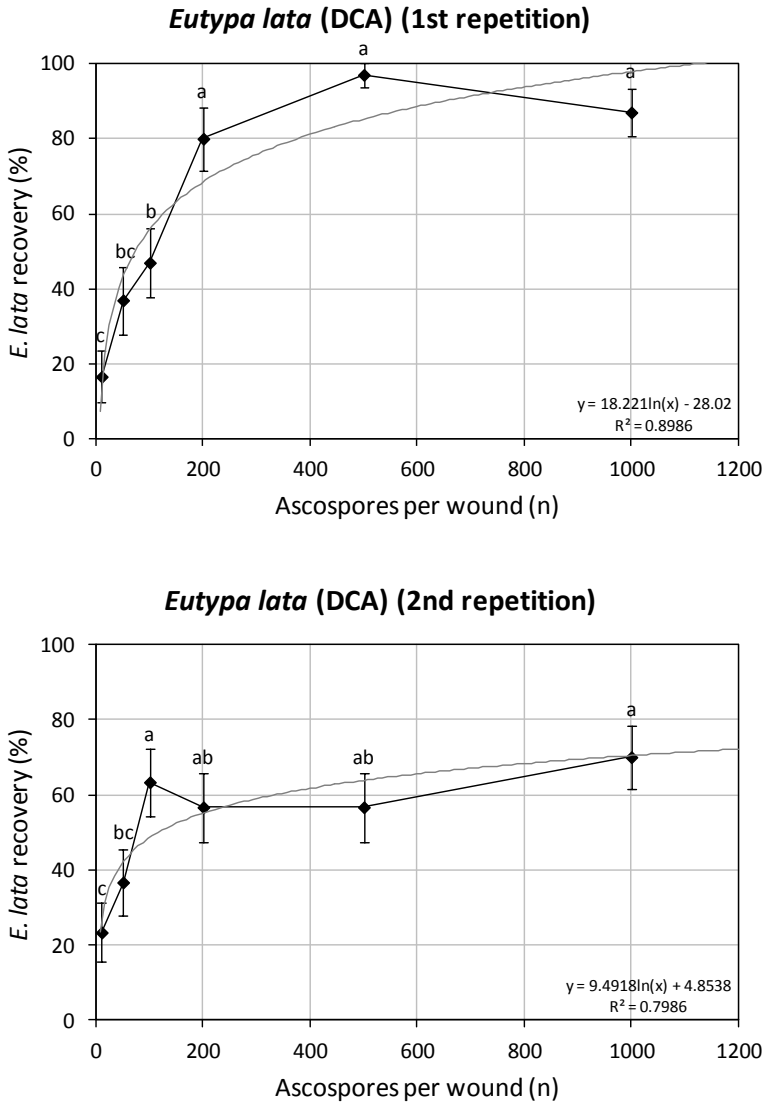

Figure 5.4. Mean percentage recovery from the two DCA experiments (cv. 'Cabernet Sauvignon') inoculated with *Eutypa lata*. Thirty replications (canes) were used in each experiment for each inoculum dosage of 10, 50, 100, 200, 500 or 1000 ascospores/wound. Significant differences among means (P<0.05) are indicated by different letters. Bars correspond to the standard error of the mean.

Regression equation analyses of mean recovery percentages of each pathogen over spore doses inoculated fitted logarithmic models with r^2-values between 0.80 to 0.99 (Figures 5.1-5.4).

5.6. Discussion

In this study, the recovery percentage of the grapevine pathogens *Diplodia seriata*, *Phaeomoniella chlamydospora* and *Eutypa lata* was evaluated in artificial inoculations of pruned canes using different inoculum doses. Fungal mycelia were recovered from vines at all doses evaluated with significant logarithmic relationships between dose rate and recovery percentage for all three pathogens. Significantly variable results occurred between repeated experiments in this study, which has also been reported in other studies (Table 5.1).

Recovery of *D. seriata* in this study was similar to that reported in a wound susceptibility study (82 %; Rolshausen *et al.*, 2010a) and a fungicide evaluation (70-80 %; Pitt *et al.*, 2012) when 1000 to 2500 conidia per wound were used. However, Serra *et al.* (2008) recorded a wide range of recovery (41-84 %) when the same dose of conidia was used, with variability between repetitions similar to the present study. Furthermore, Bester *et al.* (2007) evaluated different fungicides by inoculating 10000 conidia per wound, and the percentage recovery of the fungus in the non-treated and inoculated wounds did not exceed 40 %. However, in that study, inoculations were performed by spraying wounds with conidial suspensions, resulting in less accurate spore dosage compared with inoculating spore suspensions in a droplet. In another study, carried out to evaluate different biological control agents, Kotze *et al.* (2011) obtained 40 % recovery of *D. seriata* from an inoculated control treated with 20000 conidia per wound. In this experiment, wounds were inoculated 7 days after pruning, compared with 1 day in this study, by which time wound susceptibility may have decreased (Úrbez-Torres, 2011).

Table 5.1. Summary of previous experiments using artificial inoculation with various spore dosages of trunk disease pathogens, percentage of recovery, type of experiment, grapevine variety, type of plant material used and location of the experiment.

Pathogen	Dosage (spore/wound)	% Recovery	Type of experiment	Grapevine variety	Plant material	Location	Reference
Diplodia seriata	1000	82%	Wound Susceptibility	Chardonnay and Zinfandel	Field (vines)	California (USA)	Rolshausen *et al.* (2010a)
	1000	70-80%	Fungicide test	Semillon	Field (vines)	South Australia and New South Wales (AU)	Pitt *et al.* (2012)
	2500	41-84%	Wound Susceptibility	Sauvignon Blanc	Field (vines)	Italy	Serra *et al.* (2008)
	10000	39%	Fungicide test	Chenin Blanc	Greenhouse (canes)	South Africa	Bester *et al.*, (2007)
	20000	38%	Biocontrol test	Merlot and Chenin Blanc	Field (vines)	South Africa	Kotze *et al.*, (2011)
Phaeomoniella chlamydospora	1000	58%	Wound Susceptibility	Chardonnay and Zinfandel	Field (vines)	California (USA)	Rolshausen *et al.*, (2010a)
	3900-4200	5-25%	Wound Susceptibility	Cabernet Sauvignon	Field (vines)	France	Larignon and Dubos (2000)
	4000	15-51%	Wound Susceptibility	Sauvignon Blanc	Field (vines)	Italy	Serra *et al.*, (2008)
	10000	20-22%	Wound Susceptibility	Chenin Blanc	Field (vines)	South Africa	van Niekerk *et al.*, (2011)
	20000	58%	Wound Susceptibility	Cultivated and wild varieties	Greenhouse (potted vines)	California (USA)	Travadon *et al.*, (2013)
	20000	35%	Biocontrol test	Merlot and Chenin Blanc	Field (vines)	South Africa	Kotze *et al.*, (2011)
	100000	26-60%	Wound Susceptibility	Thompson Seedless and Cabernet Sauvignon	Field (vines)	California (USA)	Eskalen *et al.*, (2007)
	100000	94%	Pathogenicity test	Periquita	Field (vines)	South Africa	Halleen *et al.*, (2007b)
Eutypa lata	10-500	20-80%	Inoculum dose	Shiraz	Detached cane assay	South Australia (AU)	Ayres *et al.*, (2014)
	100-200	60-72%	Fungicide test	Cabernet Franc and Cabernet Sauvignon	Field (vines)	France	Lecomte *et al.*, (2004)
	100-1000	60-90%	Fungicide test	Cabernet Sauvignon	Field (vines)	France	Lecomte and Bailey, (2011)
	100-1000	30-80%	Wound Susceptibility	Grenache	Field (vines)	California (USA)	Petzoldt *et al.*, (1981)
	250	14%	Wound Susceptibility	Concord	Field (vines)	Michigan (USA)	Trese *et al.*, (1980)
	500-1000	29-74%	Fungicide test	Cabernet Sauvignon	Field (vines)	South Australia (AU)	Sosnowski *et al.*, (2008)
	500-1000	19-79%	Fungicide test	Cabernet Sauvignon	Field (vines)	South Australia (AU)	Sosnowski *et al.*, (2013)
	1000	48%	Fungicide test	Cabernet Sauvignon	Field (vines)	South Africa	Halleen *et al.*, (2010)
	1000	28%	Wound Susceptibility	Chardonnay and Zinfandel	Field (vines)	California (USA)	Rolshausen *et al.*, (2010a)
	1500	22-26%	Wound Susceptibility	Merlot	Field (vines)	France	Chapuis *et al.*, (1998)
	10000	15-20%	Wound Susceptibility	Chenin Blanc	Field (vines)	South Africa	van Niekerk *et al.*, (2011)
	20000	38%	Biocontrol test	Merlot and Chenin Blanc	Field (vines)	South Africa	Kotze *et al.*, (2011)

Previous studies with *P. chlamydospora* (Larignon and Dubos, 2000; Serra *et al.*, 2008; Rolshausen *et al.*, 2010a) which used similar spore dose ranges to the current study, reported lower recovery percentages (5-58 %) compared with 60-94 % obtained in the current study. Rolshausen *et al.* (2010a) observed a reduced wound colonization of *P. chlamydospora* when vines were artificially inoculated one week versus one day after pruning. Moreover, when using higher doses of 10^4-10^5 spores per wound, several authors obtained a large range of recovery percentages (Eskalen *et al.*, 2007; Halleen *et al.*, 2007b; Kotze *et al.*, 2011; van Niekerk *et al.*, 2011; Travadon *et al.*, 2013), but in general, they were lower than the recovery percentage obtained in the present study (Table 5.1).

In field and detached cane assays conducted with *E. lata*, the range of recovery rates was similar to those reported in other studies when the same amount of ascospores was inoculated per wound (Petzoldt *et al.*, 1981; Lecomte *et al.*, 2004; Sosnowski *et al.*, 2008; Lecomte and Bailey, 2011; Sosnowski *et al.*, 2013; Ayres *et al.*, 2014). In this study, when 200-500 ascospores per wound were inoculated, 57-97 % recovery was obtained, whereas in other studies, infection was lower than 50 % at the same or greater inoculum dosage (Trese *et al.*, 1980; Chapuis *et al.*, 1998; Halleen *et al.*, 2010; Rolshausen *et al.*, 2010a; Kotze *et al.*, 2011; van Niekerk *et al.*, 2011). These differences in recovery percentages may be due to intraspecific variation in pathogenicity, which has been previously reported for *E. lata* (Sosnowski *et al.*, 2007). Moreover, Chapuis *et al.* (1998) showed that temperature was positively correlated with epiphytic contaminant fungal populations, which may reduce the ability of *E. lata* to infect the pruning wounds and, consequently, also reduce pathogen recovery. Munkvold and Marois (1995) also reported in grapevines a strong positive correlation between temperatures after pruning and the rate of colonisation of pruning wounds by naturally occurring epiphytes which may act as competitors in wound colonisation by *E. lata*.

In the current field assay, double inoculations carried out with 200, 500 and 1000 ascospores did not produce a higher percentage of pathogen recovery, thus showing that single inoculations are sufficient to produce similar infection to double inoculations. These results were consistent with those obtained by Sosnowski *et al.* (2013) where single and double inoculations were

used without clear trends of improved recovery. With respect to the incubation period, no differences in *E. lata* recovery were found when canes were harvested at 7 and 11 months after inoculation, indicating that a shorter time of incubation before assessment can be considered, to obtain results earlier in the season. In this study, *E. lata* was recovered from 12 % of uninoculated controls in the field experiment, representing the natural disease pressure. The same percentage of natural infection was reported by Sosnowski *et al.* (2013). Luque *et al.* (2014) observed percentages of natural infections from 0.4 % to 3.2 % in case of *D. seriata* and from 0.4 to 2 % for *P. chlamydospora*. The aim of our experiments was to determine optimal spore dose ranges for each pathogen that will produce higher disease pressure than encountered under natural field conditions, in order to provide robust evaluation of treatments (*e.g.* wound protectants) without imposing unrealistically high disease pressure.

In the present and in other studies (Table 5.1), high variability in pathogen recovery was observed when the same spore doses were applied to wounds. The establishment of a pathogen in grapevines is a result of different factors including i) environmental parameters (Serra *et al.*, 2008; Sosnowski *et al.*, 2011a), ii) susceptibility of the grapevine variety (Sosnowski *et al.,* 2007; Travadon *et al.*, 2013), iii) age of the plant tissue (Trese *et al.*, 1980; van Niekerk *et al.*, 2004), iv) virulence and geographic origin of the isolate (Savocchia *et al.*, 2007; Sosnowski *et al.*, 2007) and v) the experimental conditions (*e.g.* inoculation and isolation methods; Elena *et al.*, 2014). Based on the authors' experience and results from previous studies listed in Table 5.1, inoculated control recoveries of 50-70 % are ideal for the pathogens tested here. Therefore, to achieve this range of recoveries, dose ranges of 100-1000 conidia of *D. seriata*, 100-2000 conidia of *P. chlamydospora*, and 100-500 ascospores of *E. lata* per wound would be required. Due to the high variability of recovery percentages observed in the current and previous studies discussed here, it is recommended to conduct a preliminary assessment of the optimal inoculum dosage range when planning artificial inoculations with these pathogens.

5.7. Acknowledgements

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Capítol 6

Pruning debris of grapevines as a potential inoculum source of *Diplodia seriata*, causal agent of Botryosphaeria dieback

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Enviat a *European Journal of Plant Pathology*.

6.1. Resum

En aquest estudi s'ha avaluat el paper de l'alliberament de conidis de *Diplodia seriata*, originats en restes de poda de la vinya, com a font d'inòcul potencial per a noves infeccions. Dos anys després d'una poda, es van recol·lectar restes de sarments que havien estat colonitzats de forma natural per *D. seriata*. Les variables analitzades varen ser el nombre de picnidis que contenien conidis, el nombre mitjà de conidis per picnidi i el percentatge de germinació dels conidis, que es van avaluar just en el moment de la recol·lecció dels sarments i 6, 12 i 18 mesos després. Durant l'experiment es va observar una reducció significativa del percentatge de picnidis que contenien conidis. El nombre mitjà de conidis per picnidi es va reduir significativament des de 270 conidis per picnidi, 24 mesos després de la poda, fins a 25 conidis per picnidi, al final de l'experiment. El percentatge de germinació dels conidis també es va reduir de forma significativa, del 63 % al 44 %, durant l'assaig. Els resultats confirmen la reducció de la pressió d'inòcul de *D. seriata*. Tot i això, 42 mesos després de la poda encara es van trobar picnidis que produïen conidis capaços de germinar i, per tant, de naturalesa infectiva.

6.2. Abstract

Conidia release of *Diplodia seriata* occurring in grapevine pruning debris was studied to determine its role as a potential inoculum source for new infections. Pruned canes naturally colonised by *D. seriata* were collected two years after pruning. Conidia release was evaluated at the collection time and 6, 12 and 18 months later. In each monitoring period, the percentage of pycnidia containing conidia, the average amount of conidia per pycnidium and the conidial germination percentage were assessed. A significant decrease in the percentage of pycnidia containing conidia was observed throughout the experiment. The average amount of conidia per pycnidium was reduced significantly, from 270 conidia per pycnidium 24 months after pruning, down to 25 conidia per pycnidium at the end of the assessment period. Germination percentage of conidia also decreased significantly, from 63 % to 44 %. Overall, these results confirmed the reduction in the *D. seriata* inoculum pressure through all the studied variables. However, 42 months after pruning pycnidia of

D. seriata still were capable to produce conidia able to germinate and thus to be infective.

6.3. Introduction

About 28 Botryosphaeriaceae species have been associated with the Botryosphaeria dieback of grapevine to date (Úrbez-Torres, 2011; Úrbez-Torres *et al.*, 2012; Correia *et al.*, 2013; Pitt *et al.*, 2013b; Linaldeddu *et al.*, 2014). These species have been recognized as important pathogens of grapevines (*Vitis vinifera* L.), resulting in significant economical losses in different grape growing regions in the world (van Niekerk *et al.*, 2004; Úrbez-Torres *et al.*, 2006; Úrbez-Torres, 2011). Pycnidia of different Botryosphaeriaceae species associated with canker and dieback diseases can be found in old pruning wounds, infected spurs, embedded in the bark of cordons or trunks of infected grapevines, and additionally in pruning debris left in the vineyard (Úrbez-Torres *et al.*, 2010; van Niekerk *et al.*, 2010). The airborne inoculum coming from these pycnidia is specially released during rainfall periods or during overhead sprinkler irrigation (Spagnolo *et al.*, 2014). Spore of *Diplodia seriata* De Not., *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not., *Dothiorella* spp., *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Diplodia mutila* Fr. apud Mont. and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. were observed in California during winter (Úrbez-Torres *et al.*, 2010). However spores of *D. seriata*, *D. mutila* and less frequently *N. parvum* and *Botryosphaeria quercuum* (Schw.) Sacc. were mostly detected between April and October in France (Kuntzmann *et al.*, 2009). Van Niekerk *et al.* (2010) showed that the presence of airborne inoculum of *Diplodia* and *Dothiorella* is also depending on rainfall and high relative humidity events. Regarding other species associated to grapevine trunk diseases, fruiting bodies of *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams and *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai have been observed inside cracks in the wood (Edwards *et al.*, 2001a; Rooney-Latham *et al.*, 2005) and their spore release is correlated with rainfall (Larignon and Dubos, 2000; Eskalen and Gubler, 2001). For *Eutypa lata* (Pers.:Fr.) Tul. & C. Tul., ascospores are released from mature perithecia following periods of rainfall exceeding 0.5 mm per day, mainly during winter in California (Ramos *et al.*, 1975; Petzoldt *et al.*, 1983) and from spring to fall in France (Kuntzmann *et al.*, 2009). It is widely

accepted that the airborne inocula of the grapevine trunk pathogens are responsible for the infection of grapevine pruning wounds, therefore largely contributing to the natural spread of diseases in mature vineyards (Rolshausen *et al.*, 2010a; van Niekerk *et al.*, 2011; Luque *et al.*, 2014).

Diplodia seriata is one of the most cited species occurring on grapevines worldwide, which has been frequently associated with the Botryosphaeria dieback (Úrbez-Torres, 2011; Bertsch *et al.*, 2013). It is known to produce spring bud mortality, leaf spots, fruit rot and trunk dieback, with brown necroses of the wood which appear as V-shaped necroses in cross sections of the affected plant parts (Luque *et al.*, 2009; Úrbez-Torres, 2011). This species was shown to be the most abundant in Catalonia, Northeast Spain (Luque *et al.*, 2009), where the present study was carried out. In a recent study in the same region, *D. seriata* was the most frequently species isolated from naturally infected pruning wounds (Luque *et al.*, 2014). In Catalonia, grapevine pruning debris is sometimes left in a pile close to the vineyards, without any further treatment, or just burnt when canes become dried. In some cases, canes are cleaved and incorporated into the soil with the aid of a tractor. Over time, canes which are spontaneously left on the ground and not further processed for removal or elimination can naturally develop fruiting bodies of Botryosphaeriaceae species, mainly *D. seriata* (Luque *et al.*, *unpublished*), as well as other fungi such as *Cryptovalsa ampelina* (Nitschke) Fuckel (Luque *et al.*, 2006). To the best of our knowledge, no data have been reported to date about the abundance of *D. seriata* in grapevine pruning debris and the potential role of infected canes as an inoculum source for the spread of the disease. The purpose of this research was therefore to study the evolution of the conidia release of *D. seriata* from naturally infected pruning debris as well as to assess the viability of the conidia being released. The knowledge about the role of grapevine pruning debris as a potential inoculum source would contribute to a better understanding of the pathogen life cycle and may also help in estimating the risk of disease development in the vineyard.

6.4. Material and Methods

6.4.1. Plant material

In November 2012 pruned canes were collected from a pruning debris pile in the margin of a vineyard cv. 'Cabernet Sauvignon' located in Pla del Penedès, Catalonia, Spain. Canes were pruned about two years earlier in winter season 2010-2011 although pruning date was not exactly known. Previous observations (Elena and Luque, *unpublished*) showed that 1-year old pruning debris was bearing occasionally *Diplodia seriata* pycnidia on the bark surface whereas pycnidia on 2-year old pruned canes were more abundant and thus this kind of plant material was used in our experiment. In the laboratory, canes were cut into 15 cm fragments with pruning shears and inspected for the presence of pycnidia of *D. seriata* on the bark surface with the aid of a stereomicroscope and a microscope. Only canes which were confirmed to be consistently infected by *D. seriata* were further used in the experiment. A total of four samples composed of 18 randomly selected cane fragments each were prepared and placed separately into plastic mesh bags (20 × 40 cm). Samples were placed outdoors for further monitoring of conidia production and release, on a 2 × 2 m ground area of an experimental vineyard located at IRTA research centre.

6.4.2. Assessment of conidia production and release

Samples of grapevine canes were processed at different times after collection: at the same time of collection (about 24 months after pruning) and 6, 12 and 18 months after samples collection (*i.e.* about 30, 36 and 42 months after pruning). In each monitoring period, a plastic bag was randomly selected and canes were randomly divided into two batches of nine canes each. In each fragment of the first batch of canes, fifty pycnidia occurring on the bark surface and resembling those of *Diplodia seriata* were randomly chosen and cross-sectioned to reveal their content. With the aid of a stereomicroscope each pycnidium was scored as containing *D. seriata* conidia, thus appearing to show a full content, or empty. In each of the remaining nine fragments in the sample, a known area (~0.5 cm^2) was selected and the total number of *D. seriata*-like pycnidia present in the area was counted. The bark of these areas was cut and removed with the help of a sterile scalpel and the resulting bark fragments were placed separately in Falcon® tubes (15 ml) containing 2 ml of sterile distilled

water. Tubes were left horizontally in an orbital shaker (Gyrotory Water Bath Shaker G76, New Brunswick Scientific) and kept overnight at 220 rpm, 4 °C, to promote release of conidia into the suspension. Subsequently, conidial concentration and germination percentages were determined three times per each tube. Conidial concentration was measured with a haemocytometer to estimate the average number of conidia per pycnidium. For the assessment of conidial germination, 100 µl of a conidial suspension were placed in the centre of a potato dextrose agar plate (PDA, Difco, Becton, Dickinson and Company) and the suspension was spread with a Digralsky spreader. Three plates per each conidial suspension were separately prepared. Plates were incubated at 25 °C in darkness for 24 h, and germination of 100 randomly selected conidia per plate was determined. A conidium was scored as germinated if at least the germinative hypha accounted for 1.5 times the length of the conidium.

6.4.3. Weather data

One month (31 days) before each assessment period, daily records of mean temperature, accumulated rainfall and mean relative humidity were obtained from either two automatic weather stations located near the samples. The first weather station was located 3 km away where pruning debris were collected to obtain the records for the first assessment time. A second device was located roughly 10 m away from the pruning debris in subsequent assessments (6, 12 and 18 months after cane collection).

6.4.4. Statistical analyses

Experimental data were analysed using SAS Enterprise Guide v.4.2 running on SAS System v.9.2 software (SAS Institute Inc.). Prior to the statistical analyses, data from the dependent variables (percentage of pycnidia containing conidia, amount of conidia per pycnidium and percentage of conidia germination) were checked for normality and homoscedasticity criteria and transformed if necessary. Data were analysed using one-way ANOVA to check for significant effects of the monitoring period on the dependent variables. After ANOVA, Tukey's Honestly Significant Difference test was used to detect differences among means at the 5 % significance level. Pearson correlation coefficients were calculated separately between each dependent variable and

either the length of the assessment period or the weather data when appropriate.

6.5. Results

Statistical analyses showed that the length of the monitoring period had significant effects on the percentage of pycnidia containing conidia (P<0.0001), the average amount of conidia per pycnidium (P=0.0027) and the conidial germination percentage (P<0.0001). In order to obtain normal and homoscedastic distributions, data from the estimated number of conidia per pycnidium was log-transformed whereas conidial viability was arcsine-transformed prior to the analyses. Percentage of pycnidia containing conidia at the beginning of the experiment was 64 % of observed pycnidia, which significantly fell below 50 % in the last two assessment periods, namely 42 % (36 months after pruning) and 46 % (42 months). The reduction in the number of pycnidia containing conidia was progressive along the experiment and accounted for 28.1 % at the end of assessments (Figure 6.1). No significant correlation was observed between the number of pycnidia containing conidia and the monitoring period (r=-0.87, P=0.1274).

The estimated amount of conidia per pycnidium at the beginning of the study was 270, and it was drastically reduced to 25 conidia per pycnidium at the end of the experiment. The reduction in the final number of conidia was over 90 % (Figure 6.2). Significant correlation between the amount of conidia per pycnidium and the monitoring period was found (r=-0.99, P=0.0128).

Percentage of conidial germination was significantly reduced from 64 % at the beginning of assessments to 44 % at the end of the experiment (Figure 6.3), which roughly indicated a loose in 20 % of conidia viability at the end of the experiment. However, a significant increase in conidial viability, above 80 %, was detected in the second assessment date (Figure 6.3). Correlation between percentage of conidial germination and the monitoring period was not significant (r=-0.65, P=0.3533).

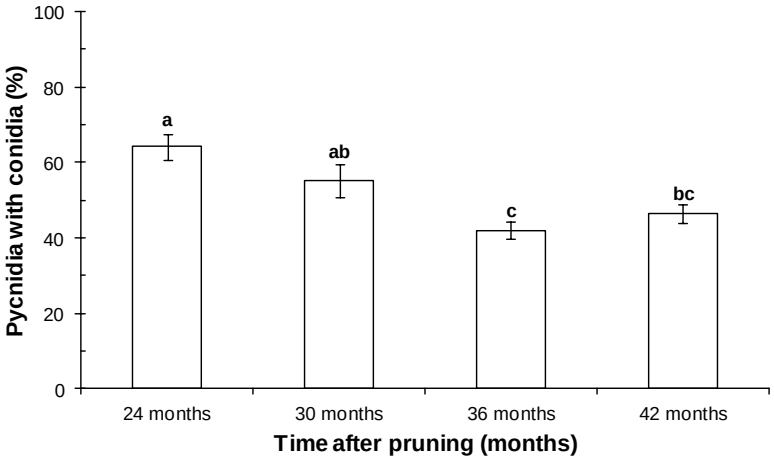

Figure 6.1. Mean percentage of pycnidia of *Diplodia seriata* containing conidia found in grapevine pruning debris at different times after pruning. Significant differences (P<0.05) among means (N=9 replications, with N=50 pycnidia each) are indicated by different letters. Bars correspond to the standard error of the mean.

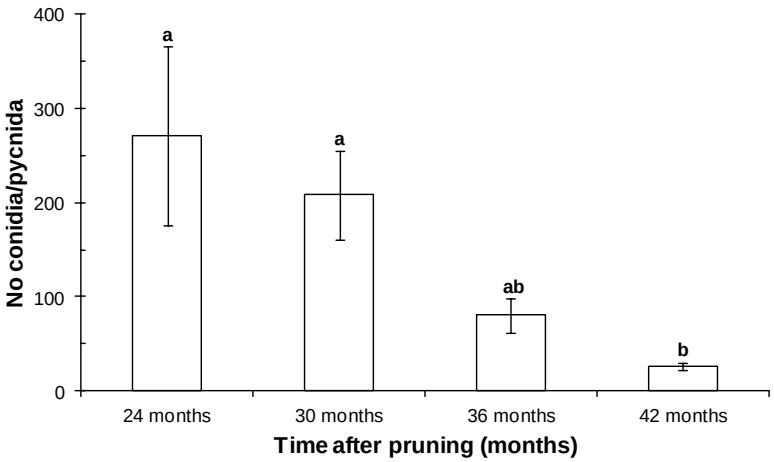

Figure 6.2. Mean amount of *Diplodia seriata* conidia per pycnidium found in grapevine pruning debris at different times after pruning. Significant differences (P<0.05) among means (N=9 replications, with N=3 pseudoreplicates each) are indicated by different letters. Bars correspond to the standard error of the mean.

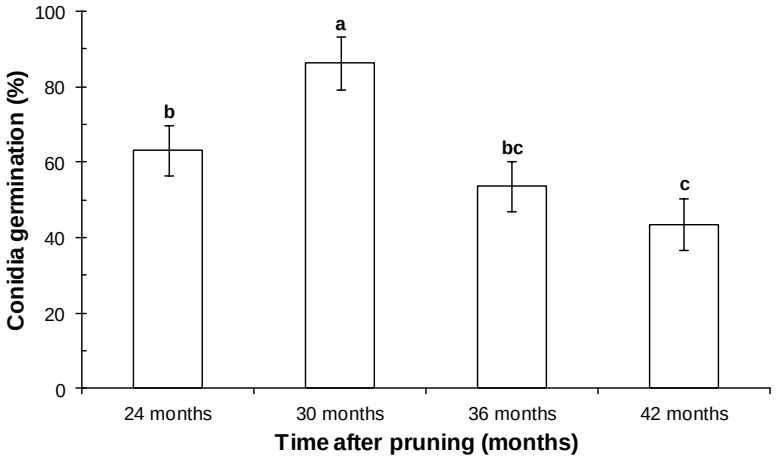

Figure 6.3. Mean germination percentage of *Diplodia seriata* conidia found in pruning debris at different times after pruning. Significant differences (P<0.05) among means (N=9 replications, with N=3 pseudoreplicates each) are indicated by different letters. Bars correspond to the standard error of the mean.

Main meteorological data recorded during the experimental period are shown in Table 6.1. Mean temperature was lower during the two assessments carried out in autumn (13 °C) compared with those done in spring (14 °C and 16 °C). Negative correlations were observed between the mean temperature and the three variables evaluated in this study, although none of them were significant (T *vs* full pycnidia: r=-0.23, P=0.7739; T *vs* conidia per pycnidium: r=-0.54, P=0.4624; T *vs* conidial germination: r=-0.30, P=0.7032). Accumulated rainfall was around 73-107 mm during the three first assessments whereas in the last one was only 7 mm (Table 6.1). Non significant positive correlations were found between rainfall values and the studied parameters (Rainfall *vs* full pycnidia: r=0.12, P=0.8769; Rainfall *vs* conidia per pycnidium: r=0.45, P=0.5455; Rainfall *vs* conidia germination: r=0.27, P=0.7336). The mean relative humidity was variable between the two assessments in autumn (75 % and 59 %), with intermediate values in spring (around 65 %) (Table 6.1). Non significant positive correlations were found between the relative humidity values and the three variables concerning inoculum production and release (Humidity *vs* full pycnidia: r=0.84, P=0.1605; Humidity *vs* conidia per pycnidium: r=0.56, P=0.4439; Humidity *vs* conidia germination: r=0.02, P=0.9791).

Table 6.1. Daily records of mean temperature (°C), accumulated rainfall (mm) and mean relative humidity (%) one month (31 days) before each assessment. Mean values are followed by the (±) standard error when appropriate.

Monitoring peridod[a]	Sampling season	Mean temperature (°C)	Accumulated rainfall (mm)	Mean relative humidity (%)
24 motnhs	Autumn	12.91±0.57	106.6	75.00±2.29
30 months	Spring	14.20±0.39	73.2	65.29±1.57
36 months	Autumn	12.57±0.70	131.3	59.10±2.22
42 months	Spring	16.35±0.23	7.2	68.39±1.40

[a]Time after pruning.

6.6. Discussion

Results obtained in this study described the evolution of the conidial inoculum of *Diplodia seriata* in grapevine pruning debris from a vineyard in NE Spain. All studied variables, namely the percentage of pycnidia containing conidia, the amount of conidia per pycnidium and the conidial viability decreased significantly between 2 and 3.5 years after pruning, which means a reduction of the inoculum pressure in the vineyard. The most sharply decrease was observed in the number of conidia present in pycnidia, suggesting that the most important part in the reduction of the inoculum pressure along time depends on conidia production. However, infective inoculum, *i.e.* viable conidia, were still detected 3.5 years after pruning, which is indicative of the long lasting period of the inoculum source occurring on grapevine pruning debris.

The first observation of the experiment was in autumn 2012 (24 months after pruning) coinciding with the beginning of the pruning season and with a major inoculum pressure resulting from a combination of higher values in all studied variables regarding fungal inoculum. A significant increase of the percentage of conidia germination was observed during the first assessment done it in spring (30 months after pruning). However, this was not observed during the second assessment also done in spring (42 months), probably due to the low rainfall recorded that season and a potential loss of viability with age. Whereas conidia germination of *Botryosphaeria* species is associated to rainfall

periods (Úrbez-Torres *et al.*, 2010; van Niekerk *et al.*, 2010) no data on decrease of conidium viability related to age is available for *D. seriata*.

Weather data were recorded one month before each assessment period, which is roughly the time needed for *D. seriata* to produce mature fruiting bodies (van Niekerk *et al.*, 2005). None of the meteorological variables were significantly correlated with the variables describing inoculum evolution. However, accumulated rainfall showed the highest values at 36 months, coinciding with a marked depletion of pycnidia recorded during the experiment. Spore-trapping studies in France and in California reported higher numbers of Botryosphaeriaceae spores trapped during rain events (Kuntzmann *et al.*, 2009; Úrbez-Torres *et al.*, 2010). Michailides and Morgan (1993) and Ahimera *et al.* (2004) reported that spores of *Botryosphaeria dothidea* seems to be primarily splash-dispersed by water, therefore the impact of water drops on the fruiting bodies would be necessary for spore dispersal. Consequently, Botryosphaeriaceae spores may not be dispersed in dry periods (Úrbez-Torres *et al.*, 2010). Amponsah *et al.* (2009) reported that spores of *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum parvum* and *Diplodia mutila* were most frequently trapped in summer in New Zealand, when rainwater run-off occurred, also indicating that rainfall provided the mechanism for conidium dispersal.

We conducted an extraction of conidia in water in a similar way that Munck and Stanosz (2008) used to quantify the abundance of conidia of *Diplodia pinea* (Desm.) Kickx and *Diplodia scrobiculata* J. de Wet, Slippers & M.J. Wingf. in cones of jack and red pines in Wisconsin, USA. We are aware that the water extraction potentially yielded both the conidia that were into the pycnidia and those which were already released from the pycnidia but present on the bark surface of cane samples. However, since we counted all the pycnidia of *D. seriata* present in the samples processed for conidia extraction we decided to express the total amount of extracted conidia as the mean number of conidia per pycnidium.

To the best of our knowledge, this is the first study aiming at quantifying the conidia release of *D. seriata* from grapevine pruning debris, and thus to

estimate the role of infected pruned canes as an inoculum source in the vineyard. Our results suggest that pruning debris left close to the vineyard becomes an important long-lasting inoculum source of *D. seriata* for new infections. Hence, elimination of pruning debris from the vineyard would be highly recommended to reduce perdurable pathogenic inoculum sources.

6.7. Acknowledgements

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Capítol 7

Natural infections of pruning wounds by fungal trunk pathogens in mature grapevines in Catalonia (Northeast Spain)

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7.1. Resum

Situació i objectius: La informació disponible sobre la infecció de les ferides de poda, que té lloc de forma natural, és escassa a la literatura. És per això que l'objectiu d'aquest estudi va ser el de determinar la micoflora que infecta les ferides de poda a dues vinyes de Catalunya. Cada vinya presentava diferents nivells de malaltia i les avaluacions es van fer en dos períodes de poda diferents.
Mètodes i Resultats: A mitjans de tardor es va realitzar una primera poda -primerenca- a les dues vinyes, deixant entre quatre i sis borrons a cada sarment. Tres mesos després es van aïllar i identificar els patògens presents en 250 sarments podats, triats a l'atzar, de cada vinya. A continuació es va procedir a fer la segona poda -tardana-, deixant dos borrons a cada sarment. Tres mesos més tard, es va repetir el mostreig de 250 sarments a cadascuna de les vinyes. Els fongs patògens identificats van ser, principalment, *Eutypa lata* (0-0.4 % de la freqüència total d'aïllaments), *Neofusicoccum parvum* (0-1.2 %), *Botryosphaeria dothidea* (0-1.6 %), *Phomopsis* spp. (0-1.6 %), *Cryptovalsa ampelina* (0-3.2 %), *Phaeomoniella chlamydospora* (0-12.0 %) i *Diplodia seriata* (0.4-68.4 %), segons les diferents èpoques de poda i vinya. Es va detectar una forta estacionalitat entre les infeccions causades per aquests patògens i l'època de la poda, amb freqüències d'aïllament més elevades després de la poda tardana en comparació amb la poda primerenca.
Conclusions: Tenint en compte les condicions ambientals i l'àrea geogràfica on es va realitzar aquest estudi, es va observar un percentatge d'infeccions naturals de les ferides de poda més baix després d'una poda primerenca, en comparació amb les mateixes dades d'una poda tardana.
Significat d'aquests estudi: Una poda primerenca, realitzada a la tardor i combinada amb d'altres mètodes de control, podria ser utilitzada per reduir les infeccions causades per fongs patògens de la fusta de la vinya a Catalunya. Tot i això, abans de generalitzar aquesta recomanació per altres zones vitivinícoles, seria recomanable avaluar el risc d'infecció en aquests llocs així com els efectes potencials d'una poda primerenca sobre la producció del cultiu.

7.2. Abstract

Background and Aims: Information on the natural infection rates of pruned canes caused by fungal trunk pathogens is scarce. This study aimed to

determine the pathogenic mycoflora infecting the pruning wounds in two vineyards in Catalonia, Spain, each with a different level of trunk diseases, and in two pruning seasons.

Methods and Results: Vines were pruned in each vineyard in mid-autumn leaving four to six buds. Three months later, pathogens were isolated and identified in 250 pruned canes chosen at random in each vineyard. Vines were then definitively pruned to two buds, and sampling for pathogen isolation and identification was repeated 3 months later. The main fungal pathogens identified in this study were *Eutypa lata* (0-0.4 % of isolations), *Neofusicoccum parvum* (0-1.2 %), *Botryosphaeria dothidea* (0-1.6 %), *Phomopsis* spp. (0-1.6 %), *Cryptovalsa ampelina* (0-3.2 %), *Phaeomoniella chlamydospora* (0-12.0 %) and *Diplodia seriata* (0.4-68.4 %). A strong seasonal effect on pathogen infections was detected for most species, with a higher isolation percentage detected after the late pruning as compared with that of the early pruning.

Conclusions: Under the environmental conditions and the geographical location of this study, our results showed that the rate of natural infection of pruning wounds was lower following early pruning (autumn) than following late pruning (winter).

Significance of the Study: Early pruning could be used in combination with other control measures, such as chemical and biological wound protectants, to reduce the infections caused by the grapevine trunk pathogens during the pruning season in Catalonia, Spain. The infection risk, however, and potential effects of the early pruning on grape production should be considered in other environments before expanding this recommendation to other grape growing regions.

7.3. Introduction

Grapevine trunk diseases, which are responsible for significant economic losses to the grape and wine industries worldwide (Creaser and Wicks, 2001; Gubler *et al.*, 2005), are caused by pathogenic fungi that promote an internal degradation of the plant woody tissues that may lead to vine death. The main grapevine trunk diseases include Petri and black foot diseases in young plants as well as Botryosphaeria dieback, Eutypa dieback and esca in mature vines. Several comprehensive articles on the symptoms and the causal agents of these diseases have been published (Carter, 1988; Carter, 1991;

Mugnai *et al.*, 1999; Larignon *et al.*, 2001b; Lecomte *et al.*, 2005b; Lecomte *et al.*, 2012; Fischer, 2006; Halleen *et al.*, 2006a; Mostert *et al.*, 2006; Surico *et al.*, 2006; van Niekerk *et al.*, 2006; Larignon *et al.*, 2009; Úrbez-Torres, 2011).

Whereas planting material used in new vineyards can be already infected with most pathogens, such as those causing Petri and black foot diseases and Botryosphaeria dieback, either systemically from infected mother vines or by contamination during the propagation process in the nursery (Gramaje and Armengol, 2011), it is widely accepted that the spread of diseases in mature vineyards depends mainly on the infection of pruning wounds by pathogens (Rolshausen *et al.*, 2010a; van Niekerk *et al.*, 2011). This has led to several studies on the susceptibility of grapevine pruning wounds to trunk pathogens, such as Botryosphaeriaceae species, including *Diplodia seriata* De Not. (Serra *et al.*, 2008), *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Úrbez-Torres and Gubler, 2011), *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips (van Niekerk *et al.*, 2011), and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Úrbez-Torres and Gubler, 2011), and *Eutypa lata* (Pers.) Tul. & C. Tul. (Moller and Kasimatis, 1980; Trese *et al.*, 1980; Petzoldt *et al.*, 1981; Trese *et al.*, 1982; Munkvold and Marois, 1995; Chapuis *et al.*, 1998; Lecomte and Bailey, 2011; van Niekerk *et al.*, 2011), *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai (Larignon and Dubos, 2000; Eskalen *et al.*, 2007; Serra *et al.*, 2008), *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams (Larignon and Dubos, 2000; Eskalen *et al.*, 2007; Serra *et al.*, 2008; van Niekerk *et al.*, 2011), and *Phomopsis viticola* (Sacc.) Sacc. (van Niekerk *et al.*, 2011). These studies examined the wound susceptibility of grapevines artificially inoculated where the grapevine cultivar, the pruning period and the inoculum load or different combinations of these factors were considered. These studies have consistently shown that the infection of wounds significantly decreases as the length of time between pruning and inoculation increases. In addition, most of the previous studies have also shown that pruning in late winter reduces the wound susceptibility to pathogens as compared with that in the early pruning done in late autumn or early winter. In contrast, Eskalen *et al.* (2007) showed that wound susceptibility to *P. chlamydospora* occurred throughout the summer season in California. Serra *et al.* (2008) also showed susceptibility to *D. seriata* to be higher in early spring in

Italy. The rate of natural infections in pruned canes (*i.e.* those not obtained through artificial inoculations), however, has not been extensively studied to date, and they can be estimated only through the spontaneous infections of the vines included as non-inoculated controls in artificial inoculations (Moller and Kasimatis, 1980; Munkvold and Marois, 1995; Larignon and Dubos, 2000; Serra *et al.*, 2008; Úrbez-Torres and Gubler, 2011). Lecomte and Bailey (2011) compared the susceptibility of grapevine pruning wounds with either natural or artificial infection with *E. lata* at two pruning periods in France. They found that natural colonisation of wounds averaged 2 % after spring pruning compared with 13 % after winter pruning, which showed a trend comparable with that of artificial inoculations.

Little information is available about the rate of natural infections caused by the fungal trunk pathogens in Spain. This study aimed to identify the pathogenic mycoflora infecting pruning wounds in two mature vineyards with a different incidence level of trunk diseases and at two pruning periods: i) after an early pruning in autumn; and ii) after a late pruning in winter. In addition, we inspected the relationship between the rate of infection and the main weather data recorded during the experimental period.

7.4. Material and Methods

7.4.1. Location and characteristics of the experimental vineyards

In autumn 2008, an experimental plot was located in El Pla del Penedès, Catalonia, Spain [UTM coordinates (Datum WGS 84): 31T 392200, 4586600], in a 30-year-old cv. 'Cabernet Sauvignon' vineyard grafted onto Richter 110 rootstock. A plot of 2400 vines in this vineyard has been monitored biannually for the evolution of the symptoms of trunk diseases since 2007 to the present. At the time this study was started (2008), about 21 % of vines had shown symptoms of trunk diseases in previous monitoring dates; therefore, this was considered as the 'diseased' vineyard. The main symptoms of trunk diseases observed during monitoring included the tiger-pattern foliar necrosis for esca, the stunted shoots with chlorotic leaves, often cupped and with tattered margins for Eutypa dieback, the reduced shoot growth with eventual chlorotic leaves for Botryosphaeria dieback, and the arm and cordon death for both Eutypa and Botryosphaeria diebacks. In autumn 2010, a second experimental

plot was located in Vilafranca del Penedès, Catalonia, Spain (UTM coordinates: 31T 391710, 4579900) in a 10-year-old cv. 'Chardonnay' vineyard grafted onto Richter 110 rootstock. The vineyard was located approximately 7 km from the first vineyard. Symptoms of trunk diseases in this vineyard had not been noticed during a biannual monitoring since 2008; therefore, this was considered as the 'healthy' vineyard, although we presumed that infected plants not showing symptoms, such as plants with latent infections, could be present. We were unable to find two vineyards in close proximity of the same cultivar but differing in the level of trunk diseases; therefore, we presumed that spontaneous infections of pruned canes would not be affected by grapevine cultivar. Both vineyards were trained as bilateral cordons with spur pruning (Royat). The experimental plots were located near an automatic weather station located in La Granada del Penedès (UTM coordinates: 31T 393660, 4580193) about 6 km from the diseased vineyard and 2 km from the healthy one. Data obtained from this weather station were considered to be representative of both vineyards. The natural fungal infections occurring in the diseased vineyard were studied for four seasons (autumn 2008 to spring 2012), while the healthy vineyard was monitored only for the last two seasons within the same experimental period (autumn 2010 to spring 2012).

7.4.2. Pruning and sampling

About 150 vines in each vineyard were pruned to four to six buds in autumn (between 9 and 18 November for all years and vineyards), simulating a mechanical prepruning, which is a common practice in this region. Three months later in winter (between 7 and 17 February for all years and vineyards), 250 pruned canes in each vineyard were chosen at random, and a 15-cm section was cut and removed from their upper end and taken to the laboratory for pathogen isolation and identification. On the same day of this sampling, all the vines were pruned definitively to two to three buds, coinciding with the traditional pruning season in the area. A longer than usual wood section (5-7 cm) was left above the top bud. Sampling for pathogen isolation and identification was repeated, following the same procedure earlier, 3 months later from the newly pruned cane end (between 11 and 24 May for all years and vineyards), so that all canes were exposed to natural infections for 3 months

after pruning. Pruning wounds were not protected physically nor chemically during the experiment.

7.4.3. Fungal isolation and identification

Cane samples were kept at 4 °C, and fungi were isolated within 48 h of sampling. Bark was removed from the upper ends of canes with a flame-sterilised scalpel to expose the inner necrotic tissues starting from the pruning wound. The length of necrosis was measured, and the 2- to 3-mm end was cut and discarded to avoid the isolation of saprophytic species. A minimum of three wood discs (5 mm in length) was obtained consecutively from the cane end with surface sterilised secateurs. Wood chips were surface-sterilised (4 minutes in 70 % ethanol), blotted on sterile filter paper to remove excessive ethanol, and plated onto Potato Dextrose Agar (PDA, Difco Laboratories) with streptomycin sulfate (Sigma-Aldrich Co.) added at 100 units per millilitre, as described by Johnston and Booth (1983). Plates were incubated at 25 °C in darkness and monitored daily for 4-11 days to check for growing fungal colonies. Fungi were identified on the basis of morphological characters of colonies and reproductive structures. Saprophytic species were identified at the family/genus level, while fungi known to be associated with trunk diseases were identified at the species level. Pathogenic fungi were confirmed by DNA sequencing from selected regions: the internal transcribed spacers ITS1 and ITS2 flanking the 5.8 s rRNA gene (ITS), and parts of the translation elongation factor 1-alpha (EF1-α) and the β-tubulin genes when applicable. The ITS region was amplified with the universal primers ITS1 and ITS4 (White *et al.*, 1990). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the EF1-α gene, while part of the β-tubulin gene was amplified with the primers Bt2a and Bt2b (Mostert *et al.*, 2006). Procedures for DNA extraction, polymerase chain reaction amplifications and DNA sequencing are detailed in Luque *et al.* (2009; 2012) and will not be included here. All regions were sequenced in both strands to clarify any nucleotide ambiguous position. The Basic Local Alignment Search Tool searches at GenBank showing high homologies with reference sequences (>97 %) confirmed the identifications. In order to estimate the potential latent infections of pathogens that could be present in the 2- to 3-mm cut ends discarded during the isolations made in February, an experiment was established as follows. In November 2012, several vines in the diseased vineyard

were prepruned as explained earlier. Over 600 pruned canes were exposed to natural infection and sampled in February. Two blocks of 160 canes were each sampled, and canes from half of each block were processed for fungal isolation and identification as explained earlier. The remaining 160 detached canes were placed into holes in 3-cm thick polystyrene boards, ensuring that the bottom of the canes extended approximately 3 cm below the boards. The boards with canes were floated on tap water in plastic trays and incubated at 22 °C for 4 weeks, as described by Ayres *et al.* (2011), except that incubation was in the dark. The water in the trays was changed weekly. After this incubation period, canes were processed for fungal isolation and identification. It was considered that the 4-week incubation period would have allowed the latent fungi existing in the cut end of canes to grow downwards into the cane to be isolated later. The frequency of fungal isolation for each group of canes was compared in order to accept or to discard the hypothesis of latent infections. This experiment was done only during the winter season (November to February) because environmental conditions in spring (after the late pruning) were enough for most of the known pathogenic fungi to grow more than 2-3 mm in 3 months (Dr Jordi Luque, *unpubluplished data*, 2009), thus letting the fungi to overpass the discarded cut end during the subsequent isolations made in May.

7.4.4. Statistical analyses

The SAS/STAT statistical package v.9.3 (SAS, Inc.) was used throughout.

Multivariate analysis: The multiple correspondence analysis was used to explore the overall relationships between the factors of the experiment (vineyard, year, pruning season) and the proportion of fungal isolations using PROC CORRESP.

Infection by individual fungi: Data of the most frequent pathogens, namely *Diplodia seriata*, *Phaeomoniella chlamydospora* and the grouped Diatrypaceae species, were analysed as a factorial model 'Vineyard' × 'Year' × 'Pruning Season'. 'Vineyard' and 'Pruning Season' were considered to be fixed effects, while 'Year' was treated as random. The second order interaction was taken as the experimental error. Non-significant (P>0.05) first order interactions were confounded with that in order to increase error's degrees of freedom. A cane was considered to be colonised with a fungal species when at least one wood

chip yielded a colony of the fungus, data thus being binomial. Pathogens were analysed differently depending on the range of per cent infection. When most values were over 5/250 successful isolations and no zeros were recorded, an analysis of variance with logit transforms was used. Normality of residuals was checked by Shapiro-Wilk's test, and homogeneity of variances by Levene's test. Means were compared with Tukey's Honestly Significant Difference (HSD) range test ($P \leq 0.05$) using PROC GLM. When several values fell below 5/250 successful isolations, Poisson regression was used. The quality of this latter model was assessed by their overall, scaled $\chi 2$ value using PROC GENMOD. Frequency of fungal isolations in the experiment on delayed isolations in 2012-2013 was analysed by Pearson's chisquared test using PROC FREQ.

Necrosis length: These data were analysed with the 'Vineyard' × 'Year' × 'Pruning Season' factorial design, without consideration of the pathogens isolated. Data were log-transformed and subjected to analysis of variance using PROC MIXED. Means were compared with Tukey's HSD range test ($P \leq 0.05$).

Correlation with weather variables: The percentage of isolation corresponding to *D. seriata*, *P. chlamydospora* and the grouped Diatrypaceae species was correlated with the weather data (namely, daily mean temperature, daily mean relative humidity and accumulated rainfall). Temperature and humidity records were averaged over different post-pruning periods: 1, 2, 4, 8 and 13 weeks. Rainfall records, instead, were accumulated and log-transformed for normality over these same periods. Because several fungal and weather variables showed a bimodal, non-normal distribution, Spearman's correlation coefficients were calculated instead of Pearson's coefficients using PROC CORR.

7.5. Results

Fungal isolations yielded positive results ranging from 46.4 to 100 % among the different seasons and vineyards, with mean values among the seasons between 74.9 and 93.2 % (Table 7.1). Overall fungal isolations were higher in spring after the late pruning and in the healthy vineyard. Saprophytic and pathogenic species not associated to grapevine trunk diseases more frequently isolated were *Alternaria* spp., *Aureobasidium pullulans* (de Bary) G. Arnaud, *Botryotinia fuckeliana* (de Bary) Whetzel, *Cladosporium* spp. and

Epicoccum spp. Other fungi less frequently identified during the experimental period were included in the genera *Acremonium*, *Arthrinium*, *Aspergillus*, *Nigrospora*, *Penicillium* and *Valsa*, and were grouped together because of their low isolation percentage and/or irregular occurrence among seasons and vineyards (Table 7.1). The percentage of isolations corresponding to unidentified taxa ranged between 1.6 and 14.4 %.

At least 13 fungal pathogens associated with grapevine trunk diseases were identified in this study (Table 7.1). One fungus in *Diatrype* and another one in *Eutypa* could not be identified at the species level, while at least two different taxa in *Phomopsis* were also unidentified at the species level. The main fungal pathogens identified during the experimental period were, in increasing order of abundance, *Eutypa lata*, *Neofusicoccum parvum*, *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not., *Phomopsis* spp., *Cryptovalsa ampelina* (Nitschke) Fuckel, *Phaeomoniella chlamydospora* and *Diplodia seriata*. The latter species was clearly the most frequently isolated, with a percentage up to 68.4 %. *Phaeomoniella chlamydospora* was isolated in a range between 0 and 12.0 %, and *C. ampelina* was isolated in a range between 0 and 3.2 %, whereas the rest of the taxa were detected in less than 2 % of the canes irrespectively of the vineyard and season (Table 7.1).

A strong seasonal effect was detected in the fungal isolations for the main pathogenic species, with a higher isolation percentage detected after the late pruning as compared with that of the early pruning, which was also shown in the factorial analysis of correspondence. On the plane defined by the two factors, a diagonal line was arbitrarily drawn that separated November pruning data points on the upper left side from February pruning data points on the lower right (Figure 7.1a). When the fungal taxa were projected on the same plane, *B. dothidea*, *Cladosporium* spp., *Dothiorella iberica* A.J.L. Phillips, J. Luque & Alves, *Dothiorella sarmentorum* (Fr.) A.J.L. Phillips, A. Alves & J. Luque, and *Botryosphaeria fuckeliana* were associated to the early pruning in November. Several taxa remained around the origin of coordinates, *i.e.* they similarly appeared in all locations and pruning periods, and included mostly saprophytic species (*Alternaria* spp., *A. pullulans*, *Epicoccum* spp., and other species) but also some infrequent pathogens (*E. lata*, *Dothiorella viticola* A.J.L. Phillips & J. Luque and *Phomopsis* spp.). Finally, there was a group of taxa more associated

with isolations coming from the late pruning in February, and included the most frequent species *C. ampelina*, *D. seriata*, *P. chlamydospora*, as well as the less frequent fungi *N. parvum*, *Eutypella citricola* Speg**.** and *Diatrype* sp. (Figure 7.1b).

Different statistical models in the analyses of the percentage isolation for the most frequent pathogens, namely *D. seriata*, *P. chlamydospora* and the grouped species of the Diatrypaceae family, were tested. Whereas data of *D. seriata* percentage isolation were logit-transformed and analysed by analysis of variance, isolation frequency of *P. chlamydospora* and Diatrypaceae was analysed by Poisson regression. Significance of effects and subsequent comparison of means are shown in Table 7.2. The seasonal influence of pruning was highly significant ($P<0.001$) for all the pathogens analysed, with a higher isolation percentage obtained in spring, after the late pruning in February. In addition, the vineyard factor was highly significant for *P. chlamydospora* and the Diatrypaceae. Whereas infections of *P. chlamydospora* were higher in the diseased vineyard (2.4 %) than in the healthy vineyard (0.3 %), the infections caused by Diatrypaceae behaved conversely (1.5 % in the healthy vineyard *vs* 0 % in the diseased vineyard). All values reported here are detransformed means obtained through the statistical analyses and may differ from the arithmetic means (not shown). A significant interaction ($P=0.021$) was detected between vineyard and pruning season for the infections caused by *D. seriata*, with the greatest difference between the pruning times in the healthy vineyard than in the diseased one. The factor year was significant for the Diatrypaceae, with more infections in the seasons 2009/10 and 2011/12 than in 2010/11. The interaction between year and pruning time for infections caused by *P. chlamydospora* was also significant mainly because of the comparatively low isolation of this pathogen after the late pruning in the diseased vineyard in 2009 (Table 7.2).

Table 7.1. Fungal isolations obtained in two vineyards showing different incidence levels of grapevine trunk diseases and pruned at two different times; on each occasion 250 canes were sampled.

	Isolation (%)											
Fungal taxa	**Vineyard 1 - Diseased**								**Vineyard 2 - Healthy**			
	November				***February***				***November***		***February***	
	2008	***2009***	***2010***	***2011***	***2009***	***2010***	***2011***	***2012***	***2010***	***2011***	***2011***	***2012***
GTD-associated fungi												
Botryosphaeriaeae	3.2	4.0	2.4	1.6	18.4	69.6	27.2	22.4	0.4	2.0	29.2	39.2
Diplodia seriata	2.4	3.2	2.0	1.6	18.4	68.4	27.2	21.2	0.4	0.4	29.2	38.0
Botryosphaeria dothidea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	1.2
Dothiorella iberica	0.4	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dothiorella sarmentorum	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dothiorella viticola	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0
Neofusicoccum parvum	0.0	0.0	0.4	0.0	0.0	1.2	0.0	0.4	0.0	0.0	0.0	0.0
Diatrypaceae	0.0	1.2	0.0	0.0	1.2	1.6	0.4	1.2	0.0	0.0	1.6	5.6
Cryptovalsa ampelina	0.0	0.4	0.0	0.0	1.2	1.6	0.4	0.8	0.0	0.0	1.2	3.2
Diatrype sp.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	1.6
Eutypa lata	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.4
Eutypa sp.	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Eutypella citricola	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4
Phaeomoniella chlamydospora	2.0	0.4	1.2	0.8	2.8	6.8	12.0	8.0	0.0	0.0	2.0	0.8
Phomopsis spp.	0.0	1.2	0.0	0.4	0.0	0.4	0.4	0.0	0.0	0.8	1.6	0.0
Other species												
Alternaria spp.	51.6	93.6	3.2	47.6	33.6	36.4	14	11.2	40.4	29.2	24.8	10.8
Aureobasidium pullulans	51.2	17.6	36.0	12.4	48.8	23.2	17.6	31.6	42.8	22.0	38.0	38.8
Botryotinia fuckeliana	1.6	1.2	0.0	1.6	0.0	0.0	0.4	0.4	1.2	7.2	1.2	0.4
Cladosporium spp.	12.8	8.8	1.2	5.2	8.4	1.2	2.8	5.6	33.2	31.6	6.8	10.8
Epicoccum spp.	3.2	7.6	0.4	0.4	24.0	2.0	2.8	0.0	0.8	0.8	2.4	0.4
Other taxa	7.2	10.8	4.0	10.8	5.6	8.8	0.8	4.0	1.2	8.0	8.2	8.0
Unidentified	12.4	13.2	1.6	4.4	6.8	5.6	8.4	10.8	3.2	8.4	8.4	14.4
Total positive reisolations	85.6	100.0	46.4	67.6	84.8	99.2	76.0	78.8	78.0	83.6	94.4	92.0
Mean values		74.9				84.7				80.8		93.2

Partial values of the taxa do not sum up to the total values at the bottom of the table, since canes could be infected by more than one fungus.

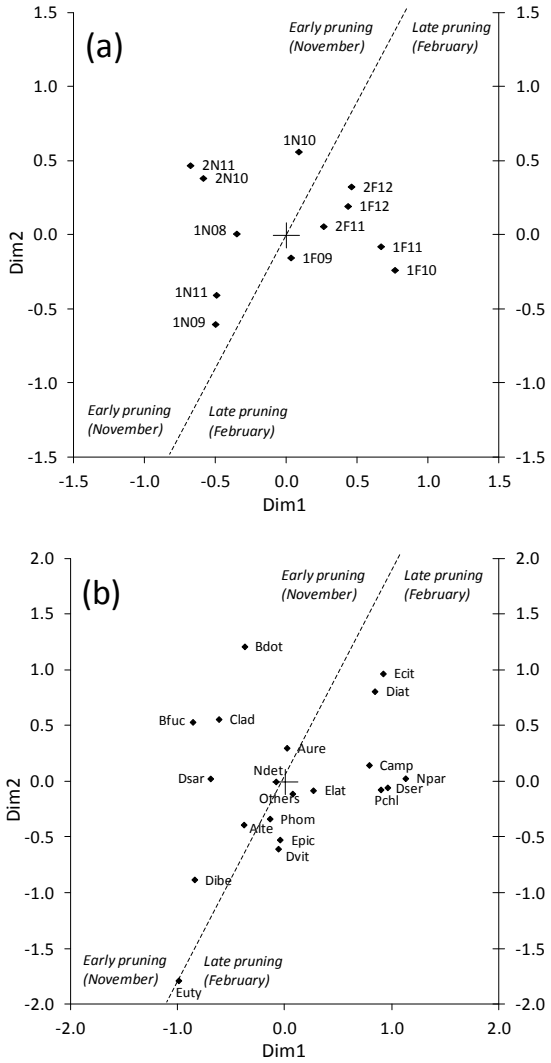

Figure 7.1. Multiple correspondence analysis of the natural infection percentage of pruning wounds occurring in two vineyards with different incidence level of trunk diseases and two different pruning periods. For the sake of clarity, results are shown in two complementary views of the same bidimensional space, on the basis of (a) location and season (factors) and (b) fungal species (variables). Legends to individual points in the graphics: (a) each code consists of: a number for vineyard location (1, Pla del Penedès, diseased vineyard; 2, Vilafranca, healthy vineyard), a letter for pruning month (F, February; N, November), followed by the two last numbers indicating the year of pruning. (b) Abbreviations of fungal taxa are as follows: Alte, *Alternaria* spp.*;* Aure*, Aureobasidium pullulans;* Bdot, *Botryosphaeria dothidea;* Bfuc*, Botryotinia fuckeliana;* Camp*, Cryptovalsa ampelina;* Clad*, Cladosporium* spp.*;* Diat*, Diatrype* sp.*;* Dibe, *Dothiorella iberica;* Dsar*, Dothiorella sarmentorum;* Dser, *Diplodia seriata;* Dvit*, Dothiorella viticola;* Ecit*, Eutypella citricola;* Elat, *Eutypa lata;* Epic*, Epicoccum* spp.*;* Euty*, Eutypa* sp.*;* Npar*, Neofusicoccum parvum;* Pchl*, Phaeomoniella chlamydospora;* Phom, *Phomopsis* spp.*;* Others, Other species (see text); Ndet*,* Nonidentified. The dashed line was arbitrarily drawn to show the pruning seasons separately.

Table 7.2. Mean values of percentage isolation of *Diplodia seriata, Phaeomoniella chlamydospora* and species of Diatrypaceae in a diseased and in a healthy vineyard that showed different incidence levels of grapevine trunk diseases and which were pruned in autumn and winter.

Fungal taxa	Vineyard		Pruning time		Year				Significant interactions
	Diseased	*Healthy*	*Autumn*	*Winter*	*2008/09*	*2009/10*	*2010/11*	*2011/12*	*Vineyard x Pruning season*
Diplodia seriata	9.4 a	5.7 a	1.1 b	36.2 a	5.4 b	17.0 a	5.6 b	5.5 b	
	F = 2.42		F = 176.32		F = 3.41				
	Effect df = 1		Effect df = 1		Effect df = 3				
	Error df = 5		Error df = 5		Error df = 5				
	P = 0.1808		*P* < 0.0001		*P* = 0.1101				
									F = 9.20
									Effect df = 1
									Error df = 5
									P = 0.0209
	Diseased	*Healthy*	*Autumn*	*Winter*	*2008/09*	*2009/10*	*2010/11*	*2011/12*	*Year x Pruning season*
Phaeomoniella chlamydospora	2.4 a	0.3 b	0.3 b	2.3 a	1.3 a	0.8 a	0.8 a	0.6 a	
	x^2 = 42.23		x^2 = 44.32		x^2 = 2.15				
	df = 1		df = 1		df = 3				
	P < 0.0001		*P* < 0.0001		*P* = 0.5416				
									x^2 = 8.57
									df = 3
									P = 0.0356
	Diseased	*Healthy*	*Autumn*	*Winter*	*2008/09*	*2009/10*	*2010/11*	*2011/12*	
Diatrypaceae	0.3 b	1.5 a	0.2 b	2.1 a	0.7 ab	1.7 a	0.2 b	0.8 a	
	x^2 = 9.64		x^2 = 24.45		x^2 = 11.22				
	df = 1		df = 1		df = 3				
	P = 0.0019		*P* < 0.0001		*P* = 0.0106				

Vineyard x Pruning season	1	2
Autumn	2.2 b	0.5 c
Winter	32.1 a	40.6 a

Year x Pruning season	2008/09	2009/10	2010/11	2011/12
Autumn	0.7 bc	0.1 c	0.4 c	0.3 c
Winter	1.0 c	2.4 b	4.4 a	2.8 ab

Interactions not included in the model

†Values correspond to detransformed means obtained through the statistical analyses and may differ from the arithmetic means (not shown). The *F* values of the analysis of variance and the χ2 values of the Poisson regressions, together with their significance levels are also given for each effect in the model. Mean values followed by different letters were significantly different according to the different statistical analyses done.

In February 2013, a total of 13.8 % of fungal isolations was obtained from canes pruned in November 2012, whereas the delayed isolations (4 weeks later) from canes of the same source detected a higher percentage isolation of 20.6 %. This difference, however, was not statistically significant (Pearson's chi-squared test, P=0.1031), thus indicating that potential latent infections occurring in the discarded 2- to 3-mm cut ends were not contributing significantly to underestimate the percentage of fungal isolations observed after the early pruning. In addition, *D. seriata* was detected twice in the first isolation (no incubation period) while only three times when isolation was delayed for 4 weeks. *P. chlamydospora* was not detected in the first round of isolations and only once after the delayed isolations. No Diatrypaceae species were isolated in this experiment.

The mean length of necroses extending from the pruning wounds ranged between 0.4 and 0.8 cm but was not significantly different (P>0.05) either between vineyards or between pruning seasons throughout the experimental period. A significant interaction (P<0.001), however, was detected between these factors because of the longer mean necrosis observed in the healthy vineyard following November pruning (0.79 cm) compared with that of the other three factor combinations (0.40-0.47 cm).

Climate conditions varied between pruning seasons. First, temperature declined in autumn**,** after the November pruning, to its yearly minimum, which coincided with the February pruning (Table 7.3). Then, it increased steadily until the second sampling period in May. Second, rainfall was stable after the November pruning and increased slightly after the February pruning. A significant correlation between the main weather variables and the isolation percentage of *D. seriata*, *P. chlamydospora* and the Diatrypaceae species was detected (Table 7.4).

Table 7.3. Mean values of temperature and relative humidity, and accumulated rainfall values at 1, 2 and 3 months in each experimental period.

Period	Month	Days	Mean T (ºC)	Mean RH (%)	Acc. Rainfall (mm)
Winter 2008-09	1	29	7.2	62	30
	2	31	6.5	71.7	99
	3	24	8.2	65.4	42
Winter 2008-09 / Totals		84	7.2	66.5	171
Winter 2009-10	1	30	12.2	66	3
	2	31	6.6	69.9	110
	3	38	5.8	69.4	58
Winter 2009-10 / Totals		99	8	68.5	171
Winter 2010-11	1	30	8.6	69	14
	2	31	7	70.7	13
	3	37	7.1	71.2	46
Winter 2010-11 / Totals		98	7.5	70.4	73
Winter 2011-12	1	30	10.5	79.4	28
	2	31	7.9	67	1
	3	31	4.8	60.8	8
Winter 2011-12 / Totals		92	7.7	68.9	38
Winter totals, all years			9.7	67.2	113
Spring 2009	1	28	8.1	66.6	5
	2	31	10.9	65.2	94
	3	31	14.4	58.2	44
Spring 2009 / Totals		90	11.3	63.2	143
Spring 2010	1	28	7.4	64.5	82
	2	31	11.2	67	37
	3	33	13.6	69.8	164
Spring 2010 / Totals		92	10.9	67.2	283
Spring 2011	1	28	9.4	73.6	125
	2	31	13.8	66.3	6
	3	33	15.4	72.4	89
Spring 2011 / Totals		92	13.1	70.7	220
Spring 2012	1	29	8.9	56.1	1
	2	31	12	65	82
	3	33	14.7	63.2	43
Spring 2012 / Totals		93	12	61.6	125
Spring totals, all years			11.8	65.7	193

Table 7.4. Spearman's correlation coefficients of the relationships between weather data and infection percentage of *Diplodia seriata* (logit transform), *Phaeomoniella chlamydospora* (log transform) and species of Diatrypaceae (log transform).

Correlation coefficient and significance	*Diplodia seriata*			*Phaeomoniella chlamydospora*			*Diatrypaceae*		
	T1	*RH1*	*LCR1*	*T1*	*RH1*	*LCR1*	*T1*	*RH1*	*LCR1*
r	**-0.6702**	-0.2328	-0.0853	**-0.6354**	-0.0442	-0.0677	**-0.5805**	-0.3115	-0.1541
p	**0.0171**	0.4665	0.7922	**0.0264**	0.8914	0.8343	**0.0478**	0.3244	0.6326
	T2	*RH2*	*LCR2*	*T2*	*RH2*	*LCR2*	*T2*	*RH2*	*LCR2*
r	-0.5079	-0.4938	-0.0922	**-0.6212**	-0.2956	-0.0249	-0.3556	-0.4958	-0.2650
p	0.0918	0.1027	0.7756	**0.0311**	0.3509	0.9387	0.2565	0.1012	0.4053
	T4	*RH4*	*LCR4*	*T4*	*RH4*	*LCR4*	*T4*	*RH4*	*LCR4*
r	-0.1975	-0. 4938	0.1058	-0.3770	-0.2956	0.2779	0.0203	-0.4958	-0.1567
p	0.5383	0.1027	0.7434	0.2270	0.3509	0.3819	0.9501	0.1012	0.6268
	T8	*RH8*	*LCR8*	*T8*	*RH8*	*LCR8*	*T8*	*RH8*	*LCR8*
r	0.5679	-0.5291	**0.6773**	0.3593	-0.3593	**0.6566**	**0.6321**	-0.5695	0.4958
p	0.0541	0.0769	**0.0155**	0.2514	0.2514	**0.0204**	**0.0274**	0.0533	0.1012
	T13	*RH13*	*LCR13*	*T13*	*RH13*	*LCR13*	*T13*	*RH13*	*LCR13*
r	**0.7549**	-0.2081	**0.7690**	0.5504	-0.1292	**0.6566**	**0.7612**	-0.2857	**0.5842**
p	**0.0045**	0.5163	**0.0035**	0.0637	0.6890	**0.0204**	**0.0040**	0.3681	**0.0461**

Numbers following abbreviations of mean temperature, relative humidity and logarithm of accumulated rainfall (LCR) refer to the weather data summarised at 1, 2, 4, 8 and 13 weeks of the experimental periods in all seasons. Significant values ($P \leq 0.05$) are shown in bold. RH, relative humidity; T, mean temperature.

Average daily mean temperature for the 13-week period after pruning was positively correlated ($P<0.010$) with the percentage isolation of *D. seriata* (logit transform) and Diatrypaceae (log transform). Accumulated precipitation over 13 weeks (log transform) also correlated highly with the percentage isolation of *D. seriata* and to a lesser degree ($0.05>P>0.01$) with the percentage isolation of *P. chlamydospora* and the Diatrypaceae. Some of these positive

correlations appeared at 8 weeks (*Diplodia seriata* × rainfall, *Phaeomoniella chlamydospora* × rainfall, and Diatrypaceae × temperature) (Table 7.4). A negative correlation with temperature ($0.05>P>0.01$) was observed for the three pathogenic taxa in the first week after pruning. For *P. chlamydospora*, this extended to temperature averaged for the first 2 weeks.

7.6. Discussion

The results obtained in this study clearly indicate that canes were naturally infected by several important grapevine trunk pathogens at rates that varied according to the pruning time (mid-autumn or late winter). Infection percentage depended on the pathogen species and the health condition of the vineyard but mainly on the time of pruning. A strong seasonal effect in pathogen infections was detected for several Botryosphaeriaceae (mainly *Diplodia seriata*) and the Diatrypaceae (mainly *Cryptovalsa ampelina*) species, and *Phaeomoniella chlamydospora*, with a higher isolation percentage of these fungi being detected following late pruning compared with that following early pruning. These seasonal differences were consistently observed over a monitoring period of 4 years in a diseased vineyard and of 2 years in a healthy one. The influence of the disease incidence in the vineyards on the natural infections of pruned canes was not clear because those of *P. chlamydospora* and Botryosphaeriaceae species were higher in the diseased vineyard, whereas infections caused by species of the Diatrypaceae were higher in the healthy vineyard. While a higher percentage of pathogen infections in the diseased vineyard could be well explained by a local higher inoculum pressure coming from the diseased vines, a higher rate of infection of Diatrypaceae in the healthy vineyard could be eventually explained only by the proximity of unknown sources of inoculum, such as a neighbouring diseased vineyard close to the experimental plot. This plot, however, is located in an extensive area of commercial vineyards, and there is no presence of native tree or shrub species nearby that could act as alternative hosts for the pathogenic inoculum of the Diatrypaceae species, as was described in Trouillas *et al.* (2010). Moreover, a greater infection because of Diatrypaceae species in this vineyard could not eventually be explained by a greater susceptibility to these pathogens of the cultivar ‘Chardonnay’ as compared with ‘Cabernet Sauvignon’ because the opposite has been reported for *Eutypa lata* (Péros and Berger, 1994).

In this study, only the most frequently isolated fungi, namely the pathogens *D. seriata*, *P. chlamydospora* and *C. ampelina* along with the saprophytes, could be statistically analysed. All other pathogen species were found in less than 2 % of samples, making it impossible to analyse by analysis of variance and logistic regression. Furthermore the high frequency of zeros made the convergence of fitting algorithms difficult, and some effects in the models could not be adjusted, for example interactions for Diatrypaceae.

Phaeomoniella chlamydospora grows slowly on culture medium compared with that of other pathogens and saprophytes, which may have resulted in an underestimation of the infection rates for this pathogen. Botryosphaeriaceae and Diatrypaceae species are known as moderate to rapid-growing fungi so no underestimation of isolation frequencies along the experiment was presumed.

To date, only Lecomte and Bailey (2011) have intentionally measured the extent of natural infection by *E. lata* in Bordeaux vineyards. Information about other pathogens or grape growing regions comes only from the uninoculated controls included in wound susceptibility experiments. For instance, Moller and Kasimatis (1980) reported 15 % of natural infections of *E. lata* in an experiment on the protection of grapevine pruning wounds with fungicides. Munkvold and Marois (1995) observed a natural infection percentage for *E. lata* ranging from 0 to 24 % while studying the length of the wound susceptibility period after pruning. Serra *et al.* (2008) observed a similar percentage of natural infections, 0-21 %, for three grapevine pathogens, namely *D. seriata*, *P. chlamydospora* and *Phaeoacremonium aleophilum*. Úrbez-Torres and Gubler (2011) recorded 0-7 % natural infections (mainly caused by *D. seriata* and *E. lata*) in an experiment on the pruning wound susceptibility of grapevines to *Lasiodiplodia theobromae* and *Neofusicoccum parvum*. A low, variable number of replicates (10-40) was commonly used in the earlier cited experiments, and thus, the natural infections rate would have been estimated with considerable experimental error. Our results obtained from a large number of samples (*N*=250) would have reduced the potential experimental error and confirmed previous reports. Lecomte and Bailey (2011) studied the susceptibility of grapevine pruning wounds to *E. lata* over a 6-year monitoring period and found that natural colonisation of wounds was highly variable

according to the year and sites with different disease incidence. The percentage of natural infections reported by Lecomte and Bailey (2011) ranged from 0 to 43 %, and mean values were about 2 % after the spring pruning (mid-May to late June) and 13 % after the winter pruning (January to February) (Dr Pascal Lecomte, *personal comment*, 2013). Not every winter, however, appeared to be equally conducive to *E. lata* infection, with 1999 and 2004 in the 9-43 % range and 2001-2003 in the 0-5 % range, similar to the spring infection. In our study, we detected *E. lata* rarely (only once in three pruning seasons), but this may reflect large differences on the geographical and weather conditions between the Atlantic climate of the Bordeaux region, France (740-1250 mm annual rainfall) and the Mediterranean climate of Catalonia, Spain (350-1000 mm). The link of *E. lata* to cooler, wetter viticultural areas has been discussed by Muruamendiaraz *et al.* (2009). Lecomte and Bailey (2011) also reported the incidence of other saprotrophic wound-colonising fungi, including *Alternaria* spp., *Epicoccum* spp., *Penicillium* spp. and *Rhizopus* spp., and remarked on the specific incidence of *Botryosphaeria obtusa* (Schwein.) Shoemaker (=*Diplodia seriata*) as one of the most common species, which is in accordance with our findings.

The correlation coefficients calculated between the mean daily temperature or the accumulated rainfall and pathogen infections showed a definite trend from negative values in the first weeks after pruning, then no significant correlations at 4 weeks, to become mostly positive and statistically significant at 8 and 13 weeks. In contrast, the coefficients corresponding to relative humidity were always negative and non-significant throughout the experimental period. While we do not have an explanatory hypothesis for the negative correlations, positive correlations possibly reflect wetter and warmer conditions, favourable for fungal growth and infection, occurring progressively after late pruning, in contrast with drier and colder conditions after early pruning. As it has been reported that wound susceptibility to trunk pathogens can be extended over long periods, from several weeks up to 3 months (Petzoldt *et al.*, 1981; Munkvold and Marois, 1995; Chapuis *et al.*, 1998; Serra *et al.*, 2008), it is not surprising that positive correlations in our study were observed 2-3 months after the pruning events, which would indicate that wound infections accumulated over the whole 3-month period after the pruning time.

Grapevines in Spain are usually pruned in autumn and winter between leaf fall and budbreak (Hidalgo, 1999), as is also commonly done in most grape growing regions in the world. The pruning period can influence crop performance, specifically phenology, grape production and quality (Winkler *et al.*, 1974; Hidalgo, 1999; Galet, 2000). Late pruning has been proposed as the most suitable period for this practice because of reduced wound susceptibility to the pathogens *D. seriata* (Serra *et al.*, 2008), *E. lata* (Petzoldt *et al.*, 1981; Munkvold and Marois, 1995; Chapuis *et al.*, 1998), *L. theobromae* (Úrbez-Torres and Gubler, 2011), *N. parvum* (Úrbez-Torres and Gubler, 2011), *P. aleophilum* (Larignon and Dubos, 2000; Eskalen *et al.*, 2007; Serra *et al.*, 2008), and *P. chlamydospora* (Larignon and Dubos, 2000; Serra *et al.*, 2008). These studies were based on artificial inoculations and may have not considered the presence of a natural pathogenic inoculum along the experimental period, and thus, the real potential risk of infections may have been biased. All of these studies, however, found that wound susceptibility decreased with age.

In South Africa, van Niekerk *et al.* (2011) observed that pruning wounds that were made and inoculated with different trunk pathogens in late winter (August) generally had a greater incidence of infection compared to the pruning wounds inoculated in mid-winter (July), which suggests, as in our study, that wounds made later in the dormant season are more susceptible to infection than wounds made earlier. In Michigan (USA), Trese *et al.* (1980) observed that pruning wounds made in late winter were also more susceptible to artificial infection by *E. lata* compared with those made in early winter. In addition, Trese *et al.* (1980) observed a low level of airborne ascospores of *E. lata* during the winter months because of subfreezing weather conditions with no rain in Michigan, which would support a low level of natural infection of *E. lata* during this period. Van Niekerk *et al.* (2011) stated that the period of wound susceptibility may not be determined necessarily by the time of year that pruning is done but rather the climatic conditions experienced after pruning, which would allow for release of inoculum from fungal fruiting bodies, the spore dispersal of pathogens, as well as the fungal infection and posterior colonisation of wounds in the pruned grapevine.

In Catalonia, Spain, traditional management practices recommend to prune late in the winter. Based on this study, it could be recommended to prune

grapevines early in the winter in order to reduce fungal infections. More research, however, would be required on the effect of such a drastic shift in the viticultural practice, especially on the crop performance. It would also be important to study the natural infection occurring in other grape growing regions in the world, so that pruning recommendations to reduce infection by trunk diseases could be made for specific regions and climates.

7.7. Acknowledgments

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Capítol 8

Seasonal susceptibility of grapevine pruning wounds and cane colonization following artificial infection with *Diplodia seriata* and *Phaeomoniella chlamydospora* in Catalonia, Spain

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8.1. Resum

L'objectiu d'aquest estudi va ser el d'avaluar el període durant el qual les ferides de poda resten susceptibles a la infecció dels fongs patògens *Diplodia seriata* i *Phaeomoniella chlamydospora* i, a més, descriure la colonització de sarments infectats artificialment amb aquests dos patògens. En el primer experiment es van realitzar dues podes, una a la tardor i una altra a l'hivern. Les ferides de poda resultants es van inocular separadament amb suspensions de conidis de cada patogen a diferents temps després de cada poda. La susceptibilitat de les ferides a tots dos patògens va disminuir a mesura que s'incrementava el temps entre la poda i el moment de la inoculació. Tot i això, el percentatge de recuperació de tots dos patògens es va situar al voltant del 10 % quan les inoculacions es van realitzar dotze setmanes després de cada poda. Les ferides de poda van restar més temps susceptibles a la infecció per *D. seriata* després de la poda tardana, a l'hivern, en comparació amb la poda primerenca, a la tardor. En canvi, no es van observar diferències entre les dues podes pel que fa a la susceptibilitat de les ferides a la infecció per *P. chlamydospora*. En el segon experiment es van podar els sarments deixant dues longituds diferents entre l'últim borró i la ferida de poda abans de ser inoculats. Els patògens es van recuperar de diferents punts d'aïllament al llarg dels sarments inoculats i després de dos períodes diferents d'incubació, per a poder estimar la colonització de cada patogen. En línies generals, *D. seriata* va mostrar una colonització dels sarments major que *P. chlamydospora*.

8.2. Abstract

The aim of this study was to evaluate the period which grapevine pruning wounds remain susceptible to infection by the pathogenic fungi *Diplodia seriata* and *Phaeomoniella chlamydospora*, and to describe the colonization of canes resulting from the artificial infection of pruning wounds with these two pathogens. In the first experiment, pruning wounds made either in fall or winter were inoculated separately with conidial suspensions of each pathogen at different times after pruning. Wound susceptibility to both pathogens decreased as increased the period between pruning and wound inoculation, but infection percentages around 10 % were still detected when pathogens were inoculated 12 weeks after pruning. Pruning wounds remained

more susceptible to infection by *D. seriata* after a late pruning in winter as compared to an early pruning in fall. No overall seasonal changes in wound susceptibility were detected for *P. chlamydospora*. In the second experiment, canes were pruned by leaving alternatively two different lengths between the top node and the pruning wound before inoculations. Pathogens were recovered at different incubation periods and from different sites along the inoculated canes to estimate the cane colonization by each pathogen. *Diplodia seriata* showed an overall higher colonization of canes than *P. chlamydospora*.

8.3. Introduction

Botryosphaeria dieback, esca and Eutypa dieback are the most destructive fungal grapevine trunk diseases in mature vineyards and are responsible for important crop losses in grape growing countries worldwide (Carter, 1991; Mugnai *et al.*, 1999; Surico *et al.*, 2006; Úrbez-Torres, 2011; Bertsch *et al.*, 2013). Botryosphaeria dieback is commonly associated to roughly 30 Botryosphaeriaceae species (Úrbez-Torres, 2011; Úrbez-Torres *et al.*, 2012; Correia *et al.*, 2013; Pitt *et al.*, 2013b; Linaldeddu *et al.*, 2014), which include virulent and weak pathogens that eventually can colonize vine tissues as endophytes (Halleen *et al.*, 2005; Aroca *et al.*, 2006; Giménez-Jaime *et al.*, 2006; Casieri *et al.*, 2009; González and Tello, 2011). *Diplodia seriata* De Not. is one of the most frequently isolated Botryosphaeriaceae species from diseased grapevines in many countries (Rovesti and Montermini, 1987; Castillo-Pando *et al.*, 2001; Larignon *et al.*, 2001a; Phillips, 2002; Auger *et al.*, 2004; van Niekerk *et al.*, 2004; Úrbez-Torres *et al.*, 2006; Savocchia *et al.*, 2007; Úrbez-Torres *et al.*, 2008; Luque *et al.*, 2009; Úrbez-Torres *et al.*, 2009; Mohammadi *et al.*, 2013; Yan *et al.*, 2013), and it causes different symptoms including bud mortality, leaf spot and chlorosis, fruit rot, shoot dieback, bud mortality, perennial cankers on grapevines and vascular necroses of the wood, that appears as V-shaped necroses in cross-sections of the affected plant parts (Úrbez-Torres, 2011). *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams is one of the most cited fungus associated with esca together with *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous (basionym: *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai) and some Basidiomycetes, mainly *Fomitiporia mediterranea* M. Fisch. in Mediterranean countries (Mugnai *et al.*, 1999; Fischer, 2002; Surico *et al.*,

2006; Bertsch *et al.*, 2013; Gramaje *et al.*, 2015). Most recognized foliar symptoms of esca are characterized by leaf interveinal discolorations that later result in large necrotic areas (Mugnai *et al.*, 1999; Surico *et al.*, 2006), although these symptoms cannot be associated with the effects of a single pathogen and they are likely to occur as a complex interaction of multiple biotic and abiotic factors which are still not fully understood (Surico *et al.*, 2006; Bertsch *et al.*, 2013). In contrast, wood symptoms caused by *P. chlamydospora* and *P. minimum* have been reported as necrotic lesions including a longitudinal brown streaking, which it looks as brown spots in cross-sectioned vine parts, as well as pink-brown or dark red-brown necrotic areas in the wood (Mugnai *et al.*, 1999). Symptoms caused by *F. mediterranea* on wood are characterized by a yellowish spongy decay of the vine woody tissues. Foliar symptoms of esca are not directly associated with those in the wood (Surico *et al.*, 2008; Luque *et al.*, 2009; Bertsch *et al.*, 2013), as the former symptoms usually appear several years after a grapevine has become infected and the wood symptoms have already developed (Bertsch *et al.*, 2013). Eutypa dieback is caused by the fungus *Eutypa lata* (Pers.) Tul. & C. Tul (Carter, 1991). In recent years, an increasing number of Diatrypaceae species have been also isolated from diseased vines and were proven to be pathogenic on grapevine (Jordan and Schilder, 2007; Trouillas and Gubler, 2010; Trouillas *et al.*, 2010; Díaz *et al.*, 2011; Trouillas *et al.*, 2011; Luque *et al.*, 2012). Foliar symptoms of Eutypa dieback, comprising stunted shoots with short internodes, small, chlorotic and cupped leaves with marginal necrosis, are thought to be caused by toxic metabolites produced by *E. lata* (Renaud *et al.*, 1989; Tey-Rulh *et al.*, 1991; Molyneux *et al.*, 2002). Wood symptoms comprise internal necrosis, visualized as wedge-shaped area of stained tissue if a cross section is made of an infected trunk or cordon, and external cankers form around sites of infection (Carter, 1991). Botryosphaeria dieback and esca are the most prevalent trunk diseases in Catalonia, Northeast Spain, where the present study was carried out. Eutypa dieback is also present in the area but not as common as the former two diseases (Luque *et al.*, 2009).

It is widely accepted that trunk diseases in mature vineyards are spread by airborne spores of the pathogens which infect pruning wounds while these remain susceptible to infection (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; van Niekerk *et al.*, 2005; Úrbez-Torres *et al.*, 2010; van Niekerk *et al.*, 2010; Luque *et al.*, 2014). The airborne inoculum has been reported to be

dispersed in vineyards after periods of rainfall (Eskalen and Gubler, 2001; Úrbez-Torres *et al.*, 2010; van Niekerk *et al.*, 2010), sprinkler irrigation (Kuntzmann *et al.*, 2009), rainwater splash impacts on plant surfaces containing fruiting bodies and spores (Baskarathevan *et al.*, 2013) and in the rainwater runoff (Amponsah *et al.*, 2009). Different studies showed that spore release of Botryosphaeriaceae species and *E. lata* coincided with the beginning of rain or irrigation events (Carter, 1991; Michailides and Morgan, 1993; Gubler *et al.*, 2005). However, the end of the spore release differed among fungi, by finishing 2 h after the end of rainfall or irrigation in case of some Botryosphaeriaceae species whereas ascospores of *E. lata* were reported to be released up to 36 h later (Pearson, 1980; Carter, 1991; Gubler *et al.*, 2005; Trouillas and Gubler, 2009). Eskalen and Gubler (2001) also showed that spread of airborne inoculum of *P. chlamydospora*, *P. minimum* and *Phaeoacremonium inflatipes* W. Gams, Crous & M.J. Wingf. is correlated with rainfall events in California. However, Larignon and Dubos (2000) observed that *P. chlamydospora* conidia were present in the vineyard throughout the whole year in France. The botryosphaeriaceous species *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips were detected in rainwater samples trough PCR-based methods thus indicating that conidia of these Botryosphaeriaceae species can be dispersed during a rainfall event (Baskarathevan *et al.*, 2013). In addition, spore-trapping studies in several countries such as California (Ramos *et al.*, 1975; Petzoldt *et al.*, 1983; Eskalen and Gubler, 2001; Úrbez-Torres *et al.*, 2010), France (Larignon and Dubos, 2000; Kuntzmann *et al.*, 2009), New Zealand (Amponsah *et al.*, 2009) and South Africa (van Niekerk *et al.*, 2010) have described the evolution of the airborne inoculum of *E. lata*, *P. chlamydospora*, *P. minimum*, *P. inflatipes*, *Phomopsis* spp., and several Botryosphaeriaceae species included in the genera *Diplodia* Fr., *Dothiorella* Sacc. and *Neofusicoccum* Crous, Slippers & A.J.L. Phillips.

Pruning wounds are often not protected because wound protection treatments are cost expensive and this is only viable in vineyards with a high cash return (Serra *et al.*, 2008; Úrbez-Torres and Gubler, 2009). Consequently, when weather conditions during the pruning season are favourable for inoculum release and spread, infections of pruning wounds are most likely to occur (van Niekerk *et al.*, 2011). Susceptibility of pruning wounds to trunk

pathogens has been previously studied through artificial inoculations in several grape growing regions such as California, Oklahoma, France, Italy, and South Africa (Moller and Kasimatis, 1978; Trese *et al.*, 1980; Petzoldt *et al.*, 1981; Munkvold and Marois, 1995; Chapuis *et al.*, 1998; Larignon and Dubos, 2000; Gubler *et al.*, 2001; Eskalen *et al.*, 2007; Serra *et al.*, 2008; Lecomte and Bailey, 2011; Úrbez-Torres and Gubler, 2011; van Niekerk *et al.*, 2011). Wound susceptibility has been assessed for *D. seriata* (Serra *et al.*, 2008), *E. lata* (Trese *et al.*, 1980; Petzoldt *et al.*, 1981; Munkvold and Marois, 1995; Chapuis *et al.*, 1998; Lecomte and Bailey, 2011; van Niekerk *et al.*, 2011), *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Úrbez-Torres and Gubler, 2011), *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips (van Niekerk *et al.*, 2011), *N. parvum* (Úrbez-Torres and Gubler, 2011), *P. inflatipes* (Gubler *et al.*, 2001), *P. minimum* (Eskalen *et al.*, 2007; Serra *et al.*, 2008), *P. chlamydospora* (Gubler *et al.*, 2001; Eskalen *et al.*, 2007; Serra *et al.*, 2008; van Niekerk *et al.*, 2011), and *Phomopsis viticola* (Sacc.) Sacc. (van Niekerk *et al.*, 2011) usually by inoculating the pathogens at different times after pruning. In general, these studies showed that wound susceptibility decreased as increased the period between pruning and inoculation of wounds. Differences in the susceptibility duration have been found among pathogens, geographic regions, grape variety, age of the vineyard, inoculation time and pruning seasons (Trese *et al.*, 1980; Petzoldt *et al.*, 1981; Chapuis *et al.*, 1998; Larignon and Dubos, 2000; Eskalen *et al.*, 2007; Serra *et al.*, 2008; Lecomte and Bailey, 2011; Úrbez-Torres and Gubler, 2011; van Niekerk *et al.*, 2011). In summary, wound susceptibility can be extended up to 4-7 weeks for most pathogens under favourable conditions (Petzoldt *et al.*, 1981; Munkvold and Marois, 1995; Chapuis *et al.*, 1998), but can be even longer, up to 12-16 weeks (Eskalen *et al.*, 2007; Serra *et al.*, 2008; Úrbez-Torres and Gubler, 2011). The decrease in wound susceptibility has been associated to the processes related to wound healing, which basically involves the drying of cane tissues below the pruning wound (Shigo, 1984; Doster and Bostock, 1988; Bostock and Stermer, 1989). This results in a dead wood area in the cane named as drying cone ("*cone de dessèchement*") by Dal *et al.* (2008) or dead wood cone ("*cone de bois mort*") by Galet (2000). Dal *et al.* (2008) stated that wound healing can affect the vascular system below the drying cone by interfering with the sap flow and may even produce an overall weakening of the vine if pruning is not conducted properly, especially in spur-pruned grapevines. However, there is no available

information to date about the influence of wound healing on the colonization of canes by pathogenic fungi.

Results obtained in a previous study on the natural infections of pruning wounds in Catalonia showed that higher infection percentages occurred in spring than in winter (Luque *et al.*, 2014), thus suggesting that pruning wounds could be overall more susceptible to pathogens after a late pruning in winter. The current study aimed at continuing this research by determining the effects of the pruning season and the wound age on the susceptibility of pruning wounds to *D. seriata* and *P. chlamydospora*. In addition, the subsequent colonization process of infected canes was studied for both pathogens by inoculating wounds made on canes with different lengths of pruned internodes.

8.4. Material and methods

8.4.1. Plant material

The experiment on the susceptibility of pruning wounds to *Diplodia seriata* and *Phaeomoniella chlamydospora* was conducted on a non-commercial vineyard located at IRTA, in Cabrils, Catalonia, Spain. Potted 7-year-old *Vitis vinifera* cv. 'Tempranillo' grafted onto Richter 110 rootstock were kept outdoors in 50 l pots filled with a peat:perlite mixture (1:1, v:v), and watered adequately to avoid water stress. The experiment on the pathogen colonization of pruned canes was conducted in a commercial 11-year-old vineyard cv. 'Chardonnay' located in El Pla del Penedès, Catalonia. Vines in both vineyards were conducted in bilateral cordons and spur pruned.

8.4.2. Fungal isolates and inoculum preparation

Diplodia seriata isolate CBS 121485 and *Phaeomoniella chlamydospora* isolate CBS 121483 were used in the experiments. Both fungi were collected in 2003 from diseased grapevines in Catalonia; *D. seriata* from a cankered cordon of a vine cv. 'Cabernet Sauvignon', and *P. chlamydospora* from wood necrotic streaking of a vine cv. 'Carignane' showing esca symptoms. Fungi were maintained at 4 °C as mycelial plugs contained in tubes filled with sterile distilled water. Inoculations were performed with conidial suspensions of each pathogen obtained as follows. A mycelial plug of *D. seriata* previously grown on

Difco™ Potato Dextrose Agar (PDA; Becton, Dickinson and Company) for 7 days at 25 °C was put on a center of a water agar plate (Bacto Agar; Becton, Dickinson and Company). Several sterile fragments (N~20) of pine (*Pinus pinea*) needles (1 cm long) were put on the media surface at about 1.5 cm surrounding the mycelial plug. Plates were incubated for 4 weeks at 25 °C under combined near UV and white fluorescent light (Philips TL-D 18W BLB, and Sylvania Standard F18W/33-640-TS cool white, respectively) in a 12/12 h photoperiod, until pycnidia formation. One day before inoculation, pine needles (N~40) with *D. seriata* pycnidia were placed in a beaker containing 30 ml sterile distilled water. The solution was kept overnight (16 h aprox.) at 4 °C in constant agitation, with the help of a magnetic stirrer, to induce conidia release from the pycnidia. Low temperature was used to prevent conidia germination. On the next day, the suspension was vacuum-filtered through a 60 µm Steriflip filter (Millipore Corporation) to get a cleaner suspension. The conidial suspension was adjusted to 2×10^4 conidia/ml using a hemocytometer. *Phaeomoniella chlamydospora* was grown on PDA plates for 3 weeks at 25 °C. On the same day of inoculations, 10 ml of sterile distilled water were added onto the *P. chlamydospora* culture, which was gently scraped with a sterile cotton stick to release conidia. The conidial suspension was collected and adjusted to 4×10^4 conidia/ml using a hemocytometer. Spore suspensions were stored at 4 °C until inoculation to avoid early conidia germination.

8.4.3. Inoculation procedures and experimental design

The experiment on wound susceptibility consisted of two independent trials, one for each pathogen, and with two different corresponding pruning dates: first pruning in mid November (early pruning) and second pruning in mid February (late pruning). In both pruning dates, a sufficient number of canes was long pruned by leaving 4-5 buds per cane and 5 cm between the pruning wound and the top node. All wounds from the whole experiment were covered with a nylon 5-µm mesh (NITEX; SEFAR) to prevent posterior natural infections (especially by *Diplodia seriata*) which could interfere with the results of the present study. On subsequent inoculation rounds, pruned canes were temporarily uncovered for inoculations and covered again with the NITEX mesh soon after. Pruning wounds were separately inoculated with either pathogen using a micropipette to provide with a drop of 50 µl of the conidial suspensions

on each wound, resulting in 10^3 conidia of *D. seriata* and 2×10^3 conidia of *Phaeomoniella chlamydospora* per wound. Canes were inoculated 1 day, and 1, 2, 4, 8 and 12 weeks after pruning. Pruned canes in an additional control group were treated similarly with 50 µl sterile distilled water instead of the pathogen inoculum. Inoculum drops placed on wounds were left to air-dry (several minutes to 1 h) before being covered again with NITEX mesh. Twenty canes per treatment were used as replicates in each combination of pruning date, pathogen, and inoculation date. Experiment was set up in a full randomized design with three canes in the same cordon of a vine treated with the two pathogens plus the control. The experiment was repeated in two consecutive seasons (2012-13 and 2013-14), with the following pruning dates: 1st repetition, on 13 Nov 2012, and 8 Feb 2013; 2nd repetition, on 13 Nov 2013, and 5 Feb 2014.

In the experiment on the pathogen colonization of pruned canes, canes were pruned in mid-winter (January). Two different lengths (2 cm and 5 cm) between the uppermost node and the pruning wound were left after pruning for a total of 40 canes per pathogen and internode length. Pruning wounds were separately inoculated with the pathogens as explained above. All pruning wounds were covered with the NITEX mesh soon after the inoculations. A control treatment was also included and treated with sterile distilled water instead of pathogens. Experiment was set up in a complete randomized design with three treated canes in the same cordon of a vine. The experiment was repeated in two consecutive years (2013-14), with the following pruning dates: 1st repetition, on 10 Jan 2013; 2nd repetition, on 15 Jan 2014.

8.4.4. Isolation procedures

In the experiment on wound susceptibility, fungal recovery was done to assess fungal infection four months after each inoculation date. Canes were cut about 20 cm below the pruning wounds and stored at 4 °C until further use. All canes were processed for fungal reisolations within a 48 h period after their collection. In the laboratory, bark of canes was removed with a sterile scalpel from the top 5 cm internode including the pruning wound. The top 2 mm of the cane including the pruning wound area was removed with sterile pruning shears and two fragments of 5 mm approximately were cut immediately below.

Fragments were surface-sterilized by soaking in 70 % ethanol for 4 minutes. After air drying on sterile filter paper to remove excess ethanol, fragments were placed on Petri dishes containing PDA amended with streptomycin sulphate at 50 $mg \cdot l^{-1}$. Plates were incubated at 25 °C until fungal colony growth allowed for pathogen reisolation and subsequent identification based on previously described morphological features (for *Diplodia seriata*, see Phillips *et al.*, 2007; for *Phaeomoniella chlamydospora*, see Crous and Gams, 2000). Recovery percentage for each pruning date, pathogen, and inoculation dates were calculated from the pathogen reisolation frequencies.

Regarding the experiment on the cane colonization, pathogen recovery was carried out at 5 and 9 months after inoculations (June and October, respectively) in half of total inoculated canes in each recovery date. The procedures for bark removal, wood chips cuttings, wood surface sterilization and plating were done as previously described. However, in order to estimate cane colonization for each pathogen, several cane sections were taken at different distances from the pruning wound (Figure 8.1). In the case of the 2-cm pruned internode, at 0.5 cm, 1.5 cm and 4.5 cm below the pruning wound. The latter point was located about 0.5 cm below the uppermost node. In the case of the 5-cm pruned internode, reisolations were attempted at the same sites as above (all of them over the uppermost node), and from a fourth site, 0.5 cm below the node and roughly about 7.5 cm below the pruning wound. Recovery percentages for each combination of pathogen, recovery date and recovery site were calculated from the reisolation frequencies.

8.4.5. Weather data

In the experiment on wound susceptibility, daily average values of temperature and relative humidity, as well as accumulated rainfall were collected from an automatic weather station located about 100 m away from the vineyard. Weather records were obtained during a period of 1 week after each inoculation event throughout the experiment.

8.4.6. Statistical analyses

Data recorded from the control groups in both experiments were used only to estimate the ratios of natural infections of pruning wounds caused by

airborne inocula of *Diplodia seriata* and *Phaeomoniella chlamydospora*, therefore they were not included in the statistical analyses. Recovery data of both pathogens obtained in each experiment were analyzed using SAS Enterprise Guide v.4.2 running on SAS v.9.2 statistical software (SAS Institute Inc.). Data of percent recoveries were transformed through the arcsine of the square root of the proportion prior the analyses. Analyses of variance for the main factors and their 2-way interaction effects on the dependent variables were performed using the Mixed Models option, where repetition of experiments was considered as a random factor and the remaining independent variables as fixed. The Residual Maximum Likelihood (REML) method was used to estimate the variance components. Separate analyses were done for each pathogen and experiment type. Means were separated using Fisher's least significant difference (LSD) test at the 5 % significance level after ANOVA. Additional independent mean comparisons were performed through Student's *t* test when appropriate. In the experiment on wound susceptibility, Pearson correlation coefficients were calculated between mycelium recovery and weather data.

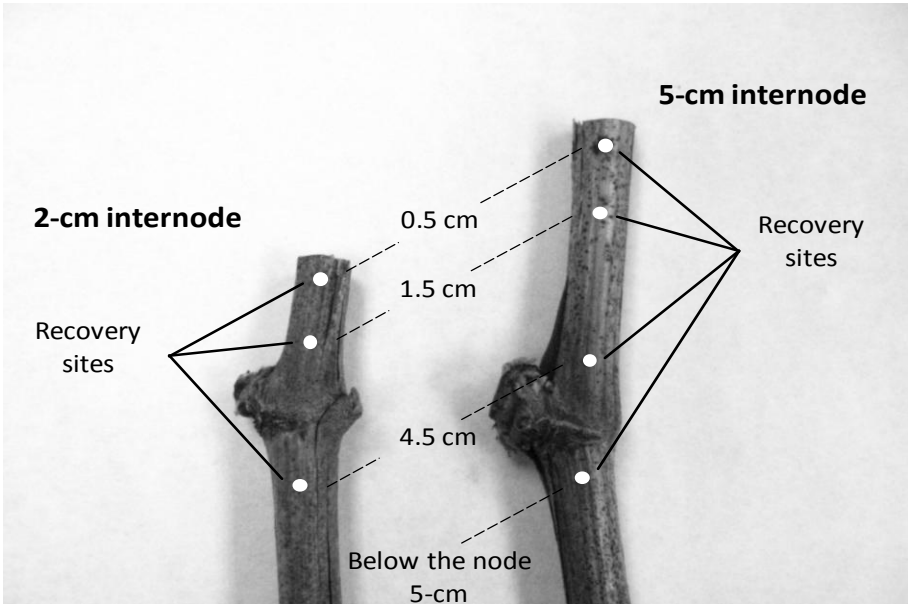

Figure 8.1. Short, 2-cm (left) and long, 5-cm (right) internodes showing the approximate sites of mycelium recovery for *Diplodia seriata* and *Phaeomoniella chlamydospora* in the grapevine cane colonization experiment.

8.5. Results

In both experiments, *Diplodia seriata* and *Phaeomoniella chlamydospora* were recovered at variable rates from artificially inoculated pruning wounds (Figures 8.2-8.5). Natural infections by *D. seriata* in the control groups of the wound susceptibility experiment ranged between 0 and 5 % in all repetitions. In the cane colonization experiment, natural infections by *D. seriata* ranged between 0 and 10 % five months after inoculation and between 55 and 70 % nine months after inoculation. Despite of the high percentages of natural infections by *D. seriata*, recovery of *P. chlamydospora* could be properly assessed in the canes artificially inoculated with this pathogen. None spontaneous infection of *P. chlamydospora* was detected in any experiment.

In the experiment on wound susceptibility, the pruning date had no significant effects on the mycelium recovery of *D. seriata* (F=2.35, P=0.3677). However, the effects of the inoculation date after pruning (F=36.53, P=0.0006) and its interaction with the pruning date (F=3.38, P=0.0052) proved to be highly significant on the mycelium recovery of *D. seriata*; therefore, the whole dataset was splitted into two subsets according to the pruning dates and data were reanalyzed. Mycelium recovery of *D. seriata* was 97.5 % when inoculated 1 day after pruning, irrespective of the pruning date (Figure 8.2). When the pathogen was inoculated 1 week after pruning, recovery percentages were around 70 % despite of the pruning date but, thereafter, the patterns of decrease in mycelium recovery were different between pruning dates (Figure 8.2). Whereas inoculation 2 weeks after pruning resulted in 65 % mycelium recovery in the case of a late pruning, fungal recovery 2 weeks after an early pruning was reduced to 35 %. The mycelium recovery progressively decreased as increased the time between pruning and inoculations, and resulted in mean values around 10 % for both pruning dates at the end of the experimental period (Figure 8.2). Mycelium recovery of *P. chlamydospora* showed a significant decrease (F=9.00, P=0.0154) in mycelium recovery dependent on the time of inoculation, whereas no statistical significances were detected for the effects of the pruning date (F=0.10, P=0.8072) and the 2-way interaction of the main factors (F=1.22, P=0.2977). In both pruning dates, mycelium recovery of *P. chlamydospora* was 75 % when wounds were inoculated 1 day after pruning. Percentages of mycelium recovery decreased along inoculation dates in a similar way despite of

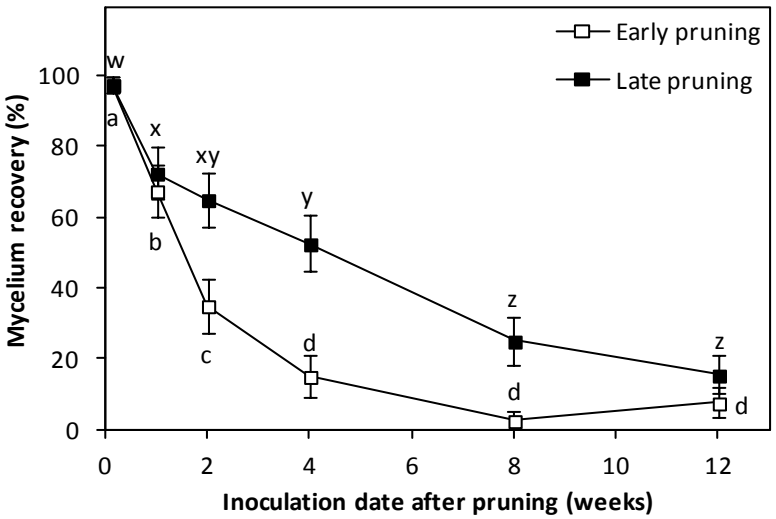

Figure 8.2. Mean percentages of mycelium recovery of *Diplodia seriata* inoculated on pruned canes of grapevine cv. 'Tempranillo' at different dates after an early pruning in November (white squares) and a late pruning in February (black squares). Forty replications were used in each combination of pruning and inoculation date. Significant differences among means (P<0.05) are indicated by different letters (*a* to *d* for the early pruning, and *w* to *z* for the late pruning). Bar errors correspond to the standard error of the mean.

the pruning date (Figure 8.3). Values corresponding to inoculations done 2 weeks after pruning were around 50 %, and about 25 % when canes were inoculated 8 weeks after pruning (Figure 8.3). Values corresponding to inoculations done 4 weeks after pruning were dissimilar between pruning dates but coincided thereafter. Recovery percentages were around 10 % when inoculations were done 12 weeks after pruning irrespective of pruning date.

Weather data for both experiment repetitions and pruning dates are shown in Table 8.1. In the first repetition (years 2012-13), mean temperature and relative humidity decreased along the inoculations dates done after the early pruning (from 15.1 to 8.2 °C, and from 71.7 to 54.6 %, respectively), whereas increased along inoculations done after the late pruning (from 8.2 to 14.8 °C, and from 54.6 to 70.3 %, respectively). Accumulated rainfall ranged from 2.1 to 7.0 mm after the early pruning and from 1.2 to 18.3 mm after the late pruning. However, evolution patterns in these variables were irregular and

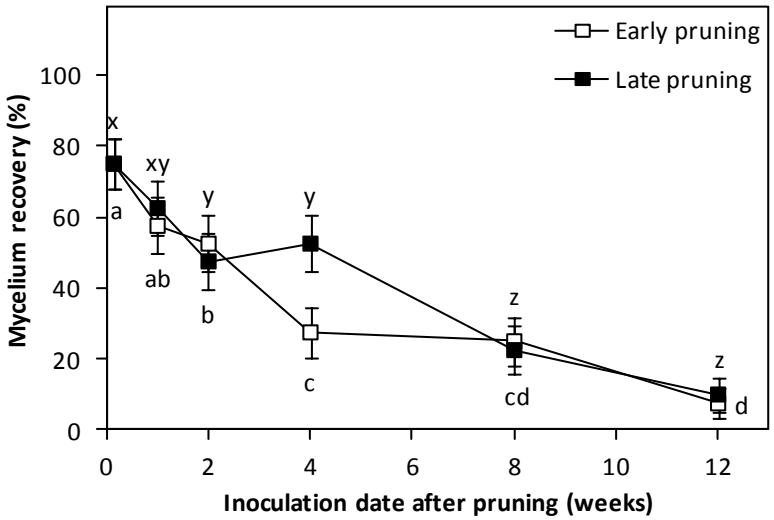

Figure 8.3. Mean percentages of mycelium recovery of *Phaeomoniella chlamydospora* inoculated on pruned canes of grapevine cv. 'Tempranillo' at different dates after an early pruning in November (white squares) and a late pruning in February (black squares). Forty replications were used in each combination of pruning and inoculation date. Significant differences among means ($P<0.05$) are indicated by different letters (*a* to *d* for the early pruning, and *x* to *z* for the late pruning). Bar errors correspond to the standard error of the mean.

showed partial low and high values within the experimental period (Table 8.1). In the second repetition (years 2013-14), evolution patterns of mean temperature and relative humidity were similar to those of the previous season, although an increase in mean temperature was noticed in the last three inoculation periods during the early pruning (Table 8.1). Mean values of temperature ranged from 8.6 to 13.5 °C during the early pruning period, and from 11.4 to 15.8 °C along the late pruning. Mean relative humidity records ranged from 50.4 to 79.0 % and from 56.7 to 71.9 % for the early and late pruning periods, respectively. Whereas accumulated rainfalls after the late pruning in both experimental repetitions were comparable (43 and 42 mm, respectively), an unusual rainy period (over 130 mm) was recorded for the week following the inoculations on 1 day after pruning in the early pruning 2013-14 (Table 8.1).

Significant positive correlations were found between recovery percentages of *D. seriata* and temperature after the early pruning carried out in 2012-13 (r^2=0.89, *P*=0.0181). However, significant negative correlations were found between recovery percentages of *D. seriata* and temperature and between recovery percentages of *P. chlamydospora* and temperature after the late pruning carried out in 2012-13 (r^2=-0.89, *P*=0.0167, and r^2=-0.81, *P*=0.0497, respectively). No other significant correlations were found between recovery percentages of pathogens and the meteorological data (Table 8.2). Additional correlations were calculated for the recovery percentages and the weather data for extended periods after inoculations (2 and 3 weeks) but no additional significant results were obtained and thus are not shown here.

Table 8.1. Mean temperature, mean relative humidity and accumulated rainfall during the one-week period after each inoculation date in the wound susceptibility experiment. Mean values are followed by the (±) standard error when appropriate.

Year	Pruning season	Inoculation date	Temperature (ºC)	Relative humidity (%)	Accumulated rainfall (mm)
2012-2013	Early pruning	1 day	15.13 ± 0.36	71.7 ± 4.9	7.0
		1 week	13.40 ± 0.37	78.1 ± 1.1	1.2
		2 weeks	8.46 ± 0.64	54.7 ± 2.6	0.0
		4 weeks	12.11 ± 1.13	65.7 ± 4.0	2.1
		8 weeks	8.24 ± 0.28	67.0 ± 3.0	2.5
		12 weeks	8.21 ± 0.64	54.6 ± 3.3	0.0
	Late pruning	1 day	8.21 ± 0.64	54.6 ± 3.3	0.0
		1 week	10.11 ± 0.31	69.0 ± 4.8	1.2
		2 weeks	6.94 ± 1.07	68.7 ± 4.3	11.1
		4 weeks	10.6 ± 0.74	65.9 ± 4.0	5.7
		8 weeks	11.05 ± 0.81	68.7 ± 4.6	18.3
		12 weeks	14.81 ± 0.38	70.3 ± 1.4	6.8
2013-2014	Early pruning	1 day	13.5 ± 0.6	66.7 ± 6.5	130.6
		1 week	8.8 ± 0.2	50.4 ± 2.9	0.0
		2 weeks	8.6 ± 0.9	51.3 ± 2.3	0.7
		4 weeks	9.9 ± 0.4	73.6 ± 2.6	0.0
		8 weeks	10.3 ± 0.5	79.0 ± 2.3	4.9
		12 weeks	11.4 ± 0.7	59.6 ± 3.2	15.5
	Late pruning	1 day	11.4 ± 0.7	59.6 ± 3.2	15.5
		1 weeks	12.3 ± 0.8	66.0 ± 3.5	8.2
		2 weeks	10.4 ± 0.3	70.4 ± 3.2	4.2
		4 weeks	12.2 ± 0.3	56.7 ± 3.2	0.0
		8 weeks	14.9 ± 0.3	71.9 ± 3.7	14.0
		12 weeks	15.8 ± 0.2	70.4 ± 1.8	0.2

Table 8.2. Correlation coefficients (*r*) and significance values (*P*) between main meteorological variables and the mycelium recovery of *Diplodia seriata* or *Phaeomoniella chlamydospora* in the wound susceptibility experiment.

Years	Pruning season	Pathogen species	Temperature		Relative humidity		Accumulated rainfall	
			r	*P*	*r*	*P*	*r*	*P*
2012-13	Early pruning	*D. seriata*	0.89	0.0181	0.67	0.1464	-0.59	0.2178
		P. chlamydospora	0.75	0.0882	0.72	0.1063	0.68	0.1397
	Late pruning	*D. seriata*	-0.89	0.0167	-0.48	0.3381	-0.51	0.3004
		P. chlamydospora	-0.81	0.0497	-0.36	0.4815	-0.60	0.2108
2013-14	Early pruning	*D. seriata*	0.30	0.5620	-0.40	0.4261	0.71	0.1113
		P. chlamydospora	0.02	0.9687	-0.45	0.3747	0.49	0.3208
	Late pruning	*D. seriata*	-0.60	0.2046	-0.67	0.1434	0.47	0.3505
		P. chlamydospora	-0.32	0.5380	-0.74	0.0900	0.62	0.1921

In the cane colonization experiment, the recovery date had no significant effect on the overall mycelium recovery of *D. seriata* and *P. chlamydospora* from inoculated canes (Table 8.3). However, significant differences in the mycelium recovery at the different recovery sites were detected for both pathogens (Table 8.3; Figures 8.4 and 8.5). Moreover, a significant interaction between the recovery date and the recovery site was detected for *D. seriata* at any length of the pruned internode, whereas no interaction between the same factors was observed for *P. chlamydospora* (Table 8.3). Mycelium recovery percentages for both pathogens significantly decreased as increased the length between the pruning wound and the recovery site (Figures 8.4 and 8.5). A visual inspection of the pruned internodes made during the reisolations showed that 25 % of the 2-cm pruned internodes and 5 % of the 5-cm pruned internodes appeared to be completely or partially dried 5 months after inoculations. However, same figures in reisolations at the end of the experimental period (9 months) were 100 and 95 %, respectively, thus indicating that effective internode drying occurred in between and coinciding with the summer season. No substantial differences were observed between the repetitions of the experiment.

Table 8.3. Analysis of variance-type statistics on the mycelium recovery percentage of *Diplodia seriata* and *Phaeomoniella chlamydospora* inoculated on grapevine pruned canes with two different pruned internode lengths.

Pathogen species	Pruned Internode	Factor	dfN	dfD	*F*	*P*
Diplodia seriata	2 cm	Recovery date (A)	1	1	13.54	0.1689
		Recovery site (B)	2	2	49.42	0.0198
		A x B	2	230	3.49	0.0322
	5 cm	Recovery date (A)	1	1	29.86	0.1152
		Recovery site (B)	3	3	17.88	0.0204
		A x B	3	307	5.01	0.0021
Phaeomoniella chlamydospora	2 cm	Recovery date (A)	1	1	1.47	0.4390
		Recovery site (B)	2	2	41.54	0.0235
		A x B	2	230	0.97	0.3809
	5 cm	Recovery date (A)	1	1	0.20	0.7309
		Recovery site (B)	3	3	45.10	0.0054
		A x B	3	307	1.29	0.2778

Five months after inoculation, mycelium recovery of *D. seriata* from the two uppermost recovery sites (0.5 and 1.5 cm) ranged between 67.5 and 90.0 % despite of the pruned internode length, but significant differences were detected between both recovery sites for each internode length (Figure 8.4). Mycelium recovery of *D. seriata* at 4.5 cm below the pruning wound was 5 % in canes with 2-cm pruned internodes and 40 % in canes with 5-cm pruned internodes, whereas no reisolation was obtained below the top node in 5-cm pruned internodes (Figure 8.4). Nine months after inoculation, no differences in mycelium recovery of *D. seriata* were found within the two and three uppermost recovery sites in canes with 2-cm and 5-cm pruned internodes, respectively; with mean values ranging from 90.0 to 97.5 % (Figure 8.4). However, recovery below the node remained significantly lower than those from upper sites even percentages had increased to 32.5 % (2-cm internode length) and 40.0 % (5-cm internode length) from the previous recovery date. Significant differences in recoveries at 4.5 cm below the pruning wound were

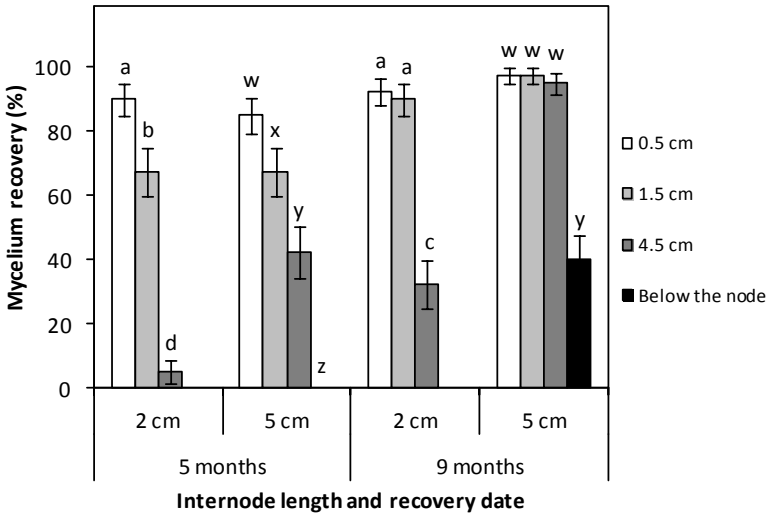

Figure 8.4. Mean percentages of mycelium recovery of *Diplodia seriata* inoculated on pruned canes of grapevine cv. 'Chardonnay' and reisolated from different recovery sites in canes of 2-cm and 5-cm internode length corresponding to two different incubation periods (5 and 9 months) after pruning. Forty replications were used in each combination of recovery site, internode length and recovery date. Significant differences (P<0.05) among means for each recovery site are indicated separately by different letters (*a* to *c* for the 2-cm internodes, and *w* to *z* for the 5-cm internodes). Bar errors correspond to the standard error of the mean.

found between the 2-cm and 5-cm pruned internodes 5 and 9 months after inoculation (t=4.33, P<0.0001, and t=7.56, P<0.0001, respectively).

Mycelium recovery of *P. chlamydospora* in the two uppermost reisolation sites ranged between 60 and 85 % despite of the internode length and recovery date, and significant differences between both recovery sites were found only in 5-cm internodes and 5 months after inoculation (Figure 8.5). Mycelium recovery percentages from the 4.5-cm recovery site were always significantly lower than values from the uppermost sites, despite of the pruned internode length and the recovery date. However, no significant differences in mycelium recovery at the 4.5-cm site between the 2-cm and 5-cm pruned internodes were detected in either recovery date (t=1.79, P=0.0770, and t=1.50, P=0.1364, respectively). Recovery percentages below the node were equal or lower than 20 % despite of the internode length and the recovery date (Figure 8.5).

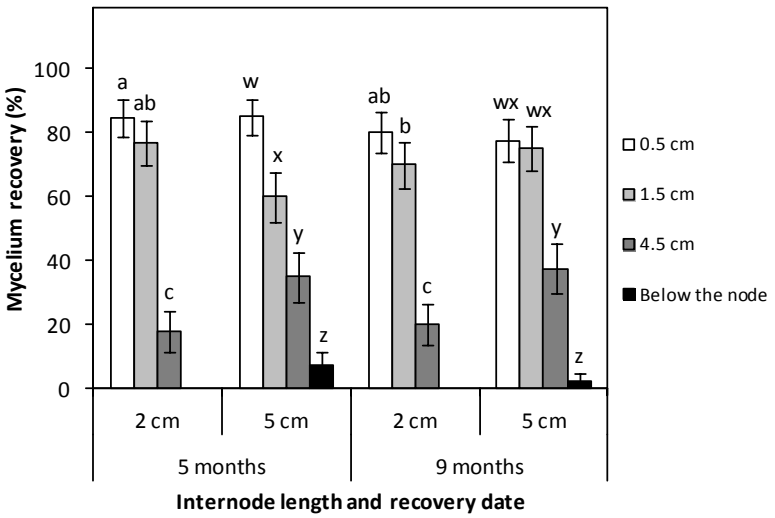

Figure 8.5. Mean percentages of mycelium recovery of *Phaeomoniella chlamydospora* inoculated on pruned canes of grapevine cv. 'Chardonnay' and reisolated from different recovery sites in canes of 2-cm and 5-cm internode length and corresponding to two different incubation periods (5 and 9 months) after pruning. Forty replications were used in each combination of recovery site, internode length and recovery date. Significant differences (P<0.05) among means for each recovery site are indicated separately by different letters (*a* to *c* for the 2-cm internodes, and *w* to *z* for the 5-cm internodes). Bar errors correspond to the standard error of the mean.

The overall mean of *D. seriata* recovery 5 months after inoculations was 54.2 % for the 2-cm pruned internodes and 48.8 % for the 5-cm pruned internodes, which includes the reisolations attempted on all processed wood chips despite of the reisolation site. At the end of the experimental period, the equivalent values were 70.8 and 80.5 %, respectively. The overall mean of *P. chlamydospora* recovery 5 months after inoculations was 59.8 % for the 2-cm pruned internodes and 46.9 % for the 5-cm pruned internodes. At the end of the experimental period, the equivalent values were 50.0 and 43.1 %, respectively.

8.6. Discussion

In this study the susceptibility of grapevine pruning wounds to the fungal pathogens *Diplodia seriata* and *Phaeomoniella chlamydospora*, as well as

the colonization of pruned canes were evaluated after artificial inoculations with spore suspensions of each pathogen. Susceptibility of pruning wounds was evaluated in two different pruning seasons, *i.e.* an early pruning in fall and a late pruning in winter, by inoculating canes at different times after pruning. Pathogen colonization of pruned canes was assessed by reisolating the pathogens from different sites along the cane at different times after inoculation. Moreover, the effect of the length of the pruned internode on the cane colonization was studied by infecting either short (2 cm) or long (5 cm) pruned internodes.

Wound susceptibility decreased as increased the time between pruning and inoculations for both pathogens. However, inoculations made 12 weeks after pruning yielded recovery percentages of *D. seriata* and *P. chlamydospora* around 10 %. These results were in accordance with the findings of previous researches, which confirmed that pruning wounds remained susceptible to these two fungi up to four months after pruning (Gubler *et al.*, 2001; Eskalen *et al.*, 2007; Serra *et al.*, 2008). However, this study and those previously cited based their conclusions on artificial inoculations of canes, in which inoculum potentials of pathogens are usually overrated as compared to the natural occurring inoculum pressure, and thus the wound susceptibility period could be overestimated. To date, no data are available on the susceptibility of wounds naturally infected and this remains to be further studied.

Seasonal differences in wound susceptibility to *D. seriata* were found to be depending on the pruning season. A rapid decrease down to 35 % infected canes was observed 2 weeks after an early pruning whereas a comparable decrease was not found until 8 weeks after a late pruning. These latter results were consistent with those reported by Serra *et al.* (2008), where in Italy pruned canes of grapevines cv. 'Sauvignon Blanc' remained susceptible to *D. seriata* up to 8 weeks after a pruning done in March. Moreover in South Africa, pruning wounds of grapevines cv. 'Chenin Blanc' remained more time susceptible to the Botryosphaeriaceae species *Neofusicoccum australe* when wounds were made and inoculated in late winter than in mid winter (van Niekerk *et al.*, 2011). However, in a study carried out in California by Úrbez-Torres and Gubler (2011) with two other Botryosphaeriaceae fungi, namely *Lasiodiplodia theobromae* and *Neofusicoccum parvum*, wound susceptibility to pathogens was significantly

higher in early winter (December-January) than in late winter (February-March). Moreover, Úrbez-Torres and Gubler (2011) observed a high rate of infection after an earlier pruning done in November. Luque *et al.* (2014) detected a higher percentage of natural infections of *D. seriata* in Catalonia, NE Spain after a late pruning done in February. These results are in accordance with those obtained here, since an overall increased period of wound susceptibility in spring would support a higher incidence of natural infections caused by *D. seriata* on grapevines in this wine region.

No seasonal differences were found in wound susceptibility in the case of *P. chlamydospora*, as the pattern of decrease in wound susceptibility was similar despite of the pruning date. Serra *et al.* (2008) also reported a decrease of susceptibility to *P. chlamydospora* along the growing season without clear differences in susceptibility between pruning dates. Larignon and Dubos (2000) showed that wound susceptibility to *P. chlamydospora* was about 7-9 weeks after a mid-winter pruning (January), whereas only 1-2 weeks after a late-winter pruning (March) in France. However, results of susceptibility duration obtained by Larignon and Dubos (2000) were highly variable among the different experimental repetitions, thus suggesting that other factors different from the host and the pathogen could be involved in the wound susceptibility duration. Gubler *et al.* (2001) observed vascular streaking in pruning wounds inoculated with *P. chlamydospora* from February to June, indicating that grapevine tissue was susceptible to the infection from dormancy to green actively growing tissue. Luque *et al.* (2014) reported a strong seasonal effect in the natural infections of pruned canes by *P. chlamydospora* in Catalonia, where infections after a late winter pruning were more frequent than those observed after an early pruning in fall. Results included in this study would indicate that wound susceptibility is similar in both pruning seasons, therefore the seasonal differences in natural infections recorded by Luque *et al.* (2014) could be related with the availability of the infective inoculum of *P. chlamydospora* in each pruning season.

Significant correlations between weather data and recovery percentages were rarely observed in this study; they were only found between temperature and pathogen recovery in the first repetition carried out in 2012-2013, whereas no significant correlations were found in 2013-2014. Correlation

coefficients between temperature and recovery percentages for *D. seriata* and *P. chlamydospora* were positive after an early pruning and negative after a late pruning, as the decrease in wound susceptibility after the early pruning coincided with a decrease in temperature recordings and the decrease in spring was coincident with increasing temperatures. Serra *et al.* (2008) observed high infection rates of pruning wounds by *D. seriata* during episodes of high temperatures and also regularly distributed rainfall in Italy, which is similar to that observed in this study. However, in California, susceptibility of pruning wounds to infection by *L. theobromae* and *N. parvum* decreased as increased the temperature in spring (Úrbez-Torres and Gubler, 2011). Other studies carried out with *Eutypa lata* in California (Trese *et al.*, 1980; Munkvold and Marois, 1995) and France (Chapuis *et al.*, 1998) also reported that pruning wounds were less susceptible under increasing temperature conditions. These results evidence that susceptibility of pruning wounds may vary among geographic regions and pathogenic fungi. Therefore, it is suggested that pruning wound susceptibility should be studied on a local or regional basis to better understand this host-pathogen interaction within the infection process.

Diplodia seriata showed an overall higher potential for cane colonization than *P. chlamydospora* as concluded from the recovery percentages of the pathogens along the infected canes and recovery dates. The higher recovery percentages of *D. seriata* at the end of the experiment (9 months) in all assessed recovery sites would support this observation. These results are in accordance with growth data of these pathogens observed *in vitro*, as it is expected that a higher *in vitro* growth rate would allow the fungus for a quantitatively rapid cane colonization. Úrbez-Torres *et al.* (2006) reported an *in vitro* growth rate of *D. seriata* of approximately 25 mm/48 h, whereas Pitt *et al.* (2013a) estimated a rate about 15-20 mm/day at 25-27 °C for the same species. Tello *et al.* (2010) estimated a growth rate for *P. chlamydospora* in the range 0.7-1.4 mm/day, whereas Whiting *et al.* (2001) reported a maximum rate of growth <2 mm/day at 25 °C. However, Troccoli *et al.* (2001) reported that *P. chlamydospora* required nine months to colonise the first 20-25 cm of grapevine stems in one-year-old potted micropropagated vines (1103 Paulsen rootstock). This is contradictory with our results but great differences in the experimental conditions of these two researches (*e.g.* plant age and variety, inoculation methods, virulence of strains) could explain those differences. In a previous

pathogenicity test with the same strains used here (but formerly named as *D. seriata* strain 398 and *P. chlamydospora* strain 454) both pathogens showed vascular necrosis of similar size (2.4 to 4.5 cm) on vines cvs. 'Macabeo' and 'Tempranillo' (Luque *et al.*, 2009), but recovery at different distances from the site of inoculation were not performed so that the cane colonization rates for both pathogens were not estimated.

The healing of pruning wounds, as characterized by the drying of the pruned internode tissues, would not have limited the cane colonization by *D. seriata*, as recovery percentages were higher at the end of the experimental period than in the first assessment. Moreover, recovery percentages were greater in lower recovery sites, thus confirming the progression of the pathogen downwards the cane. On the contrary, overall recovery percentages of *P. chlamydospora* were slightly reduced at the end of the experimental period, which suggests that cane colonization and fungal survival could be limited by cane drying. However, this hypothesis would need further research to be confirmed. Regarding the potential effect of the internode length on the cane colonization by pathogens, results showed that *D. seriata* was able to colonize over 90 % the three uppermost sites of the 5-cm internodes in 9 months. However, colonization below the node in 2-cm internodes (*i.e.*, 4.5 cm below the pruning wound) was significantly reduced as compared to the equivalent site in 5-cm internodes. This would suggest that the node may limit the rate of cane colonization by this pathogen but, on the other side, *D. seriata* reached the living tissues below the top node in similar percentages irrespective of the pruned internode length at the end of the experiment. Data on cane colonization by *P. chlamydospora* showed that pathogen recovery at 4.5 cm below the pruning wound was similar in 2-cm and 5-cm internodes at the end of the experiment. This would reject the hypothesis that the node may make difficult cane colonization by *P. chlamydospora*. This fungus is able to move actively in vessels and in the pith (Pascoe and Cottral, 2000; Troccoli *et al.*, 2001), and it is suspected that the fungus may spread into canes by movement of spores produced in vessels, although this has not been already proved (Pascoe and Cottral, 2000). However, it is important to note that, at the end of the experiment, mycelium recovery of *P. chlamydospora* below the node was significantly lower in 5-cm pruned internodes than in 2-cm internodes. This would confirm that the wood colonization rate of this pathogen may be lower

than that of *D. seriata*, and that a longer pruned internode may make difficult cane colonization by *P. chlamydospora*.

Results obtained in this study showed that pruning wound susceptibility and posterior cane colonization in grapevines may show differential features for different trunk pathogens such as *D. seriata* and *P. chlamydospora*. To the best of our knowledge, this is the first time that rates of infection at different isolation sites are assessed in artificially inoculated canes to study cane colonization of these pathogens; therefore, additional research would be needed to confirm these results in other grape growing regions. Finally, a global strategy for pruning could be optimized by pruning on dates when wound susceptibility could be lower for pathogens, and by increasing the length of the pruned internode, as longer internodes may make difficult cane colonization by slow-growing pathogens.

8.7. Acknowledgements

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Capítol 9

Viability of Botryosphaeriaceae species pathogenic to grapevine after hot water treatment

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9.1. Resum

Es va estudiar la viabilitat de vuit espècies patògenes de la família *Botryosphaeriaceae* després d'haver estat sotmeses a un tractament de termoteràpia amb aigua calenta (TAC). L'objectiu d'aquest treball ha estat el d'avaluar la validesa d'aquesta tècnica per a ser emprada com a mètode de control en els processos de propagació del material vegetal al viver. Es va realitzar un primer assaig *in vitro*, en el que fragments d'agar amb el miceli dels fongs es van introduir en tubs Eppendorf amb aigua destil·lada estèril i es van sotmetre a diferents combinacions de temperatura (50-54 °C) i temps d'exposició (15, 30 i 45 minuts) en un bany d'aigua calenta. El creixement miceliar de cada fong es va contrastar amb el del tractament control (miceli no tractat). Es van observar efectes significatius dels tractaments en la supervivència i el creixement miceliar per tots els factors analitzats (espècie, temperatura i temps), així com en les interaccions dobles dels diferents factors. En general, la supervivència dels fongs va disminuir a mesura que va augmentar la temperatura i el temps d'exposició. *Diplodia seriata*, *Neofusicoccum luteum*, *N. parvum* i *Spencermartinsia viticola* van ser les espècies més susceptibles a la temperatura, mentre que *Lasiodiplodia theobromae* i *N. vitifusiforme* van ser les més tolerants. En un segon assaig (*in planta*), els fongs van ser inoculats en sarments del portaempelt Richter 110. Els sarments inoculats es van incubar durant tres setmanes a 25 °C per a promoure la colonització del fong al sarment. Després de la incubació, els sarments van ser tractats al bany d'aigua calenta durant 30 minuts, en un rang de temperatures de 50 a 53 °C. La supervivència de tots els fongs es va reduir significativament en el tractament de 51 °C i temperatures superiors. A 50 °C, *L. theobromae* va ser l'espècie més tolerant i *N. luteum* la més susceptible. Els resultats obtinguts demostren l'eficàcia del TAC per al control d'aquests patògens en el procés de producció de planta als vivers.

9.2. Abstract

The viability of eight species of Botryosphaeriaceae pathogenic to grapevine was studied after a hot water treatment (HWT) in order to evaluate the feasibility of this technique as a potential tool to control these species during the grapevine propagation process. In a first trial (*in vitro*), mycelial plugs

contained in Eppendorf tubes with sterile distilled water were subjected to different combinations of temperature (50-54 °C) and exposure time (15, 30 and 45 minutes) in a hot water bath. Growth rates of treated mycelia were compared to untreated controls. Significant differences in survival and growth for all factors (species, temperature and time) and their 2-way interactions were observed. Fungal survival and growth generally decreased with increasing temperatures and exposure times. *Diplodia seriata*, *Neofusicoccum luteum*, *N. parvum* and *Spencermartinsia viticola* were the most susceptible species to temperature, while *Lasiodiplodia theobromae* and *N. vitifusiforme* were the most tolerant. In a second trial (*in planta*), the fungi were inoculated into grapevine canes (Richter 110 rootstock). Inoculated canes were incubated at 25 °C for 3 weeks to allow the fungi to colonize the wood and then subjected to HWT in the range of 50-53 °C for 30 minutes, and survival of fungi after HWT was assessed. Survival of all species was sharply reduced after HWT of 30 minutes at 51 °C and higher temperatures. At 50 °C, *L. theobromae* was the most tolerant species whereas *N. luteum* was the most susceptible. Results obtained in this study demonstrate the feasibility of controlling these pathogens by HWT in the nursery grapevine propagation process.

9.3. Introduction

Several species included in the Botryosphaeriaceae Theiss. & Syd. are causal agents of Botryosphaeria dieback of grapevine (*Vitis vinifera* L.), and have been reported as major trunk pathogens of grapevines worldwide (Úrbez-Torres, 2011). These pathogens cause wood necrosis in trunks and arms of infected vines, which decrease the productivity and longevity of vineyards (Úrbez-Torres, 2011; Bertsch *et al*., 2013). Although the incidence of Botryosphaeria dieback is common in 8-year and older vineyards (Larignon and Dubos, 2001b), Botryosphaeriaceae species have also been reported from grapevine nurseries. These pathogens have been isolated from grapevine rootstock mother plants, canes of scion and rootstock varieties, nursery cuttings and young grafted grapevines in nurseries (Fourie and Halleen, 2002; Halleen *et al*., 2003; Fourie and Halleen, 2004a; Giménez-Jaime *et al*., 2006; Aroca *et al*., 2010; Spagnolo *et al*., 2011; Billones-Baaijens *et al*., 2013b; Billones-Baaijens *et al*., 2013c). These findings demonstrate that Botryosphaeriaceae species are present in grapevine propagation material and in the grafted plants supplied by

nurseries, indicating that some of the current vineyard infections may have originated in the propagation process (Billones-Baaijens *et al*., 2013b). However, in some cases the presence of Botryosphaeriaceae species in grapevine propagation material was not related to the observed external symptoms (Fourie and Halleen, 2004a; Aroca *et al*., 2010; Billones-Baaijens *et al*., 2013b). Presence of these pathogens in symptomless grapevines could be explained by published reports that indicate that Botryosphaeriaceae species can behave as endophytes or latent pathogens in asymptomatic wood of symptomless plants (Slippers and Wingfield, 2007; Phillips *et al*., 2013). Therefore these pathogens could be carried into vineyards in apparently healthy plants used for vine establishment or replacement (Billones-Baaijens *et al*., 2013b).

Control of endogenous fungal pathogens in grapevines is difficult and there is an urgent need to revise the management strategies applied in nurseries to improve the phytosanitary quality of planting material (Gramaje and Armengol, 2011). Several research studies have evaluated the *in vitro* and *in vivo* effectiveness of different fungicides to control Botryosphaeriaceae spp. in vineyards, mainly when applied as pruning wound protectants (Bester *et al*., 2007; Pitt *et al*., 2010; Rolshausen *et al*., 2010a; Amponsah *et al*., 2012; Pitt *et al*., 2012). Nevertheless, the application of fungicides to control fungal trunk pathogens in the nursery process is difficult, because traditional techniques, such as chemical sprays and dips used for the control of surface pathogens, do not penetrate sufficiently into dormant grapevine cuttings to control internal infections (Gramaje and Armengol, 2011). In addition, the range of authorized products for this purpose is becoming limited in most countries.

The use of hot water treatment (HWT) at different temperature-time combinations has been reported as a promising management strategy for the control of black foot and Petri disease pathogens in grapevine propagation material (Fourie and Halleen, 2004b; Gramaje *et al*., 2008; Gramaje *et al*., 2009b; Gramaje *et al*., 2010a; Bleach *et al*., 2013; Gramaje *et al*., 2014). One of the main advantages of a HWT protocol is that the heat is able to completely penetrate the wood, killing internal pathogens, without any detrimental effects on the vine tissue (Waite and Morton, 2007). Recently, Billones-Baaijens *et al*. (2014) reported that HWT can effectively reduce the infection incidence of *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips

and *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips in artificially infected 5C rootstock cuttings, as well as any existing Botryosphaeriaceae species in naturally infected cuttings. To the best of our knowledge, no additional information on the susceptibility of Botryosphaeriaceae species to HWT is available to date. Therefore, the objective of this study was to assess the susceptibility of eight pathogenic Botryosphaeriaceae species to HWT and to evaluate the efficacy of HWT as a control strategy for Botryosphaeriaceae species in grapevine cuttings. The sensitivity of Botryosphaeriaceae species to HWT was assessed in two complementary tests, by i) evaluating the survival and growth of fungal mycelia after HWT *in vitro*, and ii) assessing fungal survival in artificially inoculated grapevine cuttings that were subjected to HWT.

9.4. Material and Methods

9.4.1. Fungal isolates

Eight Botryosphaeriaceae taxa were tested for survival and growth after HWT, namely *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not., *Diplodia seriata* De Not., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *N. luteum*, *N. mediterraneum* Crous, M.J. Wingf. & A.J.L. Phillips, *N. parvum*, *N. vitifusiforme* (Niekerk & Crous) Crous, Slippers & A.J.L. Phillips and *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous. Two isolates per taxon were used in this study except for *N. vitifusiforme*, with only one representative isolate (Table 9.1). All isolates used in the experiments were previously hyphal tip subcultured. Isolates were stored at 4 °C in sterile distilled water tubes with mycelial plugs previously grown on potato dextrose agar (PDA, Difco, Becton, Dickinson and Company). Fungi were grown for 1 week on PDA plates at 25 °C to obtain sufficient quantities of mycelium for the experiments.

9.4.2. Effect of hot water treatment on mycelial survival and growth in vitro

An experiment was carried out to check for survival and growth of isolates after treating fungal mycelia at different combinations of temperature (50 to 54 °C at 1 °C interval) and exposure time (15, 30 or 45 minutes) in a waterbath (JP SELECTA model Unitronic Vaiven; homogeneity 0.1 °C, resolution 0.1 °C). All three exposure times were tested initially at 50 °C, and temperature

Table 9.1. Botryosphaeriaceae species and representative isolates obtained from grapevine and used in this study. CBS[a] accession numbers are given for some isolates.

Species	Culture no.	CBS Accession	Geographic origin	Collector / Isolator
Botryosphaeria dothidea	JL353	CBS 121484	Caldes de Montbui, Spain	J. Luque
B. dothidea	JL380	CBS 110302	Montemor-o-Novo, Portugal	A.J.L. Phillips
Diplodia seriata	JL354		Caldes de Montbui, Spain	J. Luque
D. seriata	JL398	CBS 121485	Pacs del Penedès, Spain	J. Luque
Lasiodiplodia theobromae	JL664		Fontanars, Spain	R. Raposo
L. theobromae	JL819	CBS 124060	Marsala, Sicily, Italy	S. Burruano
Neofusicoccum luteum	JL381		Sintra, Portugal	A.J.L. Phillips
N. luteum	JL519	CBS 121482	Gandesa, Spain	J. Luque
Neofusicoccum mediterraneum	JL562		Ginestar, Spain	J. Luque
N. mediterraneum	JL763		Spain	R. Raposo
Neofusicoccum parvum	JL396	CBS 121486	L'Arboç del Penedès, Spain	J. Luque
N. parvum	JL445		Piera, Spain	J. Luque
Neofusicoccum vitifusiforme	JL563	CBS 121481	Gandesa, Spain	J. Luque
Spencermartinsia viticola	JL525	CBS 117007	Gandesa, Spain	J. Luque
S. viticola	JL571	CBS 117009	Vimbodí, Spain	J. Luque

[a] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

was increased in 1 °C intervals as fungal growth after HWT was observed in at least 10 % of cases. Agar plugs with mycelium (5 mm diam.) were obtained from the actively growing edges of 1-week-old colonies growing on PDA. Four agar plugs per isolate were placed into a 1.5 ml Eppendorf tube containing 1 ml of sterile distilled water, and treated at the above temperature-time combinations in the waterbath. Four Eppendorf tubes per isolate were used as pseudoreplicates in each temperature-time combination. After HWT, Eppendorf tubes were cooled under running tap water for 5 minutes. The agar plugs were removed from the tubes and placed in the centre of PDA plates, one plug per plate, with the mycelium facing the medium surface. Plates were incubated at 22 °C in darkness for 3 days. In addition, six non-treated mycelial plugs were plated onto PDA and incubated under the same conditions as experimental controls. After the incubation period, survival of each mycelial plug was recorded and four colony radii were measured from the colony center at perpendicular angles. Radial growth data were averaged (0.25 cm subtracted

from the mean value to account for the original agar plug size), and the relative growth compared to the controls was calculated for each isolate as

$$100 \times (T_{ijk}/C_k),$$

where T_{ij} is the mean of the four PDA plates per Eppendorf tube in each temperature-time combination *i*, pseudoreplicate *j* (*j*=1-4), and experiment *k* (*k*=1-2), and C_k is the mean of the six control PDA plates per experiment *k*.

Mean mycelium survival in each temperature-time combination was expressed as a percentage (0, 25, 50, 75 or 100 %) thus corresponding to the proportion of living mycelial plugs within the same tube. The experiment was repeated once.

9.4.3. Effect of hot water treatment on mycelial survival in planta

The survival of Botryosphaeriaceae species colonizing grapevine wood was evaluated after the *in vitro* test was completed. Extreme exposure times to HWT in the *in planta* assay were not tested because 30 minutes is the most common duration time used for HWT in grapevine nurseries. This allows temperature to uniformly equilibrate in the internal wood tissues of rootstock or scion cuttings (Gramaje and Armengol, 2011). The *in planta* test was therefore conducted at different temperatures (50 to 53 °C) and with a single 30 minutes treatment period. Dormant grapevine cane segments (20 cm long) of Richter 110 rootstock obtained from a commercial nursery located in Valencia province (Spain) were surface sterilized for 10 minutes in a sodium hypochlorite solution (1.5 % available chlorine), washed twice with sterile distilled water, and allowed to dry on sterile filter paper in a laminar flow cabinet for 1 h. Two holes (4 mm diam.) were drilled on a cane internode at 10 cm from each other. The drill was sterilized by flaming before each use. A mycelial plug (4 mm diam.) obtained from the growing edge of a 1-week-old fungal PDA colony was placed on the hole and the inoculation site was sealed with Parafilm®. For each isolate and temperature combination, four cane segments were used (eight holes in total for each pseudoreplicate). After inoculation, the ends of each cane segment were sealed with paraffin wax to prevent water loss. Cane controls were inoculated with the fungi as explained above but were not hot water treated. Cane segments corresponding to each time and temperature

combination were incubated separately in closed plastic boxes (50 × 35 × 15 cm; 26 l capacity) at 25 °C for 3 weeks in darkness, to allow for fungal colonization of the wood. The boxes were opened twice each week to prevent the onset of fermentative respiration that could affect the physiology of the cuttings and the pathogens. After the incubation period, cane segments were treated in a waterbath (PSELECTA Unitronic 320OR, standard error ± 0.1 °C) filled with distilled water and following the above described schedule. After HWT, canes were cooled for 30 minutes in a bath of tap water at room temperature, and fungal recovery was performed as follows. Two equidistant points (15 mm) were set at each side from the inoculation site. Bark at each reisolation site was removed with a sterile scalpel and a wood chip (5 mm) was cut with a sterile secateur. Each wood chip was then subdivided into two pieces, which were surface sterilized (70 % ethanol, 4 minutes) and plated on malt extract agar (MEA, Bacto Malt Extract, Becton, Dickinson and Company) supplemented with 0.5 $g \cdot l^{-1}$ streptomycin sulphate (Sigma-Aldrich). Fungi were reisolated and identified after 4 days incubation at 25 °C. Fungal survival was annotated for each individual wood chip and the percentage of recovery was calculated for each inoculation site (hole). The experiment was repeated once.

9.4.4. Statistical analyses

Mycelium survival in the *in vitro* and *in planta* experiments, and relative colony growth rates in the *in vitro* experiment were logit transformed prior to analyses. The experiments used a multifactorial design with species, temperature and exposure time (when applicable) considered as fixed factors. Experiment repetition and isolates (when applicable) were used as random factors. The *in vitro* experiments used a split plot design with temperature as the whole plot factor and time as the subplot factor. The *in planta* experiments used a completely randomized design. Analyses of variance for the main factors and their 2-way interaction effects on the dependent variables were performed using SAS Enterprise Guide v.4.2 running on SAS v.9.2 (SAS Institute Inc.), using the MIXED procedure. The Residual Maximum Likelihood (REML) method was used to estimate the variance components. Transformed data means were compared using a least significant difference test (P=0.05).

9.5. Results

9.5.1. Effect of hot water treatment on mycelial survival and growth in vitro

A preliminary statistical analyses of the whole data sets of both mycelial survival and growth showed that all fixed factors and their 2-way interactions were significant (P<0.05), with the Species factor and all 2-way interactions being highly significant (P<0.001). Therefore the whole data set was split into different species groups and the data were re-analysed. A summary of the statistical analyses results is shown in Table 9.2. Regarding mycelium survival, Temperature was significant (P<0.05) for *Botryosphaeria dothidea*, *Lasiodiplodia theobromae* and *Neofusicoccum mediterraneum*. Exposure time to hot water was significant only for *Neofusicoccum vitifusiforme*. However, the interaction Temperature × Time was significant for all species except *N. vitifusiforme* and *Spencermartinsia viticola* (Table 9.2).

The mean values of mycelium survival for all the species at different temperature and time combinations are shown in Figure 9.1. *Spencermartinsia viticola* was the most susceptible fungus to HWT since survival at 50 °C for 15 minutes was only 1.6 % and no growth was recorded at higher temperatures and longer exposure times (Figure 9.1). In a second group, survival of *Diplodia seriata*, *Neofusicoccum luteum* and *Neofusicoccum parvum* at 50 °C, 15 minutes ranged between 26.6 % and 56.3 %, but was less than 15 % for *D. seriata* and *N. parvum* when the exposure time was increased to 30 minutes, and no growth was observed for *N. luteum*. These three species were not recovered over 4 % at any time at 51 °C, and were therefore not tested at 52 °C and higher temperatures (Figure 9.1). In a third group, survival of *B. dothidea* and *N. mediterraneum* from HWT after 15 minutes was more than 67.2 % and 90.6 % respectively for temperatures equal to or lower than 52 °C. A HWT at 52 °C for 45 minutes reduced the viability of these two fungi down to 6.3 % for *B. dothidea* and 1.6 % for *N. mediterraneum*. However, a large reduction in mycelium survival was observed for *B. dothidea* and *N. mediterraneum* at 53 °C, with percentages lower than 7 % for both species at exposure times of 30 and 45 minutes (Figure 9.1). *Lasiodiplodia theobromae* and *N. vitifusiforme* were included in a fourth group that was the most tolerant to HWT (Figure 9.1). Survival of *L. theobromae* was significantly reduced at 53 °C, with survival

percentages of 75.0 % after 15 minutes, 29.7 % after 30 minutes and 3.1 % after 45 minutes, compared with the corresponding values at lower temperatures. Survival of this fungus at 54 °C was below 16 % after the 15 minutes treatment. No mycelium survival was observed at exposure times longer than 15 minutes. In contrast, survival of *N. vitifusiforme* progressively decreased with temperature and time, and mean percentages at the maximum tested temperature ranged from 40.6 % (15 minutes) to 12.5 % (45 minutes), as shown in Figure 9.1.

Regarding the effects of HWT on mycelial growth rates, Temperature was significant (P<0.05) only for *B. dothidea* and *L. theobromae* (Table 9.2). Exposure time to hot water was significant (P<0.05) for *L. theobromae* and *N. mediterraneum*. In addition, the interaction Temperature × Time was highly significant (P<0.001) for all species except *S. viticola* (Table 9.2).

The mean values of the relative mycelium growth for all the species at different temperature and time combinations are shown in Figure 9.2. *Neofusicoccum luteum* and *S. viticola* were the most susceptible fungi to HWT, since growth rates of both fungi were never greater than 1.0 % of the corresponding controls regardless of the treatment (Figure 9.2). In a second group, *D. seriata* and *N. parvum* showed relative growth rates of approx. 20 % of the controls at 50 °C for 15 minutes, but the growth rates at other Temperature × Time combinations were never greater than 2 % (Figure 9.2). In a third group, mycelial growth rates of *B. dothidea* and *N. mediterraneum* after HWT decreased progressively with increasing temperatures and exposure times, and reached negligible values (<2.5 %) at 53 °C (Figure 9.2). *Lasiodiplodia theobromae* and *N. vitifusiforme* were the species most tolerant to higher temperatures (Figure 9.2). Mycelial growth of both species was severely inhibited only at 54 °C, with mean relative growth rates below 6 % at any exposure time within this temperature. However, growth rates of *N. vitifusiforme* were never greater than 50 % whereas rates of *L. theobromae* were greater than those for *N. vitifusiforme* especially at 52 °C and lower temperatures.

Table 9.2. Summary of the statistical analyses results on the survival and growth of mycelia of eight Botryosphaeriaceae species after hot water treatment in the *in vitro* experiment. Variance components estimated through the Residual Maximum Likelihood (REML) method not shown here.

Species	Factor	Mycelium survival				Mycelium growth			
		[a]Num DF	[a]Den DF	F value	P > F	[a]Num DF	[a]Den DF	F value	P > F
Botryosphaeria	Temperature (A)	3	3	11.46	0.0377	3	3	12.97	0.0318
dothidea	Time (B)	2	2	14.64	0.0640	2	2	11.68	0.0788
	A × B	6	167	13.78	<.0001	6	167	31.95	<0.0001
Diplodia	Temperature (A)	1	1	1.67	0.4197	1	1	1.39	0.4480
seriata	Time (B)	2	2	4.92	0.1690	2	2	1.81	0.3555
	A × B	2	81	27.95	<.0001	2	81	40.13	<0.0001
Lasiodiplodia	Temperature (A)	4	4	14.69	0.0117	4	4	8.00	0.0343
theobromae	Time (B)	2	2	7.07	0.1469	2	2	21.87	0.0437
	A × B	8	210	5.91	<.0001	8	210	10.78	<0.0001
Neofusicoccum	Temperature (A)	1	1	1.43	0.4430	1	1	0.76	0.5436
luteum	Time (B)	2	2	1.22	0.4508	2	2	0.84	0.5444
	A × B	2	66	23.16	<.0001	2	66	13.20	0.0005
Neofusicoccum	Temperature (A)	3	3	9.72	0.0470	3	3	5.81	0.0911
mediterraneum	Time (B)	2	2	9.39	0.0962	2	2	23.41	0.0410
	A × B	6	167	5.63	<.0001	6	167	23.65	<0.0001
Neofusicoccum	Temperature (A)	1	1	1.44	0.4422	1	1	1.43	0.4438
parvum	Time (B)	2	2	1.72	0.3670	2	2	1.39	0.4190
	A × B	2	81	26.62	<.0001	2	81	35.15	<0.0001
Neofusicoccum	Temperature (A)	4	4	0.25	0.8976	4	4	0.31	0.8585
vitifusiforme	Time (B)	2	2	25.93	0.0371	2	2	8.77	0.1024
	A × B	8	98	1.64	0.1227	8	98	4.75	<0.0001
Spencermartinsia	Temperature (A)	1	1	1.25	0.4646	1	1	1.25	0.4646
viticola	Time (B)	2	2	1.04	0.4898	2	2	1.04	0.4898
	A × B	2	66	1.25	0.2676	2	66	1.25	0.2676

[a]: Num DF, numerator degrees of freedom; Den DF, denominator degrees of freedom.

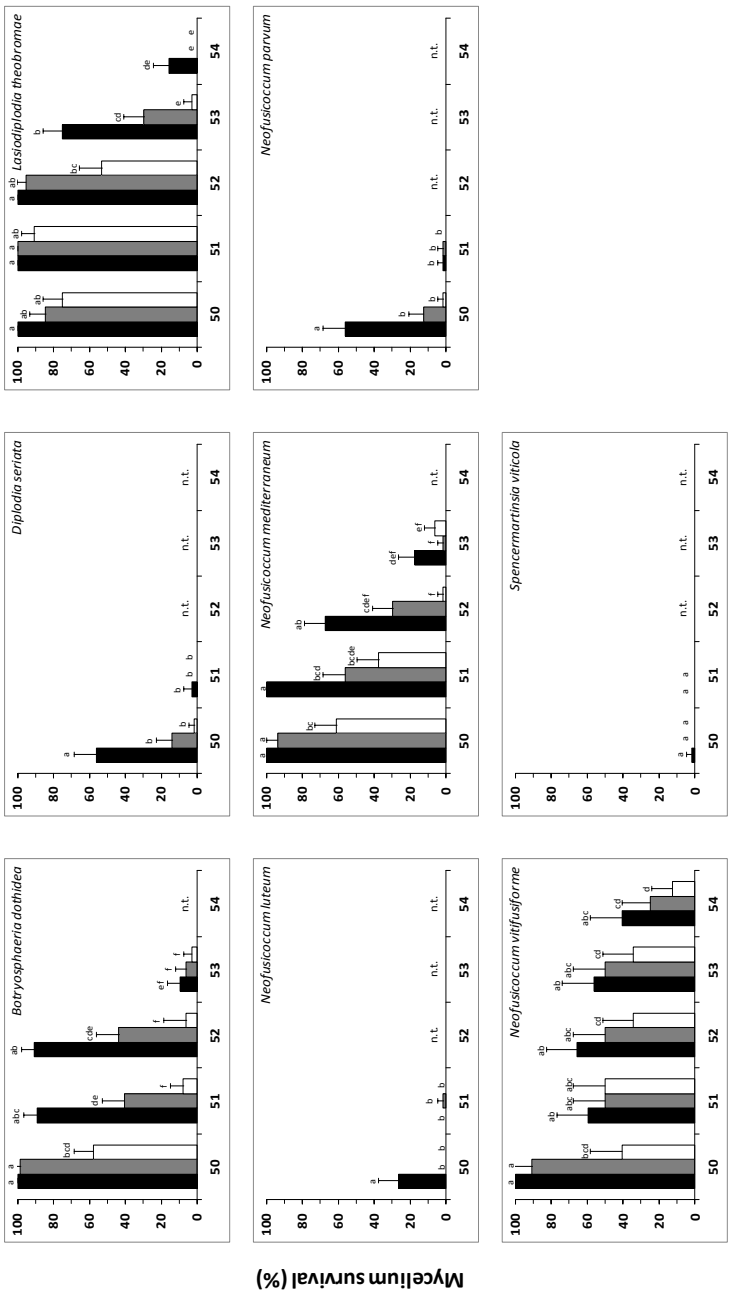

Figure 9.1. Mean mycelium survival of eight Botryosphaeriaceae species after hot water treatment in the *in vitro* experiment. Survival is expressed as the percentage of living mycelial plugs after treatments (*N*= 16 in each temperature by time combination for all species except *N. vitifusiforme*, where *N*=8). Exposure times expressed in black (15 minutes), grey (30 minutes) and white (45 minutes) bars. Mean values significantly different (*P*<0.05) according to LSD tests are indicated by different letters. n.t.=not tested.

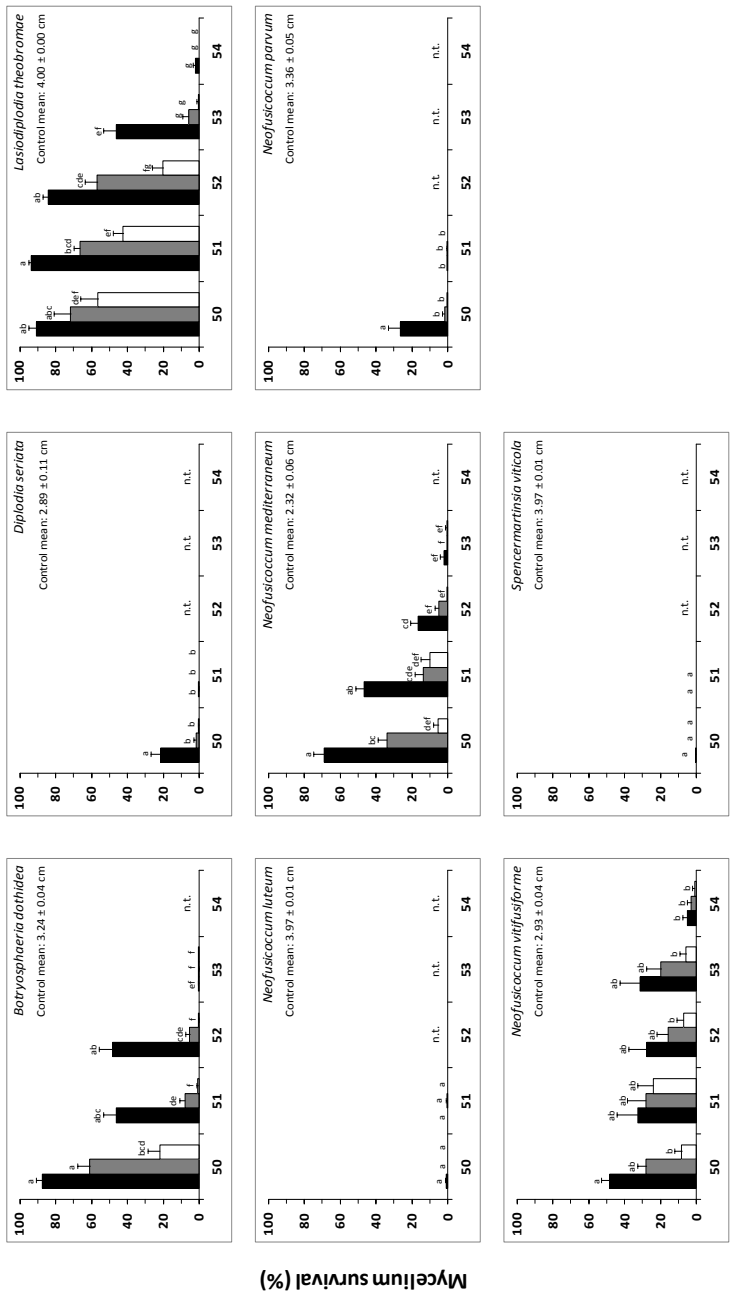

Figure 9.2. Mean relative mycelium growth of eight Botryosphaeriaceae species after hot water treatment in the *in vitro* experiment. Growth rates expressed as the relative percentage of the radial colony growth of treated mycelial plugs (N=16 in each temperature by time combination for all species except *N. vitifusiforme*, where N=8) compared to an untreated control (N=6). Exposure times expressed in black (15 minutes), grey (30 minutes) and white (45 minutes) bars. Mean values significantly different (P<0.05) according to LSD tests are indicated by different letters. The absolute mean value +/- standard error of the mean for the control group is shown at the top right corner of each graphic. n.t.=not tested.

9.5.2. Effect of hot water treatment on mycelial survival in planta

No signs of fermentation were noticed in the incubation period prior to HWT in the experiments. No botryosphaeriaceous fungi other than the inoculated species were reisolated from the untreated controls. Mean mycelial survival in control, untreated canes ranged between 39.8 % (*Diplodia seriata*) and 72.7 % (*Lasiodiplodia theobromae*) (Table 9.3). Survival of fungi after HWT was significantly dependent on Species (P=0.004), Temperature (P=0.005) and their interaction (P<0.001). Therefore, the whole data set was re-analysed in two separate 1-way analyses according to each main factor. *Neofusicoccum luteum* was not recovered from the treated canes at any tested temperature (Table 9.3), showing this to be the species most susceptible to HWT in colonised grapevine wood. In general, viability of fungi was reduced as temperature increased, with nil to very low recovery frequencies (<4 %) for all species at 51 °C and higher temperatures. However, *L. theobromae* and *Neofusicoccum vitifusiforme* were recovered occasionally from treated canes under these conditions (Table 9.3). At 50 °C, all fungi were recovered at lower frequencies than in the control treatments, but differences were statistically significant only for *N. luteum* and *N. vitifusiforme* (Table 9.3). Reduction in recovery percentages for each species at 50 °C were as follows: *Botryosphaeria dothidea* 63.5 %, *D. seriata* 37.3 %, *L. theobromae* 20.4 %, *N. luteum* 100 %, *Neofusicoccum mediterraneum* 45.3 %, *Neofusicoccum parvum* 48.6 %, *N. vitifusiforme* 61.0 % and *Spencermartinsia viticola* 76.3 %. Reduction in recovery percentages at 51 °C for *L. theobromae* was 98.9 %, and for *N. vitifusiforme* was 95.1 %.

Table 9.3. Fungal recovery of different Botryosphaeriaceae species from inoculated grapevine wood hot water treated for 30 minutes at different temperatures. Data expressed in percentages as mean values +/- standard error (SE) of the mean (*N*=16 for all species except *N. vitifusiforme*, where *N*=8). Mean values in the same column followed by different capital letters were statistically different among species. Mean values across the same row followed by different low-case letters were statistically different among temperature treatments.

Species	Treatment											
	Untreated		50 °C		51 °C		52 °C		53 °C			
	Mean ± SE	*Grouping*	*Mean ± SE*	*Grouping*	*Mean ± SE*	*Grouping*	*Mean ± SE*	*Grouping*	*Mean ± SE*	*Grouping*		
B. dothidea	49.22 ± 7.32	B a	17.97 ± 4.79	BC ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b		
D. seriata	39.84 ± 6.13	B a	25.00 ± 5.83	B ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b		
L. theobromae	72.66 ± 6.58	A a	57.81 ± 5.88	A a	0.78 ± 0.78	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b		
N. luteum	42.19 ± 6.29	B a	0.00 ± 0.00	C b	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b		
N. mediterraneum	50.00 ± 6.83	B a	27.34 ± 5.43	B ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b		
N. parvum	56.25 ± 6.55	AB a	28.91 ± 5.84	B ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b		
N. vitifusiforme	64.06 ± 9.67	AB a	25.00 ± 7.91	B b	3.13 ± 2.13	A b	0.00 ± 0.00	A b	3.13 ± 2.13	A b		
S. viticola	46.09 ± 6.16	B a	10.94 ± 3.54	BC ab	0.00 ± 0.00	B b	0.00 ± 0.00	A b	0.00 ± 0.00	A b		

9.6. Discussion

The results obtained in this study show that, depending on time and temperature, HWT can reduce the viability and mycelial growth of Botryosphaeriaceae pathogens of grapevines. In the *in vitro* experiment, responses of fungal viability and growth to different temperature and exposure time combinations were variable and highly dependent on all the experimental factors, including species, temperature and exposure time. *Diplodia seriata*, *Neofusicoccum luteum*, *Neofusicoccum parvum* and *Spencermartinsia viticola* were the most susceptible species to temperature, while *Lasiodiplodia theobromae* and *Neofusicoccum vitifusiforme* were the most tolerant. In general, the viability ranges of the eight Botryosphaeriaceae species included in the *in vitro* experiments were in accordance with previous published data on the range of cardinal temperatures for mycelial growth of these species, so that the higher are the optimal temperatures for growth the greater is the tolerance to HWT. Van Niekerk *et al*. (2004) reported optimal temperature for growth for *N. vitifusiforme* to be 30 °C. Úrbez-Torres *et al*. (2006) studied the optimum temperature for *in vitro* colony growth for seven Botryosphaeriaceae species and estimated that the optimum temperature was 30.8 °C for *L. theobromae* and *Botryosphaeria dothidea*, 29.4 °C for *N. luteum*, 28.2 °C for *N. parvum*, and 26.8 °C for *D. seriata*. Martos (2008) observed that minimum temperature for growth for several Botryosphaeriaceae species (*B. dothidea*, *D. seriata*, *N. luteum*, *N. parvum* and *N. vitifusiforme*) in different culture media was about 15 °C, whereas maximum temperature was about 35 °C. She also observed that the minimum temperature for growth for *S. viticola* was 10 °C, and this species also showed higher growth rates than any other species at 10 and 15 °C. However, growth of this species was reduced at temperatures above 20 °C. These data suggest that *S. viticola* is well adapted for growth at cooler temperatures. In addition, Martos (2008) reported that growth rates for *B. dothidea* and *N. vitifusiforme* at 35 °C were greater than those of any other Botryosphaeriaceae species, thus showing their capability for growth at high temperatures. In a study on colony growth of eight Botryosphaeriaceae species associated with grapevine, Pitt *et al*. (2013a) reported comparable results to those cited above, as they observed that *S. viticola* showed high growth rates at low temperatures (10 °C), and that optimal growth for *L. theobromae* and *B. dothidea* was at close to 30 °C. In addition, the two latter species were still able

to grow at 40 °C, whereas all other species did not. Pitt *et al*. (2013a) also reported optimal temperatures for *D. seriata* (26.6 °C) and *N. parvum* (26.8 °C), which were similar to those reported by Úrbez-Torres *et al*. (2006).

In the *in planta* experiment, responses of fungal viability were not as variable as those observed in the *in vitro* experiment. With the exception of *N. luteum*, which was completely inhibited at 50 °C, mycelium recovery of all the other species was practically suppressed at 51 °C and higher temperatures. Effects of HWT on the viability of *N. luteum* and *N. parvum* have been recently studied by Billones-Baaijens *et al*. (2014). They found that 5C rootstock cuttings artificially infected with either pathogen and hot water treated at 50 °C for 30 minutes resulted in internal infections indices of 100 % for *N. luteum* and 50 % for *N. parvum*. While the reduction in viability of *N. parvum* was comparable to the results obtained in the present study, 100 % survival of *N. luteum* observed by Billones-Baaijens *et al*. (2014) is clearly in conflict with our results. However, both pathogens were effectively controlled by treatment at 53 °C (Billones-Baaijens *et al*., 2014), in agreement with our results.

Diplodia seriata, *N. luteum*, *N. parvum* and *S. viticola*, showed similar responses to HWT both *in vitro* and *in planta*, as they were clearly inhibited at 51 °C in both types of experiments. Conversely, the other four species tested, *B. dothidea*, *L. theobromae*, *Neofusicoccum mediterraneum* and *N. vitifusiforme*, showed different viability responses among the experiments, with a higher tolerance to temperature in the *in vitro* experiments than *in planta*. We have no explanation for this differential behaviour, but it is clear that the conditions in the *in planta* experiment, *i.e.* the use of infected grapevine canes to check for fungal viability after HWT, are similar to the conditions in nurseries, where infected wood coming from infected mother vines is hot water treated to control latent wood pathogens.

Results obtained in this study provide further evidence supporting the potential of HWT as an effective control method for grapevine fungal trunk pathogens in propagation material. In Spain, Gramaje *et al*. (2008; 2009b; 2010a) determined that HWT at 53 °C for 30 minutes is needed to effectively control Petri disease pathogens, although the treatments above 51-52 °C drastically reduced conidial germination and mycelial growth of the pathogens,

while HWT protocols at 50 °C for 30 minutes may be sufficient to control black foot pathogens. However, further research is required to enhance the development of effective HWT protocols to reduce the incidence of fungal infections and ensure healthy propagation material is released from grapevine nurseries.

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Capítol 10

Discussió general

En aquesta tesi s'ha aprofundit en el coneixement de les malalties de la fusta de la vinya, aportant noves dades sobre aspectes relacionats amb la biologia dels fongs patògens, l'epidemiologia de les malalties i, finalment, el seu control. En primer lloc s'ha realitzat una caracterització del fong *Diplodia seriata* a diferents nivells -molecular, fenotípic i patogènic- per a analitzar la seva variabilitat intraespecífica (Capítol 4). Les inoculacions artificials en planta de vinya són necessàries per dur a terme diversos estudis fitopatològics -realització de proves de patogenicitat, assaigs de productes fitosanitaris-, així com qualsevol altra activitat que requereixi l'obtenció de plantes infectades. Per aquest motiu, s'ha determinat el potencial d'inòcul òptim en inoculacions artificials amb suspensions d'espores dels patògens *D. seriata*, *Eutypa lata* i *Phaeomoniella chlamydospora* (Capítol 5). En qüestions epidemiològiques, s'ha estudiat la dinàmica de l'alliberament de conidis de *D. seriata* en les restes de poda (Capítol 6), per a avaluar el paper d'aquestes restes com a font d'inòcul de noves infeccions a la vinya. També s'han estudiat les infeccions naturals de les ferides de poda (Capítol 7), així com el període durant el qual aquestes ferides resten susceptibles a la infecció dels fongs *D. seriata* i *P. chlamydospora* (Capítol 8). Finalment, en l'apartat de tècniques de control, s'ha estudiat l'efecte del tractament de termoteràpia amb aigua calenta sobre la viabilitat de diferents espècies de la família *Botryosphaeriaceae*, en condicions *in vitro* i *in planta*, per a avaluar així la potencialitat d'aquesta tècnica en el control sanitari del material de propagació (Capítol 9).

Amb l'objectiu d'aprofundir en el coneixement de la biologia de *D. seriata*, es va realitzar una caracterització exhaustiva de 83 soques d'aquesta espècie, obtingudes principalment de vinya i de diverses regions del país. Aquesta caracterització, tractada en el Capítol 4 d'aquesta tesi, va combinar els resultats obtinguts en estudis moleculars (ADN) i fenotípics (morfologia de conidis, creixement miceliar i compatibilitat vegetativa i sexual) amb els obtinguts en les proves de patogenicitat. L'anàlisi dels marcadors *Inter Simple Sequence Repeat* (ISSR) va ser una eina eficaç en l'estudi de la variabilitat intraespecífica de *D. seriata*. Mitjançant les agrupacions de l'anàlisi Bayesià i de l'anàlisi discriminant de components principals (DAPC, *Discriminant Analysis of Principal Components*), es van poder establir dos grups genètics ben diferenciats, encara que sense una segregació clara entre les soques procedents de diferents hostes o regions geogràfiques. Aquest fet dóna a entendre que hi

ha un fons genètic comú entre les soques dels diferents grups. Phillips *et al.* (2007) tampoc van trobar cap relació entre la procedència geogràfica de diverses soques de *D. seriata* i les dades de l'anàlisi de la regió *Internal Transcribed Spacer* (ITS) de l'ADN ribosomal. En el present treball, tot i que només es van diferenciar dos grups DAPC, els cinc marcadors ISSR van mostrar un percentatge de polimorfisme del 88 % en el conjunt de les soques. Aquest valor concorda amb els resultats obtinguts amb d'altres espècies de la família *Botryosphaeriaceae*, com ara *Neofusicoccum parvum* (93 % de polimorfisme, segons Baskarathevan *et al.*, 2012) i *Neofusicoccum luteum* (93 %, segons Billones-Baaijens *et al.*, 2013a), el que sembla apuntar a una gran variabilitat genètica en el conjunt de les espècies de *Botryosphaeriaceae*.

Els resultats obtinguts en l'estudi morfològic dels conidis de *D. seriata* van ser congruents amb les dades publicades en descripcions anteriors (Punithalingam i Walker, 1973; Phillips *et al.*, 2007). No es van observar diferències en quant a la llargada, l'amplada i la relació llargada/amplada dels conidis entre els conjunts de soques dels dos grups DAPC, així com tampoc entre el creixement miceliar de les soques dels dos grups. Tampoc es van observar reaccions de compatibilitat vegetativa que es corresponguessin amb els grups DAPC, fet que indicaria que els marcadors ISSR podrien tenir una diana en el genoma diferent a la que regula la compatibilitat vegetativa. La impossibilitat d'establir grups de compatibilitat vegetativa ben definits és senyal de que les barreres entre els individus són febles i que permeten el flux genètic entre ells.

Totes les soques emprades en la prova de patogenicitat (14 representants dels dos grups DAPC) van produir lesions necròtiques en sarments i plantes de vinya de la varietat 'Tempranillo' i van ser recuperades amb èxit d'aquestes zones necròtiques. Es va observar una gran variabilitat en la longitud de les necrosis causades per les diferents soques, el que no va permetre detectar diferències significatives entre els grups DAPC pel que fa aquesta variable. Així doncs, no es va trobar un component genètic que expliqués un comportament patogènic diferencial. La variabilitat observada en la virulència tampoc es va correspondre amb el creixement miceliar de les soques o la seva procedència geogràfica i/o de l'hoste. Diferents graus en la virulència també han estat observats en altres estudis amb *D. seriata*; aquesta

espècie ha estat considerada com a patògena de la vinya a Austràlia (Castillo-Pando *et al.*, 2001; Savocchia *et al.*, 2007), Xile (Auger *et al.*, 2004), França (Larignon *et al.*, 2001a), Itàlia (Rovesti i Montermini, 1987), Mèxic (Úrbez-Torres *et al.*, 2008), Sud-àfrica (van Niekerk *et al.*, 2004), Espanya (Luque *et al.*, 2009) i Estats Units (Úrbez-Torres *et al.*, 2009). En canvi, s'ha considerat un patogen feble a les regions australianes de Nova Gales del Sud i Sud d'Austràlia (Pitt *et al.*, 2013a), Xina (Yan *et al.*, 2013), Iran (Mohammadi *et al.*, 2013), Nova Zelanda (Amponsah *et al.*, 2011) i Portugal (Phillips, 2002), i com a no patogènica, a l'Est d'Austràlia (Taylor *et al.*, 2005). En aquest estudi, les lesions causades per les soques van ser significativament majors que les observades en el tractament control, però la mida mitjana d'aquestes lesions no va superar mai els 3 cm després de 12 mesos d'infecció a la planta. Aquests resultats fan pensar que *D. seriata* no és un fong especialment virulent. Tot i això, les dades obtingudes en altres estudis donen suport al caràcter patogènic de *D. seriata*. Per exemple, Luque *et al*. (2009) van aïllar aquest fong, amb una alta freqüència, de necrosis sectorials de troncs i braços de vinya amb xancres. A més, en el Capítol 7 d'aquesta tesi, en el que s'estudia la infecció natural de les ferides de poda a la zona del Penedès, es constata que *D. seriata* va ser l'espècie aïllada amb més freqüència, fet que posa de manifest que aquest fong és un patogen de la fusta a tenir en compte en condicions naturals (Luque *et al.*, 2014).

En conjunt, la caracterització de *D. seriata* ha posat en evidència la gran variabilitat d'aquesta espècie des del punt de vista molecular i patogènic, fet que ha dificultat establir possibles relacions entre la resta d'aspectes estudiats en aquest capítol. Aquest és un dels treballs de caracterització de *D. seriata* que ha comptat amb una major col·lecció de soques sotmesa a estudi (Elena *et al.*, 2015b); tot i això, en el futur seran necessaris nous treballs per a continuar ampliant el coneixement d'aquesta espècie.

Amb l'objectiu d'establir el potencial d'inòcul òptim per a realitzar inoculacions artificials amb els fongs patògens *D. seriata*, *E. lata* i *P. chlamydospora*, es va avaluar el percentatge d'infecció resultant d'inoculacions de sarments amb diferents quantitats d'espores dels patògens. Pel que fa a *D. seriata*, el percentatge d'infecció es va situar al voltant del 70 % després d'inocular entre 100 i 1000 conidis per ferida de poda, un resultat similar a l'obtingut per altres investigadors (Rolshausen *et al.*, 2010a; Pitt *et al.*, 2012).

Tot i això, en altres treballs s'han observat percentatges d'infecció menors, comparats amb el del present estudi, tot i haver utilitzat potencials d'inòcul més elevats; és el cas d'un assaig d'avaluació de fungicides (Bester *et al.*, 2007) o el d'un assaig d'agents de control biològic (Kotze *et al.*, 2011). En aquests estudis, els mètodes d'inoculació emprats van ser diferents de l'actual, fet que podria explicar les diferències observades. Així, Bester *et al*. (2007) van realitzar l'aplicació de l'inòcul mitjançant un esprai, tècnica que hauria pogut reduir el potencial d'inòcul real en la ferida, en comparació amb la deposició d'una gota d'inòcul sobre la ferida de poda, utilitzada en l'assaig actual. D'una altra banda, Kotze *et al.* (2011) van realitzar les inoculacions una setmana després de podar els sarments, període durant el qual les ferides han començat el seu procés natural de cicatrització i, en conseqüència, la susceptibilitat a aquests patògens podria haver-se reduït. En aquest sentit, el treball recollit en el Capítol 8 d'aquesta tesi, sobre la susceptibilitat de les ferides de poda als fongs patògens *D. seriata* i *P. chlamydospora*, reforça aquesta idea.

En el cas de *P. chlamydospora*, el percentatge d'infecció va ser del 60 % quan es van aplicar a cada ferida de poda entre 100 i 2000 conidis. Per a aquest patogen, els percentatges d'infecció trobats a la literatura són més variables (Larignon i Dubos, 2001a; Bester *et al.*, 2007; Serra *et al.*, 2008; Kotze *et al.*, 2011), el que indicaria una major dificultat per a uniformitzar el seu mètode d'inoculació.

Finalment, les inoculacions amb 100-500 ascòspores d'*E. lata* van resultar en un rang d'infeccions d'entre el 57 i 97 % dels sarments, tant en l'assaig de camp com en el dels sarments mantinguts en safates amb sorra. Aquests resultats van concordar amb els d'alguns estudis anteriors (Petzoldt *et al.*, 1981; Lecomte *et al.*, 2004; Sosnowski *et al.*, 2008; Lecomte i Bailey, 2011; Sosnowski *et al.*, 2013; Ayres *et al.*, 2014). Contràriament, en d'altres estudis en els que es van utilitzar concentracions de treball similars, el percentatge d'infecció obtingut va ser menor (Trese *et al.*, 1980; Chapuis *et al.*, 1998; Halleen *et al.*, 2010; Rolshausen *et al.*, 2010a; Kotze *et al.*, 2011; van Niekerk *et al.*, 2011). Aquesta disparitat podria ser deguda a la variabilitat intraespecífica d'*E. lata* (Sosnowski *et al.*, 2007) o, també, a una possible competència entre el patogen i altres fongs capaços de colonitzar les ferides de poda, dificultant-ne així la seva infecció (Munkvold i Marois, 1995; Chapuis *et al.*, 1998).

Degut a l'elevada variabilitat observada entre els percentatges de recuperació dels patògens -obtinguts en aquest i en d'altres estudis, tots ells discutits en el Capítol 5 d'aquesta tesi-, és aconsellable realitzar assajos preliminars per a establir la dosi òptima que s'usarà, posteriorment, en les inoculacions artificials amb aquests fongs. A partir dels resultats obtinguts en aquest treball, es va determinar que un percentatge d'infecció òptim podria aconseguir-se amb 1000 conidis per ferida en el cas de *D. seriata*, 500 ascòspores d'*E. lata* i 2000 conidis de *P. chlamydospora*. En els estudis d'epidemiologia del Capítol 8, sobre la infecció i la colonització de les ferides de poda, es van usar les concentracions de treball establertes en aquest estudi.

En l'àmbit dels estudis epidemiològics d'aquesta tesi, s'ha confirmat que les restes de la poda -infectades de manera natural per *D. seriata*, entre d'altres fongs- constitueixen una font d'inòcul força important i perdurable a la vinya. Durant el seguiment d'aquestes restes, realitzat entre 2 i 3 anys i mig després de la poda, es va observar una reducció progressiva del potencial d'inòcul patogen, posat de manifest per una disminució de totes les variables estudiades: el nombre de picnidis amb conidis, la quantitat mitjana de conidis per picnidi i el percentatge de germinació dels conidis. La reducció més forta del potencial d'inòcul va estar relacionada amb la disminució del nombre mitjà de conidis per picnidi, que es va fer més aparent 36 mesos després de la poda, instant marcat pel període de pluges més elevat de tot l'experiment (131 mm). Estudis previs sobre la captura d'espores d'espècies de la família *Botryosphaeriaceae* també han relacionat la dispersió aèria dels propàguls amb la coincidència de períodes de pluja (Kuntzmann *et al.*, 2009; Úrbez-Torres *et al.*, 2010).

Malgrat la reducció natural de la pressió de l'inòcul de *D. seriata* a la vinya, al cap de 3 anys i mig encara es van detectar conidis viables i, per tant, de suposada capacitat infectiva. Aquest estudi és el primer intent en descriure, quantitativament, la dinàmica de l'inòcul de *D. seriata* a les restes de la poda de la vinya. Degut a la importància d'aquestes restes en el cicle biològic del patogen i al paper que desenvolupen com a font d'inòcul per a noves infeccions, és altament recomanable retirar i eliminar les restes de la poda. Aquesta recomanació ja forma part del catàleg de pràctiques tradicionals del cultiu al nostre país, i és un exemple clar de les activitats que poden contribuir a millorar

el control de les malalties de la fusta de la vinya; en aquesta ocasió, en l'àmbit del maneig del cultiu.

Les ferides de poda són la principal via d'entrada a través de la qual els fongs patògens infecten la planta adulta, una idea que avui en dia és plenament acceptada en la comunitat científica i el sector vitícola. En el Capítol 7 s'ha estudiat el fenomen de la infecció -de forma natural-, que té lloc a les ferides de poda en dues vinyes del Penedès (Luque *et al.*, 2014). Els principals fongs patògens identificats en l'estudi van ser, en ordre d'abundància decreixent, *D. seriata*, *P. chlamydospora* i *Cryptovalsa ampelina*. Els percentatges d'infecció observats van ser molt variables en funció de l'espècie fúngica, la vinya i l'any d'estudi. No obstant això, les infeccions van estar fortament relacionades amb l'època de la poda, ja que en general van ser més elevades per a tots els patògens després d'una poda tardana -a l'hivern- que després d'una poda primerenca, a la tardor. Les correlacions entre els percentatges d'infecció i els diferents paràmetres meteorològics van revelar que, en general, la pluviometria acumulada fins a 13 setmanes després de la poda i la temperatura mitjana presentaven coeficients positius entre les diferents variables i la infecció. Aquests resultats confirmen els obtinguts per van Niekerk *et al.* (2011), els quals ja havien posat de manifest que valors més alts de temperatura i precipitació acumulada podien afavorir la infecció i colonització de les ferides per part de diverses espècies de la família Botryosphaeriaceae. En l'estudi sobre la susceptibilitat de les ferides de poda, corresponent al Capítol 8 d'aquesta tesi, es va observar que les ferides restaven més temps susceptibles a la infecció de *D. seriata* després d'una poda tardana, realitzada el febrer, moment en el que es va veure que es dóna una major infecció d'aquest fong per causes naturals. Altres autors ja havien postulat que una poda primerenca pot disminuir el risc d'infecció d'aquests patògens (Trese *et al.*, 1980; Serra *et al.*, 2008; van Niekerk *et al.*, 2011). En contrast, altres estudis han confirmat que la poda tardana és la que redueix la freqüència d'infecció a la vinya (Petzoldt *et al.*, 1981; Munkvold i Marois, 1995; Chapuis *et al.*, 1998; Larignon i Dubos, 2001a; Eskalen *et al.*, 2007; Úrbez-Torres i Gubler, 2011). Aquestes informacions contradictòries posen en evidència la gran variabilitat observada en aquests tipus d'estudis. Això fa pensar que, probablement, la susceptibilitat de les ferides de la poda a aquests patògens podria estar influenciada per determinades característiques climàtiques de cada regió vitícola, que s'haurien d'estudiar de forma local.

Segons els resultats obtinguts en aquesta tesi sobre les infeccions naturals de les ferides de poda, seria aconsellable fer un canvi en aquesta activitat en la regió del Penedès. Caldria passar de l'actual poda tardana, segons s'aconsella de forma tradicional, a una poda primerenca, que sembla reduir el risc d'infecció patògena. Aquesta recomanació és un exemple més de com una pràctica de cultiu pot contribuir a reduir el risc d'infecció i, per tant, a integrar-se en el conjunt de les mesures de control de les malalties. Tot i això, aquesta indicació no pot fer-se extensiva a la resta de regions vitivinícoles. Com ja s'ha vist anteriorment, la variabilitat observada en la resposta de la susceptibilitat de les ferides de la poda als patògens desaconsella aquesta acció. Així doncs, caldria fer estudis específics de les infeccions naturals en cada regió per a determinar, segons les condicions de cada zona, quin seria el millor moment per a realitzar la poda i reduir així el risc d'infecció.

Per avaluar la susceptibilitat de les ferides de poda a les infeccions de *D. seriata* i *P. chlamydospora*, es van inocular artificialment ferides de poda amb els dos patògens a diferents temps després d'una poda primerenca -realitzada al novembre- i després d'una poda tardana, realitzada al febrer (Capítol 8). Es va confirmar que la susceptibilitat a aquests fongs disminuïa, en conjunt, a mesura que s'incrementava el temps entre la poda i el moment de la inoculació. Aquesta reducció en la susceptibilitat estaria explicada pels fenòmens associats a la cicatrització de les ferides, conduents precisament a dificultar les infeccions patògenes (Shigo, 1984; Doster i Bostock, 1988; Bostock i Stermer, 1989). En línies generals, la susceptibilitat a *D. seriata* va semblar estar relacionada amb l'època de la poda, ja que els percentatges d'infecció van ser més alts després d'una poda tardana en comparació amb els obtinguts després d'una de primerenca. Aquesta dada és congruent amb el que es va observar en l'estudi de les infeccions naturals (Capítol 7; Luque *et al*., 2014), en el qual es van detectar unes freqüències d'infecció majors de *D. seriata* a la primavera. Estudis anteriors realitzats amb inoculacions artificials a Itàlia (Serra *et al.*, 2008) i Sud-àfrica (van Niekerk *et al.*, 2011) també van observar una major susceptibilitat de les ferides a la infecció d'aquest patogen després d'una poda tardana. En canvi, no es van observar diferències entre les dues podes, en termes de susceptibilitat, pel que fa a la infecció de *P. chlamydospora*. Serra *et al.* (2008) també van observar una disminució de la susceptibilitat de les ferides a la infecció de *P. chlamydospora* sense diferències clares entre les èpoques de

poda. En canvi, Luque *et al.* (2014) van observar que les ferides de poda eren més susceptibles a la infecció de *P. chlamydospora*, donada de forma natural, després de una poda tardana. Aquestes diferències podrien estar més aviat relacionades amb la disponibilitat d'inòcul infectiu a cada època de poda que no pas amb la susceptibilitat de les ferides al patogen. Tot i això, en el present estudi, el percentatge de recuperació de *D. seriata* i *P. chlamydospora* encara va situar-se al voltant del 10 % dotze setmanes després de la poda. Aquesta dada confirma el que ja havien apuntat altres estudis sobre el fet de que les ferides de poda poden restar susceptibles a aquests dos patògens fins a quatre mesos (Gubler *et al.*, 2001; Eskalen *et al.*, 2007; Serra *et al.*, 2008). Aquest període tan extens de la susceptibilitat de les ferides posa a prova les estratègies per a la seva protecció, que haurien de comptar amb solucions -químiques o biològiques- capaces de cobrir aquest lapse de temps tan llarg. Malauradament, a dia d'avui, la literatura científica i tècnica no és gaire extensa pel que fa a productes eficaços per a la protecció de les ferides de poda.

En l'estudi sobre la colonització de les ferides de poda en relació a la longitud de l'entrenús podat (Capítol 8), *D. seriata* va mostrar en general un major potencial per a la colonització dels sarments que *P. chlamydospora*. Després de nou mesos d'incubació, els percentatges d'infecció de *D. seriata* van ser més elevats a tots els punts d'aïllament que els de *P. chlamydospora*. Els resultats són congruents amb les dades trobades sobre el creixement miceliar d'aquests fongs, major en el cas de *D. seriata* (vegi's Úrbez-Torres *et al.* (2006), Martos (2008) i Pitt *et al.* (2013a), per a dades de *D. seriata*, i Whiting *et al.* (2001) i Tello *et al.* (2010), per a informació sobre *P. chlamydospora*). El creixement més ràpid de *D. seriata* afavoriria la colonització de l'entrenús, ja que els percentatges de recuperació obtinguts després de nou mesos d'incubació van ser més elevats que els obtinguts després de cinc mesos. En canvi,el creixement lent de *P. chlamydospora* podria dificultar la colonització, dels sarments. De fet, es van observar percentatges de recuperació menors al cap de nou mesos d'incubació que al cap de cinc mesos. Pel que fa a l'efecte de la longitud del darrer entrenús sobre la colonització del sarment, no es van observar diferències en el cas de la infecció de *D. seriata* entre els punts d'aïllament per sobre del nus, nou mesos després d'haver realitzat les inoculacions artificials i independentment de la seva longitud. Aquests resultats indiquen que una major longitud no sembla afectar la colonització de *D. seriata*.

En canvi, la colonització per sota del nus en els sarments podats a 2 cm va ser menor comparada amb la colonització del punt d'aïllament equivalent en els sarments podats a 5 cm (per sobre del nus), tot indicant que el nus actuaria com a barrera en el progrés de la colonització dels sarments. En el cas de *P. chlamydospora*, els resultats obtinguts semblen indicar que la longitud del darrer entrenús influeix en la colonització dels sarments, de manera que un entrenús més llarg dificulta el pas d'aquest fong als teixits que es troben per sota del darrer nus. En canvi, els percentatges de recuperació trobats en el punt d'aïllament a 4,5 cm de la ferida de poda van ser similars entre els sarments podats a 2 cm i 5 cm, fet que indica que el nus no seria un limitant per a la infecció d'aquest patogen. La informació obtinguda en aquest assaig podria ajudar a un control més efectiu de les infeccions de les ferides de la poda. Tot i que una poda amb un entrenús més llarg no dificultaria la infecció de *D. seriata*, sí que ho faria respecte a *P. chlamydospora*. Per extensió, semblaria lògic pensar que una poda deixant entrenusos llargs dificultaria les infeccions de fongs de creixement lent, tot i que tindria un efecte baix o nul sobre els de creixement més ràpid.

Respecte d'altres mesures de control, en el darrer estudi d'aquesta tesi (Capítol 9) es va avaluar la resposta de diferents fongs de la família *Botryosphaeriaceae* al tractament de termoteràpia amb aigua calenta (TAC), mitjançant l'estudi de la seva supervivència després del TAC en assaigs *in vitro* i *in planta*; i el seu creixement miceliar, sols en el primer dels assaigs. En l'assaig *in vitro*, *D. seriata*, *N. luteum*, *N. parvum* i *Spencermartinsia viticola* van ser les espècies més susceptibles a la temperatura, ja que per sobre de 51 °C no es va observar la supervivència de cap d'aquestes espècies, independentment del temps d'exposició a l'aigua calenta. *Botryosphaeria dothidea* i *Neofusicoccum mediterraneum* només van poder ser controlats quan la temperatura va ser superior a 53 °C, i *Lasiodiplodia theobromae* i *Neofusicoccum vitifusiforme* van ser les espècies més tolerants a la temperatura, ja que el seu miceli va sobreviure fins i tot després d'haver estat tractat a 54 °C. En l'assaig *in planta*, la supervivència de les vuit espècies es va veure pràcticament anul·lada amb una exposició de 30 minuts a 51 °C. Els resultats de l'assaig *in vitro* van posar de manifest una resposta variable de les espècies a la temperatura, el que podria estar relacionat amb el creixement miceliar dels fongs en condicions de laboratori, un aspecte tractat en estudis anteriors (van Niekerk *et al.*, 2004;

Úrbez-Torres *et al.*, 2006; Martos, 2008; Pitt *el at.*, 2013a). En contrast, resulta més difícil d'explicar perquè totes les espècies van tenir una resposta similar en l'assaig *in planta*. En qualsevol cas, les condicions experimentals d'aquest segon assaig són més properes a les condicions reals del procés de producció de planta en el viver, en el que el TAC s'aplica al material de propagació per a reduir-ne el component patogen. Aquest estudi sobre el TAC és el primer que es realitza amb un conjunt ampli d'espècies de la família *Botryosphaeriaceae*.

Gramaje *et al.* (2008; 2009b; 2010a) ja van obtenir resultats positius en el control dels patògens associats a la malaltia de Petri i el peu negre de la vinya mitjançant TAC. En aquest estudi s'ha demostrat que el TAC també pot ser una tècnica eficaç pel control d'espècies de la família *Botryosphaeriaceae*. Tenint en compte que els patògens associats a les malalties de Petri i del peu negre, conjuntament amb les espècies de *Botryosphaeriaceae*, són els fongs més freqüents en el material de propagació dels vivers, el TAC pot ser una eina molt efectiva per al seu control. En aquest sentit, la incorporació d'aquesta tècnica al procés de producció de planta pot ajudar a millorar l'estat sanitari dels plançons de vinya i, en conseqüència, a que es plantin vinyes en millors condicions sanitàries des del començament.

La lluita contra les malalties de la fusta de la vinya, en el marc d'una estratègia integrada, pretén combinar tots els conceptes, tècniques i mesures de control que, des de diferents àmbits, puguin contribuir a millorar la sanitat global del cultiu. La finalitat de tot plegat és mitigar l'impacte de les malalties i, en conseqüència, allargar la vida útil de la vinya. En aquesta tesi s'han obtingut resultats que amplien el coneixement sobre l'epidemiologia de les malalties de la fusta i que podrien tenir una aplicabilitat directa per a reduir el risc d'infecció en la vinya adulta. Es tractaria de proposar alguns canvis en el maneig del cultiu, tots ells relacionats d'una o altra forma amb la pràctica de la poda, que es deriven d'aquests coneixements adquirits. Per últim, l'estudi sobre el TAC també ha ampliat el coneixement sobre la utilitat d'aquesta tècnica pel control d'un grup de patògens en el material vegetal de propagació, els fongs de la família *Botryosphaeriaceae*, que causen greus impactes a la vinya.

Capítol 11

Conclusions generals

Versió en català

- *Diplodia seriata* presenta una gran variabilitat intraespecífica segons es va poder concloure dels estudis molecular, fenotípic, patogènic i de compatibilitat vegetativa realitzats amb 83 soques del fong.
- En la caracterització molecular de *Diplodia seriata* es va observar un alt grau de polimorfisme (88 %), però l'assignació de les soques a dos grups genètics diferenciats no va ser congruent amb els resultats dels estudis fenotípics, de compatibilitat vegetativa i de patogenicitat.
- *Diplodia seriata* és un patogen feble i variable en virulència. La seva patogenicitat vers la vinya no va estar relacionada amb la procedència geogràfica o de l'hoste de les soques, així com tampoc amb la variabilitat genètica observada.
- La inoculació artificial de les ferides de poda de la vinya amb quantitats variables d'espores dels fongs *Diplodia seriata*, *Eutypa lata* i *Phaeomoniella chlamydospora* va resultar en percentatges d'infecció variables i assimilables a corbes del tipus dosi-resposta.
- Per a assolir un rang d'infecció de les ferides de poda òptim mitjançant inoculacions artificials (entre 50 i 70 %), es va determinar que calia inocular entre 100 i 1000 conidis de *Diplodia seriata* per ferida de poda, entre 100 i 2000 conidis de *Phaeomoniella chlamydospora*, i entre 100 i 500 ascòspores d'*Eutypa lata*.
- L'alliberament de conidis de *Diplodia seriata* en restes de poda de la vinya va patir una disminució significativa entre dos i tres anys i mig després de la poda; així ho indiquen les reduccions observades en el nombre de picnidis que contenien conidis, el nombre estimat de conidis per picnidi i el percentatge de germinació dels conidis. Tot i això, tres anys i mig després de la poda encara es van detectar conidis viables, posant de manifest el rol potencial d'aquestes restes com a font d'inòcul de llarga durada a la vinya.
- En un estudi sobre les infeccions naturals de les ferides de poda realitzat a la zona del Penedès, els principals fongs patògens aïllats van ser, en ordre decreixent de freqüència: *Diplodia seriata*, *Phaeomoniella chlamydospora* i *Cryptovalsa ampelina*. En general, els percentatges d'aïllament van ser més

elevats a la primavera, després d'una poda tardana, que a l'hivern, després d'una poda primerenca realitzada a la tardor.

- La temperatura i la pluviometria acumulada en els tres mesos posteriors a la poda van correlacionar positivament i de forma significativa amb els percentatges d'infecció natural de les ferides de poda.
- La susceptibilitat de les ferides de poda a *Diplodia seriata* i *Phaeomoniella chlamydospora* va disminuir a mesura que s'incrementava el temps entre la poda i el moment de la infecció. Tot i això, el percentatge de recuperació d'aquests patògens dels sarments inoculats encara es va situar al voltant del 10 % tres mesos després de la poda, el que indica un període de susceptibilitat de les ferides relativament llarg.
- Es va detectar una susceptibilitat global major a *Diplodia seriata* després d'una poda tardana a l'hivern, en contrast amb una de primerenca a la tardor. Pel que respecta *Phaeomoniella chlamydospora*, no es van detectar canvis globals de susceptibilitat relacionats amb factors estacionals.
- El procés de cicatrització de les ferides de poda va dificultar la colonització del sarment de *Phaeomoniella chlamydospora* quan la longitud del darrer entrenús va ser major, però aquesta no va interferir en la colonització de *Diplodia seriata*.
- La tècnica de la termoteràpia amb aigua calenta, aplicada a vuit espècies de *Botryosphaeriaceae*, va reduir la viabilitat i el creixement miceliar de totes elles, posant de manifest el potencial d'aquesta tècnica per a ser usada en el control d'aquests patògens en el material de propagació del viver.
- El tractament de termoteràpia amb aigua calenta va tenir un efecte diferencial sobre la viabilitat de les soques de *Botryosphaeriaceae* segons el tipus d'assaig realitzat. Mentre que en l'assaig *in vitro* es va observar un rang de susceptibilitat variable segons la temperatura, en l'assaig *in planta* la viabilitat de tots els fongs es va reduir dràsticament a una temperatura de 51 °C o superiors i 30 minuts d'exposició.

English version

- *Diplodia seriata* showed a high intraspecific variability as confirmed by the characterization of 83 isolates from different geographic and host origins in terms of molecular, phenotypic, pathogenic and mycelial compatibility characteristics.
- The molecular study of *Diplodia seriata* revealed a high polymorphism (88 %) in the species and grouped the isolates into two different clusters. However, no relationship was found between this clustering and the results from the phenotypic, pathogenic and vegetative compatibility studies.
- *Diplodia seriata* was confirmed as a weak pathogen showing variable degrees of virulence on grapevine. Results from the pathogenicity tests showed that there was no relationship between either the geographic and host origins of isolates and the genetic variability detected.
- Artificial inoculations carried out on grapevine pruning wounds with different spore doses of *Diplodia seriata*, *Phaeomoniella chlamydospora* and *Eutypa lata* showed variable infection rates which were shown to be similar to dose-response curves.
- In order to achieve an optimal range of infection rates obtained through artificial inoculations of pruning wounds (between 50 and 70 %), it was necessary to inoculate each pruning wound with 100-1000 conidia of *Diplodia seriata* , 100-2000 conidia of *Phaeomoniella chlamydospora* and 100-500 ascospores of *Eutypa lata*.
- Release of *Diplodia seriata* conidia in pruning debris significantly decreased between 2 and 3.5 years after pruning. Pycnidia that contained conidia, mean number of conidia per pycnidia and conidia germination percentages significantly decreased during the experimental period. However, viable conidia were still detected at the end of the experiment thus revealing that pruning debris becomes an important long-lasting inoculum source.
- In a study on the natural infections of pruning wounds carried out in the Penedès wine region, the following pathogenic fungi were identified in descending order of frequencies: *Diplodia seriata*, *Phaeomoniella chlamydospora* and *Cryptovalsa ampelina*. Isolation rates were generally

higher in spring, after a late pruning carried out in winter, as compared with the lower infection rates obtained after an early pruning in autumn.

- Average temperatures and accumulated rainfall recorded over three months after pruning were seen to correlate positively with the natural infection rates of grapevine pruning wounds.
- Susceptibility of grapevine pruning wounds to the infection by *Diplodia seriata* and *Phaeomoniella chlamydospora* decreased as the time between pruning and artificial infection increased. However, infection rates of pruning wounds three months after pruning were still around 10 %, thus revealing a long period of wound susceptibility.
- Pruning wounds were in general more susceptible to infection by *Diplodia seriata* after a late pruning in winter, as compared with an early pruning done in autumn. No overall seasonal changes in terms of susceptibility were detected for *Phaeomoniella chlamydospora*.
- Natural healing of pruning wounds made cane colonization difficult for *Phaeomoniella chlamydospora* when the length of the last internodes was longer. Colonization of canes by *Diplodia seriata* was not as highly influenced by internode length as it was in the case of *P. chlamydospora*.
- The viability and mycelial growth of eight Botryosphaeriaceae species was decreased after hot water treatment in different *in vitro* and *in planta* experiments, thus showing the feasibility of this technique as a control method in the nursery propagation process.
- Depending on the experimental conditions, hot water treatment showed differential effects on the viability of Botryosphaeriaceae isolates. In the *in vitro* assay, viability of isolates varied according to the different temperature and exposure time combinations. However, *in planta*, the viability of all isolates was drastically reduced after 30 minutes at temperatures of 51 °C and higher.

Capítol 12

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Effect of the length of the pruned internode on the colonization of grapevine canes by *Diplodia seriata* and *Phaeomoniella chlamydospora*

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INTRODUCTION

Every year along autumn and winter seasons, grapevines are pruned around the world following different viticultural practices. Fresh pruning wounds can be infected by grapevine trunk pathogens through airbone spores, which become the main way for disease spread in mature vineyards. Little is known about the influence of the natural healing of the uppermost pruned cane internode on the pathogen survival and growth. Therefore, the main objective of this study was to determine the effect of the length of the pruned internode on the survival and colonization rates of canes by the pathogens *Diplodia seriata* and *Phaeomoniella chlamydospora*.

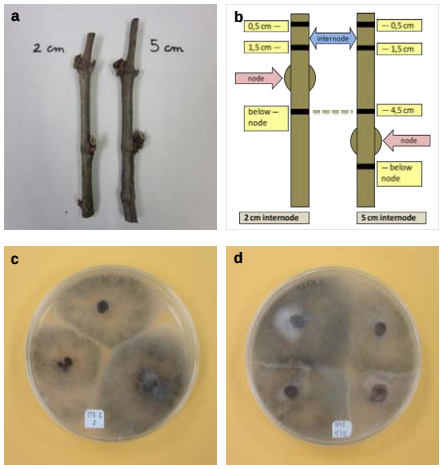
MATERIAL AND METHODS

Pathogen inoculations

Figure 1. 'Chardonnay' vineyard pruned at different internode lengths and inoculated separately with conidial suspensions of *Diplodia seriata* and *Phaeomoniella chlamydospora* (**a** and **b**).

In winter (January), 240 dormant canes in a 'Chardonnay' vineyard (Fig. 1a) were pruned leaving two different lengths between the uppermost node and the pruning wound: 2 cm or 5 cm (Fig. 2a). Conidial suspensions of *Diplodia seriata* and *Phaeomoniella chlamydospora* were prepared and pruning wounds (n=20 per treatment) were separately inoculated with the conidial suspensions at 1000 and 2000 spores per wound, respectively (Fig. 1b). Sterile distilled water was used in control treatments. The experiment was repeated twice in 2013-14.

Pathogen recovery



Five and eight months after inoculations, pathogens were recovered from cane pieces taken at different distances from the pruning wound: three or four sites depending on the internode length (Fig. 2a,b). Two common sites for fungal recovery in both groups were established at 0.5 cm and 1.5 cm below the pruning wound. An additional common site was below the bud, but at different distances from the wound for each treatment group. Wood samples were surface-sterilized in 70% ethanol for 4 minutes and plated on PDA. Recovery percentages for each pathogen, season, inter-node length, and recovery site were determined (Fig. 2c,d).

Figure 2. Canes with 2-cm and 5-cm pruned internodes (**a**). Recovery sites in 2-cm and 5-cm internodes (**b**). Recovery of *Diplodia seriata* from 2-cm (three cane pieces) and 5-cm internodes (four pieces) (**c** and **d**).

RESULTS

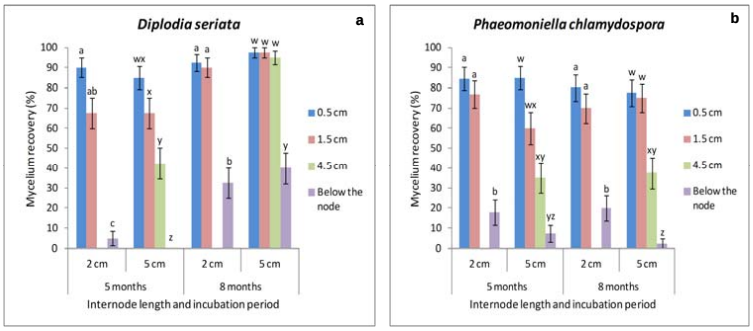


Figure 3. Mean percentages of *Diplodia seriata* (**a**) and *Phaeomoniella chlamydospora* (**b**) mycelium recovery at different recovery sites in canes (n=40) of 2 cm and 5 cm long internodes corresponding to two different incubation periods (5 and 8 months) after pruning. Significant differences (P<0.05) among means for each recovery site are indicated separately by different letters (*a* to *c* in 2-cm internodes and *w* to *z* in 5-cm internodes). Bars indicate standard error of the mean.

Five months after pruning, recovery of *D. seriata* below the node was equal or lower than 5% despite of the internode length (Fig. 3a). Eight months after pruning, recovery below the node increased to 32.5% (2-cm internode) and 40% (5-cm internode), which were not significantly different. However, recovery percentages below the node remained significantly lower than those from upper sites (Fig. 3a).

Recovery percentages of *P. chlamydospora* did not show significant differences between the two incubation periods. A significant reduction in the recovery rate was observed as the pathogen was reisolated from lower sites in the cane. The recovery percentages below the node did not exceed 20% in any case.

DISCUSSION AND CONCLUSIONS

Pathogen colonization of pruned canes seemed to depend on the fungal growth rate within the host tissues and the internode length left when pruning. Higher recovery percentages of *D. seriata* at the end of experiment (8 months) indicated that this pathogen is able to colonize grapevine tissues better than *P. chlamydospora*. Results also suggested that the uppermost node can interfere with the colonization of grapevine living tissues below the node, thus suggesting that leaving a long internode when pruning could make difficult the posterior pathogenic colonization of canes.

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Importancia de los restos de poda como fuente de inóculo de *Diplodia seriata*, agente causal del 'brazo muerto' de la vid

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INTRODUCCIÓN

El manejo de los restos de poda de la vid se suele limitar a dejar los sarmientos podados en el propio viñedo, ya sean apilados en el margen de los campos o troceados e incorporados al suelo (Fig. 1). Con el tiempo, en estos sarmientos se desarrollan cuerpos fructíferos de hongos patógenos de la vid, como *Diplodia seriata*, que se comportan como fuente de inóculo.

El objetivo de este estudio fue el de evaluar la dinámica de la liberación y la viabilidad de los conidios de *D. seriata* presentes en restos de poda de vid infectados de forma natural por el hongo, para determinar así el papel de dichos restos como fuente de inóculo del patógeno.

MATERIAL Y MÉTODOS

En Noviembre de 2012 se recogieron sarmientos podados de vid que contenían cuerpos fructíferos (Fig. 2), verificándose que correspondían a *D. seriata* mediante observación microscópica. Se prepararon cuatro muestras con 18 fragmentos de sarmientos cada una de ellas, que se dejaron en campo para su evolución de forma natural. Cada muestra se procesó en distintos períodos después de la recolección: en el mismo momento que se recogieron los sarmientos (tiempo 0) y después de 6, 12 y 18 meses.

En nueve fragmentos de cada muestra se realizó un recuento de 50 picnidios al azar para determinar si contenían conidios (Fig. 3). En los nueve fragmentos restantes se contó el número de picnidios existentes en un área determinada del sarmiento. Estas áreas se cortaron y se dispusieron separadamente en tubos con agua destilada estéril y en agitación para obtener una suspensión de conidios. Posteriormente se estimó el número de conidios por picnidio así como el porcentaje de germinación de los conidios, todo ello por triplicado (Fig. 4).

Figura 1 Restos de poda en un viñedo.

Figura 2 Picnidios de *Diplodia seriata* sobre un sarmiento procedente de los restos de una poda.

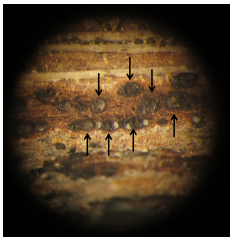


Figura 3 Corte longitudinal de picnidios de *Diplodia seriata* (flechas).

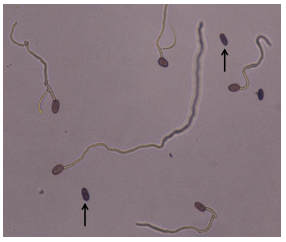
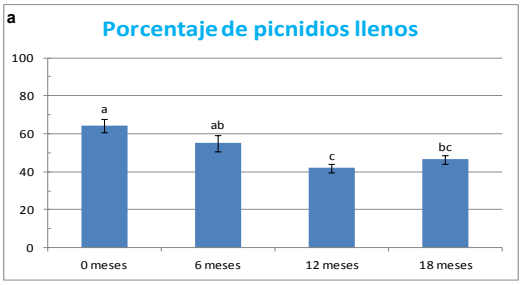
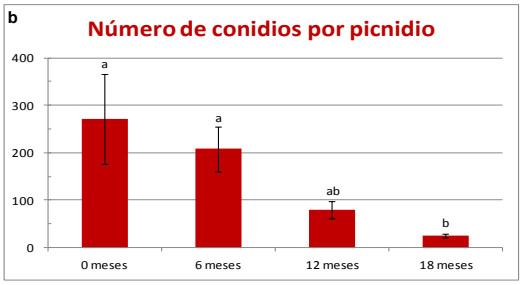


Figura 4 Aspecto de conidios de *Diplodia seriata* germinados y no germinados (flechas).

RESULTADOS





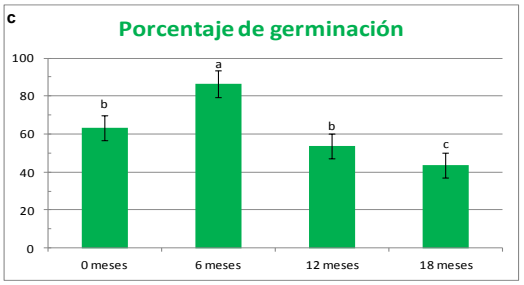


Figura 5 Valores medios del porcentaje de picnidios de *Diplodia seriata* con conidios (a), número estimado de conidios por picnidio (b), y porcentaje de germinación de los conidios (c) correspondientes a sarmientos de vid abandonados en campo después de la poda. Las diferencias significativas ($p<0,05$) están indicadas con letras distintas. Las barras de error corresponden al error estándar de la media (n=9).

- A lo largo del ensayo se observó una reducción progresiva del número de picnidios de *D. seriata* con conidios, llegándose a porcentajes inferiores al 50% a partir de los 12 meses después de la recolección (Fig. 5a).
- El número de conidios por picnidio sufrió una fuerte reducción a partir de los 6 meses, con alrededor de unos 200 conidios por picnidio, para llegar a tan sólo 25 después de 18 meses (Fig. 5b).
- El porcentaje de germinación también se vió reducido durante el periodo de evaluación, pasando del 63% en la evaluación inicial a un 44% al final del proceso, sugiriendo así una pérdida de viabilidad de los conidios con el paso del tiempo (Fig. 5c).

CONCLUSIONES

Los resultados muestran una reducción progresiva de la presión de inóculo de *Diplodia seriata*. Aún así, 18 meses después de la recolección de los sarmientos todavía existían conidios viables en los restos de poda, indicando con ello la presencia de una fuente de inóculo prolongada en el viñedo.

Este trabajo ha sido financiado con fondos del proyecto RTA2010-00009-C03 del INIA y FEDER. Georgina Elena recibe financiación de INIA para la realización de su tesis doctoral.

Susceptibilidad de las heridas de poda de la vid a la infección por conidios de *Diplodia seriata* y *Phaeomoniella chlamydospora*

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INTRODUCCIÓN

En el viñedo, la dispersión de las enfermedades de la madera de la vid depende de la infección de los cortes de poda por parte de esporas aerovagantes de los hongos patógenos causales. En la mayoría de regiones vitvinícolas mundiales, las heridas de poda no se suelen proteger con ningún agente sellante ni fungicida debido al coste económico de su aplicación. En consecuencia, los hongos pueden penetrar las heridas e invadir la madera sin mayor dificultad. El objetivo de este estudio fue el de evaluar el periodo durante el cual las heridas de poda permanecen susceptibles a la infección de los hongos *Diplodia seriata* y *Phaeomoniella chlamydospora*, mediante la inoculación artificial de los patógenos en cortes de poda y en diferentes intervalos de tiempo posteriores a la realización de la poda.

MATERIAL Y MÉTODOS

Cultivos

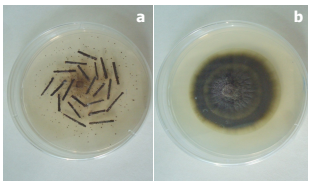

Figura 1 Cultivos de *Diplodia seriata* (a) y *Phaeomoniella chlamydospora* (b).

Diplodia seriata se cultivó en placas de Petri con agar-agua y fragmentos de hojas de pino estériles, a 25°C y con un fotoperiodo de 12/12 durante 4 semanas (Fig. 1a). *Phaeomoniella chlamydospora* se cultivó en placas de Petri con PDA, a la misma temperatura y en la oscuridad (Fig. 1b).

Periodos de poda, inoculación y reaislamiento de los sarmientos

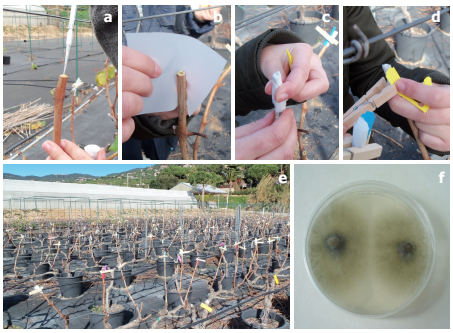

Figura 2 Inoculación con una suspensión de conidios de *Diplodia seriata* y *Phaeomoniella chlamydospora* (a). Protección de las heridas de poda con malla Nytex (b, c, d). Aspecto de la parcela experimental (e). Ejemplo de reaislamiento del hongo patógeno *D. seriata* en placa de Petri (f).

En un viñedo experimental de IRTA se llevaron a cabo una poda temprana en otoño (Noviembre) y otra tardía (Febrero). Los cortes de poda se inocularon con suspensiones de conidios de *D. seriata* y *P. chlamydospora* de forma separada (a razón de 500 y 2000 conidios por herida, respectivamente) y a diferentes periodos después de la poda: 1 día, 1 semana y 2, 4, 8 y 12 semanas (Fig. 2a). Se incluyó un tratamiento control tratado con agua destilada estéril. Una vez inoculados, los cortes de poda se protegieron con una malla de Nytex de 5 µm de luz (Fig. 2 b, c,d y e). Los reaislamientos se llevaron a cabo cuatro meses después de cada inoculación y se calculó el porcentaje de infección correspondiente a cada periodo de inoculación y hongo (Fig. 2f). El experimento se realizó por duplicado.

RESULTADOS

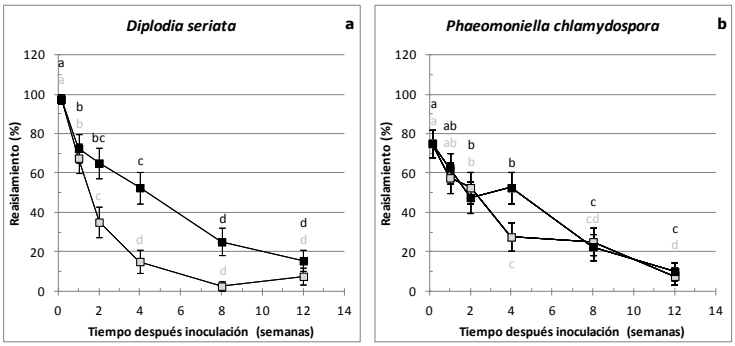

Figura 3 Valores medios del porcentaje de reaislamiento de *Diplodia seriata* (a) y *Phaeomoniella chlamydospora* (b) en inoculaciones artificiales realizadas en diferentes períodos después de una poda temprana (color gris) o tardía (negro). Las diferencias significativas ($p<0.05$) están indicadas con letras distintas. Las barras de error corresponden al error estándar de la media.

Se observaron diferencias significativas entre los porcentajes de reaislamiento de *D. seriata* según la época de poda, ya fuera temprana o tardía. En ambos casos, el porcentaje de reaislamiento fue del 97,5% en la inoculación realizada un día después de la poda (Fig 3a). Sin embargo, el porcentaje cayó por debajo del 35% a partir de las dos semanas, en el caso de una poda temprana, mientras que esta disminución no se observó hasta pasadas ocho semanas cuando se podó tardíamente (Fig. 3a). Para *P. chlamydospora* no se observaron tendencias diferenciadas entre las dos épocas de poda. El reaislamiento a las cuatro semanas de una poda tardía sufrió un ligero repunte, hasta el 52%, hecho que no se observó después de una poda temprana (Fig. 3b).

Independientemente de la época de poda y del patógeno estudiado, las inoculaciones a las 12 semanas de la poda todavía ofrecieron reaislamientos alrededor del 10% (Fig. 3b).

CONCLUSIONES

Las heridas de poda permanecen menos tiempo susceptibles a la infección de *Diplodia seriata* cuando se realiza una poda temprana, sugiriendo con ello que adelantar la poda a otoño permitiría reducir el riesgo de la infección por parte de este patógeno. En el caso de *Phaeomoniella chlamydospora*, las cuatro primeras semanas después de la poda son el periodo de máxima susceptibilidad de las heridas a su infección. Aún así, las heridas permanecieron susceptibles a los patógenos hasta 12 semanas después de la poda, aunque el porcentaje de infección medio se situó alrededor del 10%.

Este trabajo ha sido financiado con fondos del proyecto RTA2010-00009-C03 del INIA y FEDER. Georgina Elena recibe financiación de INIA para la realización de su tesis doctoral.

Optimizing techniques for evaluating *Eutypa lata* infection in grapevines

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INTRODUCTION

Artificial inoculation and subsequent isolation are widely used in studies of pathogenesis, disease epidemiology and evaluation of control methods. Regarding artificial inoculations with *Eutypa lata* on grapevines, different methods of recovery, incubation period following inoculation and spore concentrations have been published (Carter and Moller 1971; Sosnowski *et al.* 2008; Ayres *et al.* 2011; Lecomte and Bailey 2011). The aim of this study was to optimize inoculation, sterilization and incubation procedures for evaluating *E. lata* infection of grapevine wounds.

MATERIALS AND METHODS

Minimum Inoculum Potential

Figure 1. 'Shiraz' vineyard (**a**) and 'Cabernet Sauvignon' canes (**b**) inoculated with different ascospore concentrations of *Eutypa lata* (**c** and **d**).

Two experiments were conducted: a field trial in a 'Shiraz' vineyard in Australia (Fig. 1a,b) and a detached cane assay (DCA) using 'Cabernet Sauvignon' in France (Fig. 1c,d). Ascospore suspensions were prepared from naturally-infected grapevine wood and used to inoculate pruning wounds with 10, 50, 100, 200, 500 or 1000 spores. In the DCA, canes were harvested 2 weeks after inoculation and in the field, 11 months later. In order to reisolate *E. lata*, cane samples were plated on either potato dextrose agar (PDA) or malt agar (MA) for the field and DCA trials, respectively.

Recovery Methods

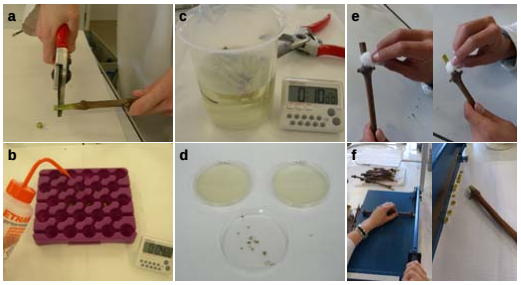

Figure 2. Methods for *Eutypa lata* recovery: two wood discs immersed in 70% ethanol for 4 min (a, b), 5 cm fragment immersed in 2.5% SH for 10 min (c, d) and canes flamed with ethanol and cut into ten discs (e, f).

A DCA using 'Cabernet Sauvignon' canes, inoculated with 150 spores/wound, was set up to compare four methods for *E. lata* recovery: 1) two discs (0.5 cm) per cane immersed in 70% ethanol for 4 minutes (Fig. 2a,b), 2) 5 cm fragment per cane immersed in 2.5% sodium hypochlorite (SH) for 10 minutes and cut in small wood chips (Fig. 2c,d), 3) canes flamed with ethanol and cut into ten discs of 0.1 cm (Fig. 2e,f), and 4) same as 3) but with bark surface disinfected by immersing the canes in 0.5% SH for 20 minutes prior to inoculation. Controls with no disinfection were included. Recovery of *E. lata* was assessed on MA 4 weeks after inoculation for techniques 1 and 2, and 2 weeks after inoculation for techniques 3 and 4.

Incubation Period

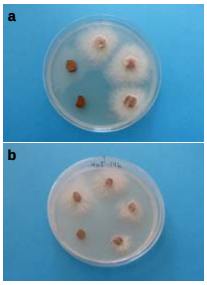

Figure 3. *Eutypa lata* recovery on malt agar after different incubation periods.

A DCA using 'Cabernet Sauvignon' canes was set up to test different incubation periods for optimum recovery of *E. lata*. Wounds were inoculated with 150 ascospores and canes harvested for recovery on MA at 2, 4 and 6 weeks after inoculation (Fig. 3).

RESULTS

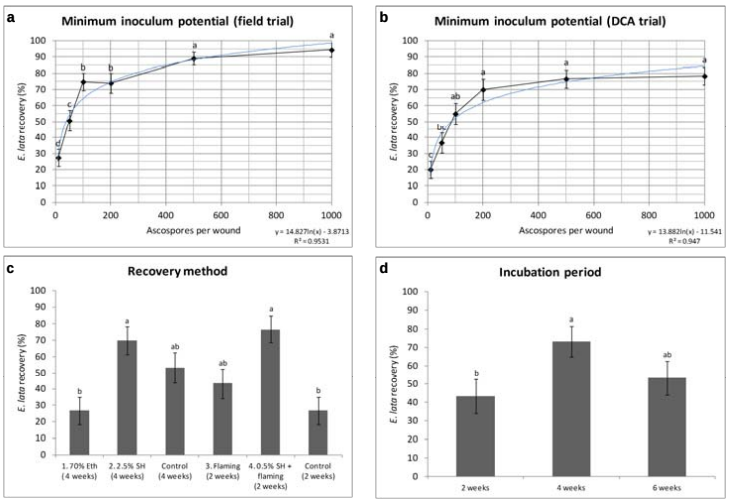

Figure 4. Mean percentages of *Eutypa lata* mycelium recovery at different inoculum doses in the field trial (n=100) and the DCA (n=60) (**a** and **b**, respectively), and after using different methods of recovery (**c**) and at different incubation periods (**d**). Significant differences among means ($P \leq 0.05$) are indicated by different letters. Bars indicate standard error of the mean.

DISCUSSION

The minimum inoculum potential for infection with *Eutypa lata* was 10 ascospores per wound, but resulting in percentages lower than 30% (Fig. 4a,b). High infection (over 75%) was achieved after inoculations of either 200 spores per wound in the field trial (Fig. 4a) or 500 spores in the DCA (Fig. 4b).

The most effective methods for *E. lata* recovery (70-75%) were; 2) immersing canes in 2.5% SH for 10 mins before plating, and 4) immersing canes in 0.5% SH prior to inoculation and then flaming with ethanol (Fig. 4c). All other methods resulted in 50% or less recovery of *E. lata*.

The greatest recovery (70%) was observed after 4 weeks incubation, although not significantly higher than after 6 weeks incubation (Fig. 4d).

CONCLUSION

Results from this study can help in optimizing procedures to evaluate infection by *E. lata* in future research.

Financial support from the INIA (Spain), under project RTA2010-00009-C03. Georgina Elena was supported by a grant from INIA.

Ayres *et al.* 2011. *Wine and viticulture* Nov-Dec, 50-53. Carter and Moller 1971. *Australian Journal of Experimental Agriculture and Animal Husbandry* 11, 684-686. Lecomte and Bailey 2011. *Vitis* 50, 35-41. Sosnowski *et al.* 2008. *Australian Journal of Grape and Wine Research* 14, 134-142.

The minimum inoculum potential of *Diplodia seriata* and *Phaeomoniella chlamydospora* needed for the artificial infection of grapevine pruning wounds

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INTRODUCTION

Artificial inoculations of grapevine canes with spore suspensions are widely used in the studies of pathogenesis, disease epidemiology, and the evaluation of control methods for pruning wound protection. A wide range in the use of spore or conidial concentrations has been found in the literature (Eskalen et al. 2007 ; Sosnowski et al. 2008 ; Philippe et al. 2010; Kotze et al. 2011). In this study, we aimed to determine the minimum inoculum potential required to infect pruning wounds by two known grapevine pathogens, *Diplodia seriata* and *Phaeomoniella chlamydospora*.

MATERIALS AND METHODS

Culture conditions

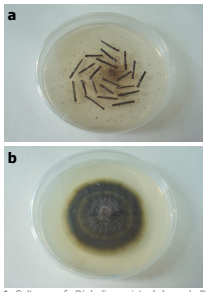

Figure 1 Cultures of *Diplodia seriata* (**a**), and *Phaeomoniella chlamydospora* (**b**).

D. seriata was grown in Petri dishes with water agar and sterile fragments of pine needles for four weeks at 25ºC with a 12/12h photoperiod (Fig. 1a). *P. chlamydospora* was grown in Petri dishes with Potato Dextrose Agar (PDA) at the same temperature in darkness (Fig. 1b).

Inoculum production and inoculation

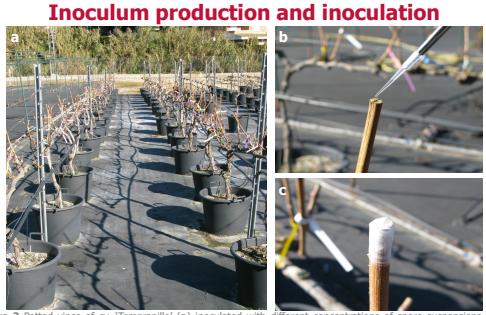

Figure 2 Potted vines of cv. 'Tempranillo' (**a**) inoculated with different concentrations of spore suspensions of the pathogens *Diplodia seriata* and *Phaeomoniella chlamydospora*. After inoculation (**b**), canes were protected (**c**).

Conidial suspensions, decreasing from $1 \cdot 10^5$ to $2.5 \cdot 10^2$ conidia·ml^{-1}, were prepared for each pathogen. Dormant canes of five-year old potted vines of cv. 'Tempranillo' were pruned leaving 6-7 buds in January (Fig. 2a). Wounds were inoculated with a drop (40 µl) of the conidial suspensions, which corresponded either to 4000, 2000, 1000, 100 or 10 conidia per wound (Fig. 2b). Wounds were wrapped with Parafilm® to favour the pathogenic infection and to avoid other fungal contaminations (Fig. 2c). A control treatment was included by using sterile water as inoculum.

Reisolation

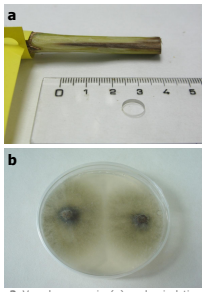

Figure 3 Vascular necrosis (**a**) and reisolation of *D. seriata* (**b**).

Four months after the inoculations, the canes were collected and taken to the laboratory for the measurement of the vascular necroses (Fig. 3a) and the reisolation of pathogens (Fig. 3b). Both variables were studied in relation to the conidia concentrations of inocula.

RESULTS

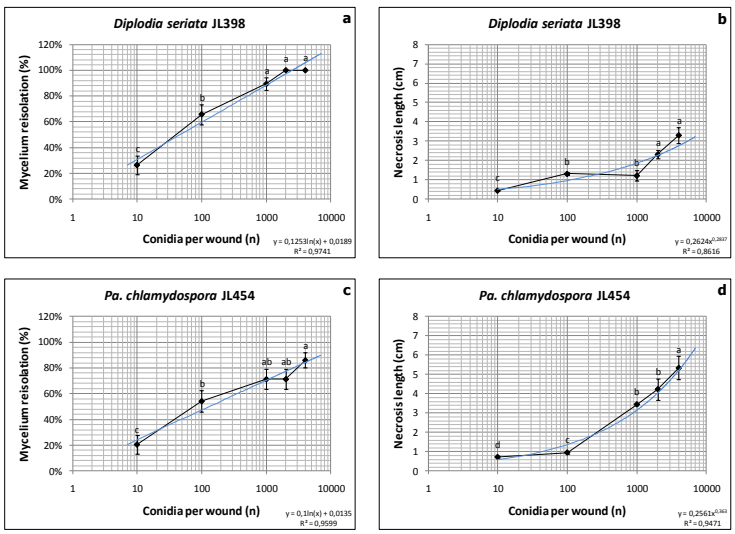

Figure 4 Mean percentages of mycelium reisolation and necrosis length for *D. seriata* (**a** and **b**, respectively) and *P. chlamydospora* (**c** and **d**, respectively) at different inoculum concentrations. A total of 38 replications (in two independent experiments) were used per conidia concentration. Significant differences among means ($p<0.05$) are indicated by different letters. Bar errors correspond to the standard error of the mean.

DISCUSSION

Positive fungal reisolations of both pathogens were obtained at the minimum concentration (10 conidia per wound), although mean percentages of infection were below 30% (Fig. 4a, 4c). Infections of *D. seriata* were above 85% when 1000 conidia per wound were used, and reached 100% at 2000 conidia or higher (Fig. 4a). Infections of *P. chlamydospora* reached 70% of canes when 1000 conidia were inoculated, but the mean infection percentage was only 86 % at the highest concentration (Fig. 4c). For both pathogens, the length of vascular necroses increased as did conidia concentration (Fig. 4b, 4d).

To ensure a significant artificial infection of canes (*i.e.* over 80%), either 500 conidia per wound of *D. seriata* or 2600 conidia of *P. chlamydospora* should be used.

Eskalen *et al.* 2007. *Plant Disease* 91, 1100-1104; Kotze *et al.* 2011. *Phitopatologia Mediterranea* 50, 247-263; Philippe *et al.* 2010. *American Journal of Enology and Viticulture* 61, 113-119; Sosnowski *et al.* 2008. *Australian Journal of Grape and Wine Research* 14, 134-142

Financial support from the INIA (Spain), under project RTA2010-00009-C03. Georgina Elena was supported by a grant from INIA.

Desarrollo de un bioensayo con agentes de control biológico frente a *Diplodia seriata* y *Phaeomoniella chlamydospora*

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INTRODUCCIÓN

La alta incidencia de las enfermedades de la madera de la vid es debida, entre otros muchos factores, a las numerosas infecciones producidas a través de las heridas de poda (Eskalen *et al.* 2007). La efectividad de los fungicidas utilizados para la protección de esta vía de entrada se ve rápidamente deteriorada (Munkvold y Marois 1995), mientras que el corte de poda continúa susceptible durante al menos 4 meses (Serra *et al.* 2008). Ante la falta de fungicidas químicos eficaces, numerosos estudios han evaluado distintos agentes de control biológico (ACB), ya que ofrecen mayor protección durante más tiempo (Kotze *et al.* 2011; Mutawila *et al.* 2011). Este estudio presenta una técnica desarrollada en laboratorio para evaluar posibles ACB frente a los hongos *Diplodia seriata* y *Phaeomoniella chlamydospora*, causantes de brazo muerto y yesca, respectivamente. Los ACB ensayados fueron *Candida sake* (CPA-1), *Pseudomonas graminis* (CPA-7) y una bacteria no identificada (Vi_19_1).

MATERIAL Y MÉTODOS

Esterilización y preparación de los sarmientos

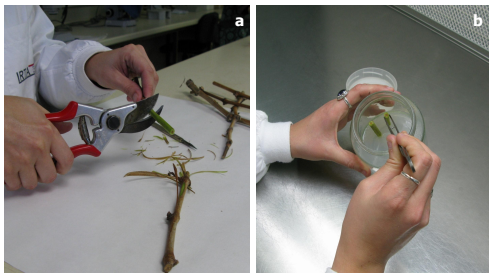

Figura 1 Sarmiento descortezado y cortado en segmentos **(a)**. Colocación de los fragmentos en frascos con agar agua al 1,5 % **(b)**.

Se cortaron sarmientos de vid previamente descortezados en segmentos de 5 cm de longitud aproximadamente (Fig. 1a). Se esterilizaron en superficie (etanol 70º, 4 minutos y posterior lavado con agua destilada, 5 minutos, 3 veces). Los fragmentos se dispusieron en posición vertical en frascos de 750 ml que contenían 150 ml de agar agua al 1,5 % (8 fragmentos por frasco) (Fig 1b).

Inoculación y reaislamiento de los ACB y patógenos

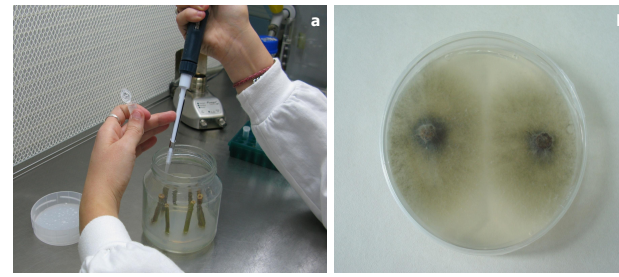

Figura 2 Inoculación de los sarmientos con el ACB y patógeno correspondientes **(a)**. Reaislamiento de *Diplodia seriata* a las dos semanas de incubación **(b)**.

Las secciones de corte de los sarmientos se inocularon separadamente con los distintos ACB (50 µL de una suspensión a 10^7 CFU/ml) y 24 horas más tarde se inocularon con los patógenos (50 µL de una suspensión a $2 \cdot 10^4$ conidios/ml) (Fig. 2a). Se incluyeron tres tratamientos control: 1) fragmentos inoculados con patógeno y sin ACB, 2) con ACB y sin patógeno, y 3) únicamente tratados con agua destilada estéril. Dos semanas más tarde se procedió al reaislamiento de los microorganismos (Fig 2b).

RESULTADOS Y DISCUSIÓN

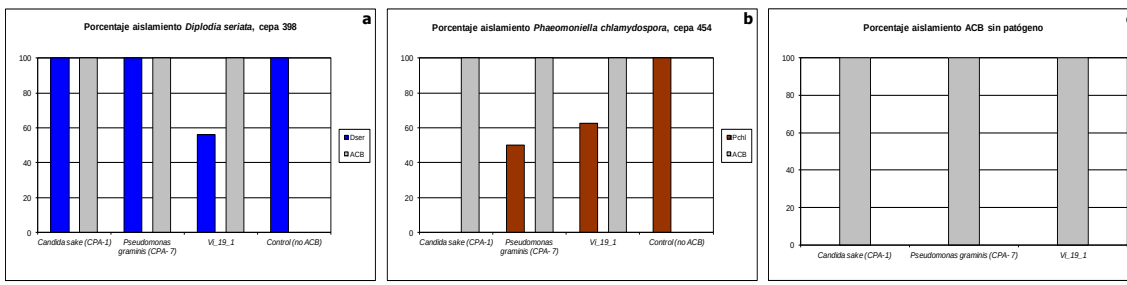

Figura 3 Porcentaje de reaislamiento de los ACB testados y de los patógenos *Diplodia seriata* (**a**) y *Phaeomoniella chlamydospora* (**b**). Porcentaje de reaislamiento de los ACB testados en los tratamientos control sin patógeno (**c**). Un total de 16 réplicas fueron usadas para cada tratamiento.

Todos los ACB se reaislaron del 100 % de los sarmientos inoculados tanto con *D. seriata* como con *P. chlamydospora* (Fig. 3a y 3b), así como del 100% de los sarmientos control sin patógeno (Fig. 3c). El grado de efectividad de estos ACB frente a ambos patógenos fue variable; Vi_19_1 fue el más efectivo contra *D. seriata* (el patógeno se reaisló en un 56 % de los sarmientos) y *C. sake* (CPA-1) el más efectivo contra *P. chlamydospora* (el patógeno no se pudo reaislar en ninguno de los casos).

Los resultados obtenidos muestran que este bioensayo permite hacer una selección preliminar, rápida y a pequeña escala, de potenciales ACB, para que éstos posteriormente puedan ser ensayados en campo frente a los patógenos de interés.

Eskalen *et al.* 2007. *Plant Disease* 91, 1100-1104; Kotze *et al.* 2011. *Phytopathologia Mediterranea* 50, 247-263; Munkvold and Marois 1995. *Phytopathology* 85, 249–256; Mutawila *et al.* 2011. *Phytopathologia Mediterranea* 50, 264-276; Serra *et al.* 2008. *Phytopathologia Mediterranea* 47, 249-256.

Financiado por el proyecto INIA RTA2010-00009-C03 y FEDER. Georgina Elena recibe financiación de INIA para la realización de su tesis doctoral.

Susceptibilidad de hongos de la familia Botryosphaeriaceae al tratamiento de termoterapia con agua caliente

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INTRODUCCIÓN

El uso de la termoterapia con agua caliente (TAC) se ha mostrado potencialmente eficaz para controlar las enfermedades de Petri y del 'Pie Negro' de la vid durante el proceso viverístico de producción de planta. Sin embargo, hasta ahora no se había estudiado el efecto de TAC sobre los patógenos de la vid pertenecientes a la familia Botryosphaeriaceae. En consecuencia, el objetivo de este estudio fue el de evaluar la susceptibilidad a TAC de ocho especies de hongos de esta familia, incluidos en los géneros *Diplodia*, *Dothiorella*, *Fusicoccum*, *Lasiodiplodia* y *Neofusicoccum*.

MATERIAL Y MÉTODOS

En un primer ensayo, fragmentos de micelio contenidos en tubos Eppendorf con agua destilada estéril se sometieron a TAC bajo distintas combinaciones de temperatura (50 a 54 °C) y tiempo de exposición (15, 30 y 45 minutos). En un segundo ensayo, los hongos se inocularon en madera del portainjerto Richter 110 previamente tratada con TAC, se incubaron a 25 °C durante 3 semanas y se sometieron de nuevo a TAC en el rango de 50 a 53 °C, durante 30 minutos. En ambos ensayos se evaluó la supervivencia de los hongos, así como el crecimiento miceliar ('in vitro') y la colonización de los tejidos vegetales ('in planta').

RESULTADOS

Supervivencia "*in vitro*"

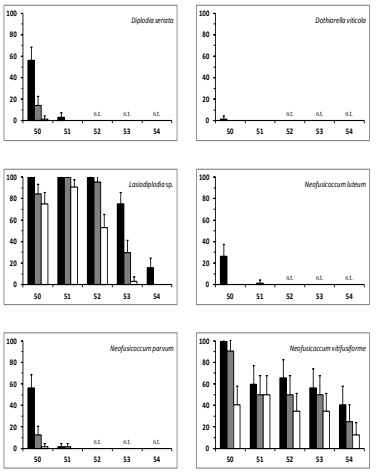

Figura 1 Valores medios del porcentaje de supervivencia de ocho especies de Botryosphaeriaceae sometidas a TAC a distintas temperaturas (50, 51, 52, 53 y 54 °C) y tiempos de exposición (15 min en color negro, 30 min en color gris y 45 min en color blanco). Las letras n.t. indican temperaturas no testadas. Las barras de error corresponden al error estándar de la media.

Crecimiento "*in vitro*"

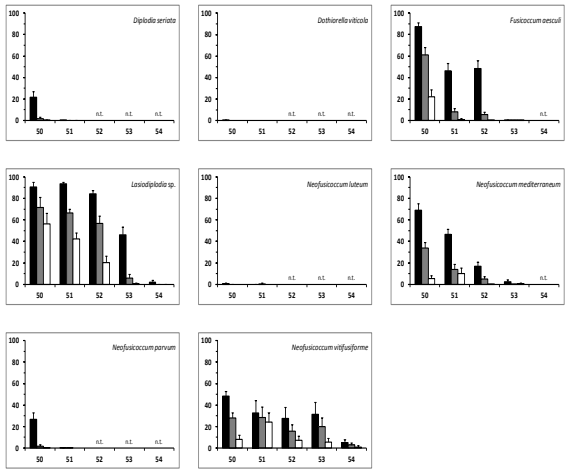

Figura 2 Valores medios del porcentaje de crecimiento relativo, respecto a un control no tratado con TAC, de ocho especies de Botryosphaeriaceae sometidas a TAC a distintas temperaturas (50, 51, 52, 53 y 54 °C) y tiempos de exposición (15 min en color negro, 30 min en color gris y 45 min en color blanco). Las letras n.t. indican temperaturas no testadas. Las barras de error corresponden al error estándar de la media.

- En el ensayo "*in vitro*" se observaron diferencias significativas en supervivencia y crecimiento para todos los factores estudiados (especie, temperatura y tiempo) y sus interacciones.
- *Diplodia seriata*, *Dothiorella viticola*, *Neofusicoccum luteum* y *N. parvum* fueron las especies más sensibles a la temperatura (Fig. 1 y 2).
- *Lasiodiplodia* sp. y *N. vitifusiforme* resultaron las especies más tolerantes (Fig. 1 y 2).

Supervivencia "*in planta*"

Colonización "*in planta*"

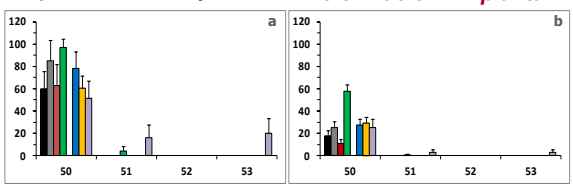

Figura 3 Valores medios del porcentaje de supervivencia (a) y colonización del sarmiento (b) de ocho especies de Botryosphaeriaceae sometidas a TAC durante 30 minutos a distintas temperaturas (50, 51, 52 y 53 °C). Las barras de error corresponden al error estándar de la media.

- En el ensayo "*in planta*", todas las especies redujeron drásticamente su supervivencia a partir de 51 °C (Fig. 3a).
- A 50 °C, *Lasiodiplodia* sp. fue la especie más tolerante, seguida de *Diplodia seriata* y *Neofusicoccum mediterraneum* (Fig. 3a y 3b).
- La especie más sensible a 50 °C fue *N. luteum*, que no mostró supervivencia alguna (Fig. 3a y 3b).

CONCLUSIONES

Los resultados obtenidos indican que el control de la mayoría de especies de Botryosphaeriaceae que infectan la madera de vid empleada en el proceso viverístico es factible mediante el uso de termoterapia con agua caliente (50-51 °C, durante 30 minutos).

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Hongos de la familia Diatrypaceae asociados a enfermedades de la madera de la vid

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INTRODUCCIÓN

En la última década, el número de especies de hongos diatripáceos asociados a enfermedades de la madera de la vid se ha visto incrementado, hasta los 14 taxones reconocidos actualmente, como consecuencia de múltiples estudios realizados en diversas zonas vitivinícolas del mundo (Farr y Rossman, 2012). Hasta la fecha, en España, tan sólo se habían citado dos de ellos: *Eutypa lata* (Arias y Moral, 1981; Armengol *et al.*, 2001; Muruamendiaraz *et al.*, 2009), agente causal de la eutipiosis, una de las enfermedades de la madera más destructivas, y *Cryptovalsa ampelina* (Luque *et al.*, 2006). Por ello, se estimó conveniente actualizar el conocimiento sobre la incidencia de las especies de la familia Diatrypaceae existentes sobre vid en nuestro país.

MATERIAL Y MÉTODOS

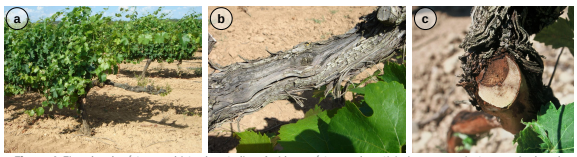

Figura 1 Ejemplos de síntomas objeto de estudio: **a**) vid con síntomas de eutipiosis, con sarmientos enanizados, de hojas pequeñas, deformadas y cloróticas; **b**) chancro en brazo; y **c**) necrosis sectorial en brazo.

Los aislados empleados en este estudio se obtuvieron de vides con síntomas de enfermedad, ya fueran afectadas por decaimiento general, chancros en brazos y tronco, y necrosis en la madera, así como de cuerpos fructíferos existentes sobre la corteza de la planta (Figura 1, Tabla 1).

Identificación y caracterización molecular

Se llevó a cabo el análisis filogenético de la secuencia completa de los espaciadores internos transcritos (ITS) del ADN ribosómico (iniciadores ITS1/ITS4; White *et al.*, 1990) y parte del gen de la β-tubulina (iniciadores Bt2a/Bt2b; Mostert *et al.*, 2006). Los análisis de inferencia Bayesiana se realizaron con ayuda del programa Mr. Bayes version 3.2 (Ronquist y Huelsenbeck, 2003). En dichos análisis filogenéticos se incluyeron además 26 secuencias de ITS y 25 de β-tubulina, obtenidos de GenBank y correspondientes a especies de Diatrypaceae ya conocidas en vid, o a taxones cuyas secuencias presentaban una alta similitud a las secuencias obtenidas en nuestro estudio.

RESULTADOS

Tabla 1 Especies de Diatrypaceae aisladas de vid y localidades de procedencia.

Especie	Aislado	Municipio	Provincia	Recol.	Variedad	Detalles
Anthostoma decipiens	JL567	Falset	Tarragona	2004	Tempranillo	Necrosis, brazo
Cryptovalsa ampelina	JL413	Vimbodí	Tarragona	2003	Garnacha tinta	Peritecio, resto de poda
Cryptovalsa ampelina	JL424	Capçanes	Tarragona	2003	Mazuelo	Peritecio, resto de poda
Cryptovalsa ampelina	JL476	Bot	Tarragona	2003	Macabeo	Chancro, brazo
Cryptovalsa ampelina	JL717	El Pla del Penedès	Barcelona	2009	Cabernet Sauvignon	Necrosis, sarmiento vivo
Eutypa lata	JL355	Caldes de Montbui	Barcelona	2002	Chardonnay	Necrosis sectorial, brazo
Eutypa lata	JL399	Pacs del Penedès	Barcelona	2003	Cabernet Sauvignon	Chancro, brazo
Eutypa lata	JL407	Mediona	Barcelona	2003	Tempranillo	Necrosis sectorial, brazo
Eutypa lata	JL411	Vimbodí	Tarragona	2003	Garnacha tinta	Necrosis sectorial, brazo
Eutypa lata	JL427	La Vilella Baixa	Tarragona	2003	Cabernet Sauvignon	Chancro, brazo
Eutypa lata	JL431	Capçanes	Tarragona	2003	Mazuelo	Necrosis sectorial, brazo
Eutypa lata	JL432	Odena	Barcelona	2003	Macabeo	Necrosis sectorial, brazo
Eutypa lata	JL479	Batea	Tarragona	2003	Garnacha tinta	Chancro, brazo
Eutypa lata	JL600	Vilajuiga	Girona	2005	Tempranillo	Necrosis sectorial, brazo
Eutypa lata	JL677	Barbastro	Huesca	2007	Cabernet Sauvignon	Chancro, brazo
Eutypa lata	JL720	Barriobusto	Álava	2006	Tempranillo	Necrosis, brazo
Eutypa lata	JL721	Lanciego	Álava	2006	Tempranillo	Necrosis sectorial, brazo
Eutypa lata	JL723	Laguardia	Álava	2008	Tempranillo	Necrosis sectorial, brazo
Eutypa lata	JL725	Bargota	Navarra	2008	Tempranillo	Necrosis sectorial, brazo
Eutypa lata	JL726	Labastida	Álava	2008	Tempranillo	Necrosis sectorial, brazo
Eutypa lata	JL727	Navaridas	Álava	2007	Tempranillo	Peritecio, brazo
Eutypa lata	JL731	La Morra	Burgos	2002	Tempranillo	Necrosis, parte desconocida
Eutypa lata	JL732	Malagón	Ciudad Real	Desc.	Garnacha tinta	Necrosis, parte desconocida
Eutypa lata	JL739	Albacete	Albacete	2010	Desconocida	Necrosis, parte desconocida
Eutypa lata	JL740	Yecla	Murcia	2009	Desconocida	Necrosis, parte desconocida
Eutypa lata	JL741	Villena	Alicante	2009	Desconocida	Necrosis, parte desconocida
Eutypa lata	JL743	La Cañada	La Rioja	2010	Tempranillo	Necrosis, parte desconocida
Eutypa lata	JL744	Azofra	La Rioja	2010	Tempranillo	Necrosis, parte desconocida
Eutypa sp.	JL488	Batea	Tarragona	2003	Garnacha tinta	Necrosis sectorial, brazo
Eutypa sp.	JL688	Barbastro	Huesca	2007	Merlot	Necrosis sectorial, brazo
Eutypa sp.	JL690	Barbastro	Huesca	2007	Chardonnay	Necrosis sectorial, brazo
Eutypa sp.	JL742	Albacete	Albacete	2010	Desconocida	Necrosis, parte desconocida
Eutypella citricola	JL583	Olèrdola	Barcelona	2004	Chenin Blanc	Necrosis, tronco
Eutypella citricola	JL734	Murcia	Murcia	2009	Desconocida	Necrosis, parte desconocida
Eutypella microtheca	JL609	Vilajuiga	Girona	2005	Tempranillo	Peritecio, resto de poda
Eutypella microtheca	JL625	Peralada	Girona	2005	Don Mariano	Necrosis, sarmiento vivo
Eutypella microtheca	JL735	Villanueva de Alcolea	Castellón	2009	Desconocida	Necrosis, parte desconocida
Eutypella microtheca	JL738	Novelda	Alicante	2009	Desconocida	Necrosis, parte desconocida

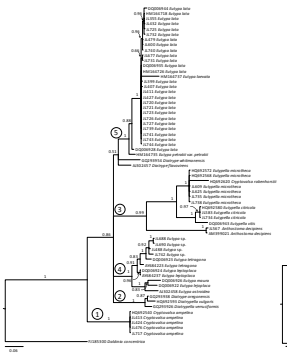

Figura 2 Árbol del consenso mayoritario obtenido a partir del análisis Bayesiano de los datos de secuencias ITS. Probabilidades posteriores en los nodos. Clados numerados en círculos.

Figura 3 Árbol del consenso mayoritario obtenido a partir del análisis Bayesiano de los datos de secuencias β-tubulina. Probabilidades posteriores en los nodos. Clados numerados en círculos.

CONCLUSIONES

- La mayoría de los 38 aislados estudiados se obtuvieron de madera infectada (esto es, de necrosis de tejidos vasculares) y de chancros de vides enfermas, lo que sugiere un posible papel patogénico de estas especies respecto la vid (Tabla 1).
- Se identificaron tres especies además de las ya conocidas *C. ampelina* (clado 1) y *E. lata* (clados 4 y 5): *Anthostoma decipiens*, *Eutypella citricola* y *Eutypella microtheca* (éstas últimas en el clado 3). Cuatro taxones adicionales no pudieron ser identificados a nivel de especie, aunque se encontraban en el clado 4, estrechamente relacionados con *Eutypa tetragona* (Figuras 2 y 3).
- *Eutypa lata* fue la especie aislada con mayor frecuencia (60 % de los aislados obtenidos). *Cryptovalsa ampelina* y *E. microtheca* ocuparon el segundo lugar en cuanto a la frecuencia de aislamientos (10 %), mientras que todas las demás especies fueron poco abundantes (Tabla 1).
- *Eutypella citricola* y *E. microtheca* se citan por primera vez sobre vid en España. Además, *A. decipiens* constituye una primera cita mundial sobre vid.

Arias y Moral, 1981. *Agricultura* 592, 827-830; **Armengol *et al.*, 2001**. *Phytopathologia Mediterranea* 40 (suppl.), S325-S329; **Farr y Rossman, 2012**. http://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm; **Luque *et al.*, 2006**. *Phytopathologia Mediterranea* 45 (suppl.), S101-S109; **Mostert et al., 2006**. *Studies in Mycology* 54, 1-115; **Muruamendiaraz *et al.*, 2009**. *Phytopathologia Mediterranea* 48, 140-144; **Ronquist y Huelsenbeck, 2003**. *Bioinformatics* 19, 1572–1574; **White et al., 1990**. PCR protocols: a guide to methods and applications. APS Press.

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